Characterization of NRAGE, a p75 Neurotrophin Receptor Interacting Protein and a Mediator of p75 Initiated Apoptosis

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CONTRIBUTION OF AUTHORS

Chapter 2

I produced almost the entirety of the physical interaction data, including the cloning of DNA constructs, in the manuscript titled "NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates Nerve Growth Factor-dependent apoptosis." I also generated the tissue distribution data, the cell cyle data, determined the correct open reading frame of NRAGE, the sequences of which was submitted to public databases. I also produced the anti-NRAGE antibodies used for immunohistochemistry by colleagues. A fragment of NRAGE was originally identified as a p75NTR interactor in a yeast-two-hybrid screen by collaborator Dr. Joe Verdi. Ms. C. Zeindler initially determined the conditions for p75NTR-TrkA co-immunoprecipitation which were used in figure 2.6. The manuscript was written by Dr. Barker, with input from me .

A. Salehi	Figures 2.1a; 2.2a,c,d; 2.3b; 2.6; 2.7
P. Roux	Figure 2.5
C. Kubu	Figures 2.3A; 2.8
A. Bhakar	Figure 2.2b
L. Tannis	Figure 2.4

Chapter 3

I generated all the data presented in the manuscript titled "NRAGE, a p75 neurotrophin receptor-interacting protein, induces caspase activation and cell death through a JNK-dependent mitochondrial pathway", with the exception of one *in vitro* caspase activity assay which was performed by our collaborator S. Xanthoudakis. I also wrote the manuscript, with input from Dr. Barker.

A. Salehi	All figures except 3.2a
S. Xanthoudakis	Figure 3.2a

Chapter 4

The compound AEG3482 was identified as a protective agent in NGF-deprived sympathetic neuron cultures at Aegera Pharmaceutics Inc. by Drs S. Morris, G. Doucet J. Durkin, and J. Gillard. I performed all the work leading to the identification of this compound as a JNK inhibitor, and a suppressor of p75NTR and NRAGE induced cell death. The possibility of involvement of Heat Shock proteins in AEG3482-mediated protection was initially proposed by Dr S. Morris, however I performed all the experiments to confirm the hypothesis, except for an HSP90 interaction assay performed by Dr. S. Morris, and confirmatory RT-PCR and transcription assays performed by Ms. K. Dickson and Dr. W. Ho, respectively. I wrote the manuscript, with input from Dr. Barker.

A. Salehi	Figures 4.2, 4.3, 4.4a,b; 4.5b, 4.6, 4.7
S. Morris, G. Doucet J. Durkin & J. Gillard	Figures 4.1, 4.5c
W. Ho	Figure 4.5a
K. Dickson	Figure 4.4c

ABSTRACT

The neurotrophins are a family of growth factors involved in the survival, development, and apoptosis of specific populations of neurons and non-neuronal cells. The pro-apoptotic action of neurotrophins is, at least partially, carried out by the p75 neurotrophin Receptor (p75NTR). However the mechanism employed by p75NTR to mediate apoptosis is poorly defined.

We used a yeast two-hybrid screen to find proteins involved in p75NTR apoptotic signaling. This screen led to to the identification NRAGE (for neurotrophin receptor-interacting MAGE homologue), as a p75NTR interacting molecule. NRAGE is a novel member of the MAGE family of proteins, a group of molecules with ill-defined function, sharing a ~200 amino-acid region of homology. We found that NRAGE binds p75NTR *in vitro* and *in vivo*, and that the ectopic expression of NRAGE promotes p75NTR-dependent death in cells normally insensitive to p75NTR signaling. Furthermore, physical and functional assays indicated that the interaction of p75NTR with NRAGE, or that of p75NTR with its co-receptor TrkA, are mutually exclusive.

To further dissect the mechanism of NRAGE mediated apoptosis, we developed an inducible recombinant NRAGE adenovirus. Induced NRAGE expression resulted in robust activation of the JNK pathway, cytosolic accumulation of Cytochrome c, activation of Caspases-3, -7 and -9 and caspase-dependent cell death. Accordingly, inhibition of the JNK pathway suppressed NRAGE-mediated caspase activation and NRAGE-induced cell death.

The well-characterized activation of JNK-dependent cell death by NRAGE or p75NTR was then used as a model system to determine the mechanism-of-action of a novel anti-apoptotic pharmaceutical, AEG3482. Our studies revealed that AEG3482 almost completely blocks JNK-dependent apoptosis induced by p75NTR or NRAGE. The apoptotic inhibitory activity of AEG3482 was attributed to its ability to induce the expression of Heat-Shock Protein-70 (HSP70), which acts as a direct inhibitor of JNK activity. Therefore AEG3482 blocks cell death through an indirect inhbition of JNK. The identical sensitivity of p75NTR- and NRAGE-initiated signaling to AEG3482 or its

analogues, provides further support for NRAGE as a mediator of p75NTR-induced apoptotic signaling.

In summary, our results show that NRAGE contributes to p75NTR-dependent cell death via JNK-dependent activation of the mitochondrial death pathway.

RÉSUMÉ

Les neurotrophines sont des facteurs de croissance qui controlent le développement, la survie, et l'apoptose des populations spécifiques des neurones et d'autres cellules. La fonction pro-apoptotique des neurotrophines est effectuée, au moins partiellement, par le récepteur de neurotrophines p75 (p75NTR). Mais le mécanisme de signalisation employée par p75NTR reste ambigu.

On a utilisé un système double hybride en levure pour trouver des nouvelles protéines participant dans la signalisation apoptotique de p75NTR. Ceci a mené à la decouverte de NRAGE (pour neurotrophin Receptor-interacting MAGE homologue). NRAGE est un nouveau membre de la famille des protéines MAGE, un groupe de molécules avec des fonctions ambigues. NRAGE interagit avec p75NTR *in vitro* et *in vivo*, et l'expression ectopique de NRAGE active l'apoptose par p75NTR dans les cellules qui ne sont pas normallement susceptible à ceci. De plus, l'interaction de p75NTR avec NRAGE, ou ce de p75NTR avec son co-récepteur TrkA, ont été trouveé d'être mutuellement exclusives.

Pour examiner le mécanisme apoptotique incité par NRAGE, on a développé un adénovirus inductible recombinant exprimant NRAGE. L'expression de NRAGE a causé une forte activation de la voie de signalisation JNK, d'accumulation de Cytochrome c dans le cytosol, d'activation des Caspase-3, -7 et –9, et la mort caspase-dépendante. En conséquence, l'inhibition de la voie de signalisation de JNK a bloqué l'activation des caspases par NRAGE, et l'apoptose associé à ceci.

Prochainement, l'activation bien-caractérisé de l'apoptose nécessitant JNK par NRAGE ou p75NTR a été utilisée comme un système modèle pour déterminer le mécanisme de fonction d'un nouveau pharmaceutique anti-apoptotic, AEG3482. Nos études ont montrées que AEG3482 a presque complètement bloqué l'apoptose dépendent de JNK initiée par p75NTR ou NRAGE. L'activité anti-apoptotique de AEG3482 a été attribuée a sa capacité d'inciter l'expression de Heat-Shock Protein-70 (HSP70), qui est un inhibiteur direct de JNK. La susceptibilité identique de l'apoptose causé par p75NTR ou NRAGE a AE3482 et

ses analogues, fournit plus d'appui pour NRAGE comme un médiateur de signalisation apoptotique incité par p75NTR.

En résumé, nos résultats suggèrent que NRAGE contribue à l'apoptose incité par p75NTR en activant un parcours mitochondrial d'apoptose qui dépend sur l'activation de JNK.

LIST OF ABBREVIATIONS

AKT	Protein kinase B
ATF2	Activating transcription factor-2
Bcl-2	B cell lymphoma-2 protein
BDNF	Brain-derived neurotrophic factor
BH	Bcl-2 homology
CARD	Caspase recruitment domain
CDK	Cyclin-dependent kinase
C. elegans	*Caenorhabditis elegans*
CED	*C. elegans* death molecule
CG	Cerebellar granule
CNS	Central nervous system
ComNGF	Commercially available NGF
CRD	Cysteine-rich domain
CRIB	Cdc42/Rac1 interactive binding
CRNF	Cysteine-rich neurotrophic factor
CSF	Corticospinal fluid
CSN	Corticospinal neurons
C-terminal	Carboxy terminal
DD	Death-domain
DED	Death effector domain
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
E1	Embryonic day one
ECD	Extracellualr domain
ER	Endoplasmic reticulum
ERK	Extra cellular-regulated kinase
ES	Embryonic stem
FADD	Fas-associated death domain-containing protein
FAP-1	Fas-associated phosphatase-1
FRS-2	Fibroblast growth factor receptor substrate-2
GAP	GTPase-activating protein
Gab-1	Grb-associated binder-1
GCK	Germinal center kinase
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GSK-3	Glycogen synthase kinase-3
GTP	Guanine triphosphate
Hid	Head-involution defective
HLA	Human leucocyte antigen
IAP	Inhibitor of Apoptosis
IBM	IAP binding motif
ICD	Intracellular domain
Ig	Immunoglobin

IKK	IκB kinase
IL-1	Interleukin-1
IRS	Insulin receptor substrate
JBD	JNK binding domain
JNK	c-Jun amino-terminal kinase
Kbp	Kilobasepair
KCl	Potassium chloride
Kd	Dissociation constant
KDa	KiloDalton
MAC	Mitochondrial apoptosis induced channel
MAG	Myelin-associated glycoprotein
MAGE	Melanoma associated antigen
MAP	Microtubule associated protein
MAPK	Mitogen-activated protein kinase
MAP2K	MAPK kinase
MAP3K	MAPK kinase kinase
MEKK	MAPK kinase kinase
MGI	Myelin-derived growth inhibitor
MHC	Major histocompatibility complex
MHD	MAGE homology domain
MLK	Mixed-lineage kinase
MOM	Mitochondrial outer membrane
mRNA	Messenger RNA
NADE	Neurotrophin receptor associated death effector
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NgR	Nogo Receptor
NIK	NF-κB inducing kinase
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
NNR5	Neurotrophin non-responsive cell line-5
NRH	Neurotrophin receptor homologue
NRIF	Neurotrophin receptor interacting factor
NT	Neurotrophin
NRAGE	Neurotrophin receptor interacting MAGE homolog
OMgp	Oligodendrocyte-myelin glycoprotein
N-terminal	Amino-terminal
p75NTR	p75 neurotrophin receptor
PAK	P21 activated kinase
PC12	Pheochromocytoma cell line
PDK-1	3-phosphoinositide-dependent kinase-1
PI3-K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PNS	Peripheral nervous system
ProNT	Proneurotrophin

PTPase	Protein tyrosine phosphatase
rAPS	Rat adaptor molecule containing PH and SH2 domains
Rb	Retinoblastoma protein
RIP-2	Receptor-interacting kinase-2
Rpr	Reaper
RSK	Ribosomal S6 kinase
SC-1	Schwann cell factor-1
SDS- PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SOS	Son of sevenless
sp75NTR	short p75NTR splice variant
TGN	Trans-Golgi network
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRADD	TNFR-associated death domain containing protein
TRAF	TNFR-associated factor
Trk	Tropomyosin-related kinase
TUNEL	Terminal transferase-mediated dUTP nicked DNA end-labeling
UNC	Uncoordianted
VSM	Vascular smooth muscle
VSM-p75	VSM cells stably transfected with p75NTR

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 Introduction to Apoptosis

Apoptosis, the most common form of programmed cell death, is central to the development and homeostasis of metazoans. For instance, during the development of the mammalian nervous system, about half of the produced neurons die by apoptosis. This death is required for the formation of appropriate connections between neurons and their targets, and for morphogenic processes such as neural tube closure (5, 6). As well, dysregulation of apoptosis leads to a variety of human pathologies including cancer, autoimmune disease and neurodegenerative disorders (7-11).

The term apoptosis was originally coined by researchers who noted two separate patterns of cell death after ischemic insult to hepatic tissue (12). One form, consistent with necrosis, was characterized by the loss of membrane integrity, morphological signs of organellar damage, and the loss of lysosomal contents. The other form, which was termed apoptosis, was characterized by preservation of membrane integrity, reduction in cell volume, plasma membrane bleb formation, morphological preservation of organellar structures, chromatin condensation, nuclear fragmentation, and budding off of cellular fragments.

Early research demonstrated that apoptosis was dependent on synthesis of new protein, suggesting that the cell plays an active role in its demise (13, 14). Thus, it is now believed that apoptosis is a proactive measure to remove excess, damaged or infected cells, without the inflammatory response which normally occurs during necrosis due to the release of cellular contents into the extracellular space.

Since the introduction of the concept of apoptosis, hundreds of genes that control the initiation, execution and regulation of apoptosis have been identified. Compelling evidence indicates that, as well as being evolutionarily conserved, the same molecules and signaling pathway mediate apoptosis initiated by a variety of insults.

1.1 Caspases, Downstream Effectors of Apoptosis

Regardless of the initial insult and the ensuing proximal signals generated, caspases normally act as downstream executioners of apoptosis. Mammalian caspases, which are so-named as they are cysteine proteases that cleave at specific aspartic acid residue, were first implicated in cell death due to their homology to the *Caenorhabditis elegans* (*C. elegans*) apoptotic gene, ced-3.

Thus far, 14 distinct mammalian caspases have been identified, seven of which are believed to play important roles in apoptosis. Caspases are initially synthesized as inactive zymogens or procaspases. Procaspases contain an N-terminal pro-domain of varying size, in addition to a central 'large subunit' and a C-terminal 'small subunit' (Figure 1.1). Unlike other protease zymogens, the removal of the N-terminal pro-domain of a caspase is unnecessary for its catalytic activation. To produce an active caspase protease, most procaspases instead undergo proteolytic cleavage at a caspase-specific aspartate residues at the junction of the small and large subunits. This cleavage takes place through autoproteolysis or by other activated caspases [reviewed in (4, 15-17)].

The catalytically active caspase consists of two identical caspase molecules. The large and small subunits from each caspase contribute four conserved surface loops to an active site, which is completed by a fifth supporting loop from the adjacent caspase. Therefore dimerization is structurally necessary for caspase activity [reviewed in (18, 19)].

According to their structure and function, caspases have been divided into two groups. 'Effector' caspases carry out the proteolysis of vital structural proteins and proteolytic activation of cell dismantling enzymes, while 'initiator' caspases mainly function to activate effector caspases by proteolytic cleavage (4, 15-17) (Figure 1.1).

Initiator caspases, such as Caspase-8 and Caspase-9 are monomeric in their pro-form. However, they contain long pro-domains, which mediate their interaction with specific adapter proteins. Exposure of cells to apoptotic stimuli results in the clustering of these adaptor proteins, bringing initiator caspases in close proximity to promote their catalytic activation (4, 18).

Effector caspases exist in a dimeric state even in their pro-form. However, they must be cleaved by initiator caspases at the junction of the large and small subunits for the active site to assume a productive conformation.

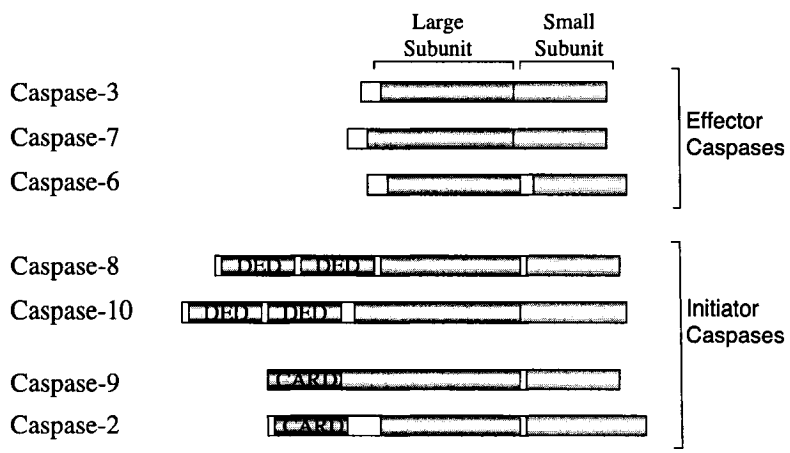

Figure 1.1 The human Caspase family. Domain structures and biological function of the human caspases are illustrated. Note that each procaspase consists of one large and one small domain and may also include caspase-recruitment domains (CARDs) and death effector domains (DEDs) [Adapted from (4)].

Thus, in brief, initiator caspases activate effector caspases, which in turn orchestrate the proteolytic dismantling of the cell. The selectivity of the cascade is due to the recognition of specific tetra or pentapeptide motifs (e.g. DEVD, IETD and VDVAD, among others) by individual caspases in their substrates which may include other caspases (4, 18, 19).

1.1.1 Intrinsic and Extrinsic Pathways of Caspase Activation

Mammals have two distinct apoptotic signaling pathways, these converge at the level of effector caspase activation, but require different initiator caspases to start the process. One pathway, called the intrinsic pathway, is triggered most often in response to growth factor deprivation, oncogene activation, cytotoxic drugs, DNA damage, and particular developmental cues. This pathway is mediated by the initiator Caspase-9, the activity of which is ultimately regulated

by the release of mitochondrial proteins. This release is controlled by the interplay of pro- and anti-apoptotic members of the Bcl-2 family (20) (Figure 1.2).

The other apoptotic pathway, termed the extrinsic pathway and mediated by the initiator Caspase-8, is activated by the binding of ligand to proteins known as death receptors (21-23) (Figure 1.2). Death receptors are members of the TNF receptor superfamily that also contain a conserved motif known as a 'death domain' in their intracellular region (23).

The intrinsic cell death pathway appears to be the primary mode of neuron elimination during development (24), while both pathways can be recruited in neuro-destructive processes such as ischemia (25).

1.1.1.1 The Extrinsic Pathway

The stimulation of death-receptors triggers apoptosis by a mechanism that depends on Caspase-8 activity (26-29). The six known death-receptors contain an intracellular globular homotypic interaction motif called a death domain (DD) (23).

Apoptotic signaling by a death receptor, such as Fas, is initiated upon binding of extracellular death-ligand, in this case FasL, to the cell-surface receptor. The death ligands are constitutively heterotrimeric, and they bind to pre-associated receptor trimers (30, 31) forming a minimally homotrimeric ligand-receptor complex.

Activated death receptors then recruit an adapter protein called Fas Associated Death Domain (FADD) (32). FADD contains two homotypic interaction domains; a DD and a related death effector domain (DED). It binds through its DED to the DED in Caspase-8 (and also Caspase-10 in humans), and through its DD directly to the DD in FAS [or in he case of TNFR1, it binds indirectly through an intermediary DD containing adapter known as TRADD (33, 34)]. The oligomeric complex, composed of Fas, FADD and Caspase-8, formed after death-ligand stimulation, is referred to as the Death Inducing Signaling Complex (DISC) (23, 35).

The induction of Caspase-8 proteolytic activity occurs when two Caspase-8 zymogens are brought into close apposition in the DISC. As each receptor trimer is believed to interact with only one Caspase-8 molecule, an active death complex probably consists of at least two activated receptor trimers. Thus, induction of cell death requires two physically linked ligand trimers (36) binding to a hexameric receptor consisting of two pre-formed trimers (30, 31).

The DISC was initially believed to lead to Caspase-8 activation via proximity induced trans-proteolysis, leading to the production of cleaved, and therefore autonomously active Caspase-8 (21, 22). However recent data suggests that dimerization alone is sufficient for activation of Caspase-8 zymogen, and that cleavage of Caspase-8 is neither necessary nor sufficient for its catalytic activity. Moreover, the initial processing of Caspase-8 zymogen occurs through intramolecular digestion rather than trans-catalysis (37-41).

Therefore, in contrast to the effector caspases, where processing is a prerequisite to activation, dimerization alone is sufficient for Caspase-8 activity. However, Cleavage of Caspase-8 at the junction of pro-domain and large-subunit may play a role in the substrate specificity of Caspase-8. The prodomain-linked Caspase-8 remains associated to the DISC where it can proteolyze membrane proximal substrates, whereas access to substrates such as effector caspases, requires cleavage of Caspase-8 from its prodomain (42, 43).

1.1.1.2 The Intrinsic Pathway

There is considerable evolutionary conservation of the apoptotic pathways. Apoptosis in the nematode *C. elegans* is orchestrated by a series of evolutionarily conserved proteins, arranged in a pathway similar, but not identical, to the intrinsic apoptotic pathway in mammals. In the nematode, four genes, ced-3, ced-4, ced-9 and egl-1, function sequentially to control the onset of apoptosis. The activation of ced-3, the only caspase in the nematode, is mediated by the adaptor ced-4 (44). In the absence of apoptotic signaling, ced-4 is constitutively suppressed by the anti-apoptotic protein ced-9 through direct physical interactions. During apoptosis, the inhibitory effect of ced-9 by ced-4 is

removed by EGL-1, which is transcriptionally activated by death stimuli (45) (Figure 1.2).

The mammalian ortholog of ced-4 is APAF-1, which serves as an adaptor protein necessary for activation of Caspase-9, the apical caspase in the intrinsic death pathway (46-49). Nematode ced-9 is homologous to the anti-apoptotic members of the Bcl-2 family in mammals (50). Egl-1 is homologous to mammalian BH3-only proteins, which physically antagonize the function of anti-apoptotic Bcl-2 family members (51).

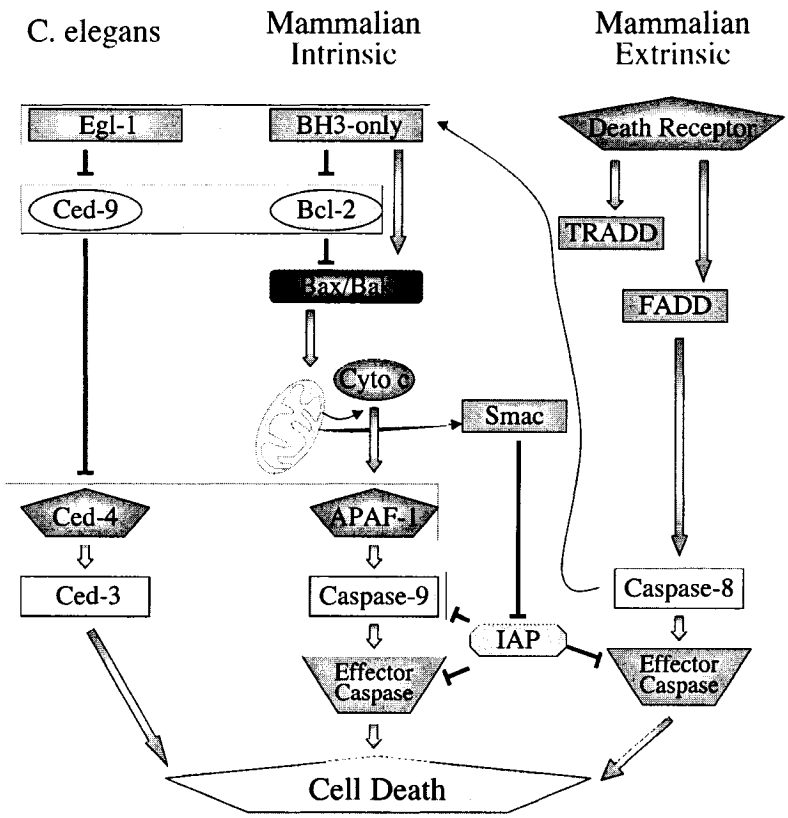

Figure 1.2 Alternate apoptotic pathways in mammals and C. elegans. Orthologues in mammals and C. elegans are encompassed in a larger square. The mammalian extrinsic pathway can cross-activate the intrinsic path through cleavage-mediated activation of the BH3-only protein Bid.

However, unlike in the nematode, the anti-apoptotic Bcl-2 family members do not have direct physical interaction with APAF-1. Instead, they modulate APAF-1 activity by regulating the release of the necessary APAF-1 co-

factor, Cytochrome c, from the mitochondria (46, 48, 49, 52). Therefore, in mammals, the mitochondrial release of Cytochrome c is a pivotal step in the activation of Caspase-9 and the mediation of the intrinsic apoptotic signal.

Cytosolic Cytochrome c acts as a co-factor for APAF-1, allowing for the cytosolic assembly of a large caspase-activating complex that has been termed the apoptosome. APAF-1 is the mammalian homologue of ced-4, but APAF-1 is more evolutionarily advanced, as in addition to a central ced-4 domain, it contains an N-terminal caspase-recruitment domain (CARD), and a C-terminal WD-40 repeat domain (WDR) (47).

APAF-1 rests in an auto-inhibited monomeric state until bound, at its C-terminal WDR, by Cytochrome c. The auto-inhibited state is likely achieved by the burial of the N-terminal CARD into the C-terminal bi-lobed WDR (53-55). Upon Cytochrome c binding to the WDR, the CARD domain is released from the WDR (53-55). The central ced-4 domain can then interact with a nucleotide, enabling it to mediate the heptamerization of APAF-1 into the seven-spoked wheel-like apoptosome complex (53-57). The central hub of the wheel-like structure is composed of CARDs, whereas nucleotide-bound ced-4 domains mediate oligomerization as well as forming the spokes that project out from the hub. These spokes connect the core of the apoptosome to the bi-lobed Cytochrome c bound WDR (54).

Cytochrome c bound APAF-1 recruits and activates Procaspase-9 through the interactions of CARDs found on both proteins. The stoichiometry of APAF-1 CARD to Procaspase-9 CARD is one-to-one (56, 58). Hence, seven procaspases are required to saturate the now exposed CARD regions on the apoptosome. The APAF-1 bound procaspases are then believed to form dimers with free inactive monomeric Procaspase-9.

Similarly to Caspase-8, Caspase-9 cleavage is neither necessary nor sufficient for its activation (59-61), with dimerization itself conferring to Caspase-9 the ability to cleave downstream targets (62). Nevertheless, Caspase-9 normally undergoes autocatalysis after binding to the apoptosome, with this cleavage being necessary for interaction with endogenous inhibitors, such as XIAP, subsequent to

the activation event (61, 63). Cleaved Caspase-9 remains bound to the apoptosome, and cleaved Caspase-9 in isolation has been found to be only marginally active (59).

Apoptosome mediated activation of Caspase-9 initiates a death signal leading to the stepwise activation of additional downstream caspases (64-66). Caspases-3 and -7 are directly activated by Caspase-9 (64), appearing to be recruited and retained within the apoptosome by Caspase-9 (67). Upon activation by the apoptosome, Caspase-3 then processes and activates Caspases-2 and -6, followed by the Caspase-6 mediated activation of Caspases-8 and -10. Thus, upon assembly of apoptosome, rapid amplification of the death signal occurs through activation of a caspase cascade, leading to the dismantling of the cell.

1.2 IAPs, Endogenous Inhibitors of Caspases

In cells, a number of controls exist to delay or alter apoptotic pathways. Members of the inhibitor of apoptosis (IAP) family of proteins, suppress apoptosis through the direct inhibition of processed caspases.

Originally identified in baculovirus (68), IAPs contain tandem repeats of an approximately 70 amino-acid motif, termed baculovirus IAP repeat domains (BIR domains), and many also contain a C-terminal RING zinc-finger domain. The conserved presence and spacing of cysteine and histidine residues within BIR domains suggests that this structure also represents a zinc-binding fold (68). Not all BIR-containing proteins appear to have anti-apoptotic functions (69-71), thus the presence of a BIR domain is necessary, but not sufficient, for inclusion in the IAP protein family.

Eight members of the mammalian IAP have been identified, including X-linked IAP (XIAP), c-IAP1, c-IAP2, NAIP and Livin/ML-IAP. The best studied member of the IAP family, XIAP, contains three N-terminal BIR domains (BIR1, BIR2 and BIR3, numbered sequentially from the N-terminus) and a C-terminal RING domain (72). c-IAP-1, and c-IAP-2 have a similar structure (73) (Figure 1.3).

1.2.1 XIAP

XIAP is the most potent inhibitor of Caspases-3 and -7 *in vitro*, while c-IAP1 and c-IAP2 have an inhibitory constant that is approximately 100-fold weaker than XIAP (74). Much of our knowledge about the molecular mechanism of IAP mediated caspase inhibition comes from crystal structure and mutagenetic studies of XIAP.

Cleavage of caspases at the linker region between the large and small subunits reveals a conserved epitope at the new N-terminus of the small subunit. This conserved motif, known as the IAP-binding motif (IBM), mediates the interaction of the cleaved caspase with a negatively charged groove in one of the BIR domains of XIAP (16).

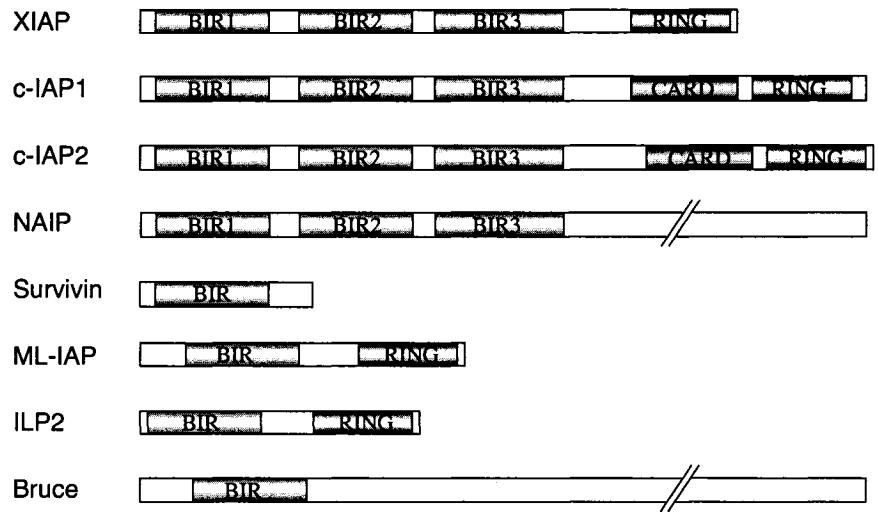

Figure 1.3 Schematic of the mammalian IAP family. IAPs are composed of one or more BIR (baculovirus IAP repeat) domain responsible for mediatitng binding to Caspsases, and could contain a RING finger domain or a Caspase-recruitment-domain (CARD) [Adapted from (4)].

XIAP binds the IBM of Caspase-9 through its BIR3 domain (61, 75-77). However this interaction is not sufficient for Caspase-9 inhibition, and the XIAP BIR3, through a different surface patch, makes contact with the Caspase-9 dimerization domain (76, 77), inhibiting dimerization, and therefore, activity of Caspase-9.

IAP residues responsible for binding to the IBM of Caspase-9 are conserved in c-IAP1 and c-IAP2; however, interaction with the Caspase-9 dimerization surface requires a sequence that is not conserved in c-IAP1 or c-IAP2 (77). This observation explains why c-IAP1 and c-IAP2 can bind to, but not inhibit, Caspase-9.

XIAP is proposed to inhibit the effector Caspases-3 and –7 through a different mechanism (78). XIAP interacts through the BIR2, and not BIR3, with the IBM at the neo-N-terminus of the Caspase-3 or -7 small-subunit, (79-81). The linker region immediately preceding the BIR2 of XIAP then occupies the active site of Caspases-3 or -7, resulting in a blockade of substrate entry (80, 82, 83).

The initial interaction of BIR2 with the IBM of Caspase-3 or –7 is crucial for XIAP function, as an isolated linker region cannot inhibit caspases (82, 84).

Therefore, XIAP inhibits Caspase-3 through steric hindrance of the active site, whereas it suppressed Caspase-9 activity through inhibition of dimerization.

1.2.2 Other IAPS

Less is known about the caspase-inhibitory mechanism of other IAPs. However the assumption that these IAPs function in a similar manner to XIAP has, of late, come into question (85). Recently, it has been proposed that effector Caspase-7 also contain an IBM at the N-terminus of the large-subunit, which is revealed after cleavage of the pro-domain (85). This large-subunit IBM mediates an interaction with c-IAP1, but not c-IAP2. A similar large-subunit IBM mediates the interaction between the *Drosophila* IAP (DIAP1) and the *Drosophila* Caspase-9 and Caspase-7 orthologues, Dronc and DrICE (85, 86).

1.2.3 Role of Ubiquitination in Regulation of IAP Activity

XIAP, c-IAP1 and c-IAP2 all contain a RING finger at the C-terminus. The RING zinc finger has been shown to have E3 ubiquitin ligase activity, directly regulating self-ubiquitination and degradation of XIAP, c-IAP1 and c-IAP2 in response to apoptotic stimuli (87). Recently, it has been established that c-IAP1, c-IAP2 and XIAP can trigger the ubiquitination of Caspase-3 and -7, suggesting that RING-dependent caspase degradation may be another manner in which IAPs inhibit apoptosis (88-91). However, as it is believed that the primary function of XIAP is the inhibition of caspase catalytic activity rather than stability, it remains unclear to what extent ubiquitination contributes to XIAP function. Accordingly, c-IAP1, c-IAP2 and XIAP have been reported to retain anti-apoptotic function even in the absence of their C-terminal RING domains (92-94). In *Drosophila*, however, DIAP1 exhibits no effect on the catalytic activity of the Caspase-9 orthologue Dronc (86), and ubiquitinating activity appears to be essential for DIAP1 function (95-100).

1.2.4 IAP Inhibition of the Extrinsic and the Intrinsic Apoptotic Pathways.

All IAPs have the ability to inhibit Caspases-3 and -7 (92, 93, 101, 102). As well, XIAP and the single-BIR-domain IAPs, Survivin and ML-IAP have been shown to inhibit Caspase-9 (92, 101-104). However, no IAP can inhibit the activity of Caspase-8 (92, 93, 101, 102, 104).

Therefore, IAP expression can inhibit the intrinsic apoptotic pathway through the inhibition of Caspase-9 and effector Caspases-3 and -7. Moreover, IAPs can inhibit the extrinsic death pathway through inhibition of effector Caspases (78, 92, 93, 104) (Figure 1.2).

Accordingly overexpression of XIAP, c-IAP-1, c-IAP-2, NAIP or Survivin has been shown to suppress apoptosis induced by a variety of stimuli including TNFα, FasL, DNA damaging agents, and growth factor withdrawal (72, 102, 105). As expected, this inhibition of apoptosis occurs downstream of Bcl-2 family mediated mitochondrial Cytochrome c release (92, 93, 103, 104, 106, 107) (Figure 1.2).

1.2.4.1 Role of IAP Proteins *in vivo*

Although, IAPs act as potent caspase inhibitors in artificial systems, their physiological significance in mammalian cells remains obscure. It has been proposed that IAPs inhibit caspases that have been inappropriately activated in the absence of a death signal. Accordingly, deletion of the *Drosophila* IAP, DIAP1, results in embryonic lethality due to widespread apoptosis (108). However, XIAP-null mice have a normal survival with no histologic or gross abnormalities (109), and *in vitro*, non-transformed cells treated with inhibitors of IAPs or derived from XIAP-knockout mice undergo chemical and ultraviolet-induced apoptosis similarly to wild-type control cells (109, 110). However, in mammalian tumor cells, inhibition of IAPs has been found to lead to massive cell death (110, 111). This particular sensitivity is believed to be due to the fact that malignant cells, through mechanisms including dysregulation of proto-oncogenes, hypoxia, and cell cycle disruption, have a drive to activate caspases (112, 113). This drive may

be blocked by IAP expression. In contrast, normal cells would be expected to have a weaker drive to activate caspases. Thus, the repression of XIAP may be more toxic to malignant cells than normal cells. In support of this hypothesis, levels of XIAP and active Caspase-3 tend to be higher in malignant cells than normal cells (114).

1.2.5 Endogenous Inhibitors of IAPs

The strongest support for the physiological importance of IAPs is the identification of a group of proteins with the primary proposed function of promoting caspase activation by blocking IAP function.

1.2.5.1 Smac

IAP-mediated inhibition of caspases can be effectively countered by a family of IBM containing proteins (16, 19), the founding member of which in mammals is the mitochondrial intermembrane protein Smac (115), also known as DIABLO (116), Smac is released into the cytosol, together with Cytochrome c, upon stimulation of apoptosis. Whereas Cytochrome c directly activates APAF-1 and Caspase-9, Smac interacts with multiple IAPs and relieves IAP-mediated inhibition of both initiator and effector caspases (115-118) (Figure 1.2). In *Drosophila*, the activity of the Smac orthologues reaper (rpr), head involution defective (hid) and grim are regulated transcriptionally rather than by mitochondrial release.

The Smac protein is synthesized in the cytosol. At its N-terminus there is a mitochondrial targeting sequence, which is removed following mitochondrial transport (115, 116). This cleavage results in the exposure of four conserved amino acids, Ala-Val-Pro-Ile, at the N-terminus of the mature Smac. This motif corresponds to the IAP-binding motif (IBM) consensus sequence in mammals and *Drosophila* (16, 19). Similar to the caspase IBMs described above, Smac IBM binds to the BIR domain groove of XIAP (79, 118, 119), through an interaction with overlapping but not identical BIR domain residues (61, 118, 119). As in the case of caspase IBMs, the interactions between Smac and IAPs require an

exposed N-terminus in the IBM, explaining why only the mature form of Smac is functional in cells.

XIAP is the predominant target of Smac/DIABLO (120-122). Smac, or its isolated IBM, can bind to either the BIR2 or BIR3 domains of XIAP, with binding to the BIR3 occurring with 5-10 fold higher affinity (123). As Smac exists in cells as a dimer (117), the dimer likely interacts with a single XIAP molecule, with each Smac molecule binding to one BIR domain. This interaction competitively inhibits the binding of Caspase-9 to BIR3, and Caspase-3 and –7 to BIR2 (79). This interaction also prevents, through steric hindrance, binding of the XIAP BIR2 N-terminal linker to Caspase-3 or –7 catalytic groove (82, 124).

1.2.5.2 HTRA2

HTRA2, like Smac, is a nuclear encoded, mitochondrial protein which is cleaved to reveal an N-terminal IBM (125). Therefore, as in the case of Smac, HTRA2 inhibits IAP activity by competing for caspase binding (126). HTRA2 also possesses a serine protease activity and can cleave and inactivate multiple IAPs (127). However, the physiological role of HTRA2 remains controversial, as studies with HTRA2 mice have shown that, *in vivo*, HTRA2 plays an IAP independent protective rather than a IAP dependent pro-apoptotic role (128).

1.2.5.3 XAF1

XAF1 is another IAP inhibitor. XAF1 is a nuclear protein that binds and sequesters XIAP in the nucleus (129). In cells, overexpression of XAF1 blocks XIAP-mediated inhibition of apoptosis (129). It remains unclear, however, if the sequestration of XIAP in the nucleus simply separates the XIAP from cytosolic caspases, or whether there are additional consequences of XIAP relocalization (129, 130).

1.3 The Bcl-2 Protein Family

In addition to its role as an energy-generating organelle, the mitochondrion has emerged as a convergence center for life and death signals (20). A key event in the intrinsic apoptotic pathway is the coordinated release of several proteins from the mitochondrial intermembrane space into the cytosol (46, 125, 131, 132). The release of Cytochrome c is a necessary event for the activation of Caspase-9 and the mediation of the intrinsic death pathway, whereas the release of IAP inhibitory molecules such as Smac and HtrA2 contributes to caspase disinhibition. In the extrinsic apoptotic pathway, however, mitochondrial Cyotchrome c release is induced downstream of caspase-activation (Figure 1.2), which amplifies the death signal and is, in certain cases, necessary for the propagation of the signal (133, 134).

Interestingly, release of different mitochondrial apoptotic factors occurs with distinct kinetics (135-138), indicating that the permeabilization of the mitochondrial outer membrane (MOM) proceeds through kinetically and perhaps mechanistically distinct steps.

Mitochondrial outer membrane permeabilization is regulated by the interplay of pro- and anti-apoptotic members of the Bcl-2 family. Bcl-2 members are characterized by the presence of several distinct motifs known as Bcl-2 homology (BH) domains designated BH-1 through BH-4 (20). Bcl-2, Bcl-X_L, Bcl-w and Mcl-1 represent anti-apoptotic members of the Bcl-2 family, characteristically containing all four BH domains. Pro-apoptotic members of this family lack the BH4 domain and can be further subdivided into multidomain (Bax and Bak) and BH3 domain-only proteins (BID, BIK, BIM, BAD, Hrk, NOXA, PUMA, etc.) (Figure 1.4).

Since many Bcl-2 proteins homodimerize, or heterodimerize with other Bcl-2 family members, it has been suggested that anti-apoptotic Bcl-2 members inhibit the function of pro-apoptotic members (or vice versa) through direct interaction (139). Indeed, in the nematode, the pro-apoptotic Bcl-2 member Egl-1 promotes ced-4 (nematode APAF-1) activation by binding to the anti-apoptotic Bcl-2 homologue, ced-9 (45) (Figure 1.2).

While some members of the Bcl-2 family, such as Bcl-2 and Bak, are constitutively localized on the mitochondrial outer membrane, these proteins and other family members have also been found in various other sub-cellular locations including the endoplasmic reticulum, the cytosol and bound to microtubules (20). The control over the subcellular localization of different Bcl-2 proteins occurs through phosphorylation, proteolysis, or interaction with other Bcl-2 members or unrelated proteins. However, regardless of their normal subcellular localization, Bcl-2 proteins appear to ultimately act at the organelle membrane to mediate cell survival and cell death signals.

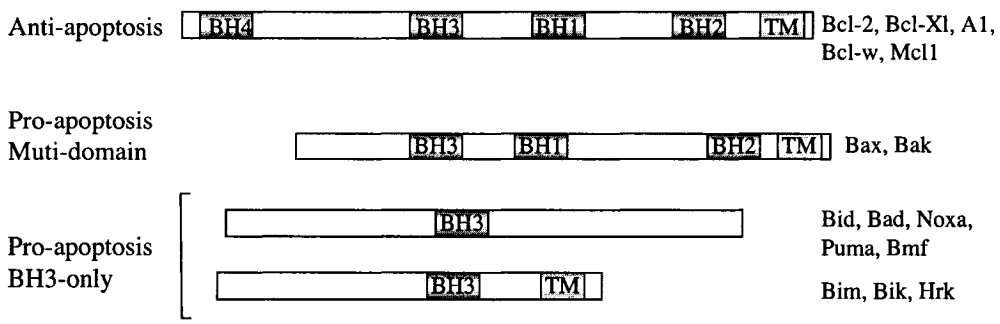

Figure 1.4 Schematic of the three subfamilies of Bcl-2-related proteins. the four Bcl-2 regions that are most highly conserved among family members (BH1–4) are indicated. Majority of members have a carboxy-terminal hydrophobic domain (TM) that may play a role in association with intracellular membranes [Adapted from (3)].

1.3.1 Multidomain Pro-Apoptotic Members BAK AND BAX

Among pro-apoptotic members, the multidomain Bak and Bax have been categorized as the last gateway of Cytochrome c release, with the main function of other Bcl-2 family members being to regulate the activity of Bak and Bax. When overexpressed either protein triggers apoptosis (20), and apoptosis induced by various cues is inhibited, *in vivo* and *in vivo*, in Bak/Bax double-knock cells (140, 141).

Bak/Bax homo-oligomerization on the mitochondrial outer membrane (MOM) is essential for MOM permeabilization (142-145). Deletion and mutagenesis studies show that the BH3 domain of Bak and Bax is necessary for their homo-oligomerization and pro-apoptotic function (144, 145). In healthy cells, Bax is predominantly localized in the cytosol or loosely attached to membranes as an inactive form. In this inactive form, the BH3 domain is masked inside the hydrophobic core of the protein. After exposure to apoptotic stimulation, Bax undergoes conformational changes, translocates and inserts into the mitochondrial outer membrane, where it oligomerizes through its now exposed BH3 domain (146), thus leading to the permeabilization of the mitochondrial outer membrane (142-145).

Despite the wealth of *in vitro* and *in vivo* investigations, the molecular mechanism responsible for Bax-dependent MOM permeabilization is still controversial and several different, though non-exclusive, hypothetical models have been proposed. In earlier models, Bax had been described as an activator of the inner mitochondrial membrane permeability transition pore (PTP) (147-149). Accordingly, Bax was believed to lead to the opening of the PTP, causing swelling of the matrix, which ruptures the outer membrane and spills Cytochrome c and other pro-apoptotic proteins into the cytosol (150, 151). In accordance with this mechanism, Bax can promote the activity of PTP components (152, 153), and inhibitors of PTP have been found to be protective in some models (154, 155). However, in some cell types, Cytochrome c release can occur in the absence of mitochondrial depolarization and without loss of outer membrane integrity, suggesting instead a more selective release mechanism, such as the formation of a Cytochrome c selective pore. PTP activation may, therefore, be a later event in apoptosis (156-159).

Hypothetically, Bax can induce the formation/opening of selective MOM pores in several ways. It could modify resident MOM channels to allow for Cytochrome c permeability. Alternatively, it could form channels on its own or in combination with other MOM proteins (160-162). Accordingly, several Bcl-2 family members, both pro- or antiapoptotic, share pore-forming domains that are

similar in structure to those observed in diphtheria toxin and colicins (163, 164). Moreover, Bax has been reported to induce channel formation in planar lipid bilayer and liposomes (144, 163-167), although the permeability of the Bax-induced pore toward Cytochrome c has not been clearly demonstrated (144, 163, 164, 166-168).

