Caspase-6a activation is regulated by Caspase-6b. Implications for a potential inhibitor of Alzheimer Disease

By
Andrea W. Lee

Department of Neurology and Neurosurgery
McGill University, Montreal

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ABSTRACT

Caspase-6 (Casp-6) is activated early in Alzheimer disease and involved in axonal degeneration, however the regulation of Casp-6 activation has not been explored. Endogenous mammalian caspase inhibitors do not exert any effect on Casp-6. Several alternatively spliced forms of caspases act as inhibitors of caspase activation. The CASP6 gene generates two transcripts from alternative splicing, CASP6α and CASP6β. We are interested in resolving the function of CASP6β, and its protein product proCaspase-6b (proCasp-6b), particularly because this splice variant contains the Casp-6 catalytic site unlike many other caspase splice variants. Here, we show that the CASP6β transcript and the proCasp-6b protein are present in many cell lines, in primary human neurons, and in human brains. Co-expression of proCasp-6a and proCasp-6b is consistently observed, and interestingly the abundance in isoform expression was different amongst the cell lines and human neurons. Purified proCasp-6b did not have caspase activity nor did it inhibit already activated Casp-6a. ProCasp-6b prevented the proteolytic activation of proCasp-6a *in vitro* and in cells. ProCasp-6b interacts directly with proCasp-6a. This work demonstrates that proCasp-6b is an inhibitor of proCasp-6a activation, likely through a novel mechanism of asymmetric dimerization. These results imply that proCasp-6b could negatively regulate proCasp-6a activation in neurons and prevent Casp6a-mediated axonal degeneration.

RÉSUMÉ

En plus de son implication dans la dégénérescence axonale des neurones, la Caspase-6 (Casp-6) est précocement activée dans la maladie d'Alzheimer. Les mécanismes moléculaires de régulation de cette activation sont cependant encore méconnus. Bien que les inhibiteurs traditionnels des caspases n'exercent aucun effet sur Casp6, ProCaspase-6b (proCasp-6b), produit par épissage alternatif, pourrait agir comme inhibiteur d'activation. En effet, le gène de la Caspase-6 peut générer 2 transcrits alternatifs nommés CASP6α et CASP6β codant respectivement 2 formes, les isoformes, proCasp-6a et proCasp-6b. Contrairement aux autres isoformes des caspases, l'existence du site catalytique sur l'isoforme proCasp-6b, suggère que cette isoforme pourrait posséder une fonction particulière sur l'activation de la proCasp-6a. Ici nous montrons, que le transcrit CASP6β et la protéine proCasp-6b sont exprimés et détectés dans un grand nombre de lignées cellulaire, dans les neurones primaires humain en culture ainsi que dans le cerveau humain. Même si la co-expression des 2 isoformes est constamment observée, il y a également des différences dans l'abondance de ces isoformes dans certaines lignées cellulaires et dans les neurones humains. L'isoforme proCasp-6b purifiée n'inhibe pas l'activité de la Casp6a préalablement activé mais interagit avec la proCasp-6a afin d'empêcher l'activation protéolytique de cette dernière *in vitro* et *in vivo*. Cette étude met en évidence un rôle d'inhibiteur de la proCasp-6b dans l'activation de la proCasp-6a et ce, vraisemblablement par un mécanisme de dimérisation asymétrique entre la proCasp-6a et la proCasp-6b. L'ensemble de ces résultats suggère que l'isoforme proCasp-6b pourrait négativement réguler l'activation de la proCasp-6a dans les neurones et ainsi empêcher la dégénérescence axonale observée dans la maladie d'Alzheimer.

PREFACE

The main focus of this thesis is characterizing the inhibitory role of splice variant, proCaspase-6b, on the activation of the cognate protease, Caspase-6a. This work is conducted on human cell lines, primary human neurons and adult brain tissues. This thesis is written in a manuscipt-based format. The thesis consists of three chapters: the introduction and literature review, the research data that was published in a scientific journal, followed by a general discussion and conclusion.

This thesis contains the following manuscript:

Lee, A.W., Champagne, N.C., Wang, X., Su, X.D., Goodyer, C., and LeBlanc, A.C. (2010). Alternatively spliced caspase-6B isoform inhibits the activation of caspase-6A. *J Biol Chem* 285(42): 31974-31984