Recent studies of MOM electrophysiological properties have revealed a, perhaps distinct, Cytchrome c interacting channel, formed in apoptotic mitochondria in a Bax dependent manner (169-171). This channel has been postulated to correspond to the Bax containing channel responsible for Cytochrome c efflux during apoptosis. This channel, referred to as MAC (for mitochondrial apoptosis-induced channel), can be immuno-depleted by Bax-specific antibodies (170), and it demonstrates very similar behavior to the lipid bilayer channels formed by recombinant Bax, suggesting Bax is a large component of the channel (170).

There is also evidence supporting the hypothesis that the Cytochrome c permeable MOM conduit is not a proteinaceous channel but rather a lipid pore, i.e. a pore with its surface covered, at least partially, by polar head groups of bent mitochondrial lipids (172, 173). Bax may induce formation of such pores through its ability to decrease planar phospholipids bilayer stability and to diminish membrane linear tension, leading to a lowered energy requirement for the formation of lipidic pores (172-174). Consistent with the proposed role of lipids in Bax pore formation, certain mitochondrial lipids such as cardiolipin are essential for Bax-induced permeabilization of artificial vesicles (144), whereas some amphiphilic cations can prevent Bax induced permeabilization (175).

1.3.2 BH3-Only proteins

BAX is expressed at basal levels in nearly all mammalian cells. Following the induction of apoptosis, the protein is translocated from the cytosol to the mitochondrial outer membrane (MOM), where it homo-oligomerizes through its BH3 domain, and behaves as a membrane-inserted protein (146, 176). Once inserted and oligomerized within the MOM, Bax can actively participate in the

release of apoptogenic factors as described above. The translocation of BAX to the mitochondria and its oligomerization, therefore, appears as critical in the implementation of apoptosis.

The N-terminal region of Bax is believed to be crucial in the regulation of BAX translocation and oligomerization; the deletion of this region increases the interaction of the protein with isolated mitochondria, and increases its pro-apoptotic activity (146, 176-179). The N-terminus is therefore considered a negative regulator of Bax translocation, masking the BH3 domain, a necessary domain for Bax homo-oligomerization.

The means by which upstream apoptotic signals induce the change in Bax conformation, leading to Bax disinhibtion, is through activation of particular BH3-only proteins. *In vitro*, Bid or Bim, but not other BH3-only proteins, induce a conformational change in Bax, relieving Bax auto-inhibition (180-183). As the BH3 domain of Bid has been found to bind the N-terminal domain Bax, the Bid mediated disinhibition of Bax maybe due to a competition between the BH3 domains of the two molecules for the N-terminal inhibitory domain of Bax (184).

1.3.2.1 Bim

Bim and Bid are activated by apoptotic pathways, and hence, connect proximal death signals to the core apoptotic pathway. In healthy cells, the pro-apoptotic activities of BH3-only proteins is kept in check by distinct mechanisms to prevent inappropriate cell death. Alternative splicing produces at least four Bim isoforms, BimEL, BimL, BimS and BimAD, which differ in pro-apoptotic potency (183, 185). In normal cells, BimL and BimEL are inactivated by sequestration by the microtubule-associated Dynein motor complex, through binding to the Dynein light chain (186). Certain apoptotic stimuli induce the release of BIM, allowing for interaction with Bax/Bak.

BimS and BimAD are regulated, not by microtubule sequestration, but transcriptionally (183). Bim transcription is upregulated in a number of cell types, including neurons after growth factor withdrawal, through activation of the

forkhead-like transcription factor FOXO3A, which is normally suppressed though AKT mediated phosphorylation (187-191).

Bim can also be suppressed post-translationally by Erk-mediated phosphorylation which targets Bim for ubiquitination and proteosomal degradation (192, 193). Finally it has been reported that, in growth-factor deprived neurons or UV irradiated fibroblasts, Bim can be activated by JNK, either through direct phosphorylation, or through transcriptional induction by the JNK activated transcription factor c-Jun (194-196).

Consistent with Bim's proposed role as a mediator of apoptosis, Bim-null mice have abnormally high number of B cells, T cells and macrophages and granulocytes (197). Also, *in vitro*, Bim-null lymphoid cells and granulocytes are highly resistant to apoptosis induced by cytokine deprivation (197).

1.3.2.2 Bid

Full-length Bid has relatively poor pro-apoptotic activity. Bid is kept inactive via an intramolecular interaction between it C-terminus and the N-terminal BH3-domain (198). Bid activity is however greatly augmented after caspase mediated proteolysis, which produces tBid (truncated Bid). caspase cleavage of Bid also exposes a glycine in Bid that can be N-myristoylated, leading to enhanced activity (199).

As Bid can be cleaved by Caspase-8, it is believed to be an important link between the extrinsic and intrinsic pathways (133, 134) (Figure 1.2). In some cell-types, this link is necessary for death-receptor induction of apoptosis. For example death-receptor induced apoptosis is somewhat defective in Bid-Null hepatocytes and fibroblasts, but not in Bid-null lymphocytes (200).

It is important to note however, that Bid can be proteolytically activated, not only by Caspase-8, but also by other caspases, such as Caspase-3 (133, 134). This might indicate that Bid functions as a general amplifier in the caspase cascade, rather than having a restricted activity in death-receptor induced signaling.

1.3.3 Anti-Apoptotic Bcl-2 Proteins, and Their BH3-Only Binding Partners

Antiapoptotic Bcl-2 family members, Bcl-2, Bcl-X_L, Bcl-w and A1, antagonize the action of pro-apoptotic members through direct protein-protein interactions. Gene targeting experiments have shown that Bcl-2 (201, 202), Bcl-X (203), Bcl-W (204, 205), and A1 (206) are required for the survival of specific cell populations. Solution structure of Bcl-X_L reveals that the BH1-BH3 domains form an elongated hydrophobic groove, which is the docking site for the BH3 domains of its pro-apoptotic binding partners (207-209). Thus, anti-apoptotic Bcl-2 members inhibit the activity of pro-apoptotic members by binding to the domain responsible for their oligomerization and pro-apoptotic activity.

As anti-apoptotic Bcl-2 members have been reported to bind to both BH3-only and multi-domain pro-apoptotic Bcl-2 members, they inhibit Bax activation in two ways. First by binding and sequestering activated Bim and Bid (208, 209), they inhibit Bim or Bid mediated activation of Bax. Second by binding activated (BH3 exposed) Bax, they inhibit Bax oligomerization and MOM pore formation. This 'double-action' of anti-apoptotic Bcl-2 members is consistent with the previous observation (210) that these molecules are able to inhibit the translocation of Bax into mitochondrial membranes (by inhibiting the Bid/Bim dependent conformational switch), as well as hinder the action of Bax once it is integrated into the membrane (by directly binding 'activated' Bax).

As mentioned, only Bid and Bim are able to interact with BAX/BAK, however all BH3 only proteins, including BAD, BIK, HRK, NOXA, PUMA and BMF, can bind with high affinity to (at least some) pro-survival Bcl-2-like molecules, and all trigger apoptosis when overexpressed (211). Both, Bcl-2 binding and apoptosis induction, require the presence of an intact BH3 domain (211).

BH3-only proteins that can bind to anti-apoptotic Bcl-2 like proteins, but not pro-apoptotic Bax-like proteins, are referred to as 'sensitizing' BH3-only molecules, as they do not directly activate Bax/Bak, but sensitize these molecules to the action of Bim /Bid by blocking the binding of Bim/Bid to anti-apoptotic

Bcl-2 members. 'Sensitizing' BH3-only members would also contribute to Cytochrome c release by preventing the direct inhibitory interaction of anti-apoptotic Bcl-2 proteins on 'activated' Bax/Bak.

1.3.3.1 The Anti-apoptotic protein, Bcl-2

Garcia and colleagues originally showed that Bcl-2 expression can support the survival of sympathetic neurons in the absence of NGF, providing the first functional evidence that the overexpression of anti-apoptotic Bcl-2 members can override the death signal induced by the withdrawal of a trophic factor (212). Subsequently, transgenic mice overexpressing Bcl-2 in nervous system were found to be protected against neuronal death during development (213), indicating the importance of the mitochondrial death pathway in developmental neural apoptosis. The Bcl-2 protein itself is particularly important for the maintenance of neurons after birth, as development of the nervous system is normal in Bcl-2-null mice, but there is loss of motor, sensory and sympathetic neurons after birth (201, 214). In accordance with the important role of Bcl-2-like molecules in the post-developmental maintenance of the nervous system, Bcl-2 overexpressing mice are resistant to neuronal injury models such as cerebral ischemia and facial nerve axotomy (215, 216).

1.3.3.2 The Anti-apoptotic protein, Bcl-X_L

Bcl-X-null embryos display massive death of immature neurons of the brain, spinal cord, and dorsal root ganglion (203). This is largely rescued by co-deletion of Bax (203), providing evidence that Bcl-X_L is an essential negative regulator of Bax mediated developmental neural apoptosis.

1.3.3.3 The BH3-Only protein, BAD

BAD binds to particular anti-apoptotic Bcl-2 members, including Bcl-X_L and Bcl-2 (217, 218). Bad is normally held in an inactive state through phosphorylation at two serine residues, serine-112 and serine-136 (219). Phosphorylation at either site promotes Bad binding to the 14-3-3 scaffold

protein, thus inhibiting its interaction with anti-apoptotic Bcl-2 proteins (219, 220). Phosphorylation at the two sites have been attributed to distinct kinases. AKT, a transducer of the growth-factor survival signal in the PI-3 kinase pathway, phosphorylates Bad at serine-136 (220), as do PAK1 and S6-kinase (221, 222). On the other hand, the protein kinases Rsk, PKA, and PAK1 are thought to mediate survival factor-induced phosphorylation of BAD at serine-112 (221, 223-226). PKA has also been shown to phosphorylate Bad at serine-155 within the BH3 domain, thereby reducing its affinity for anti-apoptotic Bcl-2 proteins (226, 227). A death stimulus, such as growth-factor withdrawal, leads to the dephosphorylation of Bad by Calcineurin or protein phosphatase 2A (228, 229). Furthermore, Phosphorylation of Bad at a distinct site by JNK and Cdc2, has been proposed to activate Bad by inhibiting its sequestration by 14-3-3 (311, 312).

In vivo, Bad is activated following traumatic spinal cord injury (230) and kainic acid induced seizure (231), and it may contribute to the observed cell death. Bad-null mice appear nearly normal, but do suffer from a higher incidence of cancer (232). Also select cell types derived from Bad-null mice display resistance to growth-factor withdrawal induced apoptosis (232).

1.3.3.4 The BH3-only protein, Hrk

Hrk is expressed mainly in the nervous and hematopoeitic systems (233-235). Duing embryogenesis, Hrk expression is induced in neuronal tissues that contain a relatively large number of apoptotic cells; the trigeminal and DRG neurons and the anterior horn of the spinal cord (236). In cultured neurons, Hrk expression is upregulated upon NGF withdrawal or treatment with amyloid-β, and its levels peak at the time when these cells are committed to die (236). Accordingly, studies in Hrk-null mice showed that Hrk is crucial for injury-induced and NGF deprivation induced apoptosis of certain neuronal populations (237).

1.3.3.5 The BH3-Only Protein, Bmf

In healthy cells, Bmf is sequestered to the cytoskeleton through an association with Dynein light chain 2 (238). Certain damage signals, such as loss of cell attachment (anoikis), unleash Bmf, allowing it to translocate and bind prosurvival Bcl-2 proteins (238).

1.3.3.6 The BH3-Only Proteins, PUMA and NOXA

Expression of both PUMA and NOXA is transcriptionally induced by p53 (239-241). Gene knock-out studies have shown that PUMA, and to a lesser extent NOXA, are essential for p53-mediated DNA-damaged-induced apoptosis in a number of cell-types, including neurons (242-244).

1.3.3.7 The BH3-Only Protein, Bik

Bik is expressed in the endothelial and hemopoietic cells (245). However, no abnormalities or resistance to apoptotic stimuli are observed in Bik-null mice, suggesting that the functions of Bik in the initiation of apoptosis mainly overlaps with those of other BH3-only proteins (245).

1.4 THE JNK MEDIATED SIGNALING PATHWAY

Cells are continually exposed to a variety of stimuli and changes in their environment. These stimuli include alterations in the amount of nutrients, growth-factors, cytokines, and adhesion to the cell matrix. In addition, cells encounter physical and chemical stimuli which generally fall into the categories of osmolarity, heat, pH, oxidative, radiation or mechanical stress. Cells respond to these cues by altering their rates of proliferation, differentiation, migration, or by executing their cell death program. Signal transduction pathways, such as the MAPK pathways, are activated by such cues, and provide the link between the stimulus and the cell's response mechanism (246). In mammals, one of the best characterized signaling pathways linking stressful stimuli to the apoptotic machinery is the JNK signaling pathway (247).

JNK belongs to the mitogen-acitivated protein-kinase (MAPK) superfamily of proteins. Three major groups of MAPK exist in mammals. The Erk and p38 families are characterized by the active-loop dual phosphorylation motifs Thr-Glu-Tyr and Thr-Gly-Tyr respectively (248, 249). The JNK family represents a third group and contains the dual phosphorylation motif Thr-Pro-Tyr (247).

Once activated, MAPKs activate their downstream effectors by phosphorylation at specific serine or threonine sites. MAPKs are themselves activated by a protein kinase cascade composed of two or more additional upstream kinases. A family of evolutionary conserved dual specificity kinases, the MAPK kinases (MAPKK or MAP2K), activate MAPKs by phosphorylation of their activation loops. MAP2Ks are, in turn, activated by a more diverse group of MAP2K kinases (MAP3K) (246).

In general, the different MAPK families, are activated by distinct stimuli and are induced by separate signaling cascades. Erks are usually activated by receptor tyrosine kinases, and relay proliferative or differentiation signals (248). JNK, and p38, are generally activated by stress stimuli and pathogenic insults (247, 249). Specifically, JNK is activated by cytokines (e.g. TNFα, IL-1) (250-252), and by a variety of cellular stress stimuli including UV light (253),

cycloheximide, anisomycin, heat shock, growth-factor withdrawal, and osmotic or oxidative stress (254), and it can relay apoptotic signals.

JNK induces mitochondrial Cytochrome c release and the intrinsic death pathway by directly modulating the activity of Bcl-2 family members through phosphorylation. JNK also activates transcription factors such as c-Jun, which, in turn, upregulate the expression of pro-apoptotic Bcl-2 proteins (discussed below).

JNK-deficient fibroblasts exhibit no defect in Fas-induced apoptosis, indicating that JNK is not involved in death-receptor mediated (extrinsic) apoptosis (255). However, it has been reported that, in some cases, JNK activity is required for the disinhibition of the TNFα receptor (but not Fas) death-inducing complex (256).

Ten isoforms of JNK family are expressed in humans (257). These ten are encoded by three closely related genes; JNK1, JNK2, JNK3. The JNK1 and JNK2 genes are expressed in most tissue, whereas JNK3 is expressed primarily in the nervous system (258).

1.4.1 JNK Mediates Activation of AP1 Transcription-Factors

The AP1 transcription-factor family member c-Jun is activated by JNK mediated phosphorylation (253, 254, 259), and in many cases, activation of c-Jun has been identified as a necessary step for mediation of JNK induced apoptosis (discussed below).

The AP1 family consists of several groups of basic-leucine zipper domain (bZIP) proteins: the Jun, the Fos, and the ATF2 subfamilies (260). Mammalian Jun family includes c-Jun, JunB, and JunD; Fos proteins are c-Fos, FosB, Fra-1 and Fra-2; and the ATF proteins that are normally included in the AP1 family are ATF-2 and ATF-a. As is the case of other bZIP transcription factors, AP1 proteins have to homodimerize, or heterodimerize with other AP1 proteins, before they can bind to their DNA target sites identified by the sequences TGACTCA, TGACGTCA, or variants thereof (260). Characteristics of different AP1 dimers are determined by their subunit composition (260).

Like other AP1 proteins, the C-terminal bZIP domain of c-Jun mediates DNA binding and dimerization, with the N-terminus of c-Jun then mediating transcriptional activation. JNK phosphorylates c-Jun at two Ser-Pro motifs within its N-terminal domain, leading to an increase in c-Jun/AP1 mediated transcriptional activity and upregulation of downstream genes (247, 260). As part of a feed-forward mechanism, the gene for c-Jun is itself a target of AP1-mediated induction, so that JNK activity leads to induction of both c-Jun phosphorylation and c-Jun protein levels (247, 261). In the literature, both, c-Jun phosphorylation and c-Jun protein upregulation, are used, almost interchangeably, to demonstrate JNK activity (see below).

The AP1 member ATF2 is also phosphorylated in its transactivation domain by JNK (260, 262). However ATF2, unlike c-Jun, is not a JNK specific target, as it can also be phosphorylated by the MAPK p38 (262).

The mechanisms that accounts for the phosphorylation-dependent upregulation of AP1 activity are unclear, but a role for the co-activator CBP/p300 has been proposed (263). Also, JNK may regulate the intrinsic histone acetylase activity of ATF2 (264).

1.4.2 *In vitro* Evidence of JNK-Mediated Apoptotic Signaling

Perhaps the best illustrated examples of the involvement of JNK in the neuronal death process come from studies of apoptosis due to growth factor withdrawal. Like all cells, neurons require trophic support. According to the neurotrophic hypothesis (265, 266), developing neurons are overproduced and thus compete for limited quantities of target-derived trophic support, with about half of all produced neurons dying in the process. This competition is necessary for the morphogenesis of the nervous system and the estabishment of correct synaptic connections.

Neurons dying due to the lack of trophic support exhibit the classical features of the intrinsic pathway of apoptosis including Cytochrome c release upstream of activation of Caspase-9 and effector caspases (267). The JNK

pathway plays a crucial part in mediating the mitochondrial release of Cytochrome c after growth factor withdrawal.

The first indication for the involvement of the JNK pathway in apoptosis came from cultures of sympathetic neurons, where c-Jun mRNA (268) and protein levels (269) were found to be upregulated in cells undergoing NGF-withdrawal induced apoptosis, whereas the level of other Jun and Fos family members remained constant (269). Microinjection of c-Jun specific antibodies (268) or dominant-negative (DN) c-Jun constructs (269) established that c-Jun activity is necessary for NGF-withdrawal induced apoptosis, whereas JunB or JunD specific antibodies had no effect (268). As well, overexpression of wild-type c-Jun was found to be sufficient to induce apoptosis in these cells (269).

Consistent with the increase in c-Jun activity, JNK, but not p38, is activated during NGF-withdrawal induced death of sympathetic neurons (270), and inhibition of the JNK pathway suppresses this death (271-273). Furthermore, JNK-dependent c-Jun phosphorylation precedes the transcriptional upregulation of the c-Jun gene (269, 270), whereas dominant-negative c-Jun inhibits the increase in c-Jun protein (269), indicating that JNK mediated phosphorylation of c-Jun leads to an increase of its transcriptional activity, which in a feed-forward mechanism, leads to the transcription of more c-Jun along with other possible death-effectors.

C-Jun-null mice die early *in utero* (274), thereby hindering a genetic analysis of c-Jun's role in NGF withdrawal induced apoptosis *in vivo*. However, cultured sympathetic neurons with a conditional deletion in the c-Jun gene demonstrate resistance to NGF-withdrawal induced death (275), providing support for the necessary role of c-Jun in NGF-withdrawal induced apoptosis.

Also, sympathetic neurons derived from JNK3-null mice display a 50% reduction in NGF-withdrawal induced c-Jun activation, and a similar reduction in NGF-withdrawal induced apoptosis (276). This partial effect is most likely due to the expression of other JNK isoforms.

The rat pheochromocytoma cell line, PC12, is commonly used as a model system in the study of trophic factor withdrawal induced death. PC12s

differentiate into sympathetic neuron-like cells once exposed to long-term NGF treatment (277). Similar to sympathetic neurons, differentiated PC12 cells undergo apoptosis once NGF is withdrawn (277). JNK is activated in PC12 cells after NGF withdrawal and this activation is necessary for the subsequent apoptotic process (271, 278).

The role of the JNK pathway has also been investigated in the growth-factor withdrawal induced death of CNS neurons. Cultures of neonatal cerebellar granule (CG) neurons can be maintained in a medium containing 10% serum and 25mM KCl (279). KCl causes membrane depolarization, providing a calcium-influx dependent survival signal, and removal of serum and KCl triggers apoptotic cell death. As in sympathetic neurons, removal of the survival signal from CG neuron cultures causes an increase in c-Jun phosphorylation and c-Jun protein levels, whereas the level of other AP1 family members do not change (280). Overexpression of c-Jun is found to be sufficient to induce apoptosis in KCl/serum maintained CG neurons. However overexpression of a c-Jun mutant where the JNK phosphorylation sites have been replaced with aspartate (thus mimicking a constitutively phosphorylated form of c-Jun) is a more potent apoptotic inducer, whereas a c-Jun mutant where the phosphorylation sites have been replaced with the unphosphorylatable residue alanine (c-JunAA) does not induce apoptosis. Furthermore, after the removal of serum and KCl, c-JunAA acts as an inhibitor of apoptosis. The observations suggest that the phosphorylation of c-Jun is both sufficient and necessary for the induction of cell death after removal of survival signal (280). Accordingly, CG neuron apoptosis is suppressed when cells are treated with a pharmacological inhibitor of an upstream JNK activator (273). Inhibition of the JNK pathway is also reported to rescue embryonal DRGs (281) and embryonal motor neurons (282, 283) from trophic factor deprivation induced apoptosis.

Fibroblasts derived from JNK1/2 double knock-out mice (DKO) mice are devoid of all JNK activity, as JNK3 has a neuronal specific expression (255). In line with the proposed key role of JNK in mediation of stress-induced apoptosis, the JNK1/2 DKO MEFs exhibit profound defects in apoptosis induced by a

number of factors (255); defects are observed in the apoptotic response to UV radiation, to the translational inhibitor anisomycin and to DNA alkylating agent methylmethanesulfonate (255). A similar defect in UV induced apoptosis is observed in MEFs derived from Cytochrome c (284), APAF-1 (285), Caspase-9 (286), or Caspase-3 (287) null mice, consistent with the requirement for the intrinsic death pathway in the mediation of JNK signaling.

1.4.3 *In vivo* Evidence of JNK-Mediated Apoptotic Signaling

1.4.3.1 Lessons from Knock-Out Mice

The *in vivo* role of JNK has been assessed in mice with deletions in one or more JNK genes. Mice deficient in JNK1, JNK2, JNK3, and JNK1/JNK3 or JNK2/JNK3 double mutants all survive normally (288). However, compound mutants lacking both JNK1 and JNK2 genes are embryonic lethal and have severe dysregulation of apoptosis in the brain (288, 289). Consistent with a pro-apoptotic role for JNK, there is a reduction of cell death in the lateral edges of hindbrain prior to neural tube closure. In contrast, increased apoptosis is found in the forebrain of the mutant (288, 289).

These observations are consistent with other studies of JNK function. Even though JNK is necessary for mediation of apoptosis after a number of insults, it is generally believed that activation of JNK also plays distinct roles that are necessary for proper cell function and survival. Accordingly, c-Jun-null mice also die early *in utero* due massive death of liver cells (274).

Whether activation of the JNK pathway will lead to apoptosis or to a different response, may depend on several factors, including the particular cellular context, the availability of specific JNK downstream effectors, the duration of JNK activation, and the nature of concurrent signaling events.

1.4.3.2 Lessons From the Use of Pharmacological Inhibitors

As JNK-DKO and c-Jun-null mice both die early *in utero* (274, 288, 289), the study of the *in vivo* pro-apoptotic roles of JNK remains mostly correlational, or relies upon the use of pharmacological inhibitors of the JNK pathway such as

SP600125 or CEP1347. The compound SP600125 is a direct inhibitor of JNK kinase activity (290, 291), while CEP1347 is an inhibitor of the MLK family, which act as MAP3Ks for JNK (271-273, 282). *In vivo* studies using these compounds have confirmed that JNK plays a necessary role in mediating both developmental and stress-induced apoptosis. For instance, JNK activity is associated with both developmental and MPTP induced death of nigrostriatal dopaminergic neurons (292-294), and treatment of mice with SP600125 or CEP1347 protects these neurons from MPTP induced apoptosis (294, 295).

The JNK pathway is also believed to play a key role in the mediation of apoptotic events following exposure of neurons to ischemic and excitotoxic stress *in vivo*. The JNK signaling pathway is activated in the brain after cerebral ischemia, or exposure to excitotoxins such as kainic acid (296-306). Accordingly, the administration of SP600125 is associated with a reduction in the ischemia-induced JNK activity and infarct volume, and an increase in the number of surviving cells (304, 307). As well, Ischemia and excitotoxin induced apoptosis and cerebral damage is markedly reduced in JNK3 null mice (303). A similar defect in excitotoxin-induced damage is observed, *in vivo* and *in vitro*, in neurons with a germ-line mutation in the c-Jun gene that replaces the JNK phosphorylation sites with alanine (308).

The above observations regarding the *in vivo* roles of JNK1, 2 and 3 genes suggest that JNK1 and JNK2 play a role in developmental cell death, as well as other physiological processes necessary for proper cell function, whereas JNK3 plays a more selective role in stress-induced neuronal apoptosis. JNK1 and JNK2 are also believed to play a role in stress-induced neuronal apoptosis, as suppression of JNK dependent apoptosis is usually not complete in JNK3 null cell (276, 303).

1.4.4 Bim and Other BH3-only Targets of JNK Signaling

The main contribution of c-Jun to JNK dependent apoptotic signaling is believed to be though the transcriptional upregulation of BH3-only proteins. Of

the BH3-only targets of JNK signaling, the Bim splice-variant BimEL has raised the most interest, as it is both transcriptionally induced by c-Jun, and directly phosphorylated/activated by JNK.

1.4.4.1 BimEL

As discussed, growth factor withdrawal induced apoptosis is a JNK dependent process in a number of neurons, including SCG (196), CG (309), and DRG neurons (196). Following growth factor withdrawal, the expression of BimEL, but not that of BAD, BID, BAX, BAK, BIK, NOXA or other BIM isoforms, is increased in SCG and CG neurons in a c-Jun and JNK dependent manner (194-196, 309).

Growth factor withdrawal also leads to the JNK dependent phosphorylation of BimEL (195). JNK phosphorylates BimEL directly and selectively at Serine-65 (195, 310). This phosphorylation site is absent in other splice variants of Bim, therefore only the BimEL isoform is phosphorylated by JNK.

Under non-apoptotic conditions, BimEL is sequestered to the cytoskeleton via binding to Dynamin-light-chain (DLC) in non-neuronal cells (186). However, in neuronal cells, BimEL is found to be associated with the mitochondria, regardless of its phosphorylation status, or the apoptotic state of the cell (194, 195). Nevertheless, through an unknown mechanism, JNK dependent phosphorylation does lead to increased pro-apoptotic activity of BimEL (195, 310), and ectopic expression of 'constitutively phosphorylated' BimEL (BimEL S65D) is more potent in inducing apoptosis than wild-type BimEL (195, 310).

The simplest hypothesis for the increased activity of phosphorylated BimEL would be that phosphorylation induces conformational changes in BimEL, allowing for more interaction between BimEL and pro- or anti-apoptotic multi-domain Bcl-2 proteins. This phosphorylation mediated conformational change has yet to be demonstrated, and it is important to note that the phosphorylation site does not correspond to the BH3 domain of the molecule (195, 310).

BimEL induction and phosphorylation play an important physiological role in mediating the JNK death signal. In SCG neurons derived from Bim-null mice or treated with Bim anti-sense oligonucleotides, growth factor withdrawal induced death is partially inhibited (194, 196). Similar observations have been made in cultures of Bim-null CG and DRG neurons following growth factor deprivation (194, 196). Importantly, in Bim-null mice, DRG programmed cell death is found to be delayed *in vivo* (194). Accordingly, in several *in vivo* studies investigating the JNK-dependent cell death induced by ischemia, expression of Bim is increased in a JNK-dependent manner (303-305).

1.4.4.2 Hrk

The partial protection from JNK-dependent apoptosis afforded to Bim-null cells suggests that other JNK targets, perhaps other BH3-only proteins, play an important role in JNK induced apoptosis. As mentioned above, no changes are observed in the expression levels of BAD, BID, BIK, NOXA, BAK or BAX, following neuronal growth factor withdrawal (309). However, Hrk expression is induced in a JNK dependent fashion in CG and SCG neuron cultures after growth factor withdrawal (309) or, *in vivo*, during the process of ischemia mediated neuronal death (305). The importance of Hrk in mediating the JNK apoptosis signal, however, has not been determined, although ectopic expression of Hrk can lead to apoptosis of growth factor maintained CG neurons (309).

1.4.4.3 BAD

Levels of BAD expression are not altered by the JNK pathway (309, 311), however JNK directly phosphorylates BAD on serine-128, leading to an increase in BAD's pro-apoptotic activity (311, 312).

The pro-apoptotic activity of BAD is normally blocked in growth-factor maintained cells by phosphorylation at serines-112 and -136, which both independently induce the association of BAD with 14-3-3 proteins (219, 220).

This association leads to the sequestration of BAD to the cytosol, and therefore its inactivation.

Phosphorylation of BAD at serine-136, and not serine-112, is induced by the kinase AKT (220). Accordingly, serine-136 is the only BAD residue phosphorylated in CG neurons maintained in IGF, as in the presence of IGF, CG neurons solely rely on the PI3-kinase-AKT pathway to maintain survival (220, 279, 313-315). Activation of the JNK pathway has been found to induce the phosphorylation of BAD at serine-128, leading to the disruption of the association of phospho-serine-136-BAD and 14-3-3, and thus the apoptosis of BAD overexpressing IGF-maintained CG neurons (311, 312). It is not yet known if serine-128 phosphorylation interferes with the interaction of 14-3-3 with serine-112 phosphorylated BAD.

1.4.4.4 Bcl-2

JNK is also believed to directly phosphorylate Bcl-2 at several sites within the unstructured loop linking the BH4 and BH3 domains, leading to the inhibtion of Bcl-2 anti-apoptotic activity (316-319). Accordingly, Bcl-2 mutant constructs lacking the JNK phosphorylation sites (317, 318), or the entire loop altogether (317), are much more effective than wild-type Bcl-2 at inhibiting death by a number of insults.

JNK dependent phosphorylation of Bcl-2 has been reported in *in vitro* kinase assays (316, 317), in cell-lines co-transfected with JNK and upstream JNK-activators (316), as well as in tumour cell lines treated with paclitaxel or other microtubule-interfering agents known to be potent activators of JNK (317-319).

The mechanism through which phosphorylation induces the inhibition of Bcl-2 anti-apoptotic activity is not known. Although it has been reported that Bcl-2 phosphorylation leads to the loss of its ability to interact with pro-apoptotic Bcl-2 members (320, 321), these observations could not be replicated in other studies (322, 323).

As JNK-mediated Bcl-2 phosphorylation has only been reported in transformed cell lines, and after treatment with non-physiological insults, the physiological relevance of this JNK-mediated event remains uncertain.

1.4.5 Signaling Upstream of JNK Activation

JNK is activated by a MAP kinase cascade, where the MAP2Ks MKK4 and MKK7 (324-329) phosphorylate and activate JNK. The MAP2Ks are in turn activated by MAP3Ks. Whereas numerous MAP3Ks (TAK1, TPL-2, and members of the MEKK, ASK and MLK families) are each capable of activating the JNK pathway, their individual physiological relevance is not clear .

1.4.5.1 MAP2Ks

The two MAP2Ks involved in JNK activation, MKK4 and MKK7 are dual specificity protein kinases that phosphorylate JNK on Thr-183 and Tyr-185, inducing the kinase activity of JNK (324-329). Interestingly, while both proteins can activate JNK by dual phosphorylation on Thr and Tyr, the two have different preference for the phosphate-acceptor sites, with MKK4 preferring Tyr185, and MKK7, Thr183 (324, 330, 331). Observations also suggest a processive mechanism for JNK phosphorylation, with phosphorylation at one site necessary for phosphorylation of the other (331, 332). Accordingly, both MAP2Ks have been found to be necessary for maximal activation of JNK (327, 328, 332-335).

MKK4 and MKK7 are also believed to mediate signaling initiated by distinct stimuli. MKK7 may mediate JNK activation by pro-inflammatory cytokines (329), whereas both MKK4 and MKK7 mediate JNK activation caused by exposure of the cell to environmental stress (327, 329). Moreover, MKK4, but not MKK7 can activate p38 *in vitro* (325), although the physiological relevance of this observation is not known.

1.4.5.2 MAP3Ks

In contrast to the JNK family and its MAP2Ks, the MAP3Ks in the JNK module are highly divergent in structure and gene number. To date, the different

MAP3Ks identified as upstream activators of the JNK pathway are: the members of the MEKK family [MEKK1 (336), MEKK2 (337), MEKK3 (337), MEKK4/MTK1 (338, 339)], the members of the MLK family [MUK/DLK/ZPK (340-343), LZK (344), MLK1 (345), MLK2/MST (346, 347), MLK3/SPRK/PTK1 (348-351)], the members of the ASK family [ASK1/MAPKKK5 (352, 353) and ASK2/MAPKKK6 (354)], TPL-2/ Cot (355, 356) and TAK1 (357).

In most cases the evidence for involvement of these MAP3Ks in the JNK pathway is based upon transfection assays (overexpression and dominant-negative experiments) and *in vitro* protein kinase assays that demonstrate phosphorylation and activation of MKK4 and/or MKK7. These data do not establish whether these MAP3Ks are physiologically relevant regulators of the JNK pathway, as promiscuity of function is often observed in transfection and *in vitro* assays. Accordingly, many of these MAP3Ks, can activate more than one MAPK pathway *in vitro*, as well as the NF-κB pathway. Whether this reflects *in vivo* function, or whether it is an artifact of overexpression is not clear. It is also unclear which MAP3Ks are relevant to specific physiological stimuli.

Knock-out animals provide a powerful strategy for clarifying the physiological roles of individual MAP3Ks, however such studies have only been reported for some MAP3K. Also the determination of redundant, but possibly important, roles played by different MAP3Ks is difficult by the use of knock-out techniques.

MEKK1-deficient mice do not have gross morphological defects except for an eyelid closure disability (358). In contrast, MEKK3 deficiency causes embryonal lethality due to failure of embryonic angiogenesis and cardiovascular development (359). Whether these defects are due to alterations in JNK signaling or changes in another path is unclear.

MEKK1-null embryonal stem (ES) cells demonstrate defects in JNK activation in response to a limited number of stimuli, including microtubule destabilizing drugs, cold shock, reovirus infection, and lysophosphatidic acid, but are not defective in response to other known JNK activators including heat-shock,

UV irradiation, anisomycin, TNFα or IL-1 (358, 360, 361). In contrast, an independently created clone of MEKK1-disrupted ES cells was found to be defective in JNK activation in response to TNFα and IL-1 (362). As in the latter case, a different strategy was used in gene targeting, the stronger phenotype may be the result of expression, within the null cells, of a large fragment of MEKK1 which may act in a dominant-negative fashion for other MEKKs.

MEKK2-deficient mast cells derived from MEKK2-null ES cells, are deficient in non-apoptotic receptor-mediated JNK activation, whereas stress induced JNK activation is still apparent (363).

In vivo and *in vitro* studies using the selective MLK inhibitor CEP1347, have implicated this MAP3K family in apoptotic JNK-mediated signaling initiated by a number of insults (271, 272, 282). CEP1347 has been found to inhibit JNK activation, and the subsequent apoptosis, induced by stimuli such as growth-factor withdrawal and MPTP induced toxicity (271, 273, 282, 294, 295). Moreover, the specific deletion of the MLK-3 gene in mouse embryonal fibroblasts (MEFs) causes a partial defect in TNFα stimulated JNK activation (364).

1.4.5.3 MAP4K

Genetic evidence from yeast and flies suggests that a group of kinases which appear to function upstream of MAP3Ks may exist in the JNK MAPK module. Ste20p is a yeast MAP4K protein that activates the MAP3K in the pheromone-responsive MAPK cascade of the budding yeast mating pathway (365, 366). A number of protein kinases with catalytic domains closely related to that of Ste20p have been identified in mammals and constitute the Ste20p family. The Ste20p family is divided into the p21-activated kinase (PAK) subfamily and the larger germinal center kinase (GCK) subfamily (367).

The PAKs contains a C-terminal catalytic domain, and an N-terminal regulatory domain with a small GTPase binding motif. PAKs have been shown to activate MAPKs (primarily JNK and p38) and to mediate apoptosis in overexpression studies (367-370).

The GCKs have N-terminal catalytic domains followed by C-terminal putative regulatory regions without conserved G protein binding sites. The 28 known human GCKs are classified in eight subdivisions (GCK I-GCKVIII) and have varied and less well characterized functions (367). Some GCKs are involved in the regulation of the JNK and p38 MAPK pathways; GCKs reported to activate JNK *in vitro* include the GCK-I subfamily members, GCK, HPK1, GLK; GCK-IV subfamily members, MINK, NIK, HGK,TNIK; and the GCK-V subfamily member, SLK (367). Those reported to activate p38 include the GCK-VI subfamily member SPAK and the GCK-VIII subfamily members TAO1 and TAO2 (367). Whereas the GCK-II subfamily member MST1, the GCK-III subfamily members MST3 and MST4, the GCK-V subfamily member LOK, and the GCK-III subfamily member SOK-1 have not been found to activate any MAPKs (367, 371, 372).

The fact that the numbers of MAP3K and MAP4K molecules are much larger than those of MAP2Ks and MAPKs raises the question why so many MAP3Ks and MAP4Ks exist. One possibility is that these upstream kinases have unique substrates which do not belong to the MAPK module. Accordingly, as mentioned, some MAP4K do not activate any MAPKs, whereas some MAP3K have been found to activate the NF-κB pathway.

The divergence of upstream kinases may also be utilized by cells to discriminate, and differentially respond, to a wide variety of extracellular stimuli such as growth factors, cytokines and physiochemical stressors, and to allow the kinases to interact with different upstream activators. For example, some, but not all, MAP3Ks and MAP4Ks can bind and be activated by the small GTPases of the Rho family.

1.4.5.4 JNK & Rho-like GTPases

Several lines of evidence indicate that small G proteins of the Rho family act as upstream activators of the JNK MAPK pathway in response to certain stimuli (373, 374). Constitutive-active forms of these proteins, in particular Rac and Cdc42, strongly activate JNK and p38 (373-375), whereas dominant-negative

mutants of Rac and Cdc42 inhibit JNK activation in response to a number of stimuli (373, 374). The PAK subfamily of MAP4Ks (367), as well as the MAP3Ks MEKK1 (376), MEKK4 (338), MLK2 and MLK3 (377) have been shown to bind Rac and Cdc42 through their Cdc42/Rac GTPase binding (CRIB) domains.

Studies using dominant-negative forms of Rac1 and Cdc42 have also shown that the activity of Rac1/Cdc42 is essential for the mediation of NGF withdrawal induced JNK activation and death of sympathetic neurons (374, 378). The membrane proximal signaling events that lead to activation of Rac/Cdc42 after neurotrophic factor withdrawal remain uncertain; however, based on data from various sources, one could synthesize the following signaling cascade. After NGF withdrawal, the small GTPases Cdc42 and Rac1 become activated (374), and bind the CRIB motif of JNK-upstream kinases that include PAKs, MEKK1, MEKK-4, MLK2, and MLK-3 (338, 342-344, 350, 351, 367, 376, 377). Activated MAP3Ks then phosphorylate and activate MKK4/MKK7, which then activates JNK. JNK activation leads to phosphorylation and transcriptional upregulation of c-Jun, which in turn induces the BH3-only molecules Bim and Hrk (196, 309). JNK mediated phosphorylation of Bim and Bad further increases the potency of these proteins (195, 310, 311), leading to Cytochrome c release, caspase activation and cell death.

1.4.6 JNK Scaffolding Molecules

It is expected that each MAP3K or MAP4K would activate specific signaling pathways in a stimulus-specific manner. However, at least *in vitro*, each MAP3K or MAP4K activates a number of different MAPK and non-MAPK signaling cascades. This raises the question of how the *in vivo* signaling specificity is achieved. The use of scaffold proteins is believed to be one manner through which this specificity is achieved.

Scaffold proteins assemble components of signaling cascades into modules. They do not necessarily contain any intrinsic enzymatic activity, however they do contain binding domains that allows for the recruitment of

different components of a specific pathway simultaneously. They thus segregate different signaling cascades and confer pathway specificity. Scaffolds would also allow for the dynamic regulation of the subcellular localization of signaling cascade components, and would permit increased processivity which is needed for double phosphorylation of MKK4/7 and JNK.

The first identified scaffold protein was Ste5p, which assembles the Ste11p-Ste7p-Fus3p MAPK module of the *S. cerevisiae* pheromone mating pathway and, in addition, couples this cascade to cytoskeletal components of the cell (379-381).

In mammalian cells, JIP1 acts as a scaffold for the JNK signaling pathway (382-384). Different stretches within the primary structure of JIP1 have been found to interact with different components of the MAPK cascade (383), with the N-terminus containing the JNK binding domain (JBD) (382, 383). Specifically, JIP1 has been reported to interact with the JNK isoforms JNK1 and JNK2, the MAP2K MKK7, and the MLK family members MLK3, LZK and DLK (383-385). Moreover, JIP proteins appear to act as dimers, so it is likely that the JIP-assembled kinase cascade functions in trans rather than in cis (384, 386).

When separated from the rest of JIP1, the JBD acts as a decoy substrate and potently and selectively inhibits JNK, but not p38 (382, 387). The JBD has thus been shown to block JNK dependent apoptosis induced by a number of insults, including NGF-withdrawal induced death of sympathetic neurons (387, 388).

JIP1, and the closely related JIP2 (384) are most highly expressed in the nervous system (382, 384). JIP1-null hippocampal neurons display reduced JNK activity and cell death in response to excitotoxic and ischemic insults *in vivo* and *in vitro* (392, 393). JIP1 has also been proposed to be necessary for JNK activity in adipose tissue (394).

JIP3 is structurally unrelated to JIP1 and JIP2. However, JIP3, like JIP1 and JIP2 binds to JNK, MKK7, and members of the MLK family (386). An alternatively spliced form of JIP3, JSAP, has been reported to be highly expressed in the nervous system (395). However, unlike JIP1-3, JSAP is reported to interact

with MKK4 and MEKK1, and the neuronally expressed JNK member, JNK3 (395).

The β-Arrestin scaffolds are implicated in JNK signaling by G protein coupled receptors (GPCR). The activated/phosphorylated receptors can recruit the scaffold protein β-arrestin-2 (396, 397), which serves as a site of assembly for a JNK signaling module (396). β-Arrestin-2 directly binds JNK3 and ASK1 at different sites and indirectly engages MKK4 (396). This scaffold protein therefore serves to recruit a JNK signaling module to activated seven transmembrane-spanning receptors.

It has also been reported that the MAP3K MEKK1 itself acts as a scaffold protein for either the JNK or ERK pathways, with components of these pathways interacting with different regions in the N-terminal regulatory domain of MEKK1 (398, 399). The ability to act as a scaffold for these two kinase cascades is consistent with its proposed role as an activator of both pathways (250, 360, 398)

1.4.7 JNK Docking Site and Molecular Interactions

In contrast to other kinases, substrate recognition by JNK, and MAPKs in general, does not take place via a transient interaction between the active site of the kinase and the phospho-acceptor site of the substrate. Rather, the recognition between MAPKs and its substrate is due to a stable interaction between regions distinct from the active site and the phospho-acceptor site (400-404). This type of interaction is referred to as docking interaction. Conceptually, the docking interaction may augment the efficiency and specificity of the enzymatic reaction.

The docking motif recognized by JNK is referred to as the delta or D domain (403, 404). The D domain consists of a cluster of basic residues and two leucines or isoleucine residues (403). For instance, JNK associates with c-Jun through the D domain at the N-terminus of c-Jun (amino acids 30-60) (403-406). This association is a prerequisite for the phosphorylation, since JunD with its incomplete docking site is not efficiently phosphorylated by JNK either *in vitro* or *in vivo*. However, JunD is phosphorylated by JNK after dimerization with c-Jun or JunB (257, 405).

It is believed that after docking, JNK then dissociates from the D domain and reassociates with the phospho-acceptor site of the substrate via its catalytic pocket. Specific recognition of the phospho-acceptor site is also critical for JNK phosphorylation, since JunB is not phosphorylated by JNKs due to the lack of prolines in the phospho-acceptor site (405). The existence of D domains in the proposed Bcl-2 targets of JNK phosphorylation has not been assessed.

Regions within JNK that mediate the docking interaction are the common docking (CD) and the ED site, which together have been proposed to form a docking groove for the D domain of JNK substrates (251, 403, 404, 407-409). Interestingly however, this docking groove is proposed to not only mediate interaction of JNK with its substrates, but also its interaction with MAP2Ks and scaffolding proteins (403, 404, 407, 409, 410). Accordingly, docking deficient mutants of JNK are both, less efficiently phosphorylated by MKK4 and less efficient kinases of c-Jun (409). Also peptides representing JNK binding sites of JIP1 and MKK4 can inhibit, not only the binding of JNK to MKK4 , but also JNK to c-Jun and ATF2 (410). This indicates that competition exists between the substrate, the scaffold, and the upstream kinase for binding to JNK.

Peptides representing JNK binding sites of JNK interactors have also been used as potent inhibitors of JNK activity *in vitro* (410, 411), and have proved to be powerful inhibitors of cell death in *in vivo* models, such as cerebral ischemia (412).

1.5 The p75 Neurotrophin Receptor

1.5.0 Introduction

The p75 neurotrophin receptor (p75NTR) is a type I transmembrane protein which, under certain circumstances, can activate the JNK mediated apoptotic pathway. p75NTR was originally identified as a receptor for the NGF ligand [Kd ≈ 1 nM; (413)], and was later discovered to bind to all members of the neurotrophin family; NGF, BDNF, NT-3 and NT-4 (also known as NT-5 or NT-4/5) with equal affinity (413-415). Neurotrophins also interact with the Trk family of receptor tyrosine kinases, with each member of the Trk family displaying selectivity for one or more neurotrophins (416). Even though, both, the Trk family and p75NTR, bind to neurotrophins, they share no sequence homology in either the ligand-binding or cytoplasmic domains, and activate distinct neurotrophin-dependent signaling pathways.

In 2001, about 15 years after the identification of p75NTR as a neurotrophin receptor, this receptor was found to bind to the pro-, unproteolytically processed, forms of the neurotrophins with higher affinity than to mature neurotrophins (417, 418). Proneurotrophins (proNTs), however, do not interact with the Trk receptors (417-419). The discovery of proNTs as the 'true' ligands of the p75NTR, ushered in a new wave of p75NTR-related research, and also forced substantial reinterpretation of previous p75NTR-related studies.

1.5.1 p75NTR Structure

p75NTR is the founding member of the TNF Receptor (TNFR) superfamily of proteins that now contains about 25 receptors (420). p75NTR, however, is an unusual member of this family as its ligands are structurally unrelated to the homotrimeric type II transmembrane ligands which typically bind TNFR family members (421). Also, TNFR proteins generally act as autonomous signaling molecules, whereas there normally is a requirement for a co-receptor for the initiation of p75NTR-mediated signaling events.

The human p75NTR gene encodes a 399 amino acid type-I transmembrane protein plus a 28 amino acid leader sequence. The intracellular domain (ICD) of p75 is palmitoylated at cysteine 279 and phosphorylated on serine and threonine residues (422, 423). It is not known if these modifications are necessary for p75NTR function, interactions, folding or localization. The other post-translational modification of p75NTR include a single N-linked carbohydrate at position 33 and several O-linked carbohydrates in extracellular juxtamembrane stalk domain (424) (Figure 1.5). Distal to the stalk domain, the extracellular domain (ECD) has four tandem cysteine-rich modules. These cysteine-rich domains (CRDs), each containing 6 cysteines forming three intrachain disulfide bridges (425), are the distinguishing features of the TNFR superfamily. In p75NTR, they are referred to as CRDs 1-4 commencing at the N-terminus.

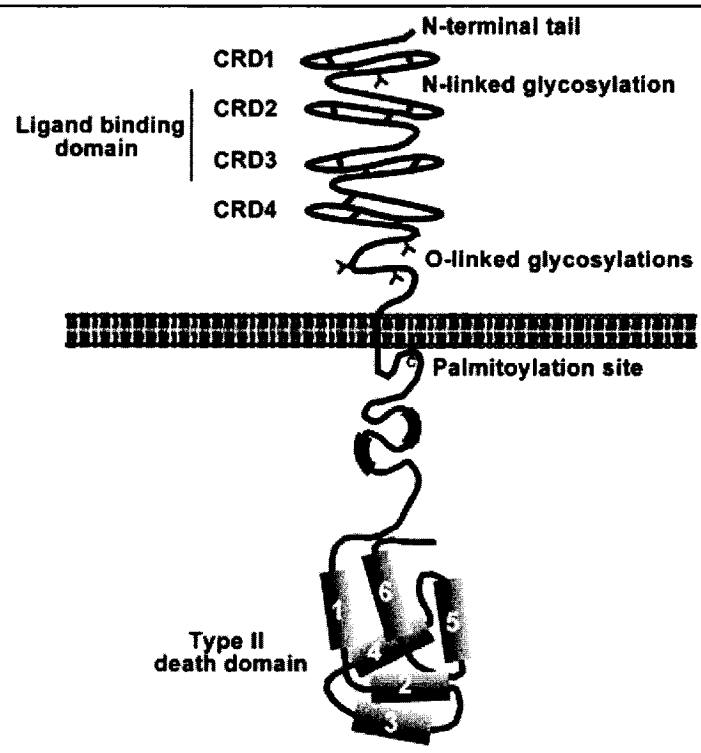

Figure 1.5. Structure of the p75NTR protein. P75NTR is a type I transmembrane receptor with an extracellular domain that contains four cysteine-rich domains (CRD), and multiple glycosylation sites. The intracellular domain contains a palmitoylation site, and a type II death domain [Adapted from (1)].

Binding of neurotrophins to p75NTR is mediated by the CRDs (426-433). A splice-variant of p75NTR, referred to as short-p75 (sp75), does not contain residues encoded by exon III. As this exon codes for CRD2-CRD4, this isoform is incapable of neurotrophin interaction (434). A soluble form of p75NTR ECD is produced by proteolytic cleavage of full-length p75NTR by a constitutive metaloprotease (435-437). The soluble ECD is produced at very high levels during development and peripheral nerve injury (435, 438), but its physiological role is unknown.

Other than the CRDs, another feature that p75NTR shares with some other members of the TNFR superfamily, is an 80 amino acid death domain (DD) in its intracellular domain. As mentioned previously, the DD in 'death receptors' such as TNFR1 and Fas serves as a homotypic protein binding module that interacts with adaptor proteins that aggregate and activate Caspase-8. However, unlike the death receptors, p75NTR does not induce Caspase-8 dependent cell death (439-441). Also, in contrast to the 'death-receptor' DDs, the DD of p75NTR does not have a propensity for homodimerization (442), and adaptor molecules that bind 'death receptor' DDs, clearly do not bind to p75NTR (440, 443)

These differences are believed to be due to the dissimilar structure of the DDs. In fact, based on structural differences, the DDs have been divided into two types. Type I DDs exist in death receptors such as TNFRI and Fas. Type II DDs, exist in p75NTR as well as non-TNFR related proteins such as UNC5, DAP kinase and Ankyrin. A comparison of the DDs of p75NTR and Fas showed, that while both domains are globular structures arranged in two bundles each containing three helices, the relative orientation of the first helix is distorted by 90^{o} in one molecule compared to the other (442, 444).

1.5.2 A Note About p75NTR Knock-Out mice

Genetic deletion in mice represents the most powerful experimental tool to assess the physiological roles of a candidate gene. Unfortunately, no full knock-out of the p75NTR gene has been produced. The original strategy used to develop p75NTR-null mice was to disrupt exon III of the p75NTR gene (445). However, a

later study showed that the p75NTR gene generates a splice variant which lacks exon III (434). Thus, both wild-type and p75NTRexonIII-/- mice produce this isoform (sp75NTR) at low levels in several tissues (434). Therefore, p75exonIII-/- mice are only hypomorphs as they still express a p75NTR gene products.

A complete gene knock-out was attempted when exon IV of the p75NTR gene was disrupted (434). However, the disrupted p75NTR gene was found to encode an aberrant transcript leading to the expression of an anomalous truncated form of p75NTR containing a small portion of the extracellular domain, and the entire transmembrane and intracellular domains of p75NTR (446). This truncated mutant of p75NTR was found to induce apoptosis when overexpressed in PC12 cells. Therefore, p75exonIV-/- mice express a gain of function mutant of p75NTR which can interfere with the correct interpretation of its phenotype.

As the expression level of sp75NTR in p75exonIII-/- animals is found to be very low to undetectable (434, 446), and therefore its contribution to the phenotype may be considered to be minimal, we will only refer this mutant (which will be termed p75NTR-null) in the following pages.

1.5.3 p75NTR as a Trk Co-Receptor

The main function of p75NTR was originally believed to be as a neurotrophin co-receptor for the Trk family. While lately, several novel functions have been ascribed to p75NTR, its modulation of Trk activity remains an important component of its biological function.

1.5.3.1 Trk Receptor Structure and Function

The Trks are tyrosine-kinase receptors that bind selectively to distinct neurotrophins and promote the survival of specific population of neurons during development. Accordingly, genetic ablation of Trk expression results in extensive loss of specific neuronal populations (447).

Trks display selectivity in their interactions with different NTs. TrkA preferentially interacts with NGF, TrkB with BDNF and NT-4, while TrkC binds to NT-3 (448-456). TrkA can also interact, with much lower affinity, with NT-3

and NT-4, while TrkB can bind to NT-3 with low affinity. In these cases, NT-3 and NT-4 are classified as non-preferred ligands (457).

Trks are type I transmembrane proteins, containing, in their ECD, leucine-rich repeats flanked by two cysteine-rich regions, followed by two Immunoglobulin (Ig)-like domains (458, 459). The second Ig-like domain is reported to be mainly responsible for neurotrophin binding (460-465), while other domains may play a modulatory role (466, 467).

Binding of NT homodimers to Trk receptors results in Trk dimerization, autophosphorylation of tyrosine residues within the activation loop, followed by autophosphorylation of seven additional tyrosine residues (468-470). These phosphorylated tyrosines act as docking sites for signaling molecules containing PTB (phosphotyrosine-binding) or SH2 domains, which regulate a number cell growth and survival signaling pathways, including the Ras, phosphatidylinositol 3-kinase (PI3-K), and phospholipase Cγ (PLCγ) cascades (416, 471).

Amongst the NT-Trk interactions, that between NGF and TrkA is the best-characterized model of how NTs signal through their cognate receptors. TrkA initiates signaling events leading to cell survival and differentiation, mainly through induction of pathways involving PI3-K mediated activation of AKT, and c-RAF and B-RAF mediated activation of Erk.

AKT is believed to be mainly responsible for signaling cell survival through phosphorylation mediated inhibtion of pro-apoptotic molecules such as Bad, Caspase-9, GSK3β , FKHRL1, and IκB (416, 472, 473), while the TrkA-mediated long term activation of Erk, leading to MEF2, Elk-1 Rsk, and CREB activation, is indispensable for mediating differentiation (416, 474).

Receptor proximal signaling events leading to PI-3K and Raf activation are exceedingly complex, and will not be discussed in detail. In brief, the recruitment of the adaptor molecule Grb2, be it directly, or via mediation of either Shc, FRS2, SH2-B or rAPS (475-477) leads to the activation of Erk through the Grb2→SOS→Ras→c-Raf path, and to the activation of AKT through the Grb2→SOS→Ras→PI3-K path or the Grb2→Gab→PI3-K path (416). The long-term activation of Erk, a peculiar characteristic of TrkA signaling necessary for

neuritogenesis, is maintained by Frs2→Crk→C3G→Rap1→B-Raf (416, 474). Other TrkA binding molecules such as IRS-1, CSK and PLCy also contribute to differentiation and survival signaling (416).

1.5.3.2 Role of p75NTR in Trk Signaling

Other than its ability to autonomously initiate signals, p75NTR can act as a neurotrophin co-receptor for Trk proteins. p75NTR can modulate both the efficiency and specificity of neurotrophins mediated Trk activation. Therefore, by modulating Trk activity, p75NTR can modify Trk mediated downstream signaling events such as cell survival and neuritogenesis (1). Astonishingly, major findings in the last few years have established that the two major signaling events, autonomously generated by the p75NTR, are also the modulation of apoptosis and axonal growth. Therefore, there is great concordance between the Trk-dependent and Trk-independent outcomes of p75NTR signaling. This, in turn, means that, in cells that co-express both p75NTR and Trk, the assignment of a particular p75NTR-mediated signaling outcome to a Trk-dependent or Trk-independent pathway is exceedingly difficult. It is with this caveat, that we attempt the following review of p75NTR-related literature.

1.5.3.2.1 p75NTR Increases NGF-Mediated TrkA Activation

Binding studies have demonstrated that p75NTR and TrkA receptors bound independently to NGF with low affinity [Kd ≈ 1 nM; (413, 451, 478)]. However, when co-expressed with p75NTR, the affinity of TrkA for NGF is increased by two orders of magnitude [Kd ≈ 10^{-11} M; (478-480)], and thus, in the presence of p75NTR, there is an increase in the level of TrkA activation at low NGF concentrations (481-483). The formation of these high-affinity NGF binding sites is believed to be necessary for neuronal competition for the subpicomolar concentrations of NGF present *in vivo*. In the case of neurons not receiving adequate trophic support, the loss of TrkA-mediated survival signals promotes the activation of a signaling cascade that ultimately results in JNK activation, loss of

mitochondrial integrity, activation of Caspase-9 and cell death [NGF-withdrawal induced death; (267, 276, 387, 388, 484-487)].

In accordance with a role for p75NTR in increasing the affinity of TrkA for NGF, cultures of p75NTR-null derived sensory and sympathetic neurons, which normally co-express p75NTR and TrkA, display an impaired ability to respond to limiting NGF concentrations (488, 489), and *in vivo*, p75NTR-null mice have a 50% loss of sensory neurons of the DRG after the peak period of naturally occurring cell death (445, 490-492). p75NTR-null mice do not, however, display a loss of sympathetic SCG neurons within the ganglia (445, 493, 494), even though sympathetic innervation to a number of targets is reduced (495), perhaps reflecting reduced TrkA neuritogenic activity.

The mechanism through which p75NTR increases the NGF binding affinity of TrkA remains controversial. Two hypothetical potential mechanisms of p75NTR-TrkA interactions have been proposed. The presentation or sequestration model postulates that NGF binds rapidly to p75NTR, leading to either an increase in the local concentration of NGF, or the presentation of NGF in a conformation more suitable for TrkA binding. In this model there is no absolute requirement for a direct interaction of p75NTR with TrkA. This model is supported by several classes of experiments. First, when a mutant NGF deficient in p75NTR binding is used, co-expression of p75NTR and TrkA does not lead to the formation of high affinity binding site (496), or increased TrkA activation (483, 496, 497). Also, disruption of the p75NTR interaction with NGF, via binding of BDNF or specific antibodies to p75NTR, results in reduced TrkA activation (481). Accordingly, the ECD of p75NTR is reportedly sufficient for the increase in ligand-induced activity of TrkA (482).

Alternatively, the conformational model proposes that binding of p75NTR to TrkA induces allosteric changes in TrkA which increase its affinity for NGF. In this model, unlike in the presentation model, NGF binding to p75NTR is not necessary for the formation of high-affinity sites. However, the conformational model, unlike the presentation model, implies the need for a ligand-independent physical interaction between p75NTR and TrkA. Accordingly, co-

immunoprecipitation studies have detected a ligand-independent interaction between p75 and Trk proteins (498-501), where the p75NTR-Trk complex is stabilized by both intracellular and extracellular interactions (498, 499). Also, in support of the conformational model, but inconsistent with the presentation model, high-affinity binding sites are still observed after co-expression of TrkA with a p75NTR mutant deficient in NGF binding (502). Accordingly, a p75NTR homologue with a similar ICD, but divergent a ECD deficient in NT binding, can cooperate with TrkA to form high-affinity binding sites (503). Also in support of the conformational model, cytosolic mutations in the ICD of p75NTR or TrkA result in the loss of high-affinity binding sites (502, 504).

1.5.3.2.2 p75NTR Increases the Specificity of TrkA Ligand Binding

p75NTR increases the specificity of TrkA for its preferred ligand NGF, both by increasing the affinity of TrkA for NGF as described above, and by decreasing the level of TrkA activation by its non-preferred ligands. When TrkA is expressed alone, high concentrations of NT-3 and NT-4 can lead to its activation (505-508). However, in the presence of p75NTR, its activation by these non-preferred ligands is greatly attenuated (489, 509, 510). Similarly, BDNF, NT3 and NT4 can all activate TrkB in the absence of p75, whereas only BDNF provides a functional response upon co-expression of TrkB with p75NTR (499).

The mechanism through which p75NTR blocks NT-3 and NT-4 mediated TrkA/TrkB activation is not known. However, it has been demonstrated that, when p75NTR and TrkA are co-expressed, treatment with a neurotrophin which binds to p75NTR with higher affinity than to TrkA (BDNF), initiates a p75NTR dependent intracellular pathway leading to TrkA serine phosphorylation (511). This phenomena, termed receptor transmodulation, has been observed with other functional receptor pairings, where phosphorylation of specific serine and threonine residues within the cytoplasmic domain of a tyrosine-kinase receptor results in inhibition of its ligand-mediated activation (512-515). Thus, it has been hypothesized that the non-preferred/non-ligand-induced p75NTR-dependent

serine phosphorylation of TrkA reduces its kinase activity in a similar manner, but this has not been demonstrated experimentally.

Other research suggests that the p75NTR-mediated decrease in Trk activation by non-preferred ligand requires neither the ICD of p75NTR (499, 501), nor ligand binding to p75NTR (501), and that, therefore, the increased specificity is proposed to be the result of an allosteric effect of the ECD or transmembrane domain of p75NTR on Trk.

1.5.3.3 Role of p75NTR in NGF-Withdrawal Induced Cell Death

Consistent with the proposed role of p75NTR as an augmenter of TrkA specificity, the level of NT-3 induced TrkA activation is increased when p75NTR function is blocked through the use of antibodies, deletion constructs or knock-out techniques (489, 509, 510). Hypothetically then, NT-3 or NT-4 could, in the absence of p75NTR, partially substitute for NGF. Accordingly, in a number of cell systems it has been observed that the inhibition of p75NTR expression leads to a delay in NGF-withdrawal induced apoptosis (493, 494, 517-519). In these cases, it is presumed that endogenously expressed NT-3 or NT-4 maintain the level of TrkA activation at a level suitable for prolongation of neuronal survival. Alternatively, in the absence of p75NTR, any endogenously expressed BDNF (or NT-3 or NT-4) can not exercise inhibitory transmodulation on TrkA activity.

p75NTR-mediated augmentation of TrkA receptor specificity is therefore proposed to be necessary for efficient NGF-withdrawal induced cell death (1, 516). Accordingly, PC12 cells (which co- express p75NTR and TrkA) selected for low p75NTR expression, die much slower after NGF withdrawal, whereas, PC12 cells selected for high p75NTR expression, die much faster (517). Also in cultures of DRG neurons, inhibition of p75NTR expression leads to a delay in NGF withdrawal induced apoptosis (518). Similarly, p75NTR-null SCG neurons have a delay in NGF withdrawal induced cell death *in vitro*, and in developmental apoptosis *in vivo* (493, 519).

Also, generic data supports a role for NT-3 in maintaining the survival of p75NTR-null NGF deprived neurons. Mice heterozygous for a NGF deletion

(NGF+/-) have 50% fewer sympathetic neurons, however neuron numbers are normal in the absence of p75NTR (p75NTR-/- NGF+/-). When NT-3 levels are reduced (p75NTR-/- NGF+/- NT3 +/-), neuron numbers decrease compared to p75NTR-/- NGF+/- NT3+/+. Therefore, one can conclude that without p75NTR, NT3 can substitute for NGF *in vivo* (494).

Also consistent with an inhibitory transmodulation role for BDNF, the rate of apoptosis of sympathetic cells maintained in a limiting concentration of NGF is increased following BDNF treatment, and the number of sympathetic cells surviving the period of naturally occurring death is increased in BDNF-null mice (493). This suggests that BDNF normally hastens sympathetic developmental death, presumably through a p75NTR dependent decrease in TrkA background activity.

1.5.3.4 An Alternative Model for the Role Of p75NTR in NGF-Withdrawal Induced Apoptosis

p75NTR has been shown to be capable of autonomous apoptotic signaling, i.e. inducing apoptosis in cell types where there is no co-expression of Trk. There has, therefore, been controversy as to whether the p75NTR-dependent acceleration of NGF-withdrawal induced apoptosis in purely due to an inhibition of Trk activity, or could be because of autonomous p75NTR apoptotic signaling.

According to the latter model, the p75NTR apoptotic pathway is constitutively active due to the autocrine presence of ligands such as BDNF. However, TrkA activation by NGF leads to a survival signal and the concurrent inhibition of the p75NTR death signal. Therefore, according to this model, NGF withdrawal leads to cell death, in large part, by unmasking the p75NTR autonomously generated death signal. Consistent with this model, NGF-dependent TrkA activation may inhibit p75NTR-induced JNK activity through an AKT dependent mechanism (520, 521). Also, Ras GTPase, which is normally activated following binding of NGF to TrkA, has been shown to suppress the JNK pathway (522).

Also, in support of a role for p75NTR autonomous signaling in NGF-withdrawal induced apoptosis are the common features shared by the two pathways, suggesting that they are one and the same. Though proximal events leading to the initiation of the death signal in either case remain unidentified, both the p75NTR-induced and NGF-withdrawal induced death pathways are dependent upon Rac-mediated activation of the JNK pathway (374, 441, 493, 523-526).

Therefore, it has been proposed that the observed BDNF potentiation of NGF-withdrawal induced cell death is due, not to inhibitory receptor transmodulation, but to the initiation of the p75NTR-dependent autonomous signaling cascade. Accordingly, BDNF also increases the rate of cell death in sympathetic cultures where survival is maintained, not by TrkA activation, but by depolarizing concentrations of KCl (493). Moreover, the deletion of p75NTR delays the naturally occurring death of sympathetic neurons even in TrkA-null animals (519), indicating a TrkA independent pro-apoptotic role for p75NTR.

It is important to note that the TrkA-dependent and independent models of p75NTR-mediated potentiation of NGF-withdrawal induced cell death are not mutually exclusive, and it is likely that both pathways play a role *in vivo*. For example, in the study mentioned above (493), where the authors concluded that the BDNF-mediated potentiation of NGF-withdrawal induced sympathetic neurons death was TrkA independent, there was a clear reduction in TrkA activation after BDNF treatment of sympathetic cells, suggesting that both mechanisms contribute to BDNF-mediated potentiation of apoptosis.

1.5.4 TrkA independent Signaling by p75NTR

1.5.4.1 p75NTR Mediates RhoA Signaling

Perhaps, the most intriguing recent advance in the field of p75NTR signaling, is the discovery of its involvement in the mediation the growth inhibitory signal generated by myelin-derived growth inhibitors (MGI).

In the adult peripheral nervous system (PNS), cut axons can reseal their ends, form growth cones, extend and reattach to their targets. In contrast, cut axons in the adult central nervous system (CNS) reform growth cones, but fail to

regrow to any significant extent. This limited ability to regenerate after injury is largely because of environmental factors preventing axonal growth (527-529).

The CNS myelin-derived growth inhibitors Nogo, MAG, and OMgp are, at least partially responsible for the block in CNS axon regeneration. While other glia- and fibroblast-derived ligands such as chondroitin sulfate proteoglycans (CSPG), Ephrins and Semaphorins also contribute to this block (527).

After binding to cognate receptors located on the neurite, most axon growth-inhibitors either repel or collapse growth cones via the Rho GTPase signaling pathway (530-532). Small GTPases of the Rho family are known regulators of the actin cytoskeleton in eukaryotic cells (533-535). In general, these molecules cycle between active (GTP-bound) and inactive (GDP-bound) states through the binding of guanine nucleotides. In resting conditions, these proteins are kept in an inactive state by guanine dissociation inhibitors (GDI). In addition, the cycling of Rho-GTPases is also regulated by either enzymes that enhance GTP-binding activity [guanine nucleotide exchange factors (GEFs)] or proteins that increase GTP hydrolysis [GTPase-activating proteins (GAPs)]. When GTP bound, Rho GTPases can interact with a series of intracellular effectors, leading to the reorganization of the cytoskeleton by regulating actin filament assembly and disassembly, and by controlling the retrograde transport of F-actin within the growth cone through modulation of actinomyosin contractility (533, 536, 537). In neuronal cell lines, activation of RhoA stimulates actinomyosin contractility and stress fiber formation, resulting in growth cone collapse, whereas the induction of Cdc42 and Rac1 leads to the extension of filopodia and lamellipodia, respectively (533, 536, 538).

1.5.4.1.1 p75NTR Associated Co-Receptors: Lingo1 and Nogo Receptor

The myelin derived growth inhibitors (MGI) Nogo, MAG and OMgp transduce their inhibitory signal via an interaction with the Nogo receptor (NgR1) on their target cells. NgR1, consists of eight leucine-rich repeats (LRRs) with N- and C-terminal caps, a 120 amino acid stalk region, and a guanine phophatidyl

inositol (GPI) anchor. Since NgR1 is GPI-linked to the cell surface, it is incapable of independent signal transduction.

NgR1 was recently found to modulate RhoA activity as part of a trimeric complex composed of Lingo1 and p75NTR. Lingo1 (539) contains 12 LRRs with N- and C-terminals caps, an Ig-like domain, and transmembrane and intracellular domains. Each component of this trimeric complex can interact with the other two independently through interactions mediated by the extracellular domain (539-542), however all three components of the complex are necessary for mediating MGI induced Rho activation (539).

NgR1 acts as the MGI binding component of the complex, and its affinity for MGI is not affected by binding to Lingo1 or p75NTR (539, 541), however binding to MGI stabilizes the interaction between p75NTR and NgR (541).

p75NTR acts as the Rho activating member of the complex. p75NTR is believed to activate Rho by sequestering, and thus inactivating, the Rho inhibitor RhoGDIα (543). The interaction between p75NTR and RhoGDIα is mediated by the fifth helix of the p75NTR death-domain (543). In neurons, this interaction is induced by binding of MGI to the endogenous trimeric complex (543), leading to the activation of Rho.

In postnatal cerebellar granule (CG) and embryonal DRG neuron cultures, the binding of p75NTR to RhoGDIα is necessary for the downstream effects of MGI, as both, MGI induced Rho activation and MGI dependent growth suppression, are not observed in p75NTR-null derived CG and DRG neurons (540, 541, 543). Furthermore, in CG and DRG neuron cultures, a synthetic peptide (PEP5), previously known to interact with the C-terminal region of the p75NTR death domain, inhibits MGI-mediated Rho activation and growth inhibition by blocking the MGI induced association of RhoGDIα with P75NTR (543). Also, overexpression of a p75NTR mutant lacking the intracellular domain in CG neurons, or treatment of cell with soluble p75NTR extracellular domain, suppresses the growth inhibitory effects of MGI, presumably by disrupting the endogenous complex (541).

The interaction of p75NTR with RhoGDIα and subsequent RhoA activation can also be reproduced by overexpression of p75NTR in HEK293 cells, which lack NgR expression (543, 544). Addition of NGF, BDNF or NT-3 to these cells inhibits RhoA activation (544), presumably by decreasing the affinity of p75NTR for RhoGDIα, suggesting that neurotrophins and MGI/NgR have opposing effects on the p75NTR- RhoGDIα interaction. However it is not clear if neurotrophins have a similar effect on the p75NTR-mediated activation of RhoA in neurons endogenously expressing p75NTR/NgR/Lingo1, as conflicting observations have been reported (540, 541, 543).

1.5.4.1.2 In vivo Roles of p75NTR-Mediated RhoA Signaling

As discussed, p75NTR mediates the MGI-induced neuritic-growth suppression in select embryonal and perinatal neuron types. However, spinal cord injury trials involving p75NTR-null adult animals found no increase in axonal regeneration compared to wild-type (545), although a delay in RhoA activation is observed in p75NTR-null mice (546). The inability of p75NTR-null adult CNS neurons to regenerate is likely due to the *in vivo* existence of NgR independent growth inhibitors (see above), and to the expression of Taj/Troy an orphan TNFR superfamily member which can functionally replace p75NTR in the MGI trimeric receptor complex (547, 548).

Taj/Troy is highly expressed in the adult CNS (547, 548) which is consistent with a role in the control of adult axonal regeneration. Although p75NTR is highly expressed during the development of a number of neuronal populations, its expression in the adult CNS is very limited (1). Even though p75NTR is reportedly upregulated post-axotomy in several neuron types including motor neurons (549, 550), Purkinje cells (551, 552) and corticospinal neurons axotomized at cortical level (553), only a slight induction of p75NTR expression is observed in a very small subset of ascending sensory axons and not in any corticospinal axons after spinal cord injury (545). Therefore, due to its lack of expression in the adult spinal cord, it is not surprising that p75NTR-null animals display no improved regeneration after spinal cord injury (545).

During development p75NTR is expressed in neuronal populations with long axonal projections. p75NTR is expressed at high level during the axonal outgrowth of sympathetic and sensory neurons, spinal cord motor neurons, Purkinje cells, basal forebrain cholinergic neurons, and retinal ganglion cells (424, 554-561). Typically, p75NTR expression levels decrease following arrival of axon at target tissue (557). Given this expression pattern, it is more likely that p75NTR is involved in regulation of axon guidance during development rather than axonal regeneration in the adult. In fact, developmental neuritic growth inhibition by p75NTR may account for the cholinergic hyper-innervation observed in the hippocampus of p75NTR-null mice (562), or for the increased sympathetic innervation of the submandibular salivary gland of these mice (563). Also, the growth of sympathetic axons within the myelinated portions of cerebellum is greater in NGF overexpressing mice lacking the expression of p75NTR compared with those with wild-type p75NTR genes (564). Also, sympathetic and cortical subplate axons innervate inappropriate targets when p75NTR is removed (495, 565, 566), and MAG-induced growth cone turning is disrupted in Xenopus axons treated with p75NTR blocking antibodies (542).

1.5.4.2 p75NTR as a Promoter of Neuritic Growth

The above observations, concerning hyper- and mis-innervation in p75NTR-null animals, reveal a growth inhibitory role for p75NTR. However, other studies have found that certain neuronal populations have stunted or delayed axonal growth in the p75NTR-null animal, indicating p75NTR may, in some contexts, play an axonal growth promoting role. Sympathetic innervation to the lateral footpads and the pineal gland is absent in p75NTR mutant mice (495). Sensory axons in forelimbs and skin above the eyes of p75NTR-null embryos are stunted and appear less arborized (567). Accordingly, *in vitro*, neurite outgrowth of p75NTR-null DRG neurons along cryosections of sciatic nerve are reduced compared to wild-type (567). p75NTR-null animals also exhibit a delay in the outgrowth of thoracic intercoastal nerves and innervation of motor axons in the forelimb (544).

A number of hypotheses have been proposed to explain the decreased neuritic growth in p75NTR-null animals. These include decreased sensitivity to the trophic effects of NGF due to disappearance of high-affinity TrkA binding sites (489, 495), increased Rho activity due to the inability of short-p75NTR, which may be still expressed in the p75NTR-null neurons, to release RhoGDIα upon encounter with NTs (544), or a defect in Schwann cell migration or myelination (567). Although none of the above hypotheses have been thoroughly substantiated, the latter hypothesis has received more attention of late.

Several studies have demonstrated the importance of Schwann cells for axonal morphogenesis and maintenance of neuronal survival. Absence of Schwann cells in the periphery leads to defasciculation (568), neuronal degeneration and ultimately neuronal loss (568-570). Moreover, Schwann cells are important for neurite outgrowth (571, 572), as Schwann cells promote axonal extension and reciprocal signaling from the axon promotes Schwann cell migration (573, 574).

In vitro, p75NTR is demonstrated to promote Schwann cell migration and myelination. Migration of Schwann cells, which do not express TrkA, is stimulated by NGF treatment, and p75NTR-null Schwann cells display reduced migration (567, 575). Also, BDNF-mediated activation of p75NTR in Schwann cells enhances the formation of myelin in Schwann cell-sensory neuron co-cultures, whereas blocking the action of endogenous BDNF inhibit this process (576). Accordingly, in p75NTR-null mice, less Schwann cells are found associated with axonal tips, and axons have a lesser degree of myelination (567, 577).

The regulation of Schwann cell activity is believed to be mediated by the ability p75NTR to activate NF-κB in Schwann cells, where it acts as a pro-survival signal (578-580). NF-κB is also required for differentiation of Schwann cells to a myelinating phenotype, and for the expression of adhesion proteins necessary for Schwann cell migration (581).

p75NTR-induced activation of NF-κB is reportedly mediated by two cytosolic interactors, TRAF6 and RIP2. Dominant negative constructs of each of

these two proteins blocks NGF-induced p75NTR-mediated NF-κB activation in Schwann cells (580, 582). Moreover, in Schwann cells where expression of either of these two molecules has been suppressed, NGF treatment does not lead to NF-κB activation (580, 583). TRAF6 and RIP2 are believed to be components of the same signaling pathway, and a physical interaction between the two molecules has been detected (580, 584).

In summary, *in vivo* data support both a neuritic growth-inhibitory and growth-promoting activity for the p75NTR receptor, depending on the particular system and developmental phase examined. These two contrasting activities are likely mediated by different signaling pathways, affecting different components of the nerve.

1.5.4.3 Pro-NGF, a Novel p75NTR Ligand

The neurotrophins, like many other growth factors, are synthesized as immature precursors that are proteolytically cleaved intracellularly, by Furin and other proconvertases, to produce mature ligands. NT prodomains were initially proposed to function only in the promotion of protein folding and the regulation of NT secretion (585, 586). However, it was recently suggested that the high degree of sequence conservation between NT prodomains of different species indicates additional biological functions involving protein-protein interactions (417). Accordingly, significant levels of proneurotrophins (proNTs) have been detected within various tissue extracts and secretions, and in culture supernatants (417, 418, 587, 588).

1.5.4.3.1 Sortilin, the Pro-NGF Co-Receptor

Recently, Sortilin, a member of the Vps10p-domain receptor family (419, 589, 590) was found to interact with proNGF and proBDNF via binding to their prodomain (418, 419). Members of the Vps10p family were previously identified as sorting receptors for molecules in the secretory pathway and on cell membranes (591, 592). In mammalian cells, Sortilin had previously been found to

play an important role in trans-Golgi network (TGN) to endosome and TGN to lysozome trafficking events (592).

Sortilin binds to the prodomain of proNGF with a Kd of ~5 nM (419) and to the prodomain of proBDNF with a Kd of ~0.4 nM (418). p75NTR binds to proNGF or proBNDF with a Kd of ~15 nM (418, 419), and TrkA interacts with proNGF with a similar Kd (419). Thus the affinity of p75NTR or Trk for proNTs is about 15 fold less than their affinity for mature NTs (418, 419).

As Sortilin and the NGF receptors, TrkA and p75, interact with different domains of proNGF, the prospect of cooperative binding to proNGF was assessed. No increase in proNGF binding is observed after co-expression of TrkA and Sortilin in 293 cells. However, after co-expression of p75NTR and Sortilin in these cells, the affinity for proNGF rises dramatically, with a Kd of ~0.16 nM, at least 30 fold higher than the proNGF affinity of either receptor when expressed alone (419), and over five-fold higher than the affinity of p75NTR for mature NGF. A similar p75NTR affinity for proNGF is found in A875 melanoma cells which endogenously express Sortillin and p75NTR (417).

Sortilin and p75NTR form a complex in the absence of proNGF, the prevalence of which is increased after proNGF treatment (419), TrkA and Sortilin do not form such a complex, which may underlie the lack of cooperative proNGF binding after TrkA and Sortilin co-expression (417). Accordingly, proNGF or proBDNF have not been found to activate TrkA or TrkB, respectively (417, 418). Therefore, in the presence Sortillin, proNTs can be viewed as ligands that bind specifically to p75NTR and not to the other class of NT receptors, the Trks. The p75NTR-Sortilin interaction, however, does not result in allosteric changes within the receptor binding domain of p75NTR, as the affinity of p75NTR for mature NGF remains the same after Sortilin co-expression (419).

Mature NGF was previously shown to induce a weak to moderate p75NTR-dependent death signal in a number of cell types which lack endogenous TrkA expression, including cultured mature oligodendrocytes (523), and cultured hippocampal neurons (525). Also, stable expression of p75NTR in a sympathoadrenal cell line [MAH cells, (500)], and vascular smooth muscle

(VSM-p75) cells (593) rendered them sensitive to death induced by mature NGF. Similiarly, mature BDNF was previously demonstrated to induce a moderate increase in the death of sympathetic neurons, which express p75NTR in the absence of TrkB (275, 493). However, the concentration of NTs required for induction of apoptosis is considered supraphysiological and 2-8 fold higher (2-8 nM) than the Kd of p75 for NT (Kd $\approx$ 1 nM). This is in contrast with other receptors, where a ligand concentration of less than one Kd is usually sufficient to produce a potent signal.

Recently, after the discovery of its ability to bind to p75NTR, proNGF was found to induce significant cell death in VSM-p75 cells and oligodendrocytes at low concentrations (417, 419, 587, 588). Both cell types co-express p75NTR and Sortilin (but not TrkA) (419), and both receptors are required for mediation of proNGF-induced apoptosis (419). Similarly, proBDNF has been shown to induce death of of VSM-p75 cells, and sympathetic neuron which co-express p75NTR and Sortilin (but not TrkB) (418). Furthermore, Schwann cells, which express p75NTR but not Sortilin, are resistant to the pro-apoptotic actions of proNGF. However these cells became sensitive to proNGF after transfection of Sortilin (419), suggesting that the expression level of Sortilin in different cell types is a determinant of their sensitivity to proNGF.

Importantly, ProNGF appears to be a vastly more potent apoptosis inducer than mature NGF, mediating cell death at concentrations up to ten fold lower than the Kd value of p75NTR/Sortillin for proNGF (Kd $\approx$ 0.16 nM) (417, 419, 587, 588). In fact, concentrations as low as .04 nM and .01 nM induce substantial cell death in VSM-p75 cells (588) and oligodendrocytes (587), respectively. Thus, proNGF appears to be at least 100 fold more apoptogenic than mature NGF. As for ProBDNF, much less is known about its pro-apoptotic properties due its very recent identification as a death inducing molecule (418). Accordingly, its apoptogenic dosage has not yet been thorougly defined, and it is not known if it is as effecient as proNGF at inducing apoptosis at ultra-low concentrations. Nevertheless, proBDNF has been reported to be at least 10-20 fold more effective at inducing cell death than mature BDNF (418).

The large difference in the potency of mature NGF and proNGF has suggested that the apoptosis that was previously associated mature NGF, may actually be due to proNGF contamination within preparations of commercially available mature NGF. Commercially available 'mature NGF', either obtained by recombinant means or harvested from the submaxillary glands of mice, has been found to contain significant, but variable level of contamination with pro-forms of NGF (587, 594). This contamination is due to the fact that even recombinant 'mature NGF' is produced form virus or plasmid encoding preproNGF expressed and proteolytically processed in mammalian or insect cell lines (595-597). This contamination has been found to make a large difference in the pro-apoptotic activity of the commercial NGF preparation. For example, p75NTR-mediated oligodendrocyte apoptosis induced by commercial submaxillary-derived NGF, was found to be largely inhibited after immunodepletion of the NGF with a proNGF specific antibody (587).

Using proNGF-immunodepleted preparations of mature NGF, the authors of the above study suggested that proNGF is at least 200 fold more potent at inducing oligodendrocyte cell death than pure mature NGF (587). However, the possibility that pro-apoptotic activity is an absolutely exclusive property of the pro-form of NGF has not been adequately addressed in the literature.

The fact that, in the presence of Sortilin, proNGF binds to p75NTR with only five-fold higher affinity, but induces apoptosis with over 200 fold more potency than mature NGF, suggests that the increased apoptotic activity of proNGF is not solely due to its higher affinity for p75NTR. proNGF binding to the p75NTR/Sortilin complex, likely initiates unique signaling events not occurring after NGF-p75 interaction.

1.5.4.3.2 NGF-Mediated Activation of p75NTR/TrkA, Revisited

Before the discovery of an independent function for proNGF, NGF was only believed to initiate p75NTR-dependent apoptosis in the absence of TrkA co-expression, as when the two receptors are co-expressed, activation of TrkA signaling by NGF leads to the suppression of p75NTR signaling (see above).

However, as proNGF can activate p75NTR and not the TrkA receptor, proNGF-mediated p75NTR activation can take place regardless of TrkA co-expression. For instance, SCG neurons which co-express Sortilin, p75NTR and TrkA, undergo p75NTR dependent apoptosis upon the addition of proNGF (417, 419). Under the same culture conditions mature NGF was found to inhibit cell death, as expected (419). It is therefore expected that, *in vivo*, the ratio of proNGF to mature NGF acts as crucial regulating factor in maintaining the balance between survival and death in p75NTR and TrkA co-expressing neurons.

It would be predicted that, in cases where the two receptors are co-expressed, the proNGF content must be considerable to tip the balance towards cell death. For NGF preparations with a proNGF content of up to 6% (submaxillary-derived NGF) are known to be potent inhibitors of cell death when applied to p75NTR/TrkA co-expressing neurons, whereas they act to induce cell death in the absence of TrkA (see above).

In the presence of Sortilin, proNGF interacts with p75NTR with five-fold higher affinity than the interaction between mature NGF and TrkA. The inefficiency of proNGF to induce cell death in TrkA/p75NTR co-expressing neurons is, therefore, likely due to high-affinity TrkA-NGF binding sites, formed upon co-expression of p75NTR, which bind NGF with a Kd of ~10^{-11} M.

Therefore, formation of high-affinity NGF binding site on TrkA can promote cell survival in two ways. First, as mentioned in the previous section, the formation of high-affinity NGF binding sites can increase TrkA activation in response to the limiting concentrations of mature NGF available *in vivo*. Furthermore, as we have just discussed, formation of high affinity NGF binding sites raises the affinity of TrkA to a higher level than the affinity of p75NTR/Sortillin for proNGF, increasing the activation level of TrkA to a sufficient level to effectively mask proNGF-p75NTR death signaling.

1.5.4.3.3 In vitro Apoptosis Induced by Products of the NGF Gene

Before the discovery of the biological role of proNGF in 2001, commercially available NGF [mostly mature NGF + up to 6% proNGF (587)]

was known to induce p75NTR dependent apoptosis in a number of cell types which lacked TrkA expression. The weak to moderate apoptosis induced by commercial NGF (comNGF) in cultures of mature oligodendrocytes, and a VSM subline stably expressing p75NTR is now believed to be mostly due to the proNGF contamination in comNGF (587).

However, comNGF also induces weak to moderate apoptosis in cultures of Hepatic stellate cells (598), hippocampal neurons (525), trigeminal neurons (599), neonatal Schwann cells (600), and p75NTR overexpressing MAH cells (500). In most cases, *in vitro* induced cell death is only observed after treatment with comNGF, and not with comBDNF or comNT-3. This may be due to the contamination of comNGF with the pro-form, which may be present in lesser amounts in comBDNF and comNT-3, as these proteins, unlike comNGF, are only produced by recombinant means, mostly from plasmids coding only the mature form (601, 602). Alternatively, the exclusisve ability of comNGF to induce apoptosis could be due to the possible co-expression of TrkB or TrkC in the target cells, leading to attenuation of BDNF or NT-3 induced p75NTR activation.

1.5.4.3.4 In vivo Roles of Pro-NGF

Due to the recent discovery of the pro-apoptotic role of proNGF, there have been only a limited number of studies where *in vivo* p75NTR dependent cell death has been causally linked to proNGF action. The most thorough of these studies deals with corticospinal neuron apoptosis following axotomy.

The corticospinal system constitutes a major central motor projection to spinal cord motoneurons (603). After axotomy at the internal capsule level, about half of adult rat corticospinal neurons of the sensory motor cortex (CSNs) undergo apoptosis (604, 605). This death is believed to be p75NTR-mediated, as levels of p75NTR, but not Trk are increased after axotomy (553, 604), and intracortical administration of a anti-p75NTR antibody almost completely blocks axotomy induced death (553). Moreover, axotomy induced death is completely blocked in p75NTR-null mice (588).

The death of CSNs is proposed to be proNGF dependent as analysis of corticospinal fluid (CSF) derived from injured brains demonstrated that there is a substantial increase in the level of secretion of proNGF after axotomy (588). However, the secretion levels of mature NGF, or pro- or mature forms of BDNF or NT-3 are not changed. Also, CSN death is inhibited after the intracortical administration of antibodies raised against the pro- or mature domain of proNGF, and is partially suppressed when experiments are carried out in NGF -/+ mice (588).

As mentioned above, cultured mature oligodendrocytes, which express Sortilin and p75NTR but not TrkA, undergo proNGF-induced death (419, 587, 588), which is completely suppressed in cells derived from p75NTR-null animals (587). *In vivo*, unlike in culture, mature oligodendrocytes do not normally express p75NTR, however after spinal cord injury, p75NTR expression level is highly induced in these cells, coinciding with massive injury induced apoptosis (587). The death of oligodendrocytes might contribute to chronic demyelination, resulting in spinal cord dysfunction (606-611). ProNGF is believed to be involved in the apoptosis of oligodendrocytes *in vivo*, as its levels are potently induced after spinal cord injury. Furthermore, post-injury spinal cord extracts induce oligodendrocyte apoptosis *in vitro*, which can be suppressed by either anti-proNGF antibody, or in p75NTR-null oligodendrocytes (587). Oligodendrocyte apoptosis is also significantly, but not completely, inhibited *in vivo* in p75NTR null animals (587).

In both injury models discussed above, there is a concomitant increase in the level of expression of p75NTR and proNGF, leading to proNGF-induced p75NTR-mediated cell death. This may be a common theme in many injury models, however due to the recent discovery of the role of proNGF, this possibility has not yet been fully investigated.