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ABBREVIATIONS

Aβ	Amyloid beta peptide
Ac	N-Acetyl
AD	Alzheimer Disease
AEBSF	4-(2-Aminoethyl)-Benzenesulfonyl Fluoride
AFC	7-Amino-4-Trifluoromethyl-coumarin
ALS	Amyotrophic Lateral Sclerosis
ANOVA	Analysis of Variance
Apaf-1	Apoptotic Protease Activating Factor-1
APP	Amyloid Precursor Protein
AS	Alternative Splicing
ATP	Adenosine Triphosphate
Bak	Bcl-2 antagonist or killer
Bax	Bcl-2 associated X Protein
BBB	Blood-Brain Barrier
BCA	Bicinchoninic Acid
Bcl-2	B Cell Lymphoma-2
BIR	Baculovirus IAP Repeat
BPA	Branch Point Adenosine
BSA	Bovine Serum Albumin
CAD	Caspase-Activated DNase
CARD	Caspase Recruitment Domain
Casp-1	Caspase-1
Casp-2	Caspase-2
Casp-3	Caspase-3
Casp-4	Caspase-4
Casp-5	Caspase-5
Casp-6	Caspase-6
Casp-7	Caspase-7
Casp-8	Caspase-8
Casp-9	Caspase-9
Casp-10	Caspase-10
Casp-12	Caspase-12
Casp-14	Caspase-14
cDNA	Complementary DNA
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
c-IAP1	Cellular-IAP1
c-IAP2	Cellular-IAP2
CNS	Central Nervous System
dATP	Deoxy Adenosine Triphosphate
DD	Death Domain

DED	Death Effector Domain
DIABLO	Direct IAP Binding Protein with Low pH
DISC	Death Induced Signaling Complex
DN	Dominant-Negative
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DR-4	Death Receptor-4
DR-5	Death Receptor-5
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EGFP	Enhanced Green Fluorescent Protein
ER	Endoplasmic Reticulum
ESS	Exonic Splicing Silencer
FADD	Fas-Associated protein with Death Domain
Fas	FS7-Associated Cell Surface Antigen
FasL	FS7-Associated Cell Surface Antigen Ligand
GST	Glutathione S-Transferase
HD	Huntington Disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hnRNA	Heterogenous Nuclear RNA
hnRNP	Heterogenous Nuclear Ribonucleoprotein
hnRNP L	Heterogenous Nuclear Ribonucleoprotein-L
HtrA2	High Temperature Requirement protein A2
Htt	Huntingtin
IAP	Inhibitor of Apoptosis Protein
ICAD	Inhibitor of Caspase-Activated DNase
ICE	Interleukin- 1β Cleaving Enzyme
ICE-LAP	ICE-Like Apoptotic Protease
IL-18	Interleukin-18
IL-1α	Interleukin 1-α
IL-1β	Interleukin 1-β
ILP-2	IAP-like Protein-2
IPTG	Isopropyl-beta-D-Thiogalactopyranoside
IVT	*In Vitro* Translated
kDa	Kilodalton
LRR	Leucine Rich Repeat
mRNA	Messenger Ribonucleic Acid
MCI	Mild Cognitively Impaired

MW	Molecular Weight
NAIP	Neuronal Apoptosis Inhibiting Protein
NCI	Non-Cognitively Impaired
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NFT	Neurofibrillary Tangle
NMD	Nonsense-Mediated Decay
NPT	Neuropil Thread
PARP	Poly(ADP-ribose) polymerase
PD	Parkinson Disease
PEI	Polyethylenimine
PIDD	p53-induced Protein with a Death Domain
PRR	Pattern Recognition Receptor
PTP	Permeability Transition Pore
PVDF	Polyvinylidene Fluoride
RAIDD	RIP Associated Ich-1/CED homologous protein with Death Domain
RIP	Receptor Interacting Protein
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
RNAi	RNA inteference
RRM	RNA Recognition Motif
RS	Serine-Rich
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
Smac	Second Mitochondrial Activator of Caspases
snRNP	Small Nuclear Ribonucleoprotein
SOD1	Superoxide Dismutase-1
SP	Senile Plaques
SR	Serine/Arginine
TBI	Traumatic Brain Injury
TLCK	Nα-Tosyl-L-Lysine Chloromethyl Ketone
TNF	Tumour Necrosis Factor
TRAIL	TNF-Related Apoptosis Inducing Ligand
U2AF	U2 Auxiliary Factor
UBC	Ubiquitin-Conjugating
XIAP	X-linked IAP

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Cysteinyl proteases, or caspases, are major molecular players of the apoptotic pathway. There are twelve human caspases identified, of which we are particularily interested in effector caspase-6 (Casp-6). Of the effector class of caspases, Casp-6 has been shadowed by the notorious Caspase-3 (Casp-3) and Caspase-7 (Casp-7). Originally thought to be a redundant executioner of apoptosis, Casp-6 is emphasized as a mediator of neuritic degeneration and causative link to Alzheimer Disease (AD) (Albrecht et al., 2007; Guo et al., 2004; Nikolaev et al., 2009).

To substantiate these claims, a proteomic analysis identified several neuronal cytoskeletal proteins as substrate targets of Casp-6, implicating the breakdown of the neuronal architecture as a consequence of active Casp-6 (Klaiman et al., 2008). From a clinical aspect, Casp-6 activation occurs early in AD, even preceding any visible pathological manifestations in the brain (Albrecht et al., 2007). In fact, some aged non-cognitively impaired (NCI) individuals had detectable amounts of Casp-6 localized to the entorhinal cortex, an area known to be initially susceptible to AD pathology. All cases of familial AD brains, an inheritable form of AD characterized by known mutations of amyloid precursor protein (APP) or Presenilin I & II proteins, tested positive for Casp-6 staining (Albrecht et al., 2009). Furthermore, a recent study showed Casp-6 dependent neuritic degeneration in human primary neurons (Sivananthan et al., 2010). Biologically, the Casp-6 mediated degeneration of the neuritic connections in the absence of immediate cell death, occurs as a slow and chronic decline, seemingly

reflective of the symptomatic progession of AD in affected individuals. As a result, it is of dire need to find ways to regulate Casp-6 expression and activity, especially since there is no effective inhibitor to this caspase.