Nonetheless, the levels of p75NTR are known to be increased in a number of injury or disease states among neurons, oligodendrocytes and schwann cells. These include hippocampal neurons after pilocarpine induced seizure (612, 613), Schwann cells after axotomy (614), and motor neurons after transection of

neonatal facial nerve (615, 616). Furthermore, absence of the p75NTR gene has been associated with reduced cell death in the above cases.

p75NTR is also induced in dying striatal and hippocampal neurons after ischemia (617-619), in Purkinje cells after axotomy (551, 552) and in Purkinje and cortical neurons following experimental allergic encephalomyelitis (620, 621). Also, increased p75NTR expression in patients with multiple sclerosis (622), amyotrophic lateral sclerosis (623) and Alzheimer's disease (624, 625) has led to the speculation that p75NTR maybe involved in these neurodegenerative diseases.

p75NTR may also play a role in the mediation of developmental cell death. Mouse and chick embryonal retinal ganglion neurons undergo a high level of apoptosis during early development, at a time where no detectable TrkA is present (626, 627). This cell death is largely suppressed in p75NTR-null mice, or by intraocular administration a p75NTR-ECD specific antibody in chick (626, 627). This apoptosis is also dependent on a product of the NGF gene, as cell death is reduced in NGF-null mice, or after administration of an NGF-specific antibody in chick (626, 627). Although these studies were conducted before the discovery of the biological roles of Sortilin and proNGF, the data are consistent with proNGF-induced p75NTR-mediated apoptotic signaling.

There is also evidence in support of a pro-apoptotic role for p75NTR in embryonal spinal cord motor neurons, as decreased apoptosis and a large increase in cell numbers are detected in the p75NTR- or NGF-null animals (627). These observations are also consistent with a role for proNGF in the apoptosis of these neurons.

p75NTR also plays a role in the apoptosis of a population of hair follicle keratinocytes during the normally occuring cyclic regression of the hair follicle (628, 629). The hair follicle (HF) represents a hair shaft producing mini-organ that shows a cyclic activity during postnatal development, with alternating periods of active growth (anagen), apoptosis driven regression (catagen) and resting (telogen) (630). More specifically, anagen referes to the period of stem-cell based regeneration of the hair shaft factory, the anagen hair bulb. At the end of anagen

the vast majority of epithelial cells, including melanocytes and different keratinocyte populations, that were directly or indirectly involved in active hair shaft production are deleted by massive and highly controlled apoptosis in the very rapid process of organ involution called catagen (630). p75NTR is expressed in a population of keratinocytes [outer root sheath (ORS) keratinocytes], which undergo massive apoptosis during catagen. The expression of p75NTR, in the absence of Trk co-expression, co-incides with the period of programmed cell death and co-localize with cells undergoing apoptosis (629). Consistent with a pro-apoptotic for p75NTR, regressing ORS keratincytes from p75NTR-null mice display significantly reduced apoptosis compared to wild-type cells (629). Accordingly, p75NTR-null mice show a significant delay in the morphological progression of catagen (629). Conversely, transgenic mice overexpressing the NGF gene products display earlier onset and accelerated progression of catagen (629), consistent with a role for proNGF in the apoptosis of ORS keratinocytes.

Cells in the somite undergo programmed cell death during embryonal development. This cell death may also be proNGF dependent as it is blocked after the *in vivo* application of an anti-NGF antibody. Also the death appears to be p75NTR dependent, as dying cells display expression of p75NTR, but not TrkA, and as the cell death is blocked by exogenous application of NT-4, which may compete for proNGF for binding to p75NTR (631).

As mentioned, proBDNF, like proNGF, has recently been found to have pro-apoptotic activity, and to be capable of potentiating sympathetic neuron death at a higher level than mature BDNF (418). Therefore, the possibility that the attenuation of SCG apoptosis in BDNF-null animals is due to lack of proBDNF rather than mature BDNF merits further investigation.

1.5.4.4 p75NTR and the JNK pathway

As mentioned, p75NTR-mediated apoptosis is dependent upon activation of the JNK pathway. Due to the recent discovery of the biological role of proNGF, the entirety of data on p75NTR-generated apoptotic signaling is based either on the use of high comNGF concentrations to initiate p75NTR dependent

apoptosis, or on the use of p75NTR signaling models where p75NTR overexpression is used to emulate p75NTR activation. As the apoptotic signaling generated by high comNGF concentrations has now been largely attributed to the proNGF contamination within preparation of comNGF (587), it is assumed that the apoptotic signaling pathways identified through the use of high comNGF concentrations are equivalent to those initiated by proNGF, although this has yet to be demonstrated.

ComNGF-induced p75NTR-mediated cell death was originally demonstrated to correlate with JNK activation in cultures of mature oligodendrocytes (523, 524). Furthermore, the comNGF-induced increase in cell death was attenuated after the ectopic expression of a dominant-negative JNK construct (526), suggesting that JNK activation is necessary for the observed cell death. Activation of the JNK pathway is also necessary for neurotrophin-dependent p75NTR-mediated death of sympathetic and hippocampal neurons *in vitro* (493, 525, 583). Activation of JNK-dependent apoptosis is also observed after the adenovirus-mediated overexpression of p75NTR in PC12 cells (441, 632).

Consistent with a JNK dependent intrinsic death mechanism, p75NTR-induced death of cultured hippocampal neurons requires activation of Caspase-9 and effector caspases, but not Caspase-8 (525, 613). Also, in PC12 cells, Caspase-9 and effector caspases, but not Caspase-8, are activated following overexpression of p75NTR (441). ComNGF dependent death of mature oligodendrocytes also did not require activation of Caspase-8 (439).

As discussed, JNK activation can induce mitochondrial release of apoptogenic factors though, either AP-1 driven upregulation of pro-apoptoic Bcl-2 proteins, and/or through phosphorylation mediated activation of these proteins. Both mechanisms seem to play an important role in p75NTR-mediated apoptosis.

Studies of Bcl-2 family phosphorylation levels have led to the determination that Bad and BimEL are phosphorylated by JNK in p75NTR overexpressing PC12 cells (310, 441). Other studies have found that c-Jun is

phosphorylated and levels of AP-1 dependent transcription are increased after p75NTR activation in a number of cells (441, 493). Furthermore, depletion of BimEL and Bad proteins prior to p75NTR overexpression leads to a block in p75NTR-induced cell death, consistent with a pro-apoptotic role played by the upregulation or phosphorylation of these proteins (310, 441).

However, whether p75NTR-mediated cell death is dependent upon AP-1 mediated transcription/translation of new proteins, such as Bim and Bad, or if phosphorylation of existing protein is sufficient for mediation of apoptosis, seems to depend on the cell type. For instance, p75NTR-mediated cell death occurs very rapidly in oligodendrocytes, with DNA fragmentation observed maximal at four hours (523, 524), suggesting a transcription/translation independent mechanism. However, p75NTR dependent cell death of SCG neurons is transcription/translation dependent (275). The cell-type specific dependence of p75NTR-mediated apoptosis on transcription/translation of new protein is perhaps related to the strength of the JNK signal, as determined by the expression levels of cell-surface p75NTR/Sortillin and other mediators of JNK signaling, and on the availability of an inactive pool of previously translated BimEL/Bad proteins.

Despite the ample data linking p75NTR to JNK activation and subsequent cell death, little is known about the receptor proximal events activating the JNK pathway, other than a requirement for an active Rac GTPase upstream of JNK (526). Rac1 is activated following comNGF treatment of oligodendrocytes, and studies using dominant-negative forms of Rac1 have indicated that Rac is essential for the mediation of p75NTR-induced JNK activity and cell death (526). Activation of Rac1 can lead to the induction of the JNK pathway via interaction with the CRIB motif of MAP4Ks and MAP3Ks including GCKs, MEKK1-4, and MLK2-3 (338, 342-344, 350, 351, 367, 376, 377). The membrane proximal signaling events that lead to p75NTR-mediated Rac activation however remain uncertain.

RhoGDIα, the p75NTR interactor responsible for p75NTR-mediated RhoA activation and neuritic outgrowth inhibition, has been reported, in other

systems, to have the ability to activate Rac (633). However for several reasons RhoGDIα is not believed to be the Rac activating intermediate in the p75NTR signaling pathway. First, interaction with neurotrophins has been reported to release RhoGDIα from p75NTR leading to the inhibition of the downstream GTPase (543, 544), whereas neurotrophin binding to p75NTR activates Rac. Second, the p75NTR cytosolic motif responsible for apoptotic signaling has been mapped to the juxtamembrane region of p75NTR (634, 635), whereas RhoGDIα is known to bind to the p75NTR death-domain (543). Finally, whereas treatment with the p75NTR interacting peptide PEP5 suppresses MGI induced neuritic growth inhibition, it does not inhibit p75NTR-induced cell death (543). Therefore, p75NTR signaling to the JNK pathway is likely mediated by a different interactor.

In the past few years numerous direct cytosolic interactors of p75NTR have been identified. Some, such as SC-1 (636), FAP1 (637) and RIP2 (580) have not been proposed to have a role in mediating p75NTR-induced apoptosis.

The p75NTR interactor NADE (638) is proposed to play a role in the p75NTR-induced death signal, as co-expression of p75NTR and NADE, but neither alone, induces apoptosis in 293 cells. However, the NADE mediated signaling mechanism has not been identified and its physiological relevance is unknown.

The p75NTR interactor NRIF may play a role in mediating the p75NTR apoptotic signal, as NRIF-null mice show a similar deficit in retinal neuron apoptosis as that observed in p75NTR-null mice (639). Also, NRIF-null sympathetic neurons display a loss of sensitivity to BDNF- or proNGF-induced cell death (640). However NRIF is not an activator of the JNK pathway (640), and it is believed that, while it may be essential for the formation of a JNK inducing complex at the level of the p75NTR, it is not sufficient for mediating p75NTR-mediated signaling.

TRAF6 is another interactor of the p75NTR which has been proposed to be involved in the mediation of p75NTR-induced apoptosis (582, 583, 641).

Furthermore, TRAF6 is known to act as a mediator of JNK signaling by several other receptors. Accordingly BDNF induced JNK activation is reduced in TRAF6-null SCG neurons maintained in KCl, compared to wild-type (583).

We do not however believe that TRAF6 acts as a direct inducer of JNK in the p75NTR pathway. TRAF6 activates the JNK pathway, independently of Rac, by association with the adaptor proteins TAB2 and Ecsit, which directly link TRAF6 to the MAP3Ks TAK1 and MEKK1 (642, 643). However, the p75NTR-mediated activation of the JNK pathway, as discussed above, requires the activation of Rac1. Therefore, TRAF6 is unlikely to be the JNK inducing mediator of p75NTR signaling. We have found that members of the TRAF family act as regulators of intracellular trafficking of p75NTR , and we believe that the suppression of p75NTR signaling in Traf-null cells is likely due to disruptions in p75NTR trafficking (644).

Therefore, the intracellular interactor of p75NTR responsible for the induction of JNK and the subsequent apoptosis has not yet been defined. In the following chapters, I will describe the discovery and the characterization of a novel p75NTR-interacting protein which we propose acts as mediator of p75NTR-apoptotic signaling.

RATIONALE AND OBJECTIVES

Rationale

As discussed, ligand binding to p75NTR leads to the initiation of a JNK-dependent intrinsic death pathway. However, the cytosolic interactor of p75NTR responsible for mediation of this signaling event has not yet been identified. To identify this molecule, we undertook a yeast-two-hybrid screen, searching for a novel interactor of the p75NTR intracellular domain. The role of this novel interactor in p75NTR-mediated apoptotic signaling will be further defined as below.

Objectives

There are three main objectives to this thesis:

1. To identify a novel interactor of the p75NTR receptor, and to confirm its role in p75NTR-mediated apoptotic signaling by assessing whether its ectopic expression will confer the ability to undergo comNGF-induced p75NTR-mediated apoptosis to cells which are normally insensitive to p75NTR apoptotic signaling (chapter 2)
2. p75NTR-induced cell death is known to be dependent on the JNK-mediated activation of the intrinsic cell death pathway. Thus, if the newly identified p75NTR interactor is a physiologically relevant mediator of the p75NTR death signal, then its overexpression should also initiate the JNK-dependent intrinsic death pathway. This possibility is assessed in chapter 3.
3. If p75NTR and its putative interactor (termed NRAGE), indeed lie on the same signaling path, then death signaling mediated by either protein should show similar sensitivity to different pharmacological inhibitors. This fact was confirmed and exploited to characterize the mechanism of action of a novel pharmacological inhibitor of apoptosis, AEG3482. Thus, the similar sensitivity of p75NTR- and NRAGE-mediated signaling to

AEG3482 and to its analogues provides further credence for the hypothesis that NRAGE lies on a p75NTR-mediated apoptotic signaling path (chapter 4).

PREFACE TO CHAPTER 2

As discussed in the previous chapter, p75NTR is known to activate the intrinsic death pathway in a JNK-dependent manner. As p75NTR does not contain an intrinsic enzymatic activity, it must activate the JNK pathway through interactions with other molecules, however no such molecules were identified at the time of this study. To search for novel interactors of p75NTR we undertook a yeast-two-hybrid screen using the intracellular domain of p75NTR as basit. This screen led to the identification of a novel molecule termed NRAGE, which we placed in the p75NTR-initiated death signaling pathway by demonstrating that its ectopic expression promotes p75NTR-dependent death in cells normally insensitive to p75NTR signaling.

It is important to note that this study was completed in 2000, one year before the discovery of proNGF as the 'true' apoptotic ligand of p75NTR. However consistent with a role for proNGF in the induction of p75NTR/NRAGE-mediated apoptosis, we only observed cell death in the presence of submaxillary-derived NGF, an NGF preparation now known to contain high levels (up to 6%) of contamination with proNGF (587) (further discussed in chapter 5).

CHAPTER 2

NRAGE, A Novel MAGE Protein, Interacts with the p75 Neurotrophin Receptor and Facilitates Nerve Growth Factor Dependent Apoptosis.

NRAGE, A Novel MAGE Protein, Interacts with the p75 Neurotrophin Receptor and Facilitates Nerve Growth Factor Dependent Apoptosis.

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2.0 Abstract

The mechanisms employed by the p75 neurotrophin receptor (p75NTR) to mediate neurotrophin-dependent apoptosis are poorly defined. Two hybrid analyses were used to identify proteins involved in p75NTR apoptotic signaling, and a p75NTR binding partner termed NRAGE (for Neurotrophin Receptor-interacting MAGE homologue) was identified. NRAGE binds p75NTR *in vitro* and *in vivo* and NRAGE associates with the plasma membrane when NGF is bound to p75NTR. NRAGE blocks the physical association of p75NTR with TrkA and conversely, TrkA overexpression eliminates NRAGE-mediated NGF-dependent death, indicating that interactions of NRAGE or TrkA with p75NTR are functionally and physically exclusive. NRAGE overexpression facilitates cell cycle arrest and permits NGF-dependent apoptosis within sympathetic neuron precursors cells. Our results show that NRAGE contributes to p75NTR-dependent cell death and suggest novel functions for MAGE family proteins.

2.1 Introduction

The neurotrophins are a family of growth factors whose functions include the regulation of neuronal survival, differentiation, axonal and dendritic growth, and the regulation of synaptic activity (645, 646). Neurotrophins exert their effects by binding two types of cell surface receptors. The Trk receptors are receptor tyrosine kinases that selectively bind different members of the neurotrophin family, with TrkA preferentially binding nerve growth factor (NGF), TrkB selectively binding brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5) and TrkC interacting only with neurotrophin (NT-3). The second class of neurotrophin receptor contains the p75 neurotrophin receptor (p75NTR), a member of the TNF receptor superfamily that binds each of the neurotrophins (647).

When p75NTR and TrkA are co-expressed, p75NTR facilitates NGF binding to TrkA and thereby enhances TrkA activation and NGF-dependent survival (481-483, 496). p75NTR increases Trk receptor ligand specificity by reducing binding of non-preferred ligands (494, 499) and by attenuating TrkA activity through intracellular signaling paths (511). The precise physical arrangement of p75NTR with the Trk receptors is not known but p75NTR and TrkA co-distribute on cell surfaces (648) and p75NTR can be immunoprecipitated with each of the three Trk receptors when both are overexpressed (498, 499).

p75NTR is widely distributed within cells that lack catalytically active Trk receptors and is capable of autonomous signaling. Numerous studies have shown that p75NTR can mediate cell death both *in vitro* and *in vivo* (518, 523, 626, 627, 634, 649), but the precise signaling events responsible for this effect are uncertain. p75NTR causes a neurotrophin-dependent increase of sphingomyelinase activity (650, 651) and of c-Jun N-terminal kinase (JNK) activity (493, 524) but neither of these has been directly linked to p75NTR-dependent cell death*. p75NTR can also increase NF-κB activation (578, 652) but p75NTR-dependent activation of this pathway appears to antagonize, rather than facilitate, cell death (579, 641). Other TNF receptor superfamily members include

* Since the publication of this study, the JNK pathway has been firmly established as a mediator of p75NTR apoptotic signaling (see chapter 1).

the apoptotic receptors Fas, DR3, DR4 and DR5, and the immunomodulatory receptors CD30, CD40 and TNF-RII (420). The apoptotic receptor family members receptors typically contain a self-associating intracellular protein-protein interaction motif known as the death domain which allows them to couple to the apoptotic caspase cascade through cytosolic adaptor proteins such as FADD and TRADD. p75NTR contains an intracellular region which resembles the death domain (653), but it shows crucial structural differences compared to the Fas death domain (442, 444) and does not self associate or bind to FADD, TRADD or RIP (443, 654). The p75NTR death domain does not mediate apoptosis when present in the context of a fas^{ECD}-$p75^{ICD}$ chimeric receptor (442, 655), indicating that the apoptotic signaling mechanism of p75NTR differs from other TNF receptor superfamily members .

Several potential p75NTR interacting proteins have recently been identified. Many TNF receptor superfamily members activate JNK and NF-κB signaling pathways through a group of interacting factors termed the TRAF proteins (656) and several TRAF family members bind the juxtamembrane region of p75NTR when both are overexpressed in 293 cells (582, 641). Two novel p75NTR interactors, termed SC-1 (636) and NRIF (639), have been recently identified, and in a surprising development, Barde's group has shown that amphiphilic sequences within the p75NTR death domain bind the RhoA GTPase (544). Determining the contribution of each of these to p75NTR-dependent cell death in appropriate physiological settings remains a major challenge.

To gain insights into p75NTR function, two-hybrid analyses were used to identify p75NTR interactors and a novel p75NTR-interacting protein termed NRAGE was identified. NRAGE binds p75NTR *in vitro* and p75NTR and NRAGE can be co-immunoprecipitated from untransfected cells that endogenously express both proteins. The subcellular distribution of NRAGE is altered by NGF binding to p75NTR and overexpression of NRAGE disrupts the physical association of p75NTR with TrkA. NRAGE facilitates growth arrest and is required for p75NTR-dependent apoptosis in sympathetic precursor cells. These data show that NRAGE is an important mediator of p75NTR signaling and provide new insights into the physiological roles of MAGE family members.

2.2 Materials and Methods

Two hybrid screening, cDNA cloning and sequence analyses. The yeast strain PJ694A (MAT Trp1-901 Leu2-3, 112 ura3-52 His3-200 gal4 gal80 Lys2::Gal1-His3 Gal2-Ade2 Met2::Gal7-lacZ) was the gift of Philip James (University of Wisconsin) and was cultured as described (657). The bait p75NTR intracellular domain was generated by PCR amplification from full length rat p75NTR cDNA and cloned into the XbaI sites of plasmid pGAD424 (Clontech). A rat neural crest cell cDNA library was produced in Lambda Zap (Stratagene) and was screened using the 0.6 kb NRAGE identified in the yeast two hybrid screen using standard hybridization techniques. Several overlapping clones were identified and sequenced. The longest of these, designated MNCM20, contained the entire predicted 775 amino acid open reading frame. The NRAGE sequence was compared with known and predicted sequences using web servers operated by the National Center for Biotechnology Information and the Institute for Genomic Research (TIGR). THC #252649 on the TIGR database was identifed as the human homologue of NRAGE and full length NRAGE human clones were obtained from Research Genetics Inc and sequenced. Alignments of human and rat NRAGE were performed on the web server operated by the National Center for Biotechnology Information and dendrograms were produced using the PROTDIST and BIONJ programs on the web server at HTTP://BIOWEB.PASTEUR.FR. NRAGE sequences deposited in GENBANK have been assigned accession numbers AF217963 (human) and AF217964 (rat).

In vitro translation and pulldown experiments. For pulldown experiments, polypeptides containing amino acids 410 to 709 of NRAGE (the 0.6 kb fragment identified from two-hybrid screening) were produced as 35S-labelled proteins using an *in vitro* transcription/translation kit (Promega) and then diluted in pull-down buffer containing 10 mM Tris-OH (pH 8.0), 150 mM NaCl, 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1μg/ml leupeptin, 5mM DTT and 5% dry skim milk powder. Diluents were precleared with unbound glutathione-Sepharose beads (Pharmacia) for 1 hour and then supplemented with glutathione beads coupled to 25 ug of appropriate GST fusion

protein. After a 1 hour incubation at 4° C, samples were washed extensively in pull-down buffer lacking 5% milk and eluted from beads by boiling in SDS-PAGE sample buffer. Samples were analyzed on parallel SDS-PAGE gels which were either Coomassie stained to confirm equivalent fusion protein loading or processed for fluorography using En3Hance (Dupont). In separate experiments, MBP-p75NTR-DD or MBP-p75NTR-ICD were diluted in pull-down buffer and then incubated for 1 hour at 4° C with GST or GST-NRAGE (amino acids 410 to 709) prebound to glutathione-S-transferase beads. Samples were washed and prepared for immunoblotting as above.

Polyclonal antibody generation Rabbit polyclonal antibodies against NRAGE were initially produced against keyhole limpet hemocyanin coupled peptides (peptide 1: EAEARAEARNRMGIGDE, peptide 2: GPWSWDDIEFELLTWDEE) derived from the COOH-terminal region of NRAGE; these were adequate for immunoblotting only. To produce antibodies for immunocytochemistry, a PCR product representing amino acids 53-295 of rat NRAGE was cloned into pGEX1, sequenced to confirm fidelity and then used to produce a GST-NRAGE fusion protein which was purified over glutathione columns and used to immunize rabbits. Antibodies were affinity purified as described (658), with anti-peptide antibodies purified on GST-NRAGE$^{410-709}$ and antibodies against the GST-NRAGE$^{53-295}$ purified on 6X-His-tagged NRAGE$^{53-295}$.

Transfections and immunoprecipitations. For immunoprecipitations involving transient transfections of 293 cells, expression plasmids for TrkA (7 μg), p75NTR (3 μg), and NRAGE$^{410-709}$ (5 μg) were co-transfected into cells on 100 mm plates by calcium phosphate precipitation. For immunoprecipitations involving COS7 cells, 5 μg of expression plasmids encoding either N-terminal myc-epitope tagged full length NRAGE, p75NTR or empty vector were transfected into cells on 100 mm plates using lipofectamine (Gibco BRL) as per the manufacturer's instructions. 100 ng of an expression plasmid driving expression of enhanced green fluorescent protein (pEGFP-N1 - Clontech) was included to monitor transfection efficiency. 48 hours after transfection, plates

were placed on ice, rinsed with ice-cold Tris-buffered saline (10 mM Tris (pH 8.0), 137 mM NaCl), and then lysed with 1 ml of CHAPs lysis buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 0.7% Chaps, 10% glycerol, 1 μg/ml leupeptin, 1μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM orthovanadate). For immunoprecipitations involving PC12nnr5 cells, cells maintained in normal growth media were first rinsed with ice-cold Tris-buffered saline (10 mM Tris (pH 8.0), 150 mM NaCl), and then lysed with 1 ml of NP-40 lysis buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, 10% glycerol, 1 μg/ml leupeptin, 1ug/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM orthovanadate). In all cases, nuclei were removed by centrifugation (10,000g, 5 minutes) and the lysates were precleared by incubation for 1 hour at 4° C with 30 μl of Sepharose beads. The beads were removed by centrifugation and supernatant was incubated with either 7 μl of REX, a polyclonal antiserum directed against the p75NTR extracellular domain , with 5 μg of MC192 or with 5 μg of control antibodies, as indicated in the Figure Legends. After incubating for two hours at 4° C, samples were supplemented with 30 μl of Protein G-Sepharose (for monoclonal primaries) or 30 μl of Protein A sepharose (for polyclonal primaries) at 4° C for 60 min. For PC12nnr5 cells, the lysates were divided into three identical aliquots prior to the addition of three isotype matched primary antibodies. In all cases, beads were pelleted and washed three times in lysis buffer, resuspended in SDS-PAGE sample buffer, and processed for immunoblot analyses as indicated in the figure legends.

Tissue lysates and subcellular fractionation. Lysates were prepared from dissected embryonic or adult tissues by solubilizing tissues in NP40 lysis buffer (1:5, w/v) and centrifuging at 10,000g for 30 minutes to remove insoluble material. For subcellular fractionation studies, PC12nnr5 cells were left untreated or treated with NGF (100 ng/ml) for various times then scraped from plates in ice-cold phosphate-buffered saline. Cells were centrifuged for 5 minutes at 2000g and then resuspended in 15 ml HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 255 mM sucrose and protease inhibitors). Cells were homogenized in a glass on teflon homogenizer with 10 strokes at 1200rpm and then triturated twice with a 25

gauge needle. The lysate was centrifuged at 19000g for 20 minutes and the pellet (LM) was retained. The supernatant was centrifuged at 180000g for 75 minutes and the resulting supernatant was designated cytosol. The LM pellet was resuspended in 9 ml HES buffer, layered on top of a 3 ml 40% sucrose cushion and centrifuged at 100000g for 60 minutes. The resulting pellet was resuspended in 400 ul HES buffer and desigated mitochondria/nuclei. The white interface at the sucrose cushion interface was collected, diluted in 15 ml HES buffer and spun at 40000g for 20 minutes. The resulting pellet was resuspended in 200 ul HES buffer and designated plasma membrane.

In situ hybridization, immunocytochemistry and BrdU labeling. In situ hybridization was performed using a DIG-labelled complementary fragment representing a 5' untranslated fragment of NRAGE (1-404 of the rat NRAGE cDNA) as previously described (659). Immunocytochemistry of PC12nnr5 cells was performed as previously described (660) and immunocytochemistry of embryos was performed essentially as previously described (661). Affinity purified NRAGE antibodies against the N-terminal region of NRAGE were used at a concentration of 0.45 ug/ml and purified MC192 was used at 2 ug/ml; equivalent concentrations of purified non-specific rabbit Ig or MOPC21 were used as negative controls on parallel slides for all immunocytochemistry. Appropriate goat FITC and Cy5 conjugates were used as secondary detection reagents together with Hoescht 33248 added at a final concentration of 200 ng/ml. EGFP tagged NRAGE was produced by cloning myc-tagged NRAGE into the XhoI site of pEGFP-C2. 293 cells were transfected with EGFP alone or with EGFP-NRAGE using Lipofectamine (Gibco) according to the manufacturer's instructions. Two days after transfection, the cells were incubated for 60 minutes in 1 mM BrdU, and were stained with anti-GFP (Clontech), and anti-BrdU (Boehringer Mannheim) according to the manufacturer's instructions. Immunocytochemical images were captured on a Zeiss Axioscop 2 microscope equipped with an Eclipse imaging system.

MAH cell transfection/apoptosis assay MAH cells and MAH^{p75NTR} cells (483) were infected with a retrovirus driving expression of full length rat NRAGE

cDNA. Cells were selected in 500 ug/ml hygromycin for 2 weeks in the presence of 5 uM dexamethasone and then pooled and expanded. To test the effect of NRAGE on viability, cells were seeded at 1 x 10^6 cells/100 mm dish, dexamethasone was withdrawn on the next day, cells were washed with Hank's buffered salt solution and placed in media with or without neurotrophins (NGF from Cederlane Laboratories, BDNF and NT-3 from Peprotech; all resuspended in PBS) but lacking dexamethasone. Cells were scored for viability using trypan blue exclusion after 24 and 48 hours. Each data point was performed in triplicate and experimental results were analyzed by multiple analyses of variance with statistical probabilities assigned using the Tukey test for multiple comparisons.

2.3 Results

A whole mouse E13–E15 yeast two-hybrid library was screened using the entire rat p75NTR intracellular domain as bait, and a single 0.9 kb clone that activated both LacZ and HIS3 transcription in the presence of the p75NTR bait was identified. This cDNA fragment was used to screen a rat neural crest cell cDNA library, and the largest clone identified (2.9 kb) was sequenced. This clone hybridized with a single 3.0 kb mRNA on Northern blots (data not shown) and therefore represents a near full-length cDNA. The largest open reading frame is 2328 nucleotides in length and encodes a 775 amino acid protein with a predicted molecular weight of 85,792 Da. We have named this protein NRAGE for Neurotrophin Receptor–interacting MAGE homolog (see below). The cDNA fragment identified in the two-hybrid screen encodes a 300 amino acid fragment and was designated $NRAGE^{410-709}$. Human NRAGE is represented in the TIGR database by a series of overlapping clones designated THC252649. Sequencing of IMAGE cDNA clones representing full-length human NRAGE revealed that the rat and human forms of NRAGE are 87% identical overall with 97% identity in the C-terminal 315 amino acids (Figure 2.1A).

BLAST searches revealed that NRAGE belongs to the MAGE family, a group of ~25 proteins distinguished by the presence of a 200 amino acid MAGE homology domain. The functional roles of most of the proteins in this class are unknown, but one family member, Necdin, has been implicated in cell cycle control. Necdin and several other MAGE family members consist of little more than the MAGE homology domain but others (nM15, MAGE-like, and KIAA1114) are larger proteins and show loose homology to NRAGE across most of its length (data not shown). A dendrogram showing the relatedness of the NRAGE MAGE homology domain to others in the family in shown in Figure 2.1B. NRAGE shows no significant homologies to proteins outside of the MAGE family but contains an unusual interspersed repeat consisting of 25 hexameric units with a WQXPXX consensus sequence (Figure 2.1A).

To verify the p75NTR-NRAGE interaction biochemically, *in vitro* pulldown assays were performed. Figure 2.2A shows that the p75NTR

intracellular domain fused to glutathione S-transferase (GST) efficiently bound *in vitro*–translated NRAGE$^{410–709}$, whereas GST alone did not bind this protein. In separate experiments, NRAGE$^{410–709}$ was fused to GST and used in pullouts with maltose binding protein (MBP) fused to either the entire p75NTR intracellular domain (MBP-ICD, amino acids 276–425) or the p75NTR death domain (MBP-DD, amino acids 329–425). Figure 2.2B shows that NRAGE-GST readily bound the MBP-ICD fusion protein but failed to associate with MBP-DD, indicating that the critical binding determinants for NRAGE association are in the p75NTR juxtamembrane domain. To test whether full-length NRAGE interacts with intact p75NTR when both are overexpressed *in vivo*, full-length Myc-tagged NRAGE was transfected into COS7 cells in the presence or absence of p75NTR, and lysates were immunoprecipitated with MC192, a p75NTR-specific monoclonal antibody. Figure 2.2C shows that NRAGE specifically coimmunoprecipitated with MC192 but only when p75NTR was present. We were concerned that these overexpression paradigms may force nonphysiological interactions and therefore confirmed the presence of a p75NTR-NRAGE complex within untransfected PC12nnr5 cells, which express p75NTR and NRAGE but lack Trk receptor expression. Figure 2.2D shows that endogenous NRAGE was specifically coimmunoprecipitated with p75NTR from PC12nnr5 cells, indicating that p75NTR and NRAGE interact under physiological conditions.

To examine the embryonic expression of NRAGE mRNA, whole-mount in situ hybridization studies were performed on rat embryos at embryonic age E11.5 using cDNA probes corresponding to the 5'-untranslated region of the rat NRAGE cDNA. Figure 2.3A shows that NRAGE is expressed at low levels throughout the embryo and is enriched in the developing brain and spinal cord. To examine NRAGE expression at later stages of development, affinity-purified NRAGE antibodies were used to immunoblot tissue extracts prepared from E18 rat embryos and from adult rats. Figure 2.3B shows that extracts from all E18 tissues show detectable levels of NRAGE expression, with highest levels within the cortex and cerebellum. However, by adulthood, NRAGE levels have decreased markedly in all tissues and are detectable only within cortex and lung.

To determine if NRAGE and p75NTR are colocalized in the developing nervous system, double-label immunofluorescence studies were performed on E14 rat. Figure 2.4A and Figure 2.4B show staining of the developing medulla oblongata with affinity-purified NRAGE antibodies compared to control antibodies and shows NRAGE is highly expressed in the proliferating and marginal zones but excluded from the mantle zone (Figure 2.4A and Figure 2.4C). p75NTR is not expressed within the proliferating zone but is abundant in the marginal and mantle zones (Figure 2.4D), and p75NTR and NRAGE are therefore initially coexpressed in the mantle zone, a region where p75NTR has been shown to mediate developmental apoptosis (627). p75NTR and NRAGE coexpression is also observed in many other regions of the developing nervous system, including the trigeminal and dorsal root sensory ganglia (data not shown) and within the facial motor nucleus (Figure 2.4E and Figure 2.4F). In motoneurons, p75NTR is present on both cell bodies and processes of the cells, whereas NRAGE is detected only within cell bodies.

To examine the subcellular compartmentalization of NRAGE, biochemical fractionation studies were performed (Figure 2.5). In untreated PC12nnr5 cells, NRAGE is located mainly in the cytosol, with lower levels present on the plasma membrane and within nuclei. However, NGF treatment results in NRAGE accumulation in plasma membrane fractions within 30 min, and these levels increase further with longer NGF exposures. Syntaxin 1 and Histone 3 levels were assessed to monitor the quality of the isolated organelles and revealed appropriate enrichment of the plasmalemmal and nuclear fractions. The compartmental distribution of p75NTR was identical to that of syntaxin 1 (data not shown) and neither p75NTR, syntaxin 1, or Histone 3 showed any recompartmentalization in response to NGF treatment.

p75NTR and TrkA receptors are coexpressed in several neuronal populations and can be coimmunoprecipitated when overexpressed in Sf9 or 293 cells (498, 499). To determine if p75NTR-TrkA complexes contain NRAGE, p75NTR and TrkA were transiently expressed in 293 cells in the absence and presence of $NRAGE^{410-709}$, the NRAGE fragment originally identified in the yeast

two-hybrid screen. p75NTR was immunoprecipitated and immune complexes were examined for the presence of TrkA by immunoblotting. When only TrkA and p75NTR were coexpressed, TrkA readily coimmunoprecipitated with p75NTR, but expression of the NRAGE fragment with the two receptors consistently prevented coimmunoprecipitation of TrkA with p75NTR (Figure 2.6). We made rigorous attempts to identify endogenous or epitope-tagged full-length NRAGE or $NRAGE^{410–709}$ within TrkA immunoprecipitates and within p75NTR-TrkA coimmunoprecipitates derived from several transfected cell lines, but NRAGE was not detected within the coimmunoprecipitates (data not shown). This indicates that p75NTR is capable of forming independent complexes with TrkA or NRAGE but that a tripartite complex containing all three molecules is not formed.

The normal cellular functions of most MAGE family members are unknown but Necdin inhibits cell cycle progression in 3T3, COS, and 293 cells (662-664). To begin to dissect the mechanism by which NRAGE may affect p75NTR-dependent signaling, NRAGE was examined for effects on cell cycle progression. To address this, an enhanced green fluorescent protein NRAGE fusion protein (EGFP-NRAGE) was transfected into 293 cells, and cells were exposed to a 1 hr bromodeoxyuridine (BrdU) pulse at either 24, 36, or 48 hr after transfection and then analyzed for BrdU incorporation by immunocytochemistry (Figure 2.7). In untransfected cells or in cells expressing a control EGFP protein, ~40% of cells incorporate BrdU, but in cells expressing the NRAGE-EGFP fusion, the BrdU labeling rate dropped below 5%–10% at each of the three time points, indicating that NRAGE overexpression strongly retards cell cycle progression.

To test if NRAGE plays a role in p75NTR-mediated cell death, we used native MAH cells, a sympathoadrenal cell line that lack p75NTR and TrkA receptors, or MAH^{p75NTR} cells, which stably overexpress the receptor (Figure 2.8A). In the absence of added neurotrophin, NRAGE overexpression had no effect on the viability of MAH cells or MAH^{p75NTR} cells, and NGF treatment of MAH cells overexpressing NRAGE did not affect survival. However, MAH^{p75NTR}

cells that were transfected with NRAGE cDNA and exposed to NGF showed a highly significant reduction in survival (Figure 2.8B), which was accompanied by DNA nucleosomal laddering, a biochemical hallmark of apoptosis (data not shown). BDNF and NT-3 had no effect on the survival of these cells, indicating that this effect was NGF specific. Finally, we tested whether MAH^{p75NTR} cells expressing NRAGE and TrkA exhibited NGF-mediated death. Figure 2.7 shows that NGF treatment of these cells did not reduce viability but instead facilitated survival, presumably through the activation of TrkA-dependent survival pathways. These data suggest that NRAGE forms part of a NGF-specific p75NTR apoptotic signaling cascade in sympathoadrenal cells and that the apoptotic effect of NRAGE is antagonized by TrkA.

2.4 Discussion

Substantial evidence supports the hypothesis that p75NTR activates autonomous signaling pathways that can result in apoptosis in a number of distinct cell types but the mechanisms responsible remain unclear (665). To identify molecules involved in p75NTR signaling, we performed yeast two hybrid screens and identified a novel protein termed NRAGE. NRAGE associates with the cytosolic domain of p75NTR in yeast, *in vitro* and in mammalian cells. The NRAGE fragment does not bind the p75NTR death domain and but rather interacts with the 80 amino acid intracellular juxtamembrane domain of p75NTR. p75NTR binds full-length NRAGE when both are overexpressed in COS7 cells and the two molecules form a complex in untransfected PC12nnr5 cells. NRAGE is the first p75NTR interacting protein shown to interact with p75NTR in untransfected cells and meets all criteria for a physiologically relevant p75NTR interacting protein.

An important physiological function of p75NTR is to modulate responses of the Trk receptors to the neurotrophins (516). When overexpressed, p75NTR and TrkA form a co-immunoprecipitable complex and it is likely that formation of a p75NTR-Trk complex is necessary for the functional interactions of these two receptors (498, 499). We have shown that $NRAGE^{410-709}$ antagonizes the formation of a p75NTR-TrkA complex and found that NRAGE is not present in p75NTR immunoprecipitates which contain TrkA. These experiments are important for two reasons. First, they strongly support the hypothesis that NRAGE is a *bona fide* p75NTR interactor. Second, they indicate that the formation of p75NTR-TrkA and p75NTR-NRAGE complexes are mutually exclusive. Our studies show that MAH cells which overexpress NRAGE and p75NTR undergo cell death in an NGF-dependent manner but when TrkA is present, the p75NTR-NRAGE apoptotic signal is suppressed. These findings show that NRAGE can contribute to the p75NTR-mediated apoptotic response and are consistent with previous studies which have suggested that signaling functions of p75NTR and TrkA are mutually antagonistic (666).

NRAGE is a member of the MAGE family, a large group of proteins which all contain a well conserved ~200 amino acid region known as the MAGE homology domain (667, 668). Many of these proteins consist of little more than the MAGE homology domain and small amount of flanking sequence but others have an extended domain structure which resembles that of NRAGE. Most of the MAGE genes are highly expressed during development but are present at much lower levels in the adult (669-672). However, expression of many MAGE genes increases markedly in numerous cancers due to gene demethylation (673, 674) and cytosolic MAGE proteins which are expressed in malignant cells are proteolyzed to peptides, transported into the endoplasmic reticulum and then presented on the cell surface as antigenic MHC-associated peptides (675-679). However, with the exception of Necdin, the normal intracellular role of members of this gene family remains unknown.

The only MAGE family member that has been functionally characterized is Necdin, originally identified in a screen designed to identify proteins involved in the neural differentiation of P19 cells (680). Overexpression of Necdin causes cell cycle arrest in NIH3T3, HEK293 and SAOS2 cells (662, 663) through mechanisms that may involve physical interactions with E2F-1 or p53 (663, 664). Necdin expression increases as neurons become post-mitotic (662, 681, 682) and it has been hypothesized to facilitate the transition from a cycling cell to a post-mitotic state. Mice lacking the Necdin gene appear to develop normally *in utero* but typically die shortly after birth due to post-natal respiratory distress (683). Necdin has been identified as a candidate gene for Prader-Willi Syndrome (PWS), a common neurogenetic disorder that results in early transient respiratory distress and hypotonia which is followed by mental retardation and hyperphagia (684-686).

Given the structural homology of NRAGE and Necdin, it is likely that NRAGE and Necdin mediate cell cycle effects through a shared mechanism. The effect of NRAGE on both cell cycle and apoptosis raises the intriguing possibility that the apoptotic effect of p75NTR may be secondary to cell cycle disruption. It is interesting in this regard that SC-1 produces growth arrest when overexpressed

(636) and that ZacI, an NRIF homologue, mediates cell cycle arrest and apoptosis (687). It is conceivable, therefore, that p75NTR-initiated apoptotic signals are transduced through a complex of molecules which may activate a cell cycle-related mechanism distinct from the apoptotic pathway used by other members of the TNF receptor superfamily. A link between apoptosis and cell cycle disruption has been shown in several neuronal death paradigms and it is becoming increasingly accepted that cell cycle disruptions may be an important mechanism for initiating neuronal death (688-691). Recent findings indicate that p75NTR-mediated death of retinal cells is associated with an increase in cyclin B2 levels and can be blocked using roscovitine, a CDK inhibitor (692), consistent with the hypothesis that p75NTR mediates apoptosis through a cell cycle dependent pathway that may involve NRAGE. p75NTR and NRAGE are coexpressed in many central and peripheral ganglia as well as in the intermediate zone, where p75NTR has been shown to play a role in developmental apoptosis (627). It may be significant that p75NTR expression in the developing spinal cord occurs in the region where neurons are undergoing their terminal mitosis, since it is conceivable that p75NTR may contribute to cell cycle exit as well as apoptosis. It is likely that studies that directly address the role of p75NTR in early nervous system development will provide new insights into the physiological role of the receptor.

In conclusion, NRAGE binds p75NTR under physiological conditions, antagonizes the association of p75NTR with TrkA, inhibits cell cycle progression and facilitates p75NTR-mediated apoptosis. The discovery of NRAGE as a p75NTR interactor is consistent with the hypothesis that p75NTR mediates neuronal apoptosis through interactions with cell cycle regulatory events.

A

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Rat :     1 MAQKPDGGAGLRGFQAEASVEDSALLVQTLMEAIQISEAPPTSQATAAASGPNASPQSSQ 60
Human     1 MAQKMDCGAGLLGFQAEASVEDSALLMQTLMEAIQISEAPPTNQATAAAS-----PQSSQ 55

Rat      61 PPTANEKADTEVSAAAARPKTGFKAQNTTTKGPN---DYSQARNAKEMPKNQPKVAFKSQ 117
Human    56 PPTANEMADIQVSAAAARPKSAFKVQNATTKGPNGVYDFSQAHNAKDVPNTQPKAAFKSQ 115

Rat     118 NATSKGPHAASDFSHAASTGKSAAKKSEMAFKGQNTTTKAGPSATYNFTQSPSANEMTNN 177
Human   116 NATSKGPNAAYDFSQAATTGELAANKSEMAFKAQNATTKVGPNATYNFSQSLNANDLANS 175

Rat     178 QPKTA-KAWNDTTKIPGADAQTQNVNQAKMA----DVGTSAGISETDGAAAQTSADGSQA 232
Human   176 RPKTPFKAWNDTTKAPTADTQTQNVNQAKMATSQADIETDPGISEPDGATAQTSADGSQA 235

Rat     233 QNVESRTIIRGKRTRKINNLNVEENSNGDQRRASLASGNWRSAPVPVTTQNPPGAPPNVL 292
Human   236 QNLESRTIIRGKRTRKINNLNVEENSSGDQRRAPLAAGTWRSAPVPVTTQNPPGAPPNVL 295

Rat     293 WQTPLAWQNPSGWQNQTARQTPPARQSPPARQTPSAWQNPVAWQNPVIWPNPVIWQNPVI 352
Human   296 WQTPLAWQNPSGWQNQTARQTPPARQSPPARQTPPAWQNPVAWQNPVIWPNPVIWQNPVI 355

Rat     353 WPNPIVWPGPIVWPNPWAWQSTPGWQSPPSWQAPPSWQSPQQWQGPPDWQLPPDWSWPPD 412
Human   356 WPNPIVWPGPVVWPNPLAWQNPPGWQTPPGWQTPPGWQGPPDWQGPPDWPLPPDWPLPPD 415

Rat     413 WSFPSDWPFPPDWIPADWPIPPDWQNLRPSPNLRSSPNSRASQNQGPPQPRDVAL    472
Human   416 WPLPTDWPLPPDWIPADWPIPPDWQNLRPSPNLRPSPNSRASQNPGAAQPRDVAL    475

Rat     473                                                           532
Human   476                                                           535

Rat     533                                                           592
Human   536                                                           595

Rat     593                                                           652
Human   596                                                           655

Rat     653     DWTAQFMEAADEALDALDAAAAEAEARAEARNRMGIGDEAVSGPWSWDDIEFELL 712
Human   656     DWTAQFMEAADEALDALDAAAAEAEARAEARTRMGIGDEAVSGPWSWDDIEFELL 715

Rat     713 TWDEEGDFGDPWSRIPFTFWARYHQNARSRFPQAFTGPIIGPSGTATANFAANFGAIGFF 772
Human   716 TWDEEGDFGDPWSRIPFTFWARYHQNARSRFPQTFAGPIIGPGGTASANFAANFGAIGFF 775

Rat     773 WVE 775
Human   776 WVE 778
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B

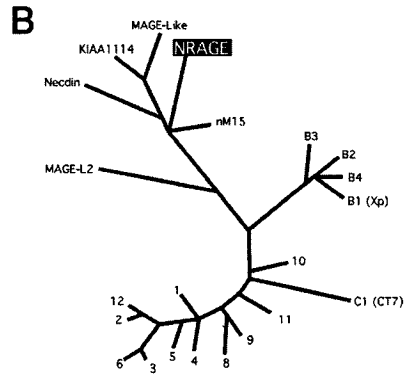


Figure 2.1. NRAGE amino acid sequence and homologies. A. Alignment of rat and human NRAGE amino acid sequences. The lightly shaded area shows the interspersed repeat domain and the darker area indicates the MAGE homology domain. The line indicates amino acids 410 to 709 which reflects the fragment initially identified by two hybrid screening. B. Dendrogram showing the relatedness of the MAGE homology domains of NRAGE and other MAGE family members.

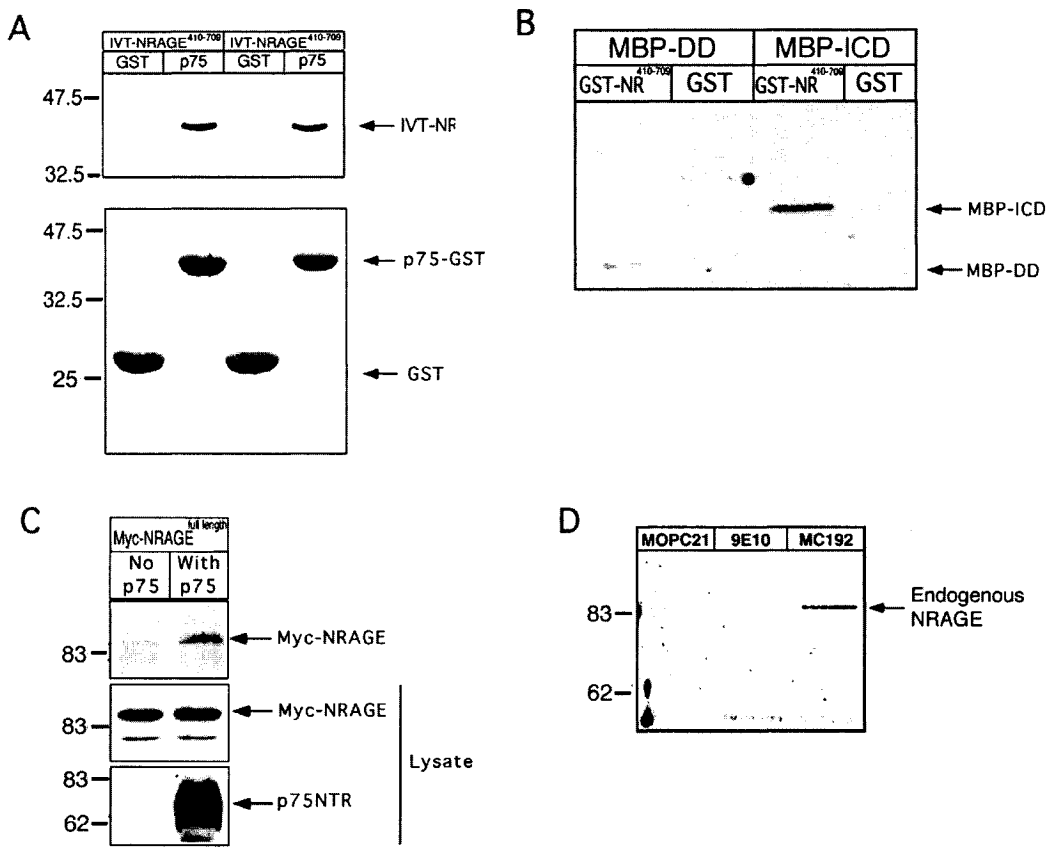


Figure 2.2. NRAGE binds p75NTR in vitro and in vivo. A. GST or GST-p75NTR-ICD fusion proteins prebound to glutathione beads were incubated with 35S-labelled NRAGE$^{410-709}$ produced by in vitro transcription/translation. Pellets were precipitated, washed, and bound proteins were visualized by fluorography after SDS-PAGE. Two repeats of identical experiments are shown. B. Equal amounts (~10 ug) of MBP-DD (lanes 1 and 2) or MBP-ICD (lanes 3 and 4) fusion proteins were incubated with GST or GST-NRAGE$^{410-709}$ (denoted GST-NR) prebound to glutathione beads. Interacting proteins were detected by Western blotting using an anti-MBP antibody. C: COS7 cells were transfected with full-length myc-tagged NRAGE in the presence and absence of p75NTR. Cells were lysed and immunoprecipitated with a p75NTR monoclonal antibody and immunoblotted with 9E10 to detect the myc-tagged protein. The top panel shows that NRAGE was co-immunoprecipitated only when p75NTR was present. Expression of NRAGE and p75NTR in cell lysates are shown in the middle and bottom panels, respectively. D: p75NTR was immunoprecipitated from lysates of PC12nnr5 cells with MC192 and immunoblotted with a polyclonal antibody against NRAGE N-terminus. MOPC21 and 9E10 were used as isotype specific antibody controls for the immunoprecipitation. Each of the experiments shown in panels A-D have been repeated a minimum of 3 times with identical results.

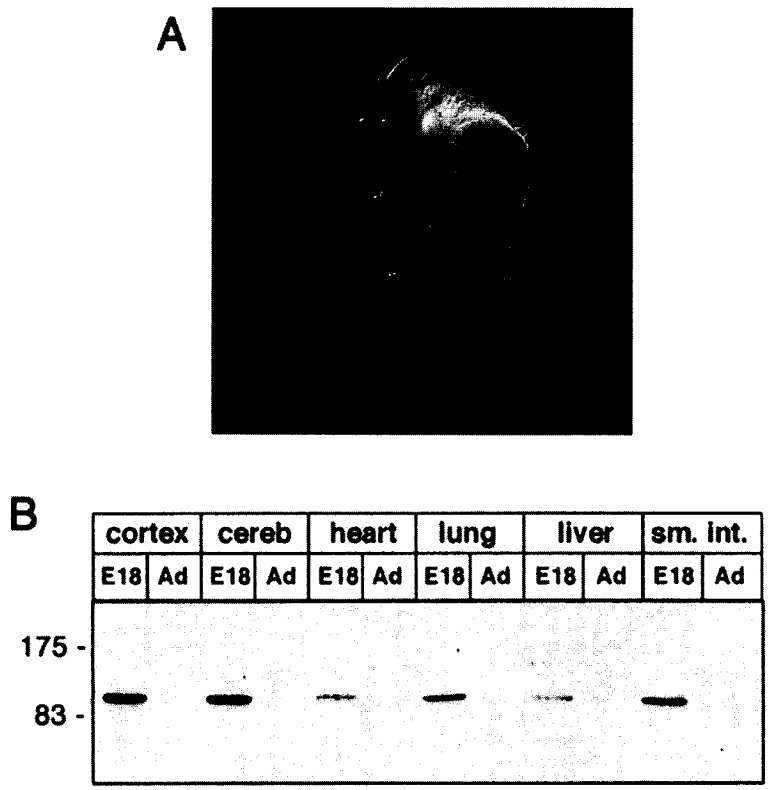


Figure 2.3. Developmental and Tissue-Specific Expression of NRAGE.
A. Whole mount in situ hybridization of E11.5 rat embryos shows that NRAGE mRNA is enriched in the developing nervous system. B. Twenty micrograms of tissues lysates derived from E18 rat embryos and adult rats (>3 months) were separated by SDS–PAGE and immunoblotted using an affinity-purified NRAGE polyclonal antibody directed against C-terminal peptide 1 (see Experimental Procedures). Expression is particularly high in the developing CNS and is reduced in all tissues with increased age.

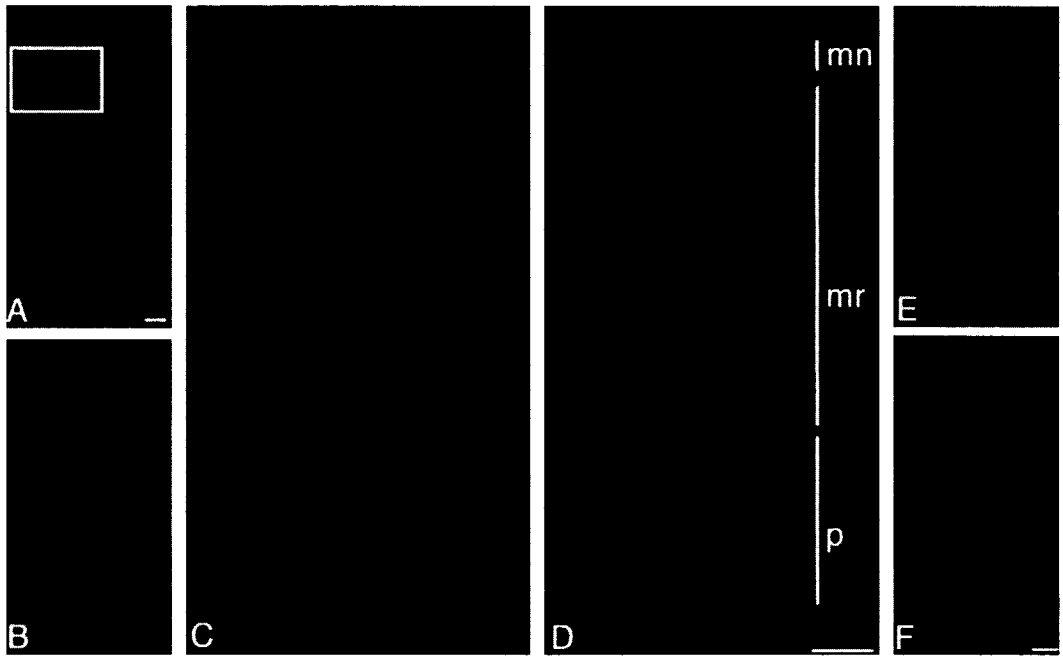


Figure 2.4. NRAGE and p75NTR Are Coexpressed in the Developing Nervous System. Double-label immunocytochemistry was performed using affinity-purified antibodies directed against NRAGE (A, D, and F) and using MC192, a p75NTR-specific monoclonal antibody (E and F). (A) through (D) show NRAGE and p75NTR within E14 medulla oblongata ([C] and [D] approximate the boxed area shown in [A]) and (E) and (F) show NRAGE and p75NTR within the E14 facial motor nucleus. (B) was stained with purified nonspecific rabbit IgG using the same conditions as for NRAGE staining. Bars in (A), (D), and (F) represent 500, 50, and 30 μM, respectively. Experiments in (A) through (H) have been repeated at least twice with identical results. Abbreviations: mn, mantle zone; mr, marginal zone; p, proliferative zone.

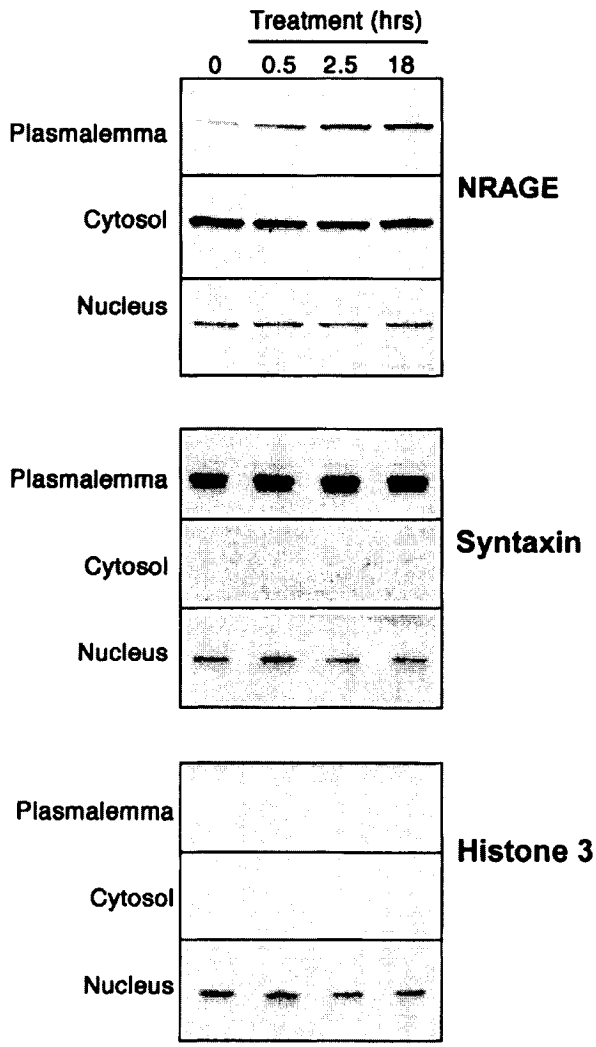


Figure 2.5. The Cellular Localization of NRAGE Changes with p75NTR Activation. PC12nnr5 cells were treated with NGF (100 ng/ml) for indicated times and then lysed in detergent-free buffer and fractionated by differential centrifugation. Fractions (20 μg) were analyzed by immunoblot using antibodies directed against NRAGE, syntaxin, or Histone 3. This experiment has been repeated three times with similar results.

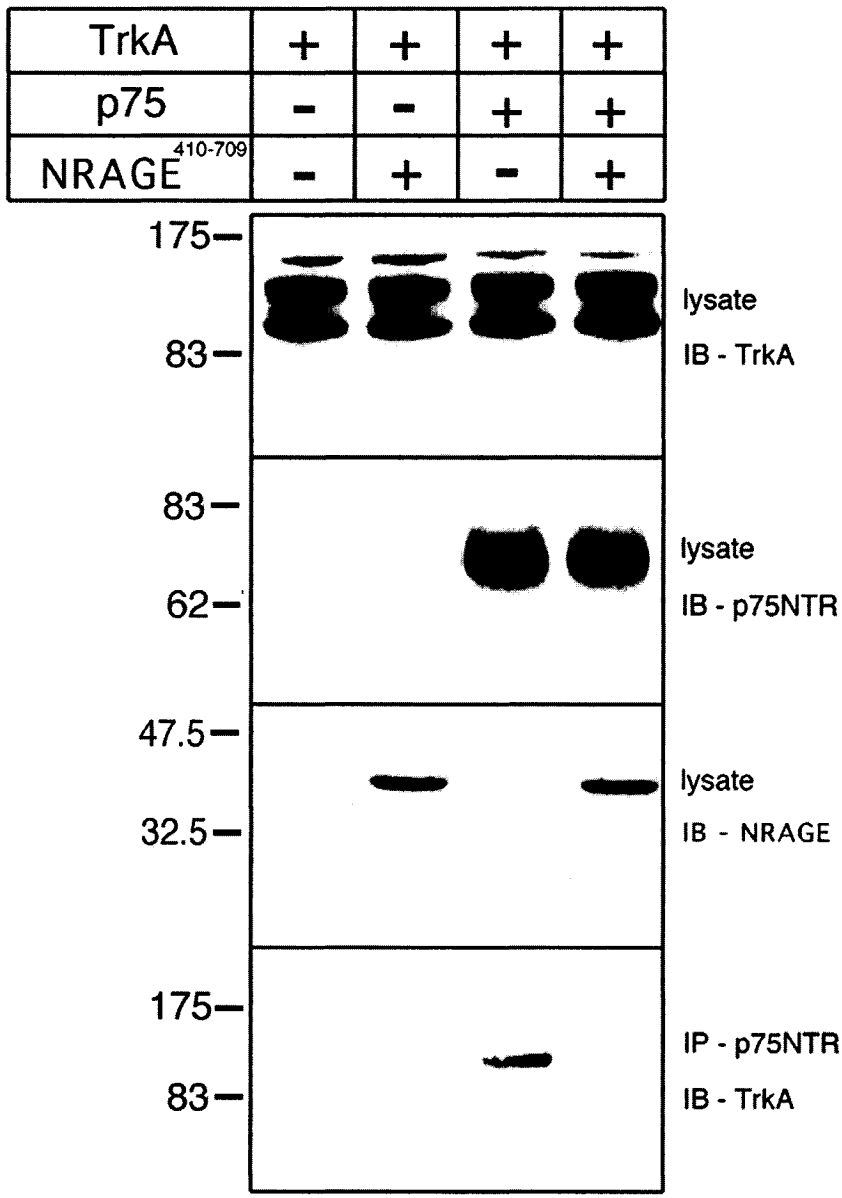
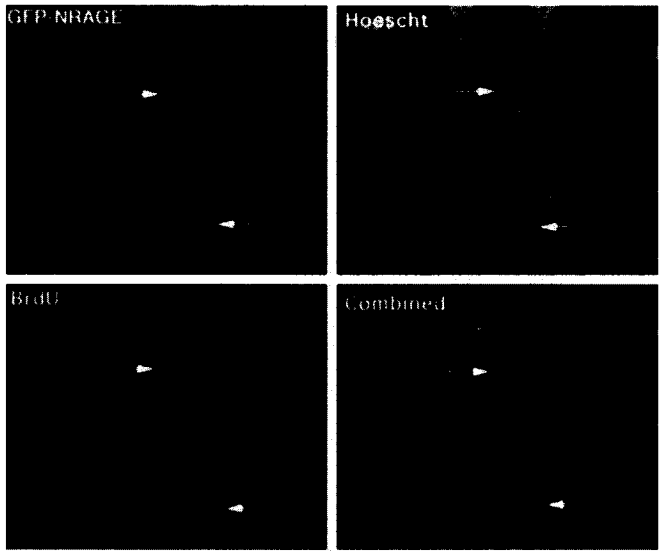


Figure 2.6. Overexpression of NRAGE disrupts a p75NTR-trkA complex. TrkA was NRAGE$^{410-709}$ and p75NTR. p75NTR was immunoprecipitated with REX and immunoprecipitates were examined for the presence of co-immunoprecipitating trkA by immunoblotting using a polyclonal anti-TrkA antibody (RTA). This experiment has been repeated four times with similar results.



	BrdU positive (%)	BrdU negative (%)
Control vector	39	61
GFP-NRAGE	4	96

Figure 2.7. NRAGE overexpression results in cell cycle arrest. A. 293 cells transfected with a GFP-NRAGE fusion protein for 36 hours were incubated with BrdU for 1 hour and then fixed for immunocytochemistry. GFP fluorescence does not withstand the conditions used for BrdU detection and therefore the NRAGE-GFP fusion protein was detected immunocytochemically using a GFP-specific antibody. GFP-NRAGE positive and negative cells are indicated by right and left arrows, respectively, in all panels. Top left panel shows GFP-NRAGE, bottom left panel shows BrdU staining and top right panel shows nuclear DNA stained with Hoescht 33258. Bottom right panel is an overlay of panels A, B and C and shows that the GFP-NRAGE containing cells are consistently BrdU negative. B. Cells were scored for BrdU labeling as a function of NRAGE expression by a naïve observer (n>200 per experiment). Experiments in A and B have been repeated three times and the result of cell counts from a representative experiment are shown.

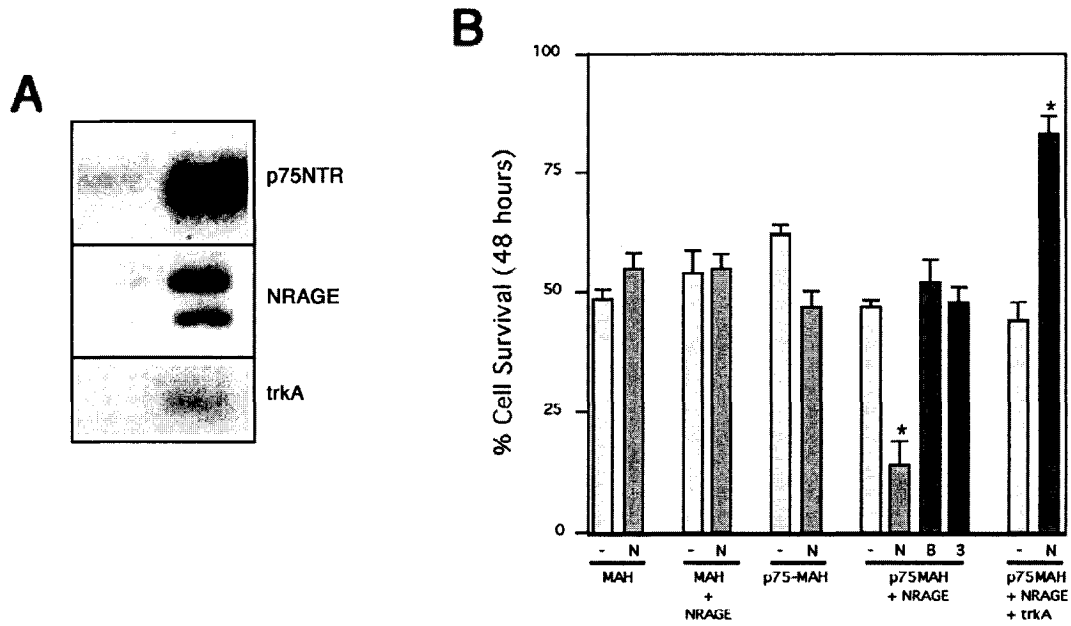


Figure 2.8. NRAGE and p75NTR overexpression results in cellular apoptosis in MAH cells. A. Untransfected MAH cells, MAH cells stably expressing p75NTR or MAH cells co-expressing p75NTR and trkA were infected with control retrovirus or with retrovirus driving NRAGE cDNA. After selection in G418, individual lines were pooled. A. Control MAH cells were compared to MAH cells expressing p75NTR, NRAGE, and trkA by immunoblot. B. Transfected MAH cells were assayed for neurotrophin-dependent survival after exposure to 100 ng/ml neurotrophin for 48 hours; 'N' = NGF, 'B' = BDNF, '3' = NT-3. Standard deviations shown; '*' = $p < 0.01$.

PREFACE TO CHAPTER 3

As discussed in Chapter 1, death induced by ligand-binding to p75NTR is known to take place through JNK-dependent activation of the intrinsic apoptotic pathway. This pathway can also be replicated, in the absence of exogenous ligand, by adenoviral mediated overexpression of p75NTR in PC12 cells (441, 632). We propose that if NRAGE indeed does lie on the p75NTR apoptotic signaling pathway, the signaling pathway initiated by NRAGE should correspond to that initiated by p75NTR. Therefore to confirm the congruency of the two pathways, in the following study we examined the signaling cascade initiated by NRAGE. As a model system to study the NRAGE-mediated signaling pathway, we adenovirally overexpressed NRAGE in a PC12 cell-line known to express p75NTR endogenously. As expected, death induced by NRAGE overexpression took place through a JNK-dependent intrinsic pathway, identical to that reported for p75NTR.

CHAPTER 3

NRAGE, a p75 Neurotrophin Receptor Interacting Protein, Induces Caspase Activation and Cell Death Through a JNK-Dependent Mitochondrial Pathway.

NRAGE, a p75 Neurotrophin Receptor Interacting Protein, Induces Caspase Activation and Cell Death Through a JNK-Dependent Mitochondrial Pathway.

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3.0 Abstract

The p75 neurotrophin receptor (p75NTR) mediates signaling events leading to activation of the JNK pathway and cell death in a variety of cell types. We recently identified NRAGE, a protein that directly interacts with the p75NTR cytosolic region and facilitates p75NTR-mediated cell death. For the present study, we developed an inducible recombinant NRAGE adenovirus to dissect the mechanism of NRAGE-mediated apoptosis. Induced NRAGE expression resulted in robust activation of the JNK pathway which was not inhibited by the pharmacological Mixed Lineage Kinase (MLK) inhibitor CEP1347. NRAGE induced cytosolic accumulation of Cytochrome c, activation of Caspases-3, -9 and -7 and caspase-dependent cell death. Blocking JNK and c-Jun action by overexpression of the JNK-binding domain of JIP1 or dominant-negative c-Jun ablated NRAGE-mediated caspase activation and NRAGE-induced cell death. These findings identify NRAGE as a p75NTR interactor capable of inducing caspase activation and cell death through a JNK-dependent mitochondrial apoptotic pathway.

3.1 Introduction

The neurotrophins are a family of growth factors involved in the survival, development, and death of specific populations of neurons and non-neuronal cells. Their effects are mediated by binding to cell surface Trk tyrosine-kinase receptors and to the p75 neurotrophin receptor (p75NTR). Roles for Trk receptors in neurotrophin-dependent growth, survival and synapse function are well established, but the precise physiological functions of p75NTR are still being defined. p75NTR can act as a co-receptor for Trk receptors, but p75NTR is also expressed in many cells that lack catalytically active Trk, where it functions autonomously to mediate neurotrophin signaling events (665).

Numerous studies over the last several years have shown that p75NTR can induce apoptosis in a variety of settings. For example, p75NTR-dependent cell death has been observed in cultured trigeminal neurons (599), embryonic hippocampal neurons (525), neonatal sympathetic neurons (493), neonatal Schwann cells (600), neuroblastoma cells (693), and within explants of otic vesicles (694). *In vivo*, overexpression of the intracellular domain of p75NTR in transgenic mouse neurons results in widespread apoptosis of peripheral and central neurons (649). Accordingly, disruption of NGF binding to p75NTR reduces apoptosis in the retina (626), and mice lacking full-length p75NTR exhibit decreased apoptosis in the developing spinal cord and retina (627) and display increased numbers of sympathetic and sensory neurons following the normal period of naturally occurring cell death (493, 695).

The precise signaling events that link p75NTR activation to apoptotic cascades remain uncertain but several findings suggest that activation of the JNK pathway may play a key role. A number of studies have shown that p75NTR-dependent apoptosis correlates with an increase in Jun kinase (JNK) activity (493, 523-525, 632), and some studies have demonstrated that blockade of the JNK pathway with chemical inhibitors (524, 525) or dominant-negative forms of JNK (526) attenuates p75NTR-dependent death in oligodendrocytes and hippocampal neurons.

Several p75NTR interacting proteins have been recently identified (665). Some of these, including NRIF (639) and NADE (638), appear to facilitate p75NTR-dependent apoptosis, but to date, p75NTR interactors have not been linked to specific apoptotic cascades. We recently demonstrated that NRAGE, a novel member of the MAGE family, binds to p75NTR under physiological conditions and facilitates p75NTR-dependent cell death (chapter 2). For the present study, we developed an overexpression paradigm to examine signaling pathways activated by NRAGE. Our data indicates 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. NRAGE-induced apoptosis correlates with MLK-independent activation of JNK and with c-Jun phosphorylation. Accordingly, blockers of JNK activity or c-Jun mediated transcription inhibit NRAGE-dependent caspase activation and reduce apoptosis. Together, these data demonstrate that the p75NTR interactor NRAGE activates a mitochondrial apoptotic cascade through a JNK- and c-Jun-dependent pathway.

3.2 Materials and Methods

Materials. zVAD-fmk was purchased from Enzyme System Products. CEP1347 was obtained from Aegera Therapeutics. Cell culture reagents were purchased from BioWhittaker, unless otherwise indicated. The JNK1 antibody (C-17, cat# sc-474) and IκBα antibody (C-12, cat# sc-371) were purchased from Santa Cruz Biotechnology. Anti-Flag antibody (M2, cat# F-3165) was obtained from Sigma. Cytochrome c antibody was purchased from Pharmingen (cat# 556433) and anti-HA antibody (12CA5, cat# 1583816) was purchased from Roche. Phospho-Thr^{183}/Tyr^{185} JNK (G9, cat# 9255), phospho-Ser^{63} c-Jun (II, cat#9261), Caspase-9 (cat# 9502), cleaved Caspase-3 (Asp175, cat#9661), cleaved Caspase-7 (Asp198; cat#9491), and cleaved PARP (Asp214; cat#9541) specific antibodies were obtained from Cell Signaling Technology. The antibody raised against the amino-terminus of NRAGE has been previously described (chapter 2). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Immunoreactive bands were detected using enhanced chemiluminescence purchased from PerkinElmer Life Sciences. All other reagents were from Sigma or ICN Biochemicals, unless otherwise indicated.

Preparation of recombinant adenovirus. Preparation of recombinant adenovirus expressing enhanced green fluorescence protein (EGFP), β-galactosidase and the myristoylated p75NTR intracellular domain (mICD) has been previously described (632). Recombinant adenoviruses expressing Flag-tagged JNK-binding domain of JIP1, Flag-tagged dominant negative c-Jun, and HA-epitope tagged MLK-3 were provided by Dr. Aviva Tolkovsky, Aegera Therapeutics, and David Kaplan, respectively, and their construction has been described elsewhere (196, 387, 696). To produce recombinant adenovirus driving the Tet-inducible expression of Myc-epitope tagged full-length NRAGE, full-length rat NRAGE cDNA was subcloned into the vector CMV 5' TetO and recombinant virus was generated and plaque purified in 293A cells. All viruses were amplified in 293A cells and purified on a sucrose gradient, as previously described (632). Viruses were then titered by optical density and using the tissue

culture infectious dose 50 (TCID) assay in 293A cells (697). Titers are expressed in term of plaque forming units.

Cell culture and infection. The PC12^{rtTA} cell line was purchased from Clontech (cat# C3015-1) and maintained in 10% CO_2 at 37° C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Clontech), 5% horse serum, 2 mM L-glutamine, 100 μg/ml penicillin/streptomycin and 100 μg/ml G418. Cells were plated 18 to 24 hours prior to infection. Where indicated, 1 μg/ml of doxycycline was added to the plates at time of infection.

Caspase activity assay. Forty-eight hours post-infection, cells were harvested on ice, washed with cold phosphate-buffered saline and caspase activity was measured at room temperature using the fluorescent substrate DEVD-amino-methyl-coumarin (AMC) at a concentration of 10 μM as described previously (698).

Cytochrome c release assay. Cytosol enriched subcellular fractions were prepared using a modification of a previously described protocol (699). In brief, five million cells were harvested, washed once in Tris-buffered saline (10 mM Tris (pH 8.0), 150 mM NaCl), once in Buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS (pH 7.4)), and then resuspended in 500 μl Buffer B (Buffer A plus 5% Percoll, 0.01% digitonin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride). A sample of this suspension was retained as total cell lysate. The remainder was incubated on ice for 15 minutes and then centrifuged at 2500 g for 10 minutes to remove intact cells and nuclei. The supernatant was then centrifuged at 15 000 g for 15 min to pellet mitochondria. The final supernatant was designated cytosol.

Immunoblotting. Cells were lysed in RIPA buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 μg/ml Aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride) and analyzed for protein content using the BCA assay (Pierce). Samples were normalized for protein content, suspended in Laemmli sample buffer, separated by SDS-polyacrylamide gel electrophoresis,

and electroblotted onto nitrocellulose. Blocking and secondary antibody incubations of immunoblots were performed in Tris-buffered saline/Tween (10 mM Tris (pH 8.0), 150 mM NaCl, 0.2% Tween 20) supplemented with 5% (w/v) dried skim milk powder. All primary antibody incubations were performed in the blocking solution, except for those involving phospho-specific antibodies which were performed in Tris-buffered saline/Tween supplemented with 5% (w/v) bovine serum albumin. Immunoreactive bands were detected by chemiluminescence (PerkinElmer Life Sciences), according to the manufacturer's instructions.

Survival assay. Analysis of cell survival was performed by MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which was added at a final concentration of 1 mg/ml for the last four hours of a two day infection. The reaction was ended by the addition of one volume of solubilization buffer (20% SDS, 10% dimethylformamide, and 20% acetic acid). After overnight solubilization, specific and non-specific absorbances were read at 570 and 690 nm, respectively. In each experiment, each data point was performed in triplicate or quadruplicate, and experimental results were analyzed by multiple analyses of variance with statistical probabilities assigned using the Tukey test for multiple comparisons. Each experiment was carried out independently at least three times. In the case of MTT assays that were accompanied by parallel LDH experiments, cells were treated as described below prior to infection.

Death assay. Analysis of cell death was performed by the lactate dehydrogenase (LDH) assay using the cytotoxicity detection kit (Roche, cat# 1644793) according to the manufacturer's instructions. In brief, prior to infection, cells were washed three times with an equal volume of $PC12^{rtTA}$ media, and then the media serum concentration was reduced by 20% by the addition of DMEM. Forty hours after infection, cell cultures were centrifuged at 500 *g*, and the supernatant was added to an equal volume of LDH reaction mixture supplied by the manufacturer (Roche). After 15-30 minute incubation, specific and non-specific absorbances were read at 490 (OD490) and 690 (OD690) nm respectively. Values reported were calculated using the following formula:

OD490 - (OD490 of media-alone control) - OD690 nm. Each data point was performed in triplicate or quadruplicate, and experimental results were analyzed by multiple analyses of variance with statistical probabilities assigned using the Tukey test for multiple comparisons. Each experiment was carried out independently at least three times.

3.3 Results

An Inducible NRAGE Adenovirus Mediates Cell Death

To investigate signaling pathways involved in NRAGE-mediated cell death, we constructed a recombinant adenovirus driving the expression of full-length NRAGE (AdNRG). Previous attempts to produce virus in which expression of an NRAGE fragment was driven by a constitutively active CMV promoter had proven unsuccessful, presumably due to a pro-apoptotic effect of NRAGE. Therefore, we designed an adenovirus that would allow for the doxycycline-inducible expression of full-length NRAGE (tagged with an amino-terminal myc epitope: myc-NRAGE) in cells co-expressing a doxycycline-regulated trans-acting factor, rtTA. For our studies, PC12 cells stably expressing the doxycycline-regulated trans-acting factor were used ($PC12^{rtTA}$). Figure 3.1A shows that NRAGE expression in AdNRG-infected cells is minimal in the absence of doxycycline, but is strongly induced following addition of 1 μg/ml of doxycycline. A small amount of 'leaky' NRAGE expression occurs, even in the absence of doxycycline, but only at high multiplicities of infection (MOIs).

To determine if NRAGE induces cell death, $PC12^{rtTA}$ cells were infected with AdNRG in the absence or presence of 1 μg/ml doxycycline. AdNRG-infected $PC12^{rtTA}$ that were treated with doxycycline displayed dose-dependent cytoplasmic condensation, cell shrinkage and detachment from the plate (data not shown). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assays performed to quantify this effect indicated that cells infected with AdNRG and treated with doxycycline displayed a significant loss in cell viability at all MOIs tested (Fig. 3.1B). In contrast, cells infected with a control virus expressing enhanced green fluorescence protein (AdGFP) showed no significant loss of viability in presence or absence of doxycycline (Fig. 3.1B). As expected (632), cells treated with a virus that constitutively expresses a myristoylated form of the p75NTR intracellular domain (mICD) showed reduced viability which was unaffected by doxycycline (Fig. 3.1B). Together, these data indicate that NRAGE overexpression induces $PC12^{rtTA}$ cell death.

NRAGE-Induced Cell Death Is Caspase-Dependent

Most forms of apoptosis converge on caspases as downstream effectors (700). To determine if caspases are activated during NRAGE-mediated cell death, a fluorogenic synthetic peptide (DEVD-AMC) cleavage assay (701) was used to assess caspase activity (Fig. 3.2A). Doxycycline treatment had no effect on caspase activity in control uninfected cells, and cells infected with a control AdGFP virus did not display increased caspase activity, relative to control uninfected cells, in either the absence or presence of doxycycline. In contrast, cells infected with AdNRG and treated with doxycycline showed a robust induction of DEVD cleavage activity.

Caspases are present within the cell in zymogen forms which, following an apoptotic signal, undergo proteolytic cleavage to produce active caspases (700). To identify specific caspases activated by NRAGE overexpression, cells were either left uninfected, or were infected with AdGFP or AdNRG, in the absence or presence of doxycycline. Cells were then lysed, and analyzed by immunoblot for levels of the intact Caspase-9 zymogen and for cleavage/activation of effector Caspases-3 and -7. Figure 3.2B shows that doxycycline-induced NRAGE expression results in the reduction in levels of the Caspase-9 zymogen, a corresponding increase in activated Caspase-3 and Caspase-7, and the cleavage of Poly(ADP-ribose) polymerase (PARP), a dual Caspase-3/7 substrate (700). Uninfected cells or cells infected with AdGFP showed no evidence of caspase activation in either the absence or presence of doxycycline. Together, these results indicate that NRAGE is a potent inducer of Caspase-9, an initiator caspase, and Caspases-3 and -7, downstream effector caspases.

To confirm that caspase activation is necessary for NRAGE-mediated cell death, viability of cells overexpressing myc-NRAGE was examined after treatment with either Boc-Asp-fmk (BAF) or zVAD-fmk, both broad-spectrum inhibitors of caspase activity (701). MTT survival and lactate dehydrogenase (LDH) death assays both revealed that NRAGE-induced death is significantly inhibited in the presence of BAF or zVAD-fmk (Fig. 3.2C), confirming a role for caspase activity in NRAGE-induced cell death. Together, these data establish that

NRAGE induces cell death through an apoptotic mechanism involving caspase activation.

NRAGE-induces caspase-independent release of Cytochrome c from mitochondria

Cleavage of the Caspase-9 zymogen requires formation of a complex consisting of Caspase-9, APAF-1 and cytosolic Cytochrome c (700). Release of Cytochrome c from mitochondria into the cytosol is a key regulatory step in this process (702). To determine if Cytochrome c is released during NRAGE-induced apoptosis, cells were left uninfected, or were infected with either AdNRG, or with a control adenovirus driving constitutive expression of β-galactosidase (AdLacZ), in the presence of doxycycline (Fig. 3.2D). Cells were then lysed, subjected to subcellular fractionation, and cytosolic fractions were analyzed for Cytochrome c levels by immunoblot. Cytochrome c was not detected in the cytosol of uninfected cells or in cells infected with AdLacZ. However, high levels of Cytochrome c were detected in the cytosol of cells expressing myc-NRAGE (Fig. 3.2D), indicating that NRAGE overexpression induces Cytochrome c release from the mitochondria.

In some models of apoptosis, Cytochrome c release may be a secondary event that occurs after caspase activation (134, 700). To determine if NRAGE-induced release of Cytochrome c from mitochondria is independent of caspase activation, the effect of the caspase inhibitor zVAD-fmk on the accumulation of cytosolic Cytochrome c was examined. Figure 3.2D shows that zVAD-fmk did not reduce NRAGE-mediated cytosolic Cytochrome c accumulation, indicating that NRAGE-induced Cytochrome c release is not a consequence of caspase activation, but instead lies upstream of caspase activation.

NRAGE activates JNK through an MLK-independent pathway

Activation of Jun kinase (JNK) and consequent transcriptional events play a necessary role in several neuronal cell death paradigms that involve Cytochrome c release (392, 703, 704) such as p75NTR-dependent apoptosis (525, 526). To

determine if JNK is activated during NRAGE-induced apoptosis, PC12^{rtTA} cells were left uninfected or were infected with AdNRG, or the control viruses AdGFP or AdLacZ in the presence or absence of 1 μg/ml of doxycycline, then lysed and analyzed by immunoblot for JNK phosphorylation status. Figure 3.3 shows that NRAGE expression induced a robust increase in JNK phosphorylation with no increase in total JNK1 protein levels. JNK activation results in phosphorylation of c-Jun which in turn leads to transcriptional activation of target promoters, including the promoter for c-Jun itself (261, 705). Therefore, to confirm that NRAGE-mediated phosphorylation of JNK was an indication of JNK signaling pathway activation, phosphorylation status and protein levels of c-Jun were determined. NRAGE overexpression resulted in a dramatic increase in both the phosphorylation of c-Jun (visualized by phospho-Jun^{Ser63} immunoblot and by slower migrating band in the total c-Jun immunoblot) and in total c-Jun levels (Fig. 3.3). In contrast, cells infected with AdGFP or AdLacZ showed no alteration in the levels or phosphorylation status of JNK or c-Jun. Under these infection conditions, EGFP is highly expressed by AdGFP and it accumulates to much higher levels than myc-NRAGE (Fig. 3.3, bottom panel), indicating that the effects of NRAGE on the JNK pathway do not reflect overexpression artifacts. Together, these data demonstrate that NRAGE specifically increases JNK activity, leading to the phosphorylation of c-Jun and activation of c-Jun transcriptional activity.

Mixed-lineage-kinases (MLKs) are MAP kinase kinase kinases (MAP3Ks) that have been identified as the upstream activators of the JNK pathway necessary for neurotrophin-withdrawal induced apoptosis (271, 272, 282, 706, 707). CEP1347, an indolocarbazole of the K252a family, is a potent inhibitor of MLKs (707), and blocks neuronal cell death in several neuronal apoptosis paradigms (271, 272, 282, 706, 707). To determine if CEP1347 blocks NRAGE-dependent activation of the JNK pathway, PC12^{rtTA} cells were infected with either AdGFP, AdNRG, or with AdMLK3, a recombinant adenovirus driving the expression of HA-epitope tagged MLK-3, and were then treated with CEP1347 or with a vehicle control. As expected, NRAGE or MLK-3 overexpression induced robust

JNK phosphorylation whereas EGFP overexpression had no discernable effect on JNK phosphorylation status (Fig. 3.4A, compare lanes 2-4). Application of CEP1347 for one hour strongly inhibited MLK3-induced JNK phosphorylation but, surprisingly, had no effect on JNK phosphorylation induced by NRAGE (Fig. 3.4A, compare lanes 3 and 7; 4 and 8). To confirm that CEP1347 was indeed ineffective in blocking NRAGE-dependent activation of the JNK pathway, cells infected with AdNRG, or with AdMLK3, were maintained in CEP1347 for the duration of the infection, and then examined for levels of c-Jun. Figure 3.4B shows that NRAGE and MLK-3 overexpression induced c-Jun expression, presumably as a result of JNK-dependent AP-1 mediated transcription. CEP1347 treatment led to an almost complete block of the MLK3-mediated increase in c-Jun protein levels, but had no effect on accumulation of c-Jun induced by NRAGE. Therefore, it can be concluded that in $PC12^{nTA}$ cells, NRAGE induces JNK activity and c-Jun accumulation through an MLK-independent pathway.

JNK Activation and c-Jun Dependent Transcription Are Necessary For NRAGE-Dependent Apoptotic Signaling.

The above observations are consistent with the hypothesis that NRAGE-mediated activation of the JNK pathway induces c-Jun mediated transcriptional events that result in mitochondrial Cytochrome c release, Caspase-9 activation, and the activation of effector Caspases-3 and -7. To directly address the requirement of JNK activation in NRAGE apoptotic signaling, we employed an adenovirus (AdJBD) encoding the JNK binding domain of the JNK-interacting protein-1, which acts to sequester JNK and thereby inhibit JNK-dependent c-Jun phosphorylation (387). To examine the role of c-Jun dependent transcription on NRAGE-induced apoptosis, we used an adenovirus encoding a c-Jun dominant negative mutant (AdΔJUN) which lacks the c-Jun transactivation domain and functions as an inhibitor of AP-1 activity (196, 269).

To determine if overexpression of either the c-Jun dominant negative mutant or the JNK binding domain of JNK-interacting protein-1 (JIP1) reduces NRAGE-mediated apoptosis, $PC12^{nTA}$ cells treated with AdNRG or AdLacZ were co-

infected with either AdΔJUN, AdJBD or AdLacZ, and their viability was then assessed by MTT survival or LDH death assays (Fig. 3.5C). These experiments were complicated by the finding that these inhibitors of the JNK pathway caused a reduction in $PC12^{rtTA}$ cell viability on their own (Fig. 3.5C, bars d-i), indicating that some basal level of JNK activity is important for viability of the PC12 cell line, as suggested by others (288, 708, 709). Nonetheless, in both assay types, myc-NRAGE expressing cells co-infected with AdΔJUN or AdJBD showed a significant decrease in cell death when compared to myc-NRAGE expressing cells treated with the control virus (Fig. 3.5C, compare k, l to j, or n, o to m). In fact, the viability of myc-NRAGE expressing cells treated with AdΔJUN and AdJBD is indistinguishable from that of cells treated with AdΔJUN and AdJBD in the absence of AdNRG co-infection (Fig. 3.5C, compare n and o to h and i), indicating that expression of these JNK pathway inhibitors completely masks the effects of NRAGE expression on viability. Therefore we conclude that the activation of the JNK pathway is required for the induction of NRAGE-mediated apoptosis.

Biochemical assays were performed in parallel to confirm that the blockade of JNK signaling attenuates the NRAGE apoptotic pathway. $PC12^{rtTA}$ cells were infected with AdNRG together with the JNK pathway inhibitors, lysed and then analyzed by immunoblot for the activation of the JNK pathway and for induction of caspases. As expected, NRAGE expression resulted in the phosphorylation and accumulation of c-Jun, the activation of Caspase-3 and the cleavage of PARP (Fig. 3.5A). As predicted, co-expression of NRAGE with the JNK binding domain of JIP1 blocked NRAGE-induced c-Jun phosphorylation and prevented the accumulation of c-Jun protein. Similarly, co-expression of NRAGE with dominant-negative c-Jun prevented the NRAGE-induced accumulation of c-Jun protein. A reduction in the level of NRAGE-induced phosphorylated c-Jun was also detected which is likely secondary to the reduction in the level of total c-Jun. Most importantly, NRAGE-dependent Caspase-3 activation and PARP cleavage were completely blocked when the JNK pathway was inhibited, demonstrating that the JNK pathway is necessary for mediation of NRAGE-induced apoptosis.