Casp-6 was identified in 1995, where two alternatively spliced (AS) protein isoforms were found (Fernandes-Alnemri et al., 1995a). The second variant, proCaspase-6b (proCasp-6b) was postulated to inhibit the cognate effector caspase form, proCasp-6a. This was based on similar dominant-negative (DN) models of inhibition in previously identified AS caspases (Alnemri et al., 1998; Friedlander et al., 1997).

Therefore, the main objective of this study is to characterize the splice variant of Casp-6 called proCasp-6b, since it retains the catalytic site of the active Casp-6 enzyme. We objectively consider the possibility that proCasp-6b may act as a redundant Casp-6 protease or that it might antagonize Casp-6a activity as a dominant-negative inhibitor. Findings from this research could potentially alleviate the pathological and clinical decline of Casp-6a mediated neurodegeneration of AD.

LITERATURE REVIEW

1.1 Apoptosis

Cellular turnover is an obligate process for proper mammalian development and healthy tissue homeostasis. Apoptosis, an organized form of programmed cell death, is primarily carried out by cysteinyl proteases termed caspases. This biochemical phenomenom was first described by Carl Vogt in 1842, but was only named and studied by electron microscopy in 1972 (Kerr et al., 1972). During apoptosis, distinct morphological changes and energy-dependent biochemical mechanisms occur. Firstly, nuclear and cytoplasmic condensation and collapse of the cell results in distinctly identifiable membrane-bound fragments (Kerr et al., 1972). The membrane-bound apoptotic bodies are subsequently phagocytosed by macrophages, parenchymal cells or neoplastic cells, and digested within phagolysosomes (Elmore et al., 2007). An important distinction that distinguishes apoptosis from necrosis or autophagy is the absence of an inflammatory reaction associated with apoptotic cell death (Elmore et al., 2007).

1.2 Cysteinyl Proteases

Proteases are major molecular players that coordinate virtually every aspect of physiology and development in the human body. They function as enzymes, capable of catalyzing the hydrolysis of polypeptide amide bonds (Chapman et al., 1997). Proteolysis is an irreversible post-translational modification widely used to generate protein diversity (Chapman et al., 1997; Olsen et al., 2006). Therefore, it is not surprising that proteases have evolved to mediate irreversible processes such as coagulation (Davie et al., 1964; MacFarlane et al., 1964), digestion and

apoptosis (Yuan et al., 1993). Functionally, all proteases operate by nucleophilic attack on the carbonyl-carbon of the amide bond of the substrate (Polgar et al., 1989). Proteases can be grouped into four major classes: aspartate, metallo, serine and cysteine (Chapman et al., 1997). More specifically, caspases utilize an active site cysteine thiol for nucleophilic attack, resulting in an acid-base hydrolysis that disrupts the covalent bond (Asboth et al., 1988).

1.2.1 Discovery/ History of Caspases

The tightly regulated proteolytic cascade of caspase activation bore resemblance to the hierarchical proteolytic model governing the blood coagulation pathway. Caspases are most notably associated at the heart of a crucial proteolytic cascade that dismantles cells during apoptosis (Miura et al., 1993; Yuan et al., 1993). In the early 90's, simultaneous reports of mammalian proteolytic activity capable of processing pro-inflammatory cytokine, pro-interleukin 1-β into its mature 17.5 kDa active cytokine heralded the subsequent identification and characterization of interleukin 1-β cleaving enzyme (ICE) (Black et al., 1989; Cerretti et al., 1992; Kronheim et al., 1992; Thornberry et al., 1992). At the time, ICE was a novel enzyme and unrelated to any known proteins. The discovery of this critical cysteine protease, later named Caspase-1 (Casp-1), was linked to apoptotic programmed cell death following the emergence of the cell death gene, *ced-3,* in *C. elegans* and the astounding sequence homology between the two (Yuan et al., 1993). This important connection prompted an assiduous search for other mammalian homologues of the ICE family. Currently, twelve human cysteine proteases have been identified: Casp-1 through Caspase-10 (Casp-10), Caspase-12

(Casp-12) and Caspase-14 (Casp-14) (Alnemri et al., 1996; Eckhart et al., 2000; Van de Craen et al., 1997).

1.2.2 Nomenclature

The expanse of caspases into mainstream research and the frenetic pace at which caspase homologues were being identified, required a unified system for name assignment. Caspases are a superfamily of **c**ysteine-dependent **asp**artate specific prote**ase**s, initially referred to as ICE family proteases (Alnemri et al., 1996). This universal nomenclature was established, using the root 'CASP' and 'caspase' to denote the gene and protein products respectively, followed