In neuronal death paradigms, JNK activation precedes mitochondrial Cytochrome c release and Caspase-9 activation. However some reports indicate that the JNK pathway can be initiated downstream of caspases (710-712). The data presented in figure 3.5A demonstrates a reduction in Caspase-3 activity and PARP cleavage in myc-NRAGE expressing cells following co-expression of inhibitors of the JNK pathway, suggesting that JNK is an activator, rather than a target, of caspases in the NRAGE-mediated apoptotic pathway. To directly address this possibility, NRAGE-dependent JNK activation was assessed in cells treated with caspase inhibitors. Although both BAF and zVAD-fmk were potent blockers of NRAGE-induced cell death (Fig, 2), neither inhibitor attenuated the NRAGE-mediated activation of c-jun or JNK (Fig. 3.5B), confirming that the observed induction of the JNK pathway lies upstream of caspase activation.

3.4 Discussion

The specific signaling mechanisms employed by p75NTR to induce apoptosis remain uncertain. We have previously demonstrated that NRAGE is a p75NTR-interacting protein that facilitates p75NTR-induced cell death (chapter 2), and in this study, we have analyzed the signaling pathways used by NRAGE to induce apoptosis. By using an inducible adenoviral expression system, we found that apoptosis induced by NRAGE occurs through an intrinsic death pathway that involves mitochondrial Cytochrome c release and cleavage of Caspase-9, an apical caspase, and activation of effector Caspases-3 and -7. We also demonstrated that NRAGE-induced apoptosis is associated with MLK-independent activation of JNK and subsequent phosphorylation and accumulation of c-Jun. Finally, using adenovirus expressing dominant inhibitory proteins, we established that the activities of JNK and the c-Jun transcription factor are critical elements required for NRAGE-induced apoptosis.

Previous studies have indicated that p75NTR, like NRAGE, also activates the JNK pathway (493, 523, 525, 632, 713). Furthermore, Harrington *et al* (526) have demonstrated that dominant-negative JNK can block p75NTR-induced apoptosis in oligodendrocytes. The precise signaling events that link p75NTR-induced JNK activation to caspase activation are not known but p75NTR-induced death can be blocked by overexpression of Bcl-X_L and appears to involve the activation of Caspases-9 and-3 but not Caspase-8 (439, 440, 634). Together, these findings indicate that p75NTR induces apoptosis through a JNK-dependent activation of the mitochondrial death pathway, thus pointing to a substantial overlap between the apoptotic pathway utilized by p75NTR and that induced by NRAGE. This overlap suggests that p75NTR and NRAGE lie on the same signaling pathway, and adds credence to the hypothesis that NRAGE is an important element in p75NTR-induced apoptosis.

A remarkable aspect of NRAGE-mediated activation of the JNK pathway is its insensitivity to the neuroprotective compound CEP1347. CEP1347 has been shown to reduce apoptosis in several neuronal apoptosis paradigms, including that induced following trophic factor withdrawal (271, 272, 282, 706, 707). Recent

studies have revealed that CEP1347 is a potent inhibitor of the MLK family, MAP3Ks which lie on the JNK pathway (707). In this study, we confirm that CEP1347 is very effective in blocking JNK activation induced by MLK3, but also show that NRAGE-dependent activation of the JNK pathway is not affected by this compound, therefore suggesting that in PC12 cells, NRAGE-mediated JNK activation occurs independently of the MLKs. Data reported by Yoon and colleagues (524) suggest a similar MLK-independence in the case of p75NTR-induced JNK activation and subsequent apoptosis of cultured oligodendrocytes. CEP1347 is a potent inhibitor of MLK activity, with maximal inhibition of MLK-induced cell death occurring at a concentration of 100 nM (707). However, Yoon and colleagues found no significant inhibition of p75NTR-mediated death at a CEP1347 concentration of 100 nM, and only a partial inhibition at a concentration of 1 μM (707), suggesting that the observed inhibition of apoptosis was due to an MLK-independent effect of CEP1347. In contrast, Friedman (525) reported a complete inhibition of p75NTR-induced death of hippocampal neurons after treatment with 200 nM of CEP1347, suggesting a role for MLK activity in the p75NTR-induced death in this cell type. These disparate results hint at the existence of cell-type specific differences in the mechanism of the p75NTR-mediated activation of JNK, presumably reflecting cell-specific expression of individual interactor proteins.

NRAGE is a member of the MAGE family of proteins. The MAGE molecules initially identified were exclusively expressed in tumor cells, germ cells or in the early embryo (2). However, subsequent studies revealed that additional family members, including NRAGE, belong to a distinct class of MAGE proteins expressed in several normal fetal and adult tissue. The molecular function of MAGE proteins still remain ill-defined but, the experiments described above suggest a novel role for MAGE proteins in regulation of cell survival through modulation of the JNK pathway. Others have recently detected an interaction between NRAGE and the anti-apoptotic protein XIAP (714) and have proposed that NRAGE may act to sequester and inactivate this protein, leading to a potentiation of caspase-mediated cell death in a promyeloid leukemic cell line.

In this setting, Bcl-2 overexpression had no effect on NRAGE activity (714), suggesting that NRAGE may act downstream of cytochome C release. Together with our results, these observations suggest that NRAGE may contribute to apoptosis through at least two distinct mechanisms.

NRAGE represents the first p75NTR interacting protein that induces cell death through the activation of the JNK pathway. NRIF, another p75NTR interacting protein (639), may play a role in mediating the p75NTR apoptotic signal, as NRIF knock-out mice show a defect similar to p75NTR knock-outs in the apoptosis of retinal neurons (639). However, mechanistic details remain to be defined and, as yet, there is no data directly linking apoptosis mediated by NRIF to that induced by p75NTR. NADE is another p75NTR interactor believed to initiate apoptosis (638). The mechanism of NADE-dependent cell death is also unknown but appears to require a member of the 14-3-3 family (715). Other interactors, such as SC-1 and RhoA have not been proposed to have a role in p75NTR-mediated apoptosis (544, 636), whereas RIP2 and FAP1 may oppose the p75NTR cell death signal by regulating components of the NF-κB pathway (580, 637). TRAF6 has been proposed as a positive regulator of the JNK pathway (580, 716) but its main function as a p75NTR interactor appears to be to promote pro-survival signals via the activation of the NF-κB pathway (582, 641). Therefore, the capacity of NRAGE to mediate JNK-dependent apoptosis indicates that this interactor may be a unique physiological mediator of p75NTR-dependant JNK and apoptotic signaling.

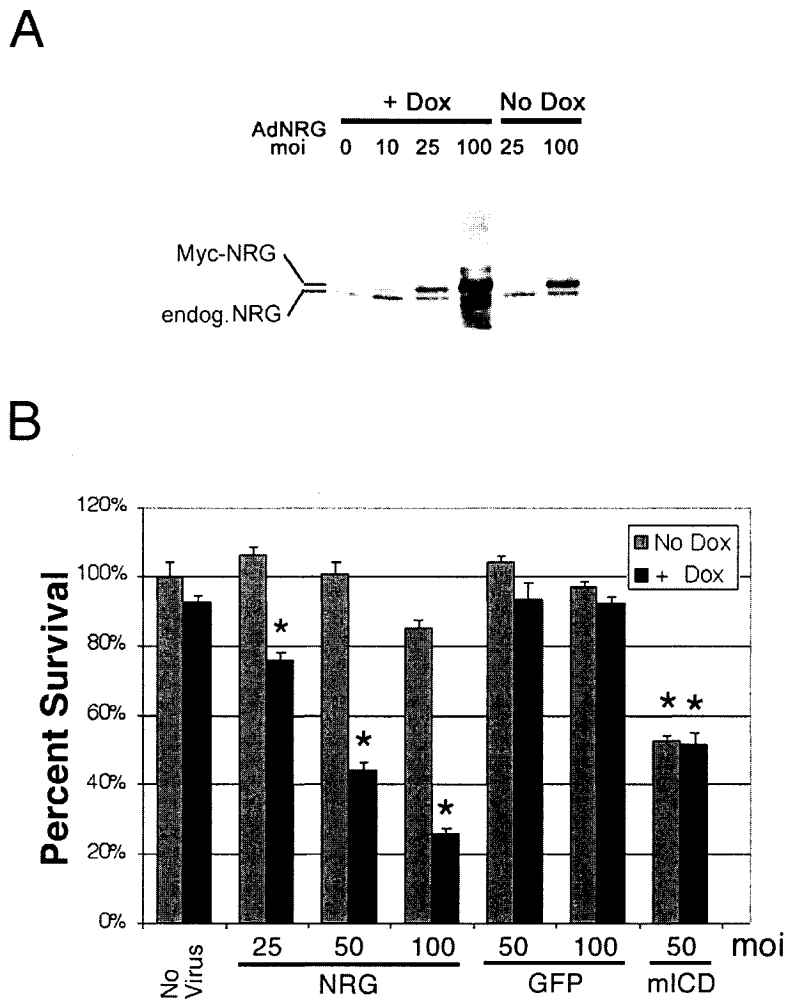

Figure 3.1. Inducible expression of NRAGE in PC12^{rtTA} cells induces cell death. A, PC12^{rtTA} cells were infected with a recombinant adenovirus (AdNRG) expressing myc-epitope tagged full-length NRAGE (myc-NRG) under the control of a Tet-responsive promoter at increasing multiplicities of infection (MOI), in the presence or absence of 1 μg/ml of doxycycline (Dox), as indicated. After 48 hours, cells were lysed and analyzed for the expression of NRAGE by immunoblotting with an NRAGE specific antibody. B, PC12^{rtTA} cells were left uninfected or were infected with AdNRG (NRG) or with AdGFP (GFP), a control recombinant adenovirus expressing the enhanced green fluorescent protein (EGFP) under the control of an unmodified CMV promoter, in the presence or absence of 1 μg/ml of doxycycline as indicated. After 48 hours, cells were assayed for survival using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described under "Experimental Procedures". PC12^{rtTA} cells infected with a recombinant adenovirus expressing the myristoylated p75NTR intracellular domain (mICD) under the control of an unmodified CMV promoter (AdmICD), were treated as above and acted as a positive control for cell death. Results are normalized relative to those obtained with no virus plus Dox, and represent the mean ± standard deviation of a representative experiment performed in quadruplicate (* = P<0.0005 relative to no virus plus Dox).

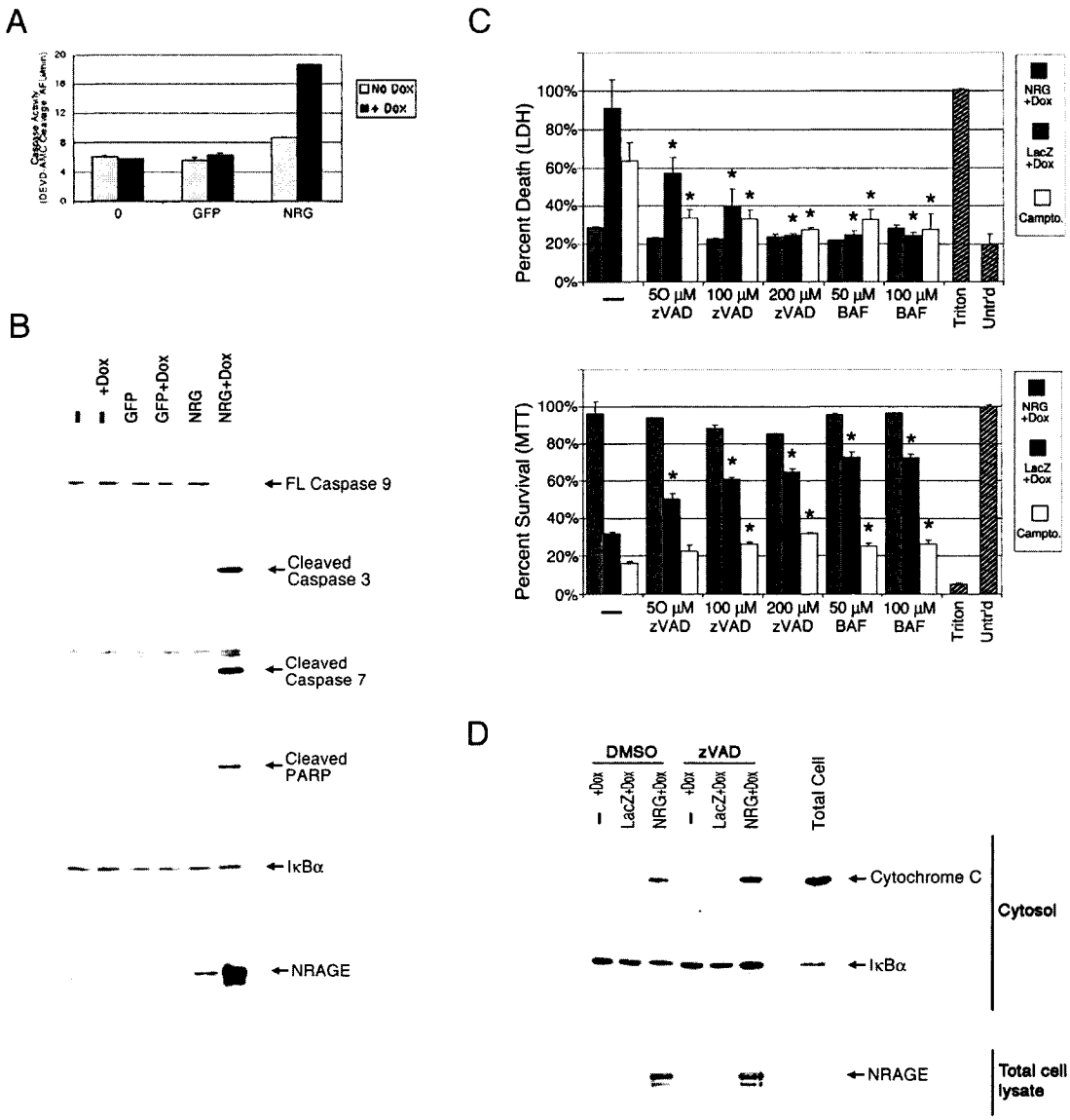

Figure 3.2. NRAGE expression induces cytochrome C release and caspase-dependent cell death. A, NRAGE expression induces caspase activity. PC12^{rtTA} cells were left uninfected, or were infected with 50 MOI of recombinant adenovirus expressing myc-NRAGE (NRG) or EGFP (GFP), in the presence or absence of 1μg/ml of doxycycline. Two days after infection, cells were lysed and 50 μg of cytosolic extracts

were assayed for caspase activity using DEVD-AMC as substrate. The cleavage activity is shown as arbitrary fluorescence units (AFU) per minute. B, NRAGE expression leads to caspase zymogen cleavage. $PC12^{rtTA}$ cells were infected and incubated under the same conditions as in A. Thirty hours after infection, cells were lysed, normalized for protein levels, and analyzed by immunoblotting for levels of NRAGE and full length Caspase-9, and, using cleavage-specific antibodies, for levels of cleaved Caspases-3 and -7 and cleaved PARP. IκBα levels were analyzed by immunoblot to confirm equal loading between lanes. C, Caspase activity is required for NRAGE-mediated cell death. $PC12^{rtTA}$ cells were infected with 50 MOI of recombinant adenovirus expressing myc-NRAGE (NRG) or β-galactosidase (LacZ) in the presence of 1μg/ml of doxycycline, or were treated with camptothecin (100 uM) as a positivc control for caspase-dependent cell death. In addition, cells were incubated with increasing concentrations of zVAD-fmk (zVAD) or Boc-Asp-fmk (BAF), broad spectrum caspase inhibitors, as indicated. After 40 hours, cells were assayed for survival using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and for death using the lactate dehydrogenase (LDH) assay, where untreated cells (Untr'd) and cells treated with 1% Triton X-100 (Triton) were used to delineate the output range of the assays. Results are normalized relative to those obtained with 1% Triton for the LDH assay, or untreated cells for the MTT assay, and represent the mean ± standard deviation of a representative experiment performed in triplicate (* = $P<0.0025$ relative to LacZ plus Dox at 0 μM caspase inhibitor for all gray bars, * = $P<0.0025$ relative to AdNRG plus Dox at 0 μM for all black bars, * = $P<0.0025$ relative to Campto. at 0 μM caspase inhibitor for all white bars). D, NRAGE expression results in caspase independent accumulation of cytochrome C in the cytosol. $PC12^{rtTA}$ cells were left uninfected, or were infected with 50 MOI of AdNRG (NRG) or, as a control, with 50 MOI of recombinant adenovirus expressing β-galactosidase under the control of an unmodified CMV promoter (LacZ). Cells were maintained in 1 μg/ml doxycycline for thirty hours after infection and the cytosolic fraction was then isolated as described in "Experimental Procedures". This procedure was performed in the absence (DMSO) or presence (zVAD) of 100μM of zVAD-fmk as indicated. The cytosolic fractions were normalized for protein content and were subjected to gel electrophoresis, along with a sample of the total cell lysate (Total Cell) obtained from the uninfected cells maintained in the presence of doxycycline, and containing the same amount of protein, followed by immunoblotting with a Cytochrome C specific antibody. The level of IκBα was also analyzed by immunoblotting with an IκBα specific antibody to demonstrate equal loading of the cytosolic fractions. In the bottom panel of D, total cell lysate samples obtained prior to the fractionation procedure were normalized for protein content and immunoblotted with an NRAGE-specific antibody.

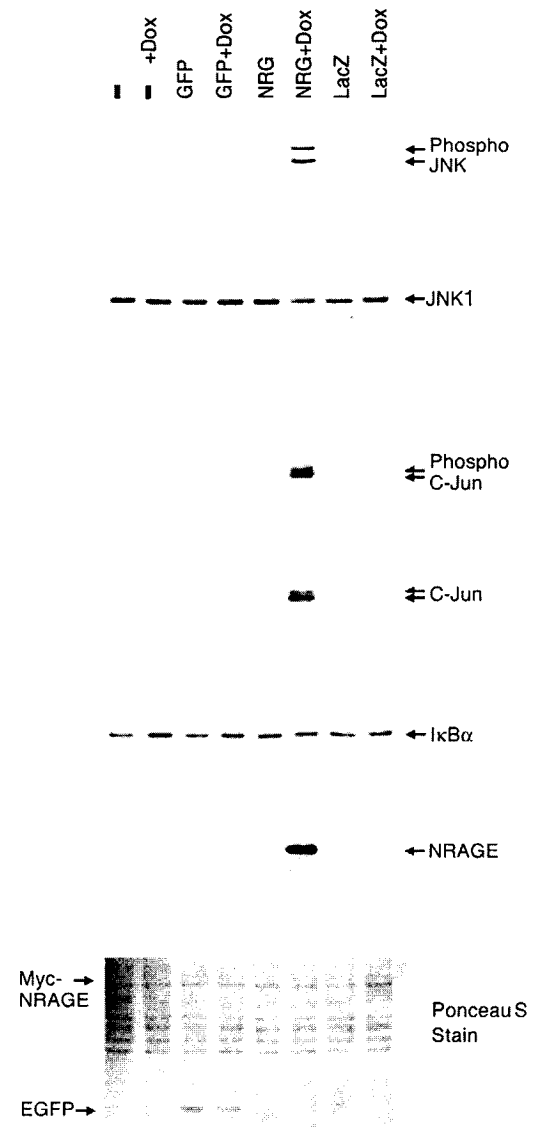

Figure 3.3. NRAGE expression induces the activation of the JNK pathway. PC12^{rtTA} cells were left uninfected, or were infected with 50 MOI of recombinant adenovirus expressing myc-NRAGE (NRG), EGFP (GFP) or β-galactosidase (LacZ) in the presence or absence of 1μg/ml of doxycycline as indicated. Thirty hours after infection, cells were lysed, normalized for protein levels, and were analyzed for levels of phospho-Thr^{183}/Tyr^{185}-JNK and total JNK, phospho-Ser^{63} Jun and total c-Jun, as well as IκBα and NRAGE by immunoblotting. Total proteins on the immunoblot were shown by Ponceau staining of a protein blot to confirm equal loading and allow for comparison of myc-NRAGE and EGFP expression levels.

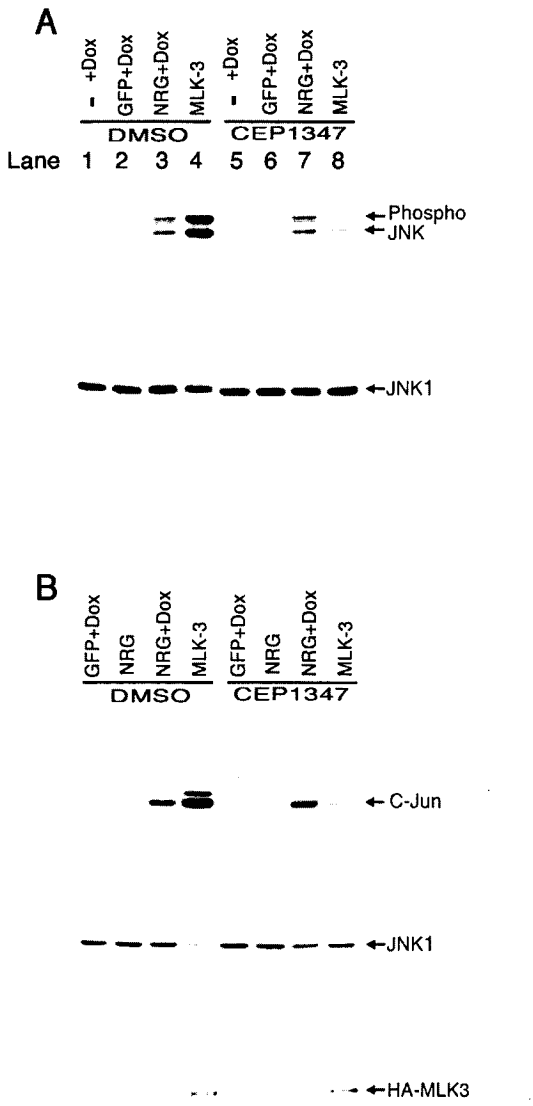

Figure 3.4. NRAGE mediated JNK activation is not blocked by CEP1347. A, Effect of short-term CEP1347 treatment on JNK phosphorylation induced by NRAGE or MLK-3. $PC12^{rtTA}$ cells were left uninfected, or were infected with 50 MOI of recombinant adenovirus expressing myc-NRAGE (NRG), or HA-epitope tagged Mixed Lineage Kinase-3 (MLK-3) or EGFP (GFP), in the presence or absence of 1 μg/ml of doxycycline as indicated. Twenty-nine hours post-infection, cells were treated with 200nM CEP1347, or an equivalent volume of DMSO, for an additional 60 min and were then lysed, normalized for protein content, and analyzed for levels of phospho-Thr^{183}/Tyr^{185}-JNK and total JNK1 by immunoblotting. B, Effect of long-term CEP1347 treatment on c-Jun protein accumulation induced by NRAGE or MLK-3. $PC12^{rtTA}$ cells were treated as above and incubated in the presence of 200nM CEP1347, or an equivalent volume of DMSO, beginning at the time of infection. Thirty hours later, cells were lysed, normalized for protein content, and were analyzed for levels of total c-Jun, JNK and HA-epitope tagged MLK-3 by immunoblotting.

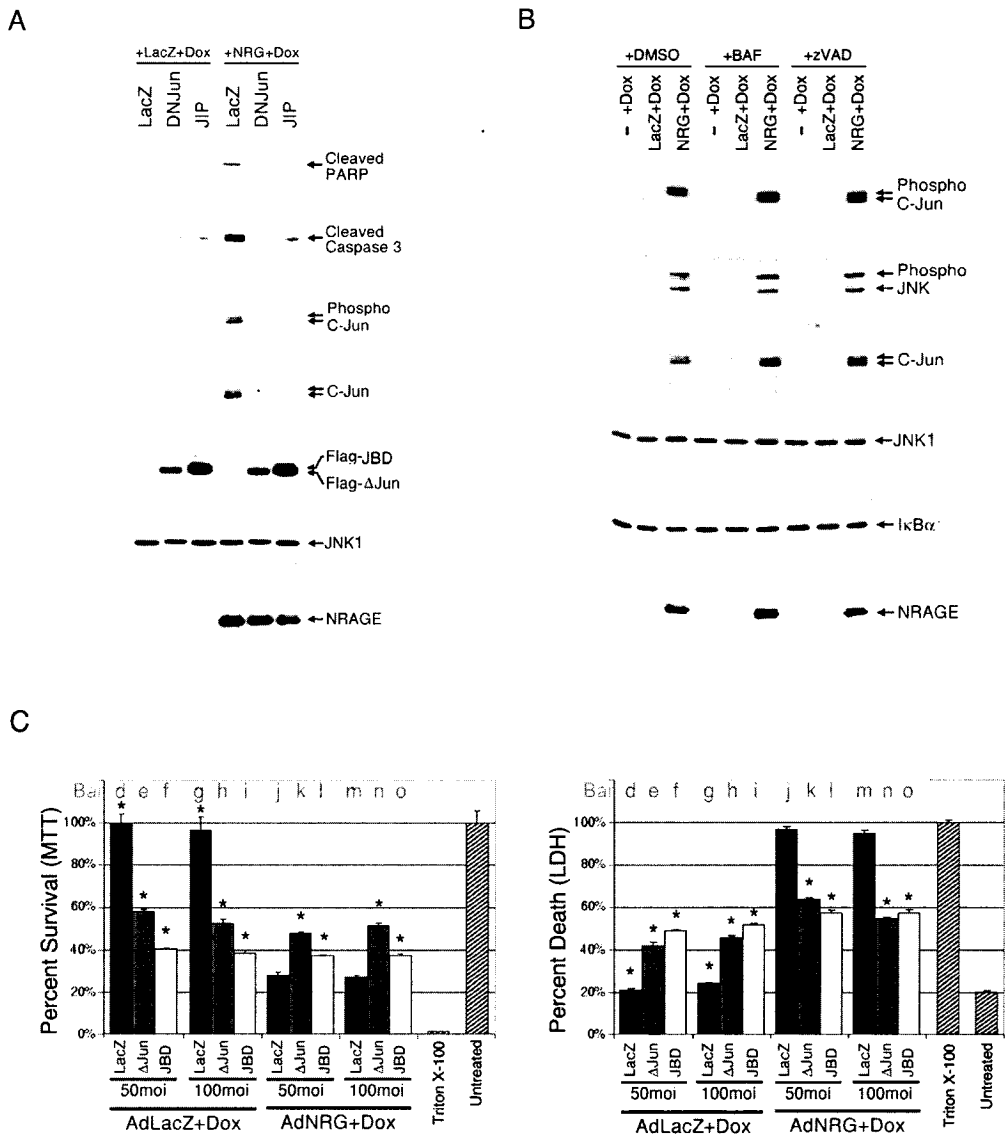

Figure 3.5. JNK activation is necessary for NRAGE mediated caspase activation and cell death. A, PC12^{rtTA} cells were infected with 50 MOI of recombinant adenovirus expressing myc-NRAGE (NRG) or β-galactosidase (LacZ) in the presence of 1 μg/ml of doxycycline. Cells were also infected with 50 MOI of recombinant adenovirus expressing Flag-tagged dominant-negative c-Jun (ΔJun), Flag-tagged JNK binding domain of JIP (JBD), or control adenovirus (LacZ). Thirty hours after infection, cells were lysed, normalized for protein levels, and analyzed for levels of cleaved Caspase-3, cleaved PARP, phospho-Ser^{63} Jun, total c-Jun, Flag-tagged dominant-negative c-Jun (Flag-ΔJUN), Flag-tagged JNK binding domain of JIP1 (Flag-JBD) and NRAGE by immunoblotting. Note that Flag-tagged dominant-negative c-Jun is not detected by the c-Jun antibody, as it lacks the amino-terminal epitopes recognized by this antibody. B, The JNK pathway does not lie downstream of caspase activation in the NRAGE-mediated

apoptotic pathway. $PC12^{rtTA}$ cells were left uninfected or were infected with 50 MOI of recombinant adenovirus expressing β-galactosidase (AdLacZ) or myc-NRAGE (AdNRG), treated with 1 μg/ml of doxycycline, and were incubated in the presence of either 100 μM zVAD-fmk (zVAD), 100 μM Boc-Asp-fmk (BAF) or vehicle (DMSO), as indicated. Thirty hours after infection, cells were lysed, normalized for protein levels, and analyzed for levels of phospho-Ser^{63} c-Jun and total c-Jun, phospho-Thr^{183}/Tyr^{185}-JNK and total JNK, IκBα and NRAGE by immunoblotting. C, NRAGE-mediated cell death is blocked by inhibitors of the JNK pathway. $PC12^{rtTA}$ cells were infected with 50 MOI of recombinant adenovirus expressing β-galactosidase (AdLacZ) or myc-NRAGE (AdNRG) together with 50 or 100 MOI of recombinant adenovirus expressing Flag-epitope tagged dominant-negative c-Jun (ΔJUN), Flag-epitope tagged JNK binding domain of JIP1 (JBD), or the control protein β-galactosidase (LacZ) and treated with 1 μg/ml of doxycycline as indicated. After 40 hours, cells were assayed for survival using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and for death using the lactate dehydrogenase (LDH) assay, where untreated cells (Untr'd) and cells treated with 1% Triton X-100 (Triton) were used to delineate the output range of the assays. Results are normalized relative to those obtained with 1% Triton for the LDH assay, or with untreated cells for the MTT assay, and represent the mean ± standard deviation of a representative experiment performed in triplicate (*$P<0.0005$ relative to AdNRG+Dox+50 MOI AdLacZ for bars d-f, j-l, *$P<0.0005$ relative to AdNRG+Dox+100 MOI AdLacZ for bars g-i, m-o).

PREFACE TO CHAPTER 4

As discussed in the previous chapters, death induced by NRAGE or by p75NTR is known to take place through JNK-dependent activation of the intrinsic apoptotic pathway. The pharmacological compound AEG3482 was originally identified by our collaborators as an inhibitor of trophic-factor withdrawal induced death of sympathetic neurons, However its mechanism of action was unidentified. As the apoptotic cascade induced by p75NTR and NRAGE is now well defined, we used the apoptotic signaling induced by these molecules as a model system to characterize the mechanism of function of AEG3482. We found that AEG3482 indirectly inhibits JNK activity through the transcriptional upregulation of HSP70, which acts as an endogenous inhibitor of JNK activity.

The similar sensitivity of p75NTR- and NRAGE-mediated signaling to AEG3482, or to its analogues, provides further support for the hypothesis that NRAGE lies on a p75NTR-mediated apoptotic signaling path.

CHAPTER 4

AEG3482 is a Novel Anti-Apoptotic Compound that Inhibits Jun Kinase Activity and Cell Death Through Induced Expression of Heat Shock Protein 70.

AEG3482 is a Novel Anti-Apoptotic Compound that Inhibits Jun Kinase Activity and Cell Death Through Induced Expression of Heat Shock Protein 70.

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Running title: AEG3482 inhibits JNK activation and cell death by inducing HSP70.

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4.0 Abstract

The Jun kinase (JNK) pathway plays a central role in the induction of apoptosis in neuronal and non-neuronal cells during development and after injury. In this study, we describe a novel group of small-molecule inhibitors of JNK-dependent apoptosis. The parental molecule, AEG3482, was identified in a screening effort designed to detect compounds that reduce NGF-withdrawal induced apoptosis of neonatal sympathetic neurons. AEG3482 completely blocked apoptosis induced by the p75 neurotrophin receptor (p75NTR) or its cytosolic interactor, NRAGE, and mechanistic studies revealed that AEG3482 treatment strongly attenuated JNK and Caspase-3 activation. AEG3482 does not function as a direct kinase inhibitor, but instead induces production of heat shock protein 70 (HSP70), previously shown to act as an endogenous inhibitor of JNK. We further show that AEG3482 induces HSP70 by binding HSP90, and thereby inducing HSF1-dependent expression of HSP70 mRNA, and we establish that accumulation of HSP70 is required for the AEG3482-induced blockade of JNK signaling. These studies indicate that AEG3482 inhibits JNK activation and apoptosis by a unique mechanism involving induced expression of HSP70 proteins.

4.1 Introduction

Multicellular organisms eliminate superfluous cells during development through the process of apoptosis. Most forms of apoptosis converge on caspases as downstream effectors, and a significant body of work indicates that caspases are activated by one of two main pathways. The intrinsic pathway involves induced permeabilization of the outer mitochondrial membrane, leading to the release of several pro-apoptotic factors, including Cytochrome c and SMAC, that cooperate to facilitate activation of initiator Caspase-9, which in turn leads to activation of effector caspases, such as Caspase-3. The extrinsic pathway is initiated by ligand binding to cell surface death receptors, which induces the assembly of a death-inducing signaling complex that facilitates activation of the initiator Caspase-8. There can be extensive cross-talk between these pathways, and activation of the extrinsic apoptotic pathway often leads to induction of the intrinsic pathway (717).

Activation of Jun kinase (JNK) has emerged as a central event in neuronal apoptosis (718, 719) The role of JNK, mechanisms of its activation, and the subsequent events leading to apoptosis have been particularly well elucidated in sympathetic neurons withdrawn from nerve growth factor (NGF). NGF withdrawal results in the activation of small GTPases, such as Rac1 (374), and the subsequent activation of a JNK signaling module that ultimately results in phosphorylation of transcription factors that include c-Jun (268-270, 387). The phosphorylated transcription factors function in part to facilitate production of BH3-domain-only proteins, which are pro-apoptotic members of the Bcl-2 family (194, 196, 309). Induction of BH3-domain-only proteins leads to the release of mitochondrial contents and thereby initiates the intrinsic apoptotic cascade.

The p75 neurotrophin receptor (p75NTR) and its downstream interacting partner, NRAGE, can initiate signaling events leading to neuronal apoptosis (1). Work by ourselves and others have established that, in both primary neurons and PC12 cells, p75NTR- and NRAGE-induced cell death occurs through induction of the JNK pathway, BH3-domain-only protein activation, and release of mitochondrial contents [chapters 2 & 3;(441)].

Because of the central role of JNK in neuronal cell death, considerable attention has been focused on developing strategies to attenuate JNK signaling. One widely pursued strategy is to develop chemical inhibitors of JNK activity. Several kinase inhibitors that target elements of the JNK activation pathway have emerged (290, 707), and some of these compounds function as anti-apoptotic compounds within *in vitro* and *in vivo* models (294, 720). Another approach is to induce the expression of endogenous proteins that attenuate JNK pathway signaling. Several proteins that directly modulate JNK signaling have been identified (382, 721-724), the best characterized of which is heat shock protein-70 (HSP70). HSP70 expression is often induced in cells exposed to stressful stimuli, and its role of reducing stress-induced damage through its chaperone function is well established (725). Independent of its chaperone function, HSP70 can directly bind and inhibit JNK, and thereby reduce apoptosis induced by a variety of insults (726-730). It is conceivable, therefore, that induction of HSP70 would provide an effective means of blocking apoptotic JNK signaling.

In the present study, a search for novel compounds that block JNK-induced cell death was conducted. High throughput screening for small-molecule inhibitors of NGF-withdrawal induced sympathetic neuron apoptosis led to the identification of a novel compound, AEG3482. AEG3482 also blocked p75NTR- and NRAGE-induced apoptosis of the PC12 neuronal cell line, correlating with the suppression of p75NTR- and NRAGE-mediated JNK signaling. The suppression of JNK signaling and apoptosis by AEG3482 were mediated through induced HSP70 expression that occurred via an HSF1-dependent pathway. Loss of function experiments, in which HSP70 levels were reduced by RNAi knockdown, confirmed that HSP70 accumulation was required for the AEG3482-mediated suppression of JNK signaling. Collectively, these studies demonstrate that AEG3482 is a potent anti-apoptotic compound that blocks the JNK signaling pathway through increased expression of HSP70.

4.2 Materials and Methods

Materials. AEG3482, AEG19940, AEG33691, AEG33733 and AEG40011 were produced at Aegera Therapeutics. Antibodies directed against JNK1 and IκBα were from Santa Cruz Biotechnology; those against c-Jun, phospho-c-Jun and cleaved Caspase-3 were from Cell Signaling Technology; those against HSP25, HSP40, and HSP70 were from Stressgen; and that against Actin was from ICN. Antibodies directed against NRAGE, and p75NTR have been previously described [chapter 2; (649)]. The PC12^{rtTA} cell line was purchased from Clontech. HSP70 specific small interfering RNAs consisted of a 'SmartPool' mixture was purchased from Dharmacon. Recombinant adenovirus driving expression of b-galactosidase, p75NTR and NRAGE have been previously described (632).

Cell culture, infection, transfection and immunoblotting. Sympathetic neurons were maintained and deprived of NGF as previously described (493, 731). PC12^{rtTA} cells were maintained and infected as previously described [chapter 3; (649)]; for NRAGE, 1 mg/ml doxycycline was added to all plates at time of infection. Wildtype and HSF1 null immortalized mouse embryonic fibroblasts (MEFs) were maintained in DMEM containing 10% fetal calf serum, 1 mM β-mercaptoethanol, 1% non-essential amino acids, 1% L-glutamine, 1% antimycotic solution and 1% sodium pyruvate (all from GibcoBRL). Sympathetic neuron survival was assessed using the MTS assay according to the manufacturer's instructions (Promega). Analysis of PC12 cell death was determined using a lactate dehydrogenase (LDH) assay (Roche) as per the manufacturer's instructions. Transfections using plasmids or RNAi were performed using Lipofectamine 2000. Immunoblotting was performed as previously described [chapter 3; (649)].

Detection of endogenous phosphorylation of GST-c-Jun. The cDNA region corresponding to amino acids 2-79 of human c-Jun was cloned into a mammalian GST expression vector (732) and this was used to transfect PC12 cells. Lysates of transfected cells were prepared and incubated with 20 ml of glutathione-conjugated beads (Pharmacia) for 1 hour at 4° C. Beads were washed

three times, resuspended in Lammeli sample buffer and incubated at 100°C for 5 minutes. The level of GST-c-Jun phosphorylation was assessed by immunoblotting with a phospho-c-Jun specific antibody.

RT-PCR. PC12 cells were treated with increasing concentrations of AEG3482 for 18 hours and mRNA was isolated using RNEasy Mini kits (Qiagen). cDNA was generated using the Omniscript RT kit (Qiagen) and random hexamers (Roche) as primers. PCR was performed using primer pairs directed against rat HSP70, HSP25 and actin (primer sequences and PCR conditions available upon request).

Transcriptional assays. MEFs were transfected with pGL3B-HSP70 or with the corresponding parental vector. AEG3482 (40 μM) was added to the cells the next day and cells were harvested 48 hours after transfection. Transcriptional assays were performed using a luciferase assay system purchased from Promega.

Statistical analysis. For quantitation, each condition was performed in triplicate or quadruplicate, and results were analyzed by multiple analysis of variance with statistical probabilities assigned using the Tukey test for multiple comparisons.

4.3 Results

The synthetic compound AEG3482 inhibits NGF-withdrawal induced death in SCG neurons.

Survival of neonatal primary sympathetic neurons is dependent on trophic support provided by NGF (725). To identify novel pharmacological inhibitors of neuronal apoptosis, a high throughput screen was developed to identify compounds that block NGF-withdrawal mediated death of primary sympathetic neurons. Over 17,000 natural, synthetic and semisynthetic compounds were analyzed for their ability to inhibit NGF-withdrawal induced apoptosis. One of the most promising compounds to emerge from this screen was an imidazothiadiazole sulfonamide of 281 Daltons, which was designated AEG3482 (Figure 4.1A). Survival of sympathetic neurons withdrawn from NGF was strongly enhanced in the presence of AEG3482, with an EC_{50} of ~20 μM (Figure 4.1B).

AEG3482 inhibits p75NTR- or NRAGE- induced apoptosis of PC12 cells.

We have previously shown that adenoviral-mediated overexpression of p75NTR, or its cytosolic interactor, NRAGE, leads to extensive JNK-dependent apoptosis of PC12 cells and primary cortical neurons [chapters 2 & 3; (441)]. Because the signaling pathways that lead to p75NTR- or NRAGE-induced cell death have been unambiguously established in PC12 cells, this system was employed to determine the mechanism of action of AEG3482. We previously produced an adenovirus that drives NRAGE expression via a doxycycline inducible element (constitutive NRAGE expression is cytotoxic - see chapter 3 for details) and for the experiments described below, a PC12 subline ($PC12^{rtTA}$) that stably expresses the doxycycline activated transcription factor, rtTA, was used.

$PC12^{rtTA}$ were infected with adenoviruses encoding p75NTR (Adp75), NRAGE (AdNRG), or as a control, LacZ (AdLacZ), and were concurrently exposed to increasing concentrations of AEG3482 for a period of 40 hours. Cell death was assessed using an LDH release assay. Overexpression of NRAGE or p75NTR led to extensive death of $PC12^{rtTA}$ cells, which was strongly attenuated by co-treatment with AEG3482 in a dose-dependent manner (Figures 4.2A, B).

Treatment with 40 μM AEG3482 reduced p75NTR- or NRAGE-induced cell death by greater than 90%. At the highest concentration tested (80 μM), the compound effectively inhibited apoptosis, but also exerted a slight toxic effect in cells infected with the LacZ control. These results indicated that the $PC12^{tTA}$ overexpression paradigm provided a convenient and biochemically tractable system for analyzing the mechanism of action of AEG3482.

AEG3482 inhibits p75NTR- or NRAGE-mediated JNK activation.

Activation of JNK activity is necessary for the induction of p75NTR- or NRAGE-initiated caspase cleavage and cell death [chapters 3; (441)]. Therefore, the effect of AEG3482 on the activity of JNK was assessed by analyzing alterations in the phosphorylation level of the JNK target, c-Jun, and in the cleavage status of Caspase-3. Figure 4.2C shows immunoblots of lysates of $PC12^{rtTA}$ cells that were infected with AdNRG, or the control virus, AdLacZ, in the presence of increasing concentrations of AEG3482. In the absence of the compound, NRAGE expression resulted in robust c-Jun phosphorylation and Caspase-3 cleavage. Levels of c-Jun protein were also increased, presumably due to the auto-activation of c-Jun transcription by phosphorylated c-Jun protein (261). Treatment with AEG3482 strongly attenuated each of these effects; a significant decrease in c-Jun phosphorylation and Caspase-3 cleavage was detectable at 10 μM AEG3482 and was virtually complete at 40 μM. Figure 4.2D shows that similar results were obtained in cells infected with Adp75, and treated with increasing concentrations of AEG3482. Thus, the suppression of JNK signaling and Caspase-3 cleavage mediated by AEG3482 occurred with a dose-dependency similar to the anti-apoptotic activity of AEG3482.

AEG3482 inhibits PC12 cell death induced by paclitaxel and cisplatin.

The ability of AEG3482 to inhibit apoptosis in response to a variety of other insults, including the DNA damaging agent, cisplatin, the microtubule disruptor, paclitaxel, and the topoisomerase inhibitor, doxorubicin, was investigated. Previous studies have demonstrated that cell death induced by

paclitaxel is dependent on JNK activation (317, 733, 734), and Figure 4.3 shows that apoptosis induced by low (10 μM) and high (50 μM) concentrations of paclitaxel are effectively reduced by AEG3482. Cisplatin-induced apoptosis has been reported to be JNK-dependent when used at low concentrations (10 μM), but JNK-independent at concentrations >25 μM (735), and consistent with this, AEG3482 protected cells exposed to 10 μM, but not 50 μM, cisplatin (Figure 4.3). Doxorubicin-induced apoptosis is JNK-independent (735), and AEG3482 treatment did not offer any protection against doxorubicin-induced cell death. Collectively, these results are consistent with the hypothesis that AEG3482 inhibits apoptosis by blocking the JNK pathway.

AEG3482 treatment induces HSF1-dependent expression of HSP70.

The structure of AEG3482 does not resemble previously identified kinase inhibitors, and AEG3482 has no effect on JNK activity *in vitro* (data not shown). The conclusion that AEG3482 does not directly suppress JNK activity is supported by the observation that the JNK inhibitory effects of AEG3482 occurred in cells subjected to extended (i.e.; 24 hour), but not short term (i.e.; 1 hr hour), incubation with the compound (Figure 4.4A). These results suggest that AEG3482 suppresses apoptotic signaling through alternate means, possibly by enhancing transcription or translation of an endogenous factor which inhibits JNK activity. Several studies have established that heat shock protein 70 (HSP70) can bind and inhibit JNK (726-730). Therefore, the possibility that AEG3482 promotes HSP70 production was explored.

To address this possibility, HSP protein levels were assessed in $PC12^{rtTA}$ cells infected with either AdLacZ, Adp75, or AdNRG, and at the same time, exposed to AEG3482. Figure 4.4B shows that treatment of $PC12^{rtTA}$ cells with increasing concentrations of AEG3482 resulted in a robust increase in HSP70 and HSP25, but not HSP40, in cells infected with either LacZ or with Adp75. Cells infected with AdNRG showed enhanced expression of HSP70 even in the absence of AEG3482. However, treatment of cells infected with AdNRG pushed HSP70 levels substantially higher that those observed with AdNRG alone.

To test if the accumulation of HSP70 and HSP25 induced by AEG3482 reflected increased steady state levels of mRNAs encoding these proteins, rtPCR analyses were performed. RNA isolated from PC12 cells, treated with increasing concentrations of AEG3482 for 18 hours, was subjected to semi-quantitative rtPCR using primers specific for HSP25, HSP70 and HSP90 mRNA. Figure 4.4C shows that treatment with the compound elevated HSP25 and HSP70 mRNA, but had no effect on HSP90 mRNA levels, or on actin mRNA, which served as control.

Transcriptional regulation of HSP70 and HSP25, but not HSP40, is mediated by the heat shock factor 1 (HSF1) transcription factor. In unstimulated cells, HSF1 is maintained largely in an inactive, latent state through an interaction with its binding partner, HSP90. Induction of cell stress results in the interaction of HSP90 with misfolded proteins and disrupts its association with HSF1, allowing it to bind and activate HSP70 and HSP25 promoters (736). The specific effect of AEG3482 on HSP25 and HSP70 production led us to hypothesize that AEG3482 binds HSP90, causing it to release HSF1 which then binds and activates HSP70 and HSP25 promoters. In initial experiments, the activity of HSP70 transcriptional reporter constructs transfected into PC12 cells were found to be stimulated by AEG3482 (data not shown) To specifically address whether HSF1 plays a role in this response, the effect of AEG3482 on mouse embryonic fibroblasts (MEFs) derived from either wild type or HSF1 null mice was examined (737). Figure 4.5A shows that the transcriptional activity of the HSP70 promoter reporter construct was strongly induced by AEG3482 in wild-type MEFs, but remained at baseline levels in MEFs lacking HSF1. The examination of protein levels of endogenous HSPs also showed corresponding changes, with strong induction of HSP70 and HSP25 in the wild type cells but not in MEFs lacking HSF1 (Figure 4.5B).

These data indicate that AEG3482 mediates activation of HSP proteins through an HSF1-dependent pathway, and are consistent with the hypothesis that AEG3482 directly binds HSP90. To test this hypothesis, AEG3482 was chemically crosslinked to Sepharose beads and used as affinity reagents in

pulldown experiments. AEG3482 beads or control beads were incubated with cell lysates, washed extensively, and bound proteins eluted and analyzed by SDS-PAGE and immunoblotting. Figure 4.5C shows that AEG3482 beads, but not control beads, bound HSP90 and that this interaction was strongly attenuated by excess unbound AEG3482. Collectively, these data indicate that AEG3482 specifically binds HSP90, thereby facilitating HSF1-dependent expression of HSP70 and HSP25.

AEG3482 inhibition of JNK activity is mediated by the induction of HSP70.

Previous studies have shown that HSP70, but not HSP25, is capable of blocking JNK activation (726-730), and we therefore focused on the functional interaction between AEG3482, HSP70 induction, and JNK pathway inhibition. To address this, the cellular effects of three analogs of AEG3482, which differed from the parental compound in a single functional group (for AEG19940, R^1 = morpholino; for AEG33691, R^1 = OCH2CO2CH3; for AEG33733, R^1 = N(H)COP) were analyzed. One of these compounds (AEG19940), like AEG3482, induced HSP70 production, whereas two others (AEG33691 and AEG33733) did not (Figure 4.6B). Only the compounds that induced HSP70 production (AEG3482 and AEG19940) were effective in preventing Adp75- or AdNRG-induced cell death (Figure 4.6B). The compounds were assessed for their ability to reduce AdNRG-induced c-Jun phosphorylation, and as before, only compounds that induced HSP70 production (AEG3482 and AEG19940) reduced JNK activation (Figure 4.6C). Thus, using these four related compounds, a strong correlation emerged between the induction of HSP70, the inhibition of JNK activity, and cell death.

Loss of function experiments were then used to establish a direct causal link between the induction of HSP70 by these compounds and their JNK inhibitory activity. For this, RNA interference directed against HSP70 was used to block its accumulation following treatment with AEG3482. The expectation was that if the induction of HSP70 by the compounds was required for JNK inhibition, then blocking the accumulation of HSP70 should reduce the JNK inhibitory

effect. Figure 4.7A shows that transfection with HSP70 siRNA modestly attenuated expression of HSP70 induced by AEG3482. This relatively poor knockdown likely reflects the low transfection efficiencies that can be achieved in $PC12^{rtTA}$ cells. Despite this, a small but consistent attenuation in the ability of AEG3482 to block c-jun phosphorylation was observed in cells transfected with HSP70 RNAi, consistent with the hypothesis that HSP70 induction is required for this effect.

To establish a firmer causal link between HSP70 induction and JNK suppression, and to circumvent the problem of low-transfection efficiency of the $PC12^{rtTA}$ cells, a method was developed to assess JNK pathway activation specifically in the RNAi transfected subpopulation of $PC12^{rtTA}$ cells, using a mammalian expression vector driving production of a fusion protein containing GST fused to amino acids 2 – 79 of c-Jun. We anticipated that the GST-c-Jun fusion protein would be readily recoverable from lysates, and that its phosphorylation status would provide a sensitive read-out reflecting the level of JNK pathway activation in the subpopulation of cells which were transfected. Although overall transfection efficiencies are low in $PC12^{rtTA}$ cells, the GST-c-Jun fusion plasmid and the HSP70 siRNA would be efficiently co-transfected, thereby providing an accurate assessment of the effect of HSP70 depletion on JNK signaling To validate the GST-c-Jun fusion protein as an *in vivo* reporter of JNK activity, $PC12^{rtTA}$ cells transfected with GST-c-Jun were treated with TNFα or exposed to hyperosmotic shock, two stimuli commonly used to activate the JNK signaling pathway (718). GST-c-Jun was then recovered from lysates and its phosphorylation status determined by immunoblotting. Figure 4.7B shows that, as predicted, both TNFα and hyperosmotic shock cause a dramatic increase in phosphorylation of GST-jun which is readily detected by immunoblot. Thus, the phosphorylation status of the GST-jun fusion protein provided a sensitive read-out that reflects the level of JNK pathway activation in transfected cells.

The above method was then used to determine if HSP70 accumulation was required to block NRAGE-induced JNK activity. AEG3482 treatment itself significantly increased expression of the GST-c-Jun fusion construct,

complicating the interpretation of results (data not shown). As an alternative, AEG19940 was used since it robustly induced HSP70 production (see Figure 4.6) without altering GST-Jun expression levels. Figure 4.7C shows that, in cells transfected with control siRNA, infection with AdNRG dramatically increases phosphorylation of GST-c-Jun, whereas AEG19940 strongly inhibited this effect. In cells transfected with RNAi directed against HSP70, the ability of AEG19940 to block GST-jun phosphorylation was almost completely lost, indicating that HSP70 accumulation plays a crucial role in the JNK inhibition elicited by this series of compounds.

4.4 Discussion

The present study indicates that the anti-apoptotic activity of AEG3482 and its analogs arises from their ability to block activation of the JNK pathway. Structurally, AEG3482 does not resemble known kinase inhibitors, and its time course of its action suggested that it may function by facilitating production of an endogenous JNK pathway antagonist. HSP70 can act as such an endogenous inhibitor of JNK activity (726-730) and our data show that AEG3482 is a potent inducer of HSP70 production and demonstrates that HSP70 accumulation is required for the observed AEG3482-mediated attenuation of JNK signaling. To our knowledge, this compound is the first shown to inhibit JNK activation through a mechanism involving induced production of HSP70.

HSPs are highly conserved proteins that are induced by a wide variety of chemical and physiological stresses (738). It is well established that HSPs play crucial protective roles in stress responses, and that they can suppress apoptosis induced by heat shock, chemotherapeutic agents, nutrient withdrawal, ionizing radiation or TNFα (738). Induction of HSPs with sublethal stresses gives rise to stress tolerance, and in several models, HSP70 has been identified as being the main HSP responsible for resistance to future insults (729, 739-742). The protective nature of HSP70 and other HSPs was originally attributed exclusively to their role as molecular chaperones that prevented stress-induced protein misfolding and aggregation, and that accelerated refolding (725). However, recent findings have demonstrated that, in addition to this function, HSP70 suppresses apoptosis by directly inhibiting components of the JNK signaling pathway (726-730). This inhibition involves direct binding of HSP70 to JNK (727). The precise HSP70-JNK binding domains have not been identified, but available data suggest that HSP70 binds JNK at, or close to, the docking groove where interactions with both JNK targets and activators occur. It is likely in this manner that the HSP70-JNK association attenuates the interaction of JNK with upstream MKKs and downstream targets (727). Inhibition of JNK by HSP70 does not appear to be directly related to its chaperone function since HSP70 mutants that lack

chaperone function still inhibit JNK; furthermore, HSP70 can inhibit JNK activation even in the absence of stress-induced protein damage (726-728).

Our data show that AEG3482 exposure results in the accumulation of HSP25 and HSP70 mRNA and protein in PC-12 cells. Transcriptional regulation of these HSPs normally requires the action of the HSF1 transcription factor and we therefore asked if AEG3482 induces HSF1 activity. Transcriptional reporter assays demonstrated that AEG3482 does indeed activate HSF1 transcriptional activity, and comparison of wild-type MEFs with those lacking HSF1 established that AEG3482 induces HSP25 and HSP70 production through an HSF1-dependent pathway. HSF1 is normally maintained in a latent form by virtue of its association with HSP90 (736), and the above data led us to hypothesize that an association of AEG3482 with HSP90 releases HSF1 and thereby facilitates HSP25 and HSP70 transcription. Consistent with this, we found that HSP90 binds AEG3482 in pullout assays.

HSP90 is composed of three main domains. The C-terminal domain contains the HSP90 dimerization site as well as docking sites for various co-chaperones. The central domain contains a large hydrophobic surface that is involved in the binding of HSP90 client proteins and the N-terminal region contains the molecule's ATPase domain (743). Unlike other chaperones, most known client proteins of HSP90 are molecules involved in the regulation of survival and growth (744). Geldanamycin is a benzoquinone ansamycin that binds to the ATP-binding pocket of HSP90 (743-745). This leads to allosteric changes in HSP90 that result in the release of HSF1 and subsequent expression of HSP25 and HSP70 (736). Because this mechanism is similar to that which we propose for AEG3482 and its analogs, it is reasonable to expect that the cellular effects of AEG3482 and geldanamyin treatment will be similar. However, although a few reports indicate that geldanamycin does confer protection to cells from protein damaging stress *in vitro* and *in vivo*, the majority of studies have shown that geldanamycin is cytotoxic and kills variety of normal and transformed cells, including PC12 cells (744, 746). The toxic effect of geldanamycin is likely due to the fact that geldanamycin not only releases bound HSF1 but, by occupying the

ATP binding pocket, renders HSP90 nonfunctional and therefore reduces the stability and activity of HSP90 client proteins (736, 744). As many client proteins of HSP90 are molecules involved in the regulation of survival and growth, including the key survival kinases AKT and RAF (747-750), disruption its chaperone activity will have dire cellular consequences. Indeed, the activity and stability of both AKT and RAF is reduced following treatment with geldanamycin (749, 751-755).

There are major structural and mechanistic differences between geldanamycin and AEG3482 and it appears unlikely that AEG3482 binds to the N-terminal ATP binding domain of HSP90, as does geldanamycin. Rather, our working hypothesis is that AEG3482 interacts with a portion of the peptide-binding domain of HSP90 and facilitates HSF-1 release while retaining chaperone activity. Consistent with this possibility, we have observed that, unlike geldanamycin, AEG3482 treatment enhances activation of AKT and Raf, both of which are HSP90 client proteins (data not shown). A similar enhancement of AKT and Raf activity is observed during the misfolded protein response, where as above, misfolded proteins interact with the peptide-binding domains of HSP90 and cause the release of HSF-1.

In conclusion, we have identified a class of compounds that inhibit JNK activation by inducing production of HSP proteins. We anticipate that AEG3482 and its analogs will serve as useful tool for basic research, and believe that they have therapeutic potential for the treatment of acute and chronic neurological disorders.

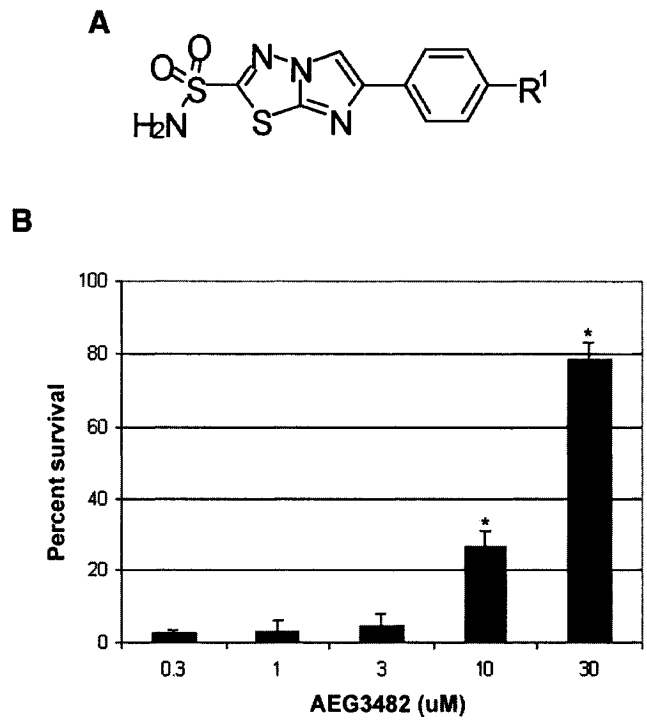


Figure 4.1. The synthetic compound AEG3482 inhibits NGF-withdrawal induced cell death. A, Molecular structure of AEG3482 and its analogues. The compound AEG3482 was isolated in a high-throughput screen for novel inhibitors of NGF-withdrawal induced death of sympathetic neurons. In AEG3482, R^1 = H. B, AEG3482 inhibits NGF-withdrawal induced death of SCG neurons. Cultures of rat SCG neurons (see Materials and Methods) initially maintained in 10 ng/ml of NGF were withdrawn from NGF and maintained in media containing AEG3482 at the concentrations indicated. After two days in AEG3482, cell viability was assayed using an MTS assay. (* = $P<0.01$ relative to 0 μM AEG3482). Experiments in B were performed three times with identical results.

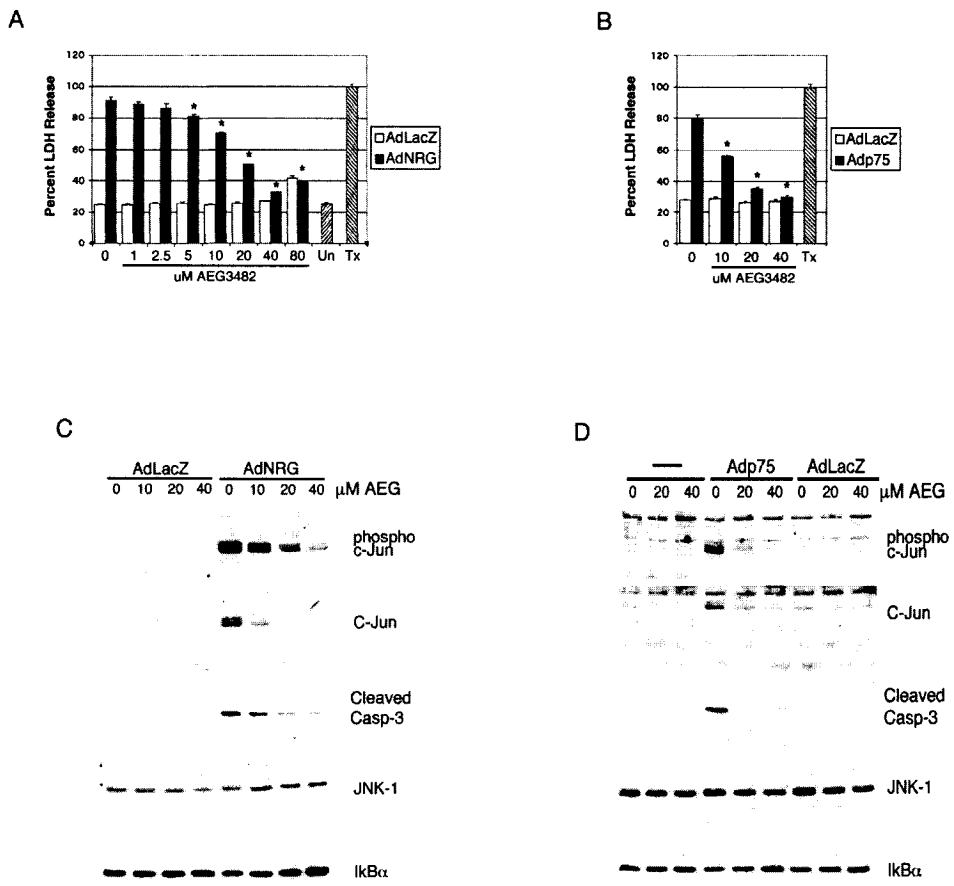


Figure 4.2. AEG3482 inhibits p75NTR or NRAGE induced JNK activation and cell death. A, B: AEG3482 inhibits NRAGE (A) or p75NTR (B) mediated cell death. PC12^{rtTA} cells were infected with recombinant adenoviruses expressing full-length NRAGE (AdNRG) at 5 MOI, full length p75NTR (Adp75) at 50 or 100 MOI, or the control protein β-galactosidase (AdLacZ) (at 5 MOI in (A) and 50 MOI in (B)). At time of infection, the cells were treated with an increasing concentration of AEG3482, or with vehicle alone, as indicated. After 40 hours, cells were assayed for death using the lactate dehydrogenase (LDH) assay; untreated cells (Un) and cells treated with 1% Triton X-100 (Tx) were used to delineate the output range of the assay. Results are normalized relative to those obtained with 1% Triton (Tx), and represent the mean ± standard deviation of a representative experiment performed in triplicate (* = $P<0.005$ relative to 0 µM AEG3482). C, D: AEG3482 inhibits NRAGE- and p75NTR-induced JNK activation. PC12^{rtTA} cells were infected with AdNRG at 5 MOI (C), with Adp75 at 50 MOI (D), or with AdLacZ at an equivalent MOI for each experiment, as indicated. At time of infection, the cells were treated with increasing concentrations of AEG3482, or with vehicle alone, as indicated. Thirty hours after infection, cells were lysed, normalized for protein content, and analyzed for levels of phospho-Jun, total c-Jun, cleaved caspase-3, JNK, IκBα, NRAGE (C) and p75NTR (D). by immunoblotting. Experiments in Panel A-D were performed three times with essentially identical results.

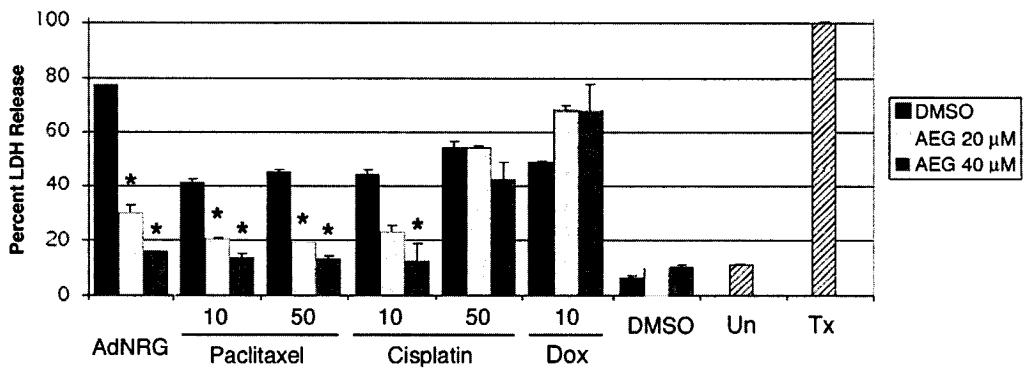


Figure 4.3. AEG3482 inhibits JNK-dependent cell death induced by chemotherapeutic drugs. Cells were infected with AdNRG at 5 MOI or treated with paclitaxel, cisplatin, or doxorubicin (Dox) at the indicated μM concentrations. At the time of treatment, AEG3482 or vehicle was added to the cultures, as indicated. After 40 hours, cells were assayed for death using the LDH assay. Results are normalized relative to those obtained with 1% Triton (Tx), and represent the mean ± standard deviation of a representative experiment performed in triplicate (* = $P<0.01$ relative to 0 μM AEG3482 for each insult). Experiments were performed twice with identical results.

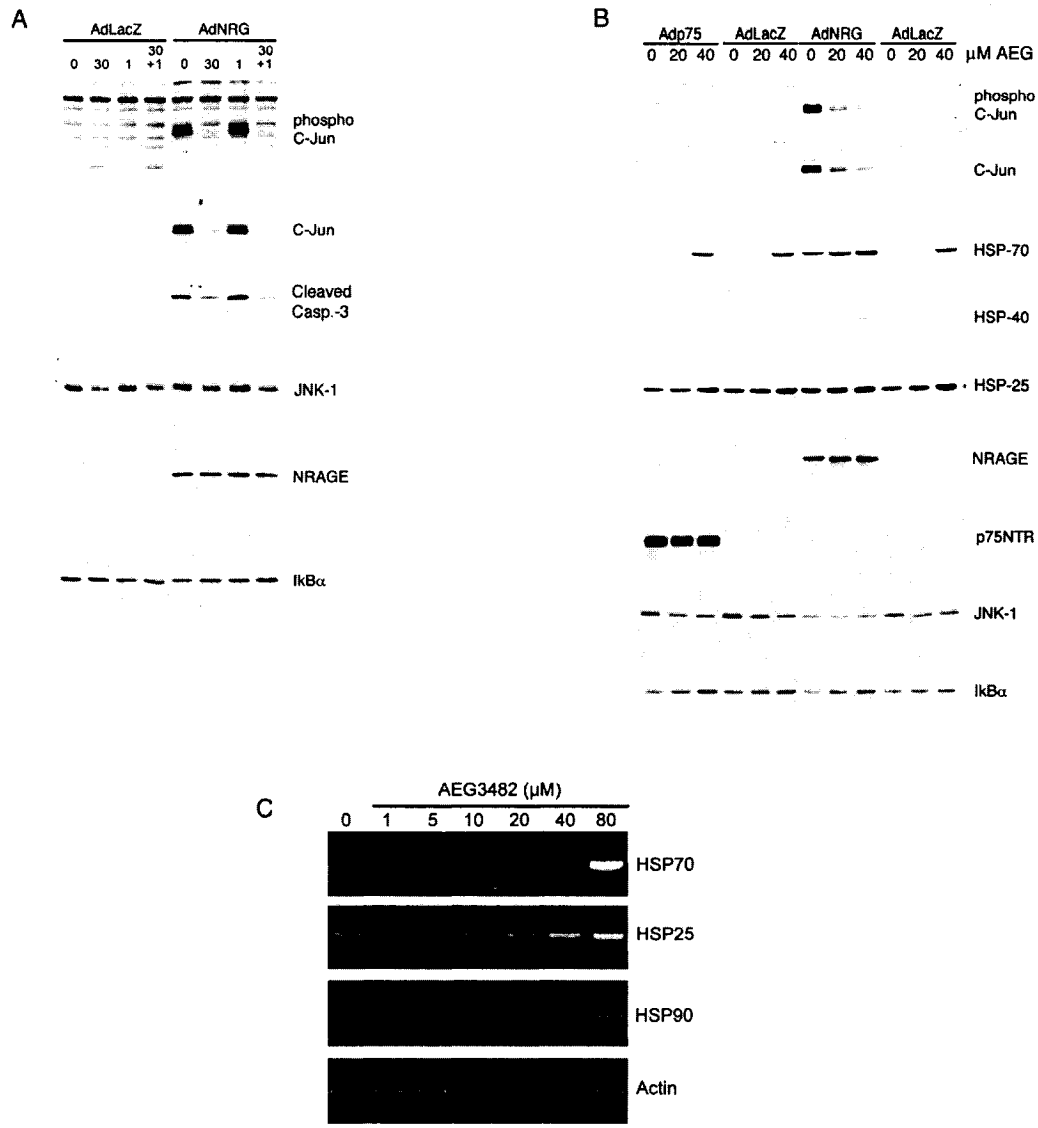


Figure 4.4. AEG3482 treatment induces expression of HSP70. A: PC12 cells were infected with 5 MOI of AdNRG (NRG) or AdLacZ (LacZ) as indicated. Cells were left untreated (0) or treated with 40 μM AEG3482 at time of infection (30), one hour before lysis (1), or at both times (30+1). Cells were lysed thirty hours after infection, normalized for protein content, and analyzed for levels of phospho-Ser^{63} c-Jun and total c-Jun, total JNK, IκBα, and NRAGE by immunoblotting. Lysates from untreated cells are indicated by '-'. B: PC12^{rtTA} cells were infected with AdNRG at 5 MOI, Adp75 at 50 MOI, or the control virus AdLacZ at 50 MOI. At time of infection, the cells were treated with increasing concentrations of AEG3482, or with vehicle alone, as indicated. Thirty hours after infection, cells were lysed, normalized for protein content, and analyzed for levels of c-Jun phosphorylation, HSP70, HSP25, HSP40, NRAGE, p75NTR, JNK-1 and IκBα, as indicated. C: RNA isolated from PC12^{rtTA} cells treated with increasing concentrations of AEG3482 for 18 hours was analyzed by rtPCR using primers specific to HSP70, HSP25, HSP90 or actin, as indicated. Experiments in Panel A and B were performed three times and that in Panel C was performed twice, with essentially identical results.

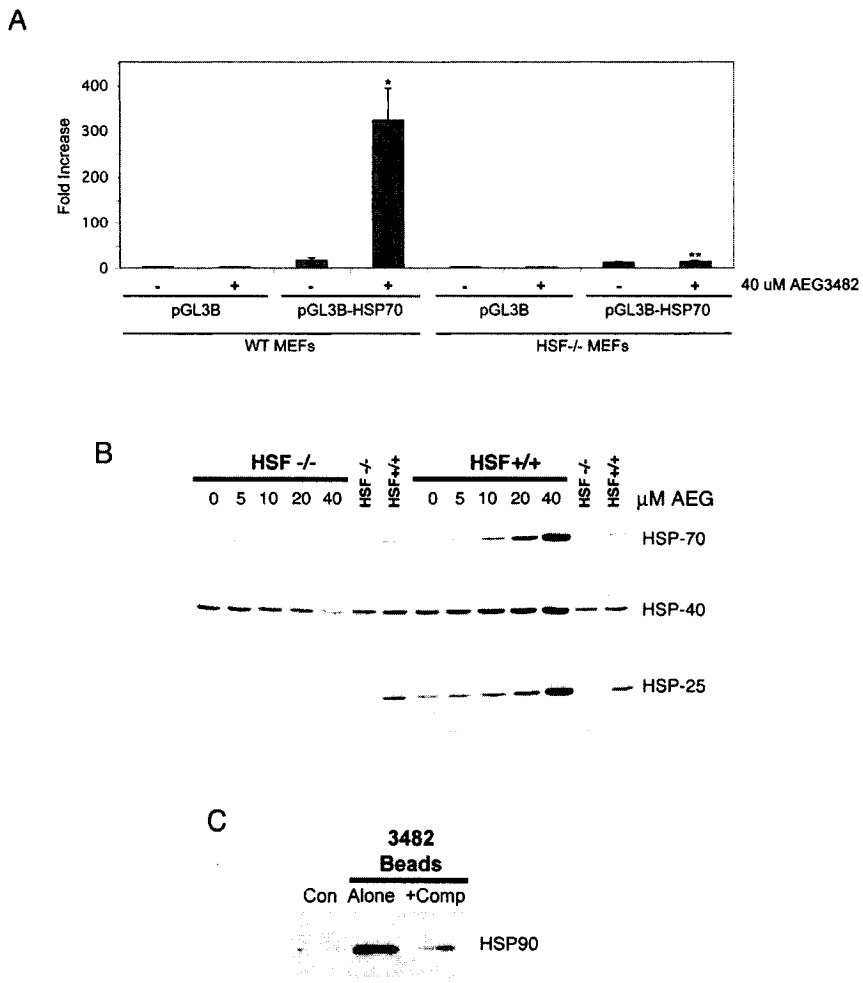


Figure 4.5. AEG3482 binds HSP90 and causes HSF1-dependent expression of HSP70 and HSP25. A: Wild-type or HSF1 null MEFs were transfected with pGL3B-HSP70, an HSP70 promoter reporter construct, or parental vector (pGL3B), then treated with 40 μM of AEG3482 for 24 hours and analyzed for luciferase content as described in Materials and Methods (* = P<0.005 relative to 0 μM AEG3482; ** = P<0.005 relative to wild-type MEFs treated with 40 μM AEG3482. B: Wild-type or HSF1 null MEFs were treated with increasing concentrations of AEG3482 for 24 hours, cells were lysed, normalized for protein content, and analyzed for levels of HSP70, HSP40, and HSP25 as indicated. C: AEG3482-conjugated Sepharose beads were incubated with PC12 cell lysate for 2 hours, washed, nd levels of bound HSP90 were analyzed by immunoblot. Con = Control beads, alone = AEG3482 beads alone, comp = AEG3482 beads + competing AEG3482 (at 80 μM). Experiments in Panel A - C were performed three times with identical results.

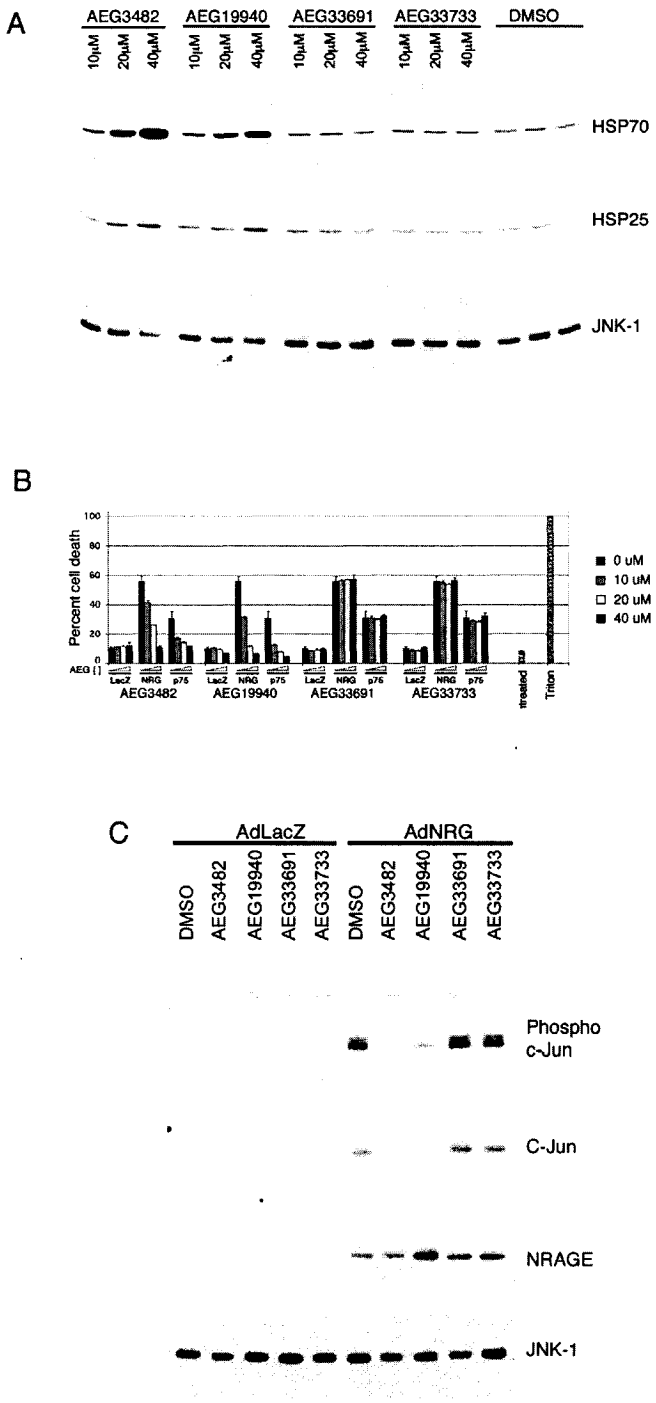


Figure 4.6. AEG3482 analogues inhibit JNK activation and induce expression of HSP70. A: PC12^{rtTA} cells were infected with 5 MOI of AdNRG, 50 MOI of Adp75 or 50 MOI of the control virus AdLacZ. At time of infection, the cells were treated with an increasing concentrations of AEG3482, or its analogues, AEG19940, AEG33691, AEG33733, or with vehicle alone, as indicated. Thirty hours after infection, cells were

lysed, normalized for protein levels, and analyzed for levels of HSP70, HSP25 and JNK-1, as indicated. B: $PC12^{rtTA}$ cells were treated as above and after 40 hours infection were assayed for death using the lactate dehydrogenase (LDH) assay. Results are normalized relative to those obtained with 1% Triton, and represent the mean ± standard deviation of a representative experiment performed in triplicate (* = $P<0.025$ relative to '0' for each AEG concentration). C: $PC12^{rtTA}$ cells were infected with 50 MOI of LacZ or 5 MOI of AdNRG50 and at the time of infection, were treated with 40 □M AEG3482, or its analogues, AEG19940, AEG33691, AEG33733, or with vehicle alone, as indicated. Thirty hours after infection, cells were lysed, normalized for protein levels, and were analyzed for phosphorylated c-Jun, total c-Jun, NRAGE and JNK-1, as indicated. Experiments in Panel A and C were performed twice and that in B were performed twice, with essentially identical results.

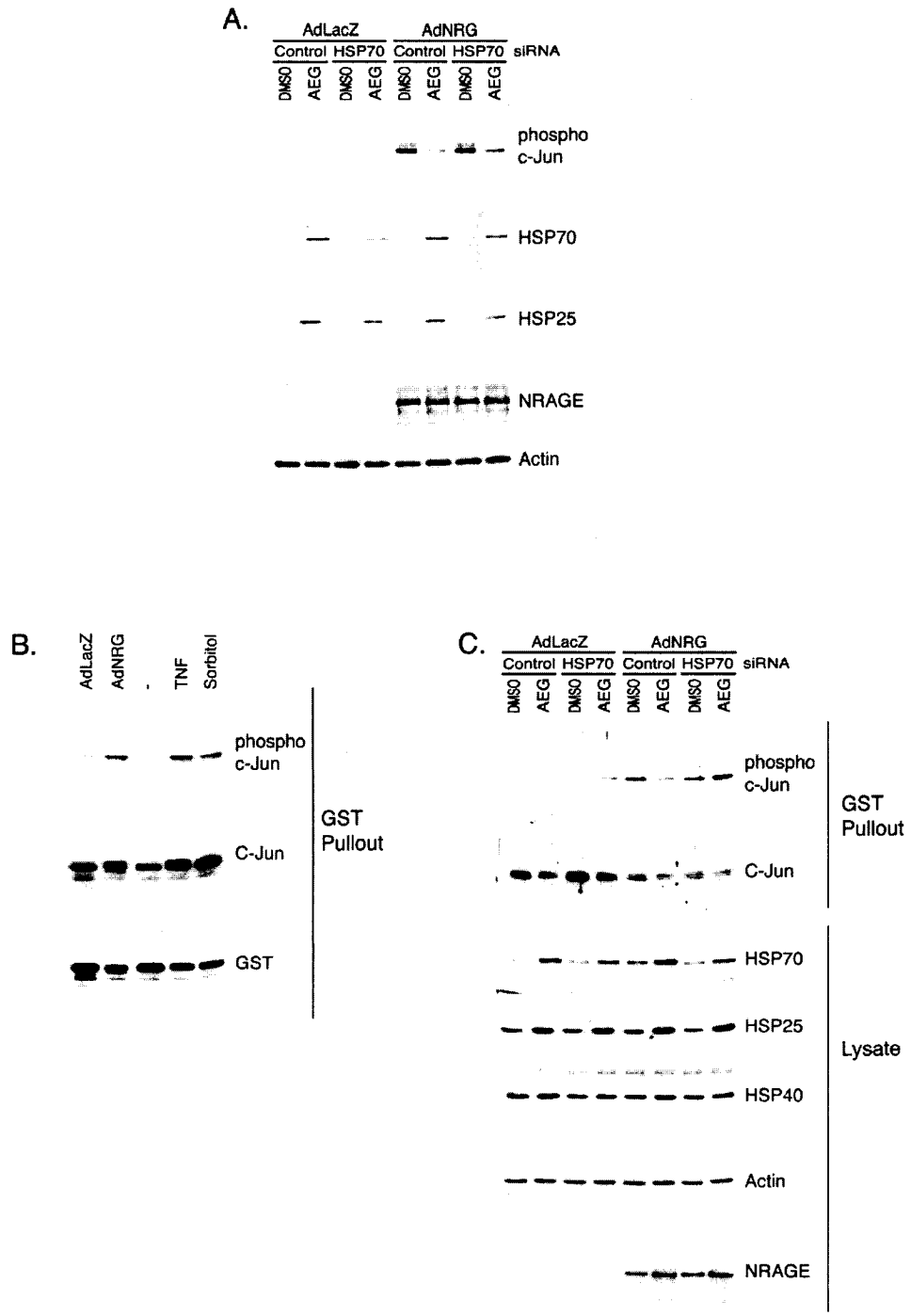


Figure 4.7. AEG3482 analogues inhibit JNK activation and induce expression of HSP70. A: PC12^{rtTA} cells were transfected with RNAi directed against HSP70 or with control RNA and then 24 hours later were infected with 5 MOI of AdNRG or 50 MOI of the control virus AdLacZ. At time of infection, the cells were treated with 40 □M AEG19940 or with a vehicle control, as indicated. After 30 hours, cells were lysed,

normalized for protein levels, and analyzed for phosphorylated Jun, HSP70, HSP25 NRAGE and actin levels. B: $PC12^{rtTA}$ cells were transfected with an expression construct driving GST-Jun. After 24 hours, cells were infected with 5 MOI of AdNRG or 50 MOI of the control virus AdLacZ or were left uninfected. After a further 24 hours, uninfected cells were left untreated or were exposed to 10 ng/ml TNF or 300 mM sorbitol for 15 minutes. Cells were then lysed, normalized for protein levels, and GST-jun was recovered by glutathione pullout and analyzed for phosphorylation using a antibody directed against c-Jun pSer63 by immunoblotting. Equivalent pullout of GST-Jun was confirmed by immunoblotting with an antibody directed against c-Jun and GST. C: $PC12^{rtTA}$ cells were co-transfected with an expression construct driving GST-Jun and with RNAi directed against HSP70 or with control RNA, as indicated. After 24 hours, cells were infected with 5 MOI of AdNRG or 50 MOI of the control virus AdLacZ. At time of infection, the cells were treated with 40μM AEG19940 or with a vehicle control, as indicated. After 30 hours, cells were lysed, normalized for protein levels, and GST-jun was recovered by glutathione pullout and analyzed for phosphorylation using an antibody directed against c-Jun pSer63 by immunoblotting. Equivalent pullout of GST-Jun was confirmed by immunoblotting with an antibody directed against total Jun. Lysates levels of HSP70, HSP25, HSP40, NRAGE and actin was determined by immunoblot. Experiments in A and C were performed three times and experiment in B was performed twice, all with essentially identical results.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 Major Findings

1. We cloned a novel member of the MAGE family of proteins, termed NRAGE (for Neurotrophin Receptor MAGE homologue), and demonstrated that it interacts with p75NTR in yeast, in co-transfected cells, as well as in cell endogenously expressing both proteins. Moreover, the interaction between NRAGE and p75NTR is direct, as a bacterially expressed p75NTR fragment can interact with *in vitro* translated NRAGE. Furthermore, our data suggests that the region within NRAGE responsible for p75NTR binding is the MAGE-homology domain and flanking sequences, which interacts with the juxtamembrane cytosolic domain of p75NTR. The interaction of NRAGE, or its homologues, with p75NTR has now been confirmed by two independent groups. NRAGE homologues constitute the only cytosolic p75NTR interactors to have been independently isolated by two different groups.

2. We determined that NRAGE participates in p75NTR-mediated apoptotic signaling by demonstrating that ectopic expression of NRAGE promotes p75NTR-dependent death in cells normally insensitive to p75NTR-mediated signaling.

3. Death induced by overexpression of NRAGE in a PC12 subline was found to take place through the JNK-dependent activation of the intrinsic death pathway. This pathway is identical to the one activated following ligand binding to p75NTR, or following overexpression of p75NTR in PC12 cells. Thus the ability to activate the JNK pathway provides support for NRAGE as a physiologically relevant mediator of p75NTR signaling.

4. AEG3482 was found to act as a potent inhibitor of both p75NTR- and NRAGE-mediated apoptotic signaling. The apoptotic inhibitory activity of AEG3482 was attributed to its ability to derepress HSF1, and activate HSF1-dependent expression of HSP70. Induced HSP70 was found to act as an inhibitor of JNK activity, as has been previously suggested. Therefore AEG3482 blocks

cell death through indirect inhibition of JNK. The identical sensitivity of p75NTR- and NRAGE-initiated signaling to AEG3482, or to its analogues, provides further support for NRAGE as a mediator of p75NTR-induced apoptotic signaling.

5.2 The MAGE family of proteins

As discussed in Chapter 2, we identified the novel protein NRAGE (for Neurotrophin Receptor interacting MAGE homologue), as a p75NTR intracellular domain interacting molecule, through the use of a yeast two-hybrid screen. NRAGE is a member of the MAGE (for melanoma associated antigen) family of proteins, and other than homology to the MAGE family, NRAGE does not bear any sequence similarity to other known proteins.

Members of the MAGE family are defined by a unique ~200 amino-acid conserved domain, known as the MAGE homology domain (MHD). The majority of MAGE proteins are composed of the MHD and relatively short flanking sequences, whereas longer MAGE proteins, such as NRAGE (775 residues in rat), usually contain the MHD towards their C-terminus (2).

Genes of the MAGE family are divided into two groups, termed group I and group II, with NRAGE (also known as MAGE-D1) belonging to the second group. This division is based on sequence homology, expression pattern, gene organization and chromosomal localization (2).

5.2.1 Group I MAGE Proteins

MAGE proteins belonging to the group I are characteristically not expressed in most adult tissues except the testis. However they are highly expressed, at both the mRNA and protein levels, in a variety of tumor types (667). In fact, more than half of human cancers of various histological type express at least one group I MAGE gene (667).

Demethylating agents induce the expression of group I MAGE proteins in cells that normally do not express these proteins, suggesting that MAGE genes are developmentally repressed though methylation of the promoter (673, 674, 756). It has thus been proposed that genetic instability in cancer cells, which is known to cause the loss of methylating control (757), results in the preferential expression of MAGE genes (673, 674, 756).

In the normal adult, the expression of group I MAGE proteins is restricted to the testis (758-762). Expression of some members has also been reported in the

placenta (758) and fetal ovaries (763). Within the gonads, Group I MAGE expression is restricted to the germ cells, with different MAGE genes expressed at distinct stages of gamete development (759, 763-765).

Due to their specific expression in tumours and germ cells, group I MAGE proteins have been hypothesized to play a role in cell division, differentiation or migration. However, no data supporting such function has been obtained and MAGE proteins have not been found to induce oncogenic transformation *in vitro*. Nevertheless, due to their cancer specific expression, group I MAGE proteins have gathered interest as a diagnostic marker for cancer.

Group I MAGE proteins have also been investigated as possible targets for anti-cancer immunotherapy. MAGE genes were initially identified as a collection of genes encoding tumor-rejection antigens expressed on human cancer cells (766). Like other proteins within the cell, as MAGE proteins undergo normal degradation, the resulting short peptides are transported into the ER lumen where they can bind HLA molecules. These fragments of the MAGE proteins are then presented on cell surface as MHC-class I antigens, and are recognized as 'foreign' by autologous cytotoxic T-lymphocytes. In contrast, fragments of proteins which are expressed normally in the tissue, are regarded as 'self' (667). Therefore, Group I MAGE proteins present ideal targets for cancer vaccine development.

All human group I MAGE genes map to the X-chromosome, and are further split into three subdivisions based on their specific chromosomal locations. MAGE-A genes are clustered at Xq28; MAGE-B genes at Xp21, and MAGE-C genes at Xp26-27 (762). Most group I MAGE genes are short, containing little more than the MHD, and their genetic structure is characterized by a large terminal exon encoding the entire open reading frame (762) (Figure 5.1).

5.2.2 GROUP II MAGES

Group II MAGE genes map to chromosomal locations distinct from the group I gene clusters. Also, unlike group I members, they are expressed in various normal tissues (2, 762). Their MHD can also be phylogenetically separated from

those of group I members and, furthermore, group II members contain a ~40 amino-acid region of homology immediately C-terminal to the MHD, that is lacking from group I MAGE proteins (Figures 5.1, 5.2). We have termed this region of homology the CCM (for conserved-domain C-terminal to the MHD).

Group II MAGEs are further split into several subdivisions based on chromosomal location and sequence homology. The four MAGE-D proteins, namely NRAGE (also known as MAGE-D1), MAGE-D2, -D3, and-D4, map to Xp11. Some additional group II MAGEs are also X-linked, whereas others such as Necdin, MAGE-G1, MAGE-F1 and MAGE-L2, map to various autosomal chromosomes (762). Like group I MAGES, most group II MAGEs are encoded by a single exon, however MAGE-D proteins are encoded by multiple exons (762).

Across species, group I MAGEs demonstrate little evolutionary relationship. For instance, MAGE-A genes in mice are much closer to each other than to their human orthologues, and a MAGE-C cluster does not exist in the mouse genome. This indicates rapid recent evolution of group I genes. In contrast, group II MAGEs show strong evolutionary conservation, and an unambiguous orthologue exist for each member in the mouse and human genome. For instance, the MHDs of the individual mouse MAGE-D genes bear over 99% identity to their human orthologues. These genes are much closer to their human orthologues, than to other MAGE-D members in mice (762).

Outside the MHD and the CCM, only sporadic regions of loose homology are observed within members of group II MAGE proteins, with most proteins containing long stretches of unshared sequence (Figure 5.2). For instance, NRAGE/MAGE-D1 contains an interspersed repeat domain (IRD) -consisting of 25 tandem repeats of the hexapeptide WQXPXX- amino-terminal to the MHD. This unusual motif is not present in other MAGE-D proteins, or in any protein in the NCBI database, but it is well conserved between the NRAGE/MAGE-D1 proteins in mouse, rat and human (2).

MAGE-D genes contain 13 exons, with 11 encoding the protein. In contrast, all other MAGE genes are characterized by a less complex genomic

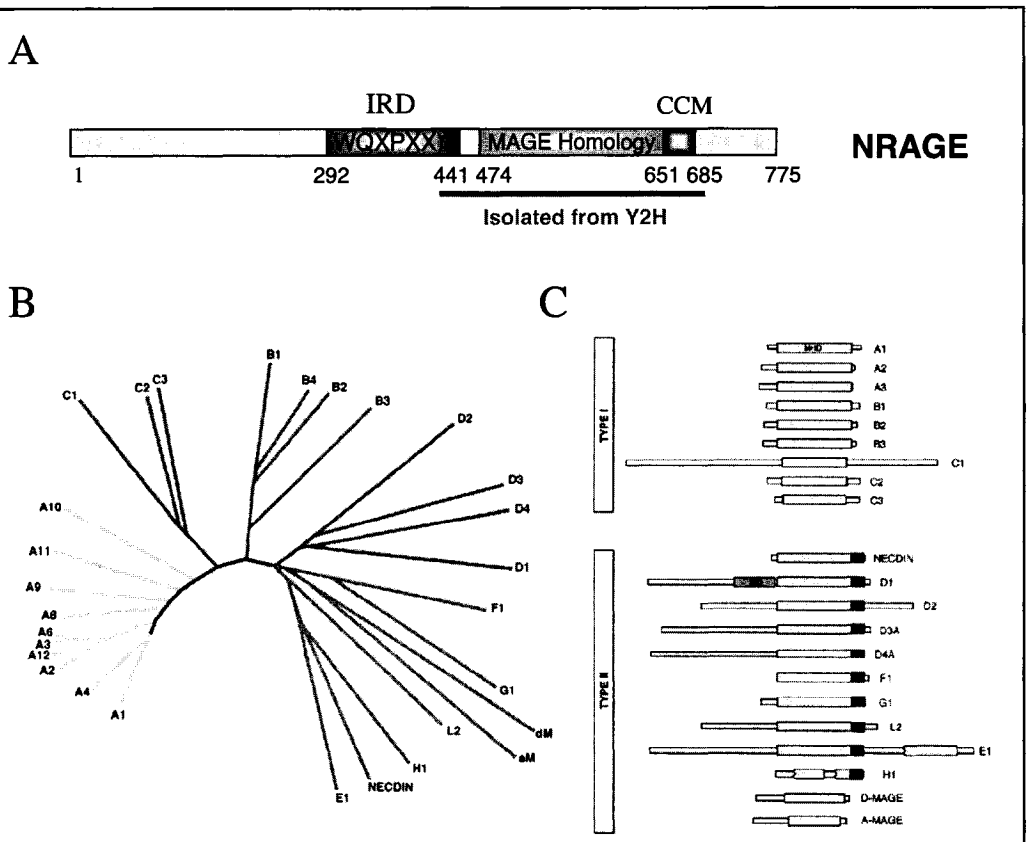


Figure 5.1 The MAGE family of proteins. A, schematic representation of NRAGE with its unique interspersed repeat domain (IRD), and its conserved MAGE-homology domain (MHD) and conserved-domain C-terminal to the MHD (CCM). B, phylogenetic relationship of group I and group II MAGE proteins. C, Schematic representation of group I and group II MAGES. The MHD is represented by the light colored boxes, the CCM is represented by the dark colored boxes. [Adapted from (2)].

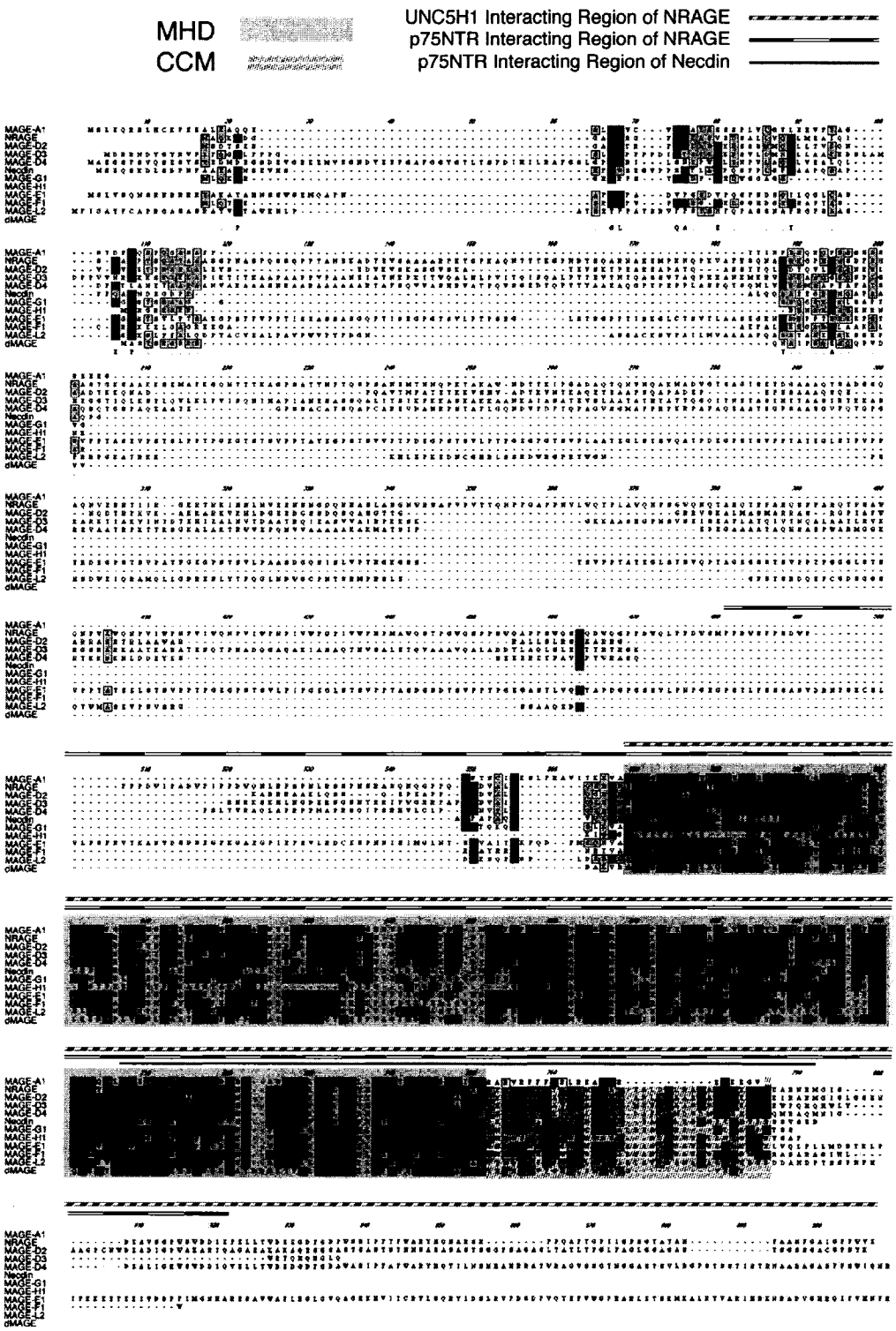
structure, most of which contain a large terminal exon carrying the complete coding sequence. This suggests that MAGE-D genes are the ancestral genes which gave rise to the first member of other MAGE subdivisions by retroposition (762, 767, 768). Some MAGE genes produced by retroposition, such as Necdin, are highly evolutionary conserved, and important non-redundant biological activities have been assigned to them (see below). The evolutionary recruitment of retrogenes to novel activities is a process referred to as exaptation (769).

Gene duplication has also clearly contributed to the emergence of the multigenic MAGE subfamilies. Some of these duplications, such as those among the evolutionary non-conserved group I MAGEs, are believed to have occurred

recently. In contrast, the duplication events that produced different members of the MAGE-D family appears to be much older. These genes have significant differences within the MHD domain and bear very divergent regions outside this domains, however they are highly conserved between orthologues in human and rodents. This clearly indicates that the MAGE-D genes have evolved independently for a long-time before the phylogenetic separation of the two species. The fact that very different MAGE-D genes are evolutionary conserved suggests that these genes exert important but distinct functions.

Consistent with an ancestral role, a zebrafish MAGE gene with a genomic structure similar to that of mammalian MAGE-D genes has been reported (762). Furthermore, single MAGE-like genes have been identified in the genome of *Drosophila melanogaster* and *Arabidopsis thaliana*.

Figure 5.2 Alignment of MAGE protein sequences. Group II MAGE proteins, along with group I member MAGE-A1 were aligned using ClustalW. Residues identical in more than 50% of the sequences are given a dark grey background, and those with more than 50% simlarity have a medium grey background. Other regions of interest are indicated as below.



5.3 Group II MAGEs as p75NTR interacting molecules

We identified NRAGE as a p75NTR interacting molecule in a GAL4-based yeast two-hybrid screen. In this screen, a bait gene, that is the p75NTR intracellular domain (ICD), was expressed as a fusion to the GAL4 transcription-factor's DNA-binding domain (DNA-BD), while a cDNA library was expressed as a fusion to the GAL4 activation domain (AD) (770, 771). When the bait and the library-derived fusion proteins interact, the DNA-BD and AD are brought into proximity, thus activating transcription of a reporter gene.

Using the yeast-two-hybrid technique and *in vitro* interaction assays, we found that the p75NTR-interacting region of NRAGE is located towards its C-terminus, between amino-acids 410-709. This 300 amino acid region contains the MHD and CCM plus an additional ~50 amino acids at the N-terminus (Figure 5.2). As discussed above, the MHD and CCM are highly conserved among members of group II MAGE proteins (Figure 5.2). Therefore, it would not be unexpected if other group II MAGE proteins were also found to interact with p75NTR.

Indeed, two years after our report of an interaction between NRAGE and p75NTR, the Fainzilber group reported that they had isolated the group II MAGE protein Necdin as a p75NTR ICD interactor in a yeast-two-hybrid screen (772). Further tests revealed that the group II MAGE protein MAGE-H1 also interacts with the p75NTR ICD (772). The yeast two-hybrid technique used for these screens was not the GAL-4 based system used previously by us, but rather the Ras-rescue based system (RRS).

In the RRS system (773), the cDNA library is cloned downstream of a myristoylation signal, localizing these proteins at the yeast plasma-membrane. The bait gene, in this case the p75NTR ICD, is fused to a Ras GEF deletion mutant. As the yeast strain used in the assay has a mutation in the endogenous Ras GEF, there is a need for the exogenously supplied Ras GEF for growth. However, to be effective, the exogenous RasGEF has to be localized at the membrane though an interaction between the fused p75NTR fragment and a gene within the

myristoylated cDNA library. Thus a positive interaction can be assessed by the growth of a yeast colony (773).

Therefore, group II MAGE proteins have been isolated as p75NTR interacting molecules using two very different screens. The RRS system may be a more appropriate technique for detecting interactions with the p75NTR ICD than the GAL-4 based technique, as the binding occurs in a membrane proximal region of the cytoplasm instead of the nucleus.

Fainzilber and colleagues confirmed the interaction between p75NTR and Necdin or MAGE-H1 in transfected Cos7 cells. The interaction with Necdin was also confirmed with endogenous proteins expressed in DRG neurons (772). In a later study they also repeated our observation of an endogenous interaction between NRAGE and p75NTR in PC12 cells (774).

A study by an independent group (775), soon after, confirmed the interaction between Necdin and p75NTR. Furthermore, they found that the group II MAGE protein MAGE-G1 also interacts with p75NTR. These interactions were confirmed in an *in vitro* binding assay, in co-transfected Cos-7 cells, and in the P19 embryonal carcinoma cell line which endogenously express these proteins.

NRAGE and Necdin, have been found to associate with p75NTR, not only at the plasma membrane, but also in endosomes internalized after p75NTR binding to NGF (774). Therefore, it has been proposed that Necdin and NRAGE form part of a p75NTR signaling endosome (774).

In summary, the group II MAGE proteins NRAGE, Necdin, MAGE-H1 and MAGE-G1 have all been found to interact with p75NTR. From the interaction data reported by the various groups, the p75NTR interacting domain of these proteins likely maps to the MHD C-terminus and the CCM (see Figure 5.2). There is great conservation between group II MAGE proteins in this region and no obvious dissimilarities can be observed between group II MAGEs reported to interact with p75NTR, and those where no interaction has yet been reported. Therefore, it may be assumed that all group II MAGEs can interact with p75NTR *in vitro*. Whether such an interaction would be permitted by the MAGE's expression pattern and subcellular localization is yet to be determined. As group I

MAGEs lack the CCM, it is unlikely that they retain the ability to interact with p75NTR, however this possibility has not been directly tested. Even if such an interaction can take place *in vitro*, it is unlikely that it would have physiological significance due to the extremely restricted expression pattern of group I MAGEs (although a role in tumour cells can not be ruled out).

Our knowledge about the biological roles of the confirmed p75NTR interactors, MAGE-G1 and MAGE-H1, is extremely limited. However, much more is known about Necdin and NRAGE.

5.3.1 NECDIN

The group II MAGE protein Necdin is perhaps the best characterized member of the MAGE family. It is a 321 amino acid (in humans) protein composed of a highly evolutionarily conserved MHD and CCM, and a more loosely conserved N-terminus. Its highest expression levels are found in the nervous system, where it is expressed by subpopulations of post-mitotic neurons in specific regions (776), such as the hypothalamus (681, 683, 772, 776, 777), pons, medulla, midbrain, thalamus (681), DRG (683, 772, 777-779), and SCG (777). It is also reported to be expressed, at lower levels, in several non-neural tissues (683).

5.3.1.1 Necdin and Prader-Willi Syndrome

The importance of Necdin in human nervous system development has been dramatically underscored by the finding that deletion of the Necdin gene contributes to the phenotype of patients with the multigenic Prader-Willi syndrome (PWS) (684-686, 780, 781).

PWS is a common neurogenetic disorder which results in a high rate of neonatal respiratory depression, hypotonia and failure to thrive. Survivors are affected by hyperphagia, obesity, hypogonadotropic hypogonadism and mild to moderate mental retardation (782).

PWS is a classic genomic imprinting disorder and patients invariably have genetic abnormalities of the 15q11-q13 chromosomal region that results in

inactivation of genes normally expressed only from the paternal allele. These genetic abnormalities fall into three categories; deletion of the paternal chromosomal region, maternal uniparental disomy, or imprinting mutations (782). Several imprinted, paternally expressed genes have been identified within the PWS candidate region, one of which is Necdin. As PWS is invariably associated with the loss of paternal specific expression of several genes, PWS likely represents a contiguous gene syndrome, with deficiencies of several genes contributing to the phenotype (782).

In the mouse like in humans, the Necdin gene is a paternally expressed, maternally imprinted gene contained in a region of conserved synteny to the PWS region. Moreover, Necdin is the first gene from the PWS deletion interval whose single deletion in mice results in a phenotype mimicking some aspect of PWS. Namely, two independently produced lines of mice with a null mutation in the paternal Necdin allele exhibit high rates of perinatal hypotonia and respiratory distress. Depending on genetic background, up to 98% die within 30 hrs after birth due to respiratory failure (683, 776).

The respiratory defect in Necdin-null mice is associated with abnormal neuronal activity within the putative respiratory rhythm-generating center, the pre-Botzinger complex, perhaps reflecting loss or perturbation of rythmogenic neurons within the medullar pre-Botzinger complex, or perturbation of neurons that normally modulate the activity of the rythmogenic neurons (783). Therefore, in patients with PWS, it is likely that Necdin deficiency contributes to the observed respiratory abnormalities through a similar suppression of central respiratory drive.

Hypothalamic deficiencies have been suggested to underlie a number of symptoms of PWS (784). Hypothalamus of PWS patients has a significantly reduced number of oxytocin-expressing neurons in the paraventricular nucleus (PVN), which could trigger the hyperphagia and obstetric problems observed in these subjects (784). Also, in PWS patients, alterations in the LHRH neurons are thought to be responsible for the decreased level of sex hormones and hypogonadism observed in these patients (784). Similar neuronal deficiencies are

also observed in Necdin-deficient mice, which exhibit over a 25% reduction in the number of hypothalamic oxytocin and LHRH-producing neurons (776). Therefore Necdin-deficiency likely contributes to the hypothalamic abnormalities in PWS patients.

5.3.1.2 Interactors of Necdin

The molecular events that lead to the neuronal deficiencies in Necdin-null mice remain to be identified. Nevertheless, Necdin has been found to interact with a number of cell-cycle and differentiation regulatory proteins within the cell. As it is possible for the deficiencies observed in Necdin-null mice to be caused by defects in neuronal proliferation and differentiation, these proposed interactions may have important physiological significance.

It has demonstrated that Necdin can physically interact with E2F-1 and p53 (663, 664). p53 protein can arrest cell cycle progression, or trigger apoptosis, by acting as a transcription factor to a large number of genes involved in the regulation of cell cycle and cell death. p53 can also induce death in a transcription-independent manner (785). Necdin overexpression, in turn, inhibits both p53 dependent transcription and p53 dependent apoptosis *in vitro* (664).

E2F-1 regulates the transcription of several genes, the products of which are required for the transition from G1 to the S phase of the cell cycle, and for DNA metabolism (786). However, apart from its proliferative function, high levels of deregulated E2F-1 are capable of inducing apoptosis in a cellular context-dependent manner (786). Necdin overexpression, in turn, inhibits E2F1-dependent transcription *in vitro* (663).

Necdin has also been found to interact with centrosome associated proteins Fez1 and BBS4 (787). The centrosome plays a pivotal role in mitosis as the cell's microtubule organizing center. As well, in post-mitotic neurons, the centrosome is crucial for axonal elongation and particle transport. Namely, axonal growth depends on microtubule nucleation at the centrosome, and on the translocation of microtubules that are released from the centrosome into the axon in a Dynein-dependent manner (788).

The Necdin interactor Fez1 colocalizes with the centrosome and its functions are known to include the regulation of transport by the microtubule motor protein Kinesin (789). Null-mutations of the Fez1 orthologue in *C. elegans* have a defect in axonal bundling which could arise from deficiencies microtubule-dependent transport (789-793).

The Necdin interactor BBS4 is proposed to be an essential protein for proper functioning of the centrosome, as BBS4-deficient cells have a loss of microtubule anchoring at the centrosome (794). Thus, BBS4-null cells are defective in cytokinesis and are prone to apoptosis (794). Consistent with a microtubular defect, BBS4-null animals display a deficiency in the sensory cilia of photoreceptors and olfactory neurons associated with distorted microtubule bundles (795).

Thus, in brief, Necdin has been proposed to interact with a number of proteins that regulate cell division, apoptosis, and differentiation/neurite-outgrowth, deficits in which could underlie the phenotype of Necdin-null animals. However, the physical and functional interactions of these proteins with Necdin have only been demonstrated in settings where both proteins are overexpressed, and the physiological relevance of these interactions has yet to be demonstrated *in vivo*.

Nevertheless, consistent with a role in cell division, ectopic expression of Necdin has been found to lead to cell cycle arrest in a number of cell lines including NIH 3T3, HEK293, SAOS2 and U2OS cells (662-664). Also, Necdin overexpressing PC12 cells display accelerated neurite-outgrowth in response to NGF (772). Moreover, ectopic Necdin expression also induces neurite-outgrowth in neuroblastoma cells (796). Whether, the accelerated differentiation observed after Necdin expression is due to hastened cell cycle arrest, or differentiation per se, is not known.

In vivo, Necdin-null embryos display a number of misrouted or stunted axon projections and tracts. These include a reduced axonal bundle leading into the anterior commissure, abnormalities in the corpus callosum, optic tract, and thalamocortical, serotonergic, catecholaminergic projections (787). The molecular

basis for these defects are not yet known, however, they are consistent with malfunction of the centrosome.

5.3.1.3 Necdin as a p75NTR Interactor

If Necdin is indeed a physiologically relevant interactor of p75NTR, it is expected that there would be overlap in the phenotype of mice null for either protein. Accordingly, there is great overlap, both spatially and temporally, in the expression patterns of the two proteins in post-mitotic neurons. For instance, p75NTR and Necdin genes are co-expressed early in development in the thalamus, in the preoptic and septal area of the basal telencephalon, in the diagonal band of Broca, in the striatum, in sympathetic ganglia, in dorsal root ganglia, and in the inner nuclear layer of the retina giving rise to the retinal ganglion cell layer (779). Also, in the telencephalic neuroepithelium, from E13 to E18, the expression of p75NTR in the preplate and the subplate (566) is similar to Necdin expression (779). Moreover, both Necdin and p75NTR expression have been reported in the adult hypothalamus as well as in LHRH producing neurons (776, 797).

Axons of Necdin-null sympathetic neurons extend slower in response to NGF, and are defective in directional growth (787). Projections of these neurons also appear thinner due to lack of axonal bundling. These defects are reminiscent of sympathetic projections in the p75NTR-null, where there is drastic reduction in the innervation of certain targets, and projections reportedly appear to be much thinner (495). Similarly, in NGF overexpressing animals, p75NTR has been proposed to play a role in promoting the directional pattern of sympathetic neurite growth, as sympathetic axons of p75NTR-null mice grow more randomly within the ganglionic neuropil (565). Additionally, growth cones in both Necdin-null SCG neurons and p75NTR-null subplate neurons have been found to have an aberrant morphology (566, 787).

Interestingly, both p75NTR-null and Necdin-null embryos have reduced thalamic projections in to the intermediate zone of the cortex (566, 787). Defects in axon bundling have been proposed to be the underlying factor in both cases

(566, 787). Consistent with this thalamocortical defect, both p75NTR and Necdin-deficient mice exhibit altered spatial learning (776, 798).

As the molecular causes underlying the above phenotypic alterations have not been investigated, it is not known if the perceived similarities in the p75NTR-null and Necdin-null mice are due to defects in the same signaling pathway. However, the phenotypes are consistent with a defect in the proposed p75NTR→Necdin→Centrosome axis. Alternatively, as Rho regulation by p75NTR has been proposed to play a role in axon growth and guidance, Necdin binding to p75NTR may be contribute to RhoA regulation, via modulation of the p75NTR-RhoGDIα interaction, or by interacting with other Rho regulatory proteins.

5.3.2 NRAGE

We found that NRAGE can act as mediator of the NGF-initiated p75NTR-dependent apoptosis of the sympathoadrenal precursor MAH cells (chapter 2). NRAGE, like p75NTR, can initiate JNK-dependent mitochondrial apoptosis (chapter 3), consistent with its proposed role as a mediator of p75NTR signaling. Accordingly, in both cases, cell death is blocked by a newly characterized inhibitor of the JNK pathway, AEG3482 (chapter 4).

Due to the potent suppression of NRAGE-induced apoptosis by several inhibitors of the JNK pathway, including the JNK-binding-domain (JBD) of JIP-1, dominant-negative c-Jun, and AEG3482, we believe that activation of the JNK pathway is the primary, if not the only, manner in which NRAGE induces apoptotic cell death.

5.3.2.1 Proposed Interaction of NRAGE with XIAP

In 2001, an independent group (714) found that NRAGE interacts with the endogenous caspase-inhibitor XIAP and, contrary to our observations, proposed that NRAGE-initiated apoptosis is mediated by NRAGE-dependent degradation of XIAP, leading directly to increased caspase-activity and thus increased apoptosis. Accordingly, NRAGE-mediated potentiation of cell death was

proposed to occur downstream of the mitochondria, as it was not blocked by anti-apoptotic Bcl-2 members. Whereas, in our model, NRAGE-mediated potentiation of cell death occurs upstream of mitochondrial release of Cytochrome c.

There are, however, many flaws in the above mentioned study. First, while NRAGE overexpression was shown to induce degradation of endogenous XIAP, this decrease in XIAP levels was never causally linked to the NRAGE-induced potentiation of apoptosis. Therefore the two phenomena could be unrelated. In fact, as discussed in an earlier chapter, whereas XIAP overexpression is known to lead to the inhibition of apoptosis (799-801), suppression of XIAP expression does not lead to increased apoptosis in non-transformed mammalian cells (see chapter 1). For instance, XIAP-null mice display no obvious apoptotic phenotype (109). Therefore, in a physiological setting, XIAP degradation would not lead to increased apoptosis.

Also, it was claimed that NRAGE-induced potentiation of cell death occurs downstream of the mitochondria as it is not blocked by overexpression of Bcl-2. However, the experiment addressing this point was fundamentally unsound as it did not compare cell death induced by NRAGE alone to that induced by co-expression of NRAGE and Bcl-2 (instead it compared death occurring in the presence of Bcl-2 alone to that induced by NRAGE in the presence of Bcl-2).

Nevertheless, the physical interaction data in this report appears valid. NRAGE was found to interact with XIAP in yeast, co-transfected 293 cells, and a 32D cell line stably overexpressing NRAGE. NRAGE was found only to interact with XIAP and not other mammalian IAPs including c-IAP1, c-IAP2 and NAIP. Moreover, the interaction took place through the interspersed-repeat domain (IRD) of NRAGE, which is unique to this protein. Therefore the proposed NRAGE-XIAP interaction is unique to these two proteins, and not shared by other members of their respective protein families.

Interestingly, XIAP has been proposed as a modulator of the JNK signaling pathway. Overexpression of XIAP can block TNFα-induced activation of the JNK pathway through an unknown mechanism. XIAP is capable of interacting with the JNK-activating kinase TAK1 (802, 803), but, curiously, this

interaction appears to result in activation rather than inhibition of JNK signaling (803). Nevertheless, the possibility that regulation of XIAP activity by NRAGE has a modulatory effect on the intensity or time-course of JNK activity merits further study.

5.3.2.2 Proposed Interaction of NRAGE with Msx and Dlx

The IRD of NRAGE has also been found to interact with some members of *meshless* (Msx) and *distaless* (Dlx) families of proteins. These interactions are direct as they occur *in vitro* using recombinant proteins, as well as in co-transfected cells. Interactions have been confirmed with Msx family members Msx1 and Msx2 (804, 805), and weaker interactions have been observed with Dlx5, and Dlx7 (804).

Msx and Dlx form two distinct, but closely related, subfamilies of homeobox genes that play essential roles in the morphogenesis of several systems, especially that of the musculoskeletal structure. Genetic, cellular, and biochemical evidence suggests that Msx and Dlx genes play important roles in early, middle, and late stages of craniofacial, axial, and appendicular skeletal formation. Initially, the differential expression patterns of Msx and Dlx genes confer spatial information to the mesenchyme of branchial arches and limbs. At later stages of embryonic development, these proteins support the formation of more-defined skeletal structures, primarily by regulating epithelial-mesenchymal signaling (806).

Accordingly, targeted gene disruption of Msx1 and especially Msx2 results in numerous developmental alterations that include defects in the calvarial bones of the skull, chondrogenic craniofacial bone abnormalities, complete failure of tooth development, and defects in endochondral bone formation and mammary gland development (807-809). Genetic overexpression of Msx2 in mice leads to craniosynostosis (premature fusion of skull bones) and orofacial bone abnormalities (810).

The expression pattern of Dlx5 also suggests a role in craniofacial development (811, 812) and limb initiation (813). Accordingly, Dlx5-null mice

exhibit craniofacial abnormalities affecting derivatives of the first four branchial arches, severe malformations of the vestibular organ, a delayed ossification of the roof of the skull and abnormal osteogenesis (811). Dlx1 and Dlx2 pattern the dentition, and the null mice exhibit perinatal lethality and ectopic skull components (814-816). Genetic disruption of the Dlx-7 gene has not been yet reported.

Gain-of-function analysis of Msx1 function in cell culture and *in vivo* model systems suggest that Msx1 acts as a negative regulator of differentiation (817-819). This is achieved through its ability to repress the transcription of differentiation genes such as *MyoD* (817), and to regulate the expression and activity of cell cycle molecules such as Cyclin D1 and CDK4 (818). In contrast, Dlx proteins act as transcriptional activators necessary for the differentiation of various cell types (820). In some cases Dlx-induced transcriptional activation has been attributed to its ability to antagonize the functions of certain Msx proteins through direct interaction (820).

NRAGE interacts with Msx and Dlx proteins through binding to their weakly homologous N-terminal region (804, 821). This interaction has been found to lead to an increased in Dlx5 transcriptional activity in an artificial reporter system (804).

Whether the proposed interaction of NRAGE with Msx/Dlx proteins has any physiological significance is not known. However, NRAGE-null animals do not display any anomaly in the formation and ossification of the skeleton, including in the craniofacial structures, limbs, vertebrae and ribs (822), inconsistent with perturbed Msx/Dlx function. As NRAGE binding to Msx/Dlx proteins occurs though its distinctive IRD, functional redundancy cannot explain the lack of phenotype in this case. Therefore, currently, no data exists in support of a functional *in vivo* interaction between NRAGE and Msx/Dlx.

5.3.2.3 UN5H

It has recently been reported that NRAGE directly interacts with the transmembrane receptor UNC5H1, but not with its homologues UN5H2 and

UN5H3 (823). The UNC5H family is the mammalian orthologue of the Uncoordinated-5 (UNC5) gene in *C. elegans*. UNC5H proteins, similarly to DCC, act as receptors for the axon-guidance molecule Netrin/UNC6 (824). However, whereas DCC mediates Netrin-1 induced growth-cone attraction, a complex of UNC5 and DCC mediates repulsion (825). There is also evidence suggesting that short range repulsion by Netrin-1 may be mediated by UNC5 alone (826, 827).

Other than their role in axon guidance, both DCC and UNC5H receptors have been found to initiate apoptosis, and the binding of Netrin to these receptors has been found to cause an inhibition of apoptotic signaling (823, 828-833). Accordingly, in Netrin-1-null mice, expression of either DCC or UN5H colocalizes with areas of apoptotic precerebellar neurons, whereas apoptosis is absent in the same DCC/UNC5H-expresing area in wild-type mice (832, 834).

The mechanism through which DCC and UN5H activate apoptotic pathways is still not fully understood. However, the DCC induced cell death has been found to be Caspase-9 dependent (830), and DCC itself has been found to indirectly interact with Caspase-9 in the absence of Netrin-1 (830). As, DCC induced activation of Casapase-9 is independent of the mitochondria (830), it has been suggested that the interaction of DCC with Caspase-9 leads to the activation of this caspase through a novel mechanism (830).

The mechanism of UNC5H death induction has also not been fully defined. However, it has been demonstrated that , upon overexpression, UNC5H1 is much more potent at inducing cell death than either UNC5H2 or UNC5H3 (823). Similarly UNC5H1, but not the other homologues, interact with NRAGE. The NRAGE domain responsible for binding to UNC5H1 maps to the same C-terminal region, containing the MHD and CCM, known to interact with p75NTR (823) (Figure 5.2).

As in the case of p75NTR, NRAGE has been proposed to mediate apoptotic signals initiated by UNC5H1 (823). The domain within UN5H1 responsible for apoptotic signaling maps to the NRAGE interaction region (823), and furthermore, UNC5H1 is incapable of inducing death in a cell line expressing

very low amounts of NRAGE, whereas ectopic expression of NRAGE restores the ability to undergo UN5H1-induced apoptosis (823).

Intriguingly, both p75NTR and UNC5H1 are type I transmembrane proteins containing a C-terminal type II death domain. Also, both p75NTR and UNC5H play roles in axonal growth and apoptosis. Moreover, the regions within these receptors responsible for both the interaction with NRAGE and the induction of apoptosis have been mapped to a juxtamembrane region containing a PEST motif [chapter 2; (635, 823)]. As both receptors interact with the same region within NRAGE [chapter 2; (823)], it is likely that interaction of NRAGE with either receptor leads to similar signaling events.

Furthermore, the overexpression of p75NTR, just like that of UNC5H1 is known to induce apoptosis in the absence of exogenously added ligand *in vitro* (441, 632). Therefore the abilities of both p75NTR and UNC5H1 to induce ligand-independent cell death, be it in an artificial system, could stem from their intracellular homology and/or their interaction with similar cytosolic interactors e.g. NRAGE.

5.3.2.4 NRAGE as a Proapoptotic Molecule *in vivo*

Recently, null mice for the NRAGE gene were constructed. Consistent with a role for NRAGE in the mediation of the p75NTR generated death signal, NRAGE-null mice mirror several of the apoptotic deficits displayed in p75NTR-null mice. As mentioned in chapter 1, p75NTR plays a necessary role in the apoptosis of hair follicle keratinocytes during the normally occurring regression of the hair follicle (629). Accordingly, p75NTR-null mice showed a significant delay in the morphological progress of hair follicle regression (629). NRAGE-null mice have also been reported to display a similar delay of follicular regression (822).

Also as discussed in chapter 1, p75NTR is necessary for apoptosis of embryonal spinal cord motor neurons, and p75NTR-null mice display an over 50% reduction in the number of dying motor neurons early in development (627).

NRAGE-null mice display a similar reduction in motor neuron cell death, consistent with a role for NRAGE in mediation of p75NTR signaling (822).

p75NTR also acts as an apoptotic molecules in other cell types such as sympathetic neurons, corticospinal neurons or oligodendrocytes (493, 523, 588). It however remains to be determined whether NRAGE also acts as a necessary mediator of p75NTR signaling in these situations.

Interestingly however, NRAGE-null mice display reduced apoptosis in DRG neurons during the period of target dependent cell death. This is surprising as p75NTR is believed to play an overall pro-survival role in the DRG neurons during this period (445, 490-492, 518), presumably through promotion of NGF-dependent activity of TrkA. Two explanations could be offered for the contrasting effects of p75NTR and NRAGE on DRG neuron survival. One possibility is that the p75NTR→NRAGE→death axis is intact in DRG neurons, but is offset by a p75NTR/TrkA→survival signal, with the overall result of a pro-survival role for p75NTR.

Alternatively, the pro-apoptotic role of NRAGE in DRG neurons is p75NTR-independent, and due to an unrelated signaling pathway. In fact, generally speaking, the NRAGE expression pattern encompasses that of p75NTR, but also includes many tissues and cell types that do not express p75NTR (835, 836) . Thus, we do not believe that the sole function of NRAGE is to act as an apoptotic adaptor molecule for p75NTR. Rather, we think of NRAGE as a more general apoptotic regulator, which can be harnessed by p75NTR, or other apoptosis signaling molecules such as UNC5H1, to mediate their effects.

5.3.2.5 Other Possible Roles of NRAGE

The ability of p75NTR to regulate neuronal outgrowth, together with the neurite-outgrowth abnormalities in mice deficient for the NRAGE homologue Necdin, and the reported interaction of NRAGE with the axon guidance receptor UNC5H1, raise the possibility that NRAGE may play a role in regulation of neuritic outgrowth/guidance. The high expression of NRAGE in neurons is consistent with such a role (836).

Hypothetically, NRAGE could modify axonal morphology through an as yet undefined mechanism. For instance, NRAGE could indirectly modulate Rho activity by modifying the ability of p75NTR to act as a displacement factor for RhoGDIα. Alternatively, the previously characterized NRAGE-induced JNK signal could play a role in NRAGE-mediated regulation of axonal morphology. In fact, the JNK pathway has been previously proposed to be necessary for growth and maintenance of neurites both *in vivo*, and *in vitro*.

JNK activity is induced during initial neurite outgrowth, and during neuritic regeneration after axotomy, in a number of neuronal populations both *in vivo* and *in vitro* (837-839). As well, JNK activity is induced in PC12 cells during NGF-induced differentiation (270). Accordingly treatment of differentiating PC12 cells, or axotomized adult nodose and dorsal root ganglion neurons, with pharmacological inhibitors or dominant-negative JNK constructs leads to a reduction in neurite outgrowth (837, 840).

The role of JNK in growth and maintenance of neurites has been partially attributed to the phosphorylation mediated activation of transcription-factors such as c-Jun, ATF-2 and Elk-1. For instance, ectopic expression of constitutively active c-Jun has been found to lead to neuritic outgrowth in PC12 cells (841, 842).

JNK also phosphorylates a number of cytoskeletal elements *in vivo*. These include the neurofilament heavy chain (843) and the microtubules associated proteins (MAPs), MAP2 and MAP1B (844), molecules that play important roles in maintenance of neuritic extensions (845, 846). JNK-mediated phosphorylation of MAPs is essential for MAP-mediated microtubule polymerization and stabilization (844). Accordingly, microtubules are abnormally short in JNK1-null mice, where several neuronal populations exhibit progressive degeneration of axonal and dendritic processes (844). Consistent with an important role in neurite extension/maintenance, the brain contains a high level of background JNK activity, which is, for the most part, localized to neuritic processes (839).

The circumstances under which activation of the JNK pathway leads to neuritic growth, rather than apoptosis, are not well defined. However factors such as the developmental stage of the affected cells, the time course of JNK

activation, and activation of concurrent pro-survival signaling paths are believed to play crucial role in this determination (842).

It remains to be determined whether NRAGE-mediated JNK activation can lead to a neuritic outgrowth response. However, as mentioned, Necdin has been proposed to play a role in axonal growth *in vivo*. Moreover, PC12 cell lines stably overexpressing either Necdin or JNK3 show a similar increase in neuritic outgrowth response when treated with NGF (772, 847).

5.4 Final Words

The observations described above present NRAGE as an important cytosolic interactor of p75NTR, and as mediator of p75NTR-induced JNK activation and subsequent apoptosis. However the exact mechanism of NRAGE 'activation' by p75NTR remains to be determined. We have detected an interaction between NRAGE and a JNK-scaffolding molecule, and believe that NRAGE-mediated activation of the JNK pathway may be, at least partially, due to the promotion of assembly of an active JNK signaling axis.

A flurry of recent findings has suggested that the signaling events generated by the p75NTR receptor complex is determined by the type of co-receptor and extracellular ligand involved in the complex. For instance, when in a complex with TrkA, p75NTR potentiates NGF-dependent TrkA activation, whereas p75NTR binding to NT-3 or BDNF inhibit activation of TrkA. Alternatively, when in a complex with NgR and Lingo1, treatment with myelin-derived growth inhibitors (MGIs) leads to a p75NTR dependent activation of RhoA. On the other hand, when complexed with Sortilin, treatment with proNGF leads to the activation of a cell death pathway. The propensity of p75NTR to form heteromeric complexes is supported by the recent crystal structure of NGF complexed with the ECD of p75NTR (433). Binding of dimeric NGF to a single chain of p75NTR results in changes in the NGF dimer that prevent binding to other p75NTR molecules, therefore NGF blocks, rather than facilitates the formation of p75NTR dimer, thus allowing for the recruitment of another receptor to the complex (433).

Although, due to its recent discovery, the nature of the apoptotic pathway initiated by proNGF and mediated by the Sortillin-p75NTR complex, has not been reported, it is believed that this pathway is one and the same as the pathway previously observed after treatment of p75NTR expressing cells [now known to also co-express Sortilin (419)] with commercial 'mature' NGF [comNGF, now known to contain a significant degree of proNGF (587)], i.e. the JNK pathway (see chapter 1).

Our data are also consistent with a model where NRAGE mediates proNGF-induced, Sortillin/p75NTR-mediated, JNK activation. Prior to the discovery of the biological roles of Sortilin and proNGF, we showed (chapter 2) that NRAGE mediates comNGF-induced death of MAH cells, an immortalized line of rat embryonic sympathoadrenal precursor cells. In the peripheral nervous system, sympathoadrenal precursor cells differentiate into sympathetic neurons or chromaffin cells (848). The expression level of Sortilin in MAH cells has not yet been addressed, however sympathetic neurons and chromaffin tumour derived PC12 cells both express high levels of Sortilin [(419); Barker, unpublished observation], therefore it is likely that Sortilin would also be expressed in the precursor of these cell types, the MAH cells.

We also observed that p75NTR/NRAGE-mediated cell death only takes place in the presence of comNGF, and not in the presence of comBDNF or comNT-3 (chapter 2). The comNGF used in this assay was submaxillary gland derived, whereas the BDNF and NT-3 were recombinant proteins derived from vectors encoding only the mature portion of these proteins (602). As submaxillary derived NGF is now known to contain a significant concentration of proNGF (587), and no cell death was observed in the presence of the mature recombinant neurotrophins, our observations are consistent with a pro-apoptotic role for proNGF. We therefore propose that NRAGE can mediate proNGF-induced Sortillin/p75NTR-mediated activation of the JNK pathway (Figure 5.3).

The requirement for p75NTR heteromerization also suggest an alternate manner in which NRAGE may promote p75NTR-mediated pro-apoptotic action, unrelated to its direct activation of the JNK pathway. We observed that the expression of NRAGE can disrupt the p75NTR-TrkA complex, whereas overexpression of TrkA can inhibit the apoptosis induced by p75NTR/NRAGE. These observations support a model where NRAGE and TrkA compete for interaction with p75NTR. Therefore, the recruitment of NRAGE to p75NTR can disrupt the pro-survival p75NTR-TrkA complex, perhaps, in favour of a pro-apoptotic p75NTR-Sortillin complex (Figure 5.3).

In a similar manner, NRAGE, or other MAGE family members, may disrupt the p75NTR-NogoR-Lingo1 complex, therefore suppressing the ability of p75NTR to respond to MGIs with the displacement of RhoGDIα and the activation of the growth-inhibitor RhoA. The ability to disrupt a functional p75NTR-NogoR-Lingo1 signaling complex may underlie the pro-differentiation/growth function observed for MAGE family members both *in vitro* and *in vivo*.

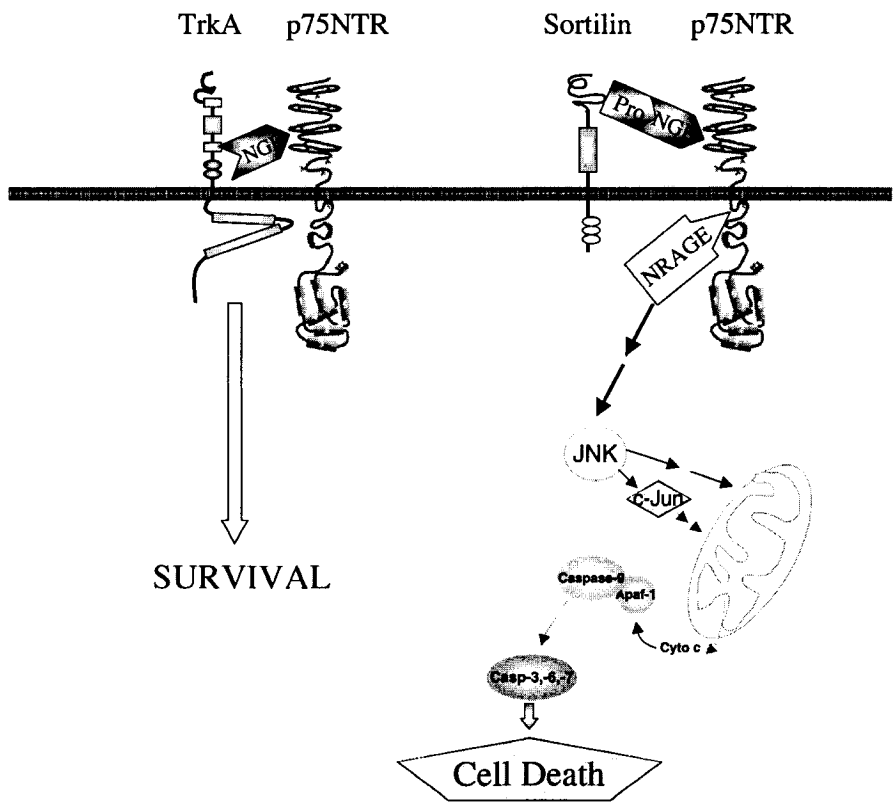


Figure 5.3 Schematic model of p75NTR/NRAGE signaling. When associated with TrkA, p75NTR acts as a component of a high-affinity receptor-complex for NGF. However, p75NTR binding to NRAGE, leads to the dissociation of the p75NTR/TrkA complex, promoting the association of p75NTR with Sortillin. This association allows for the proNGF-induced, NRAGE-mediated, activation of the JNK apoptotic signaling pathway.

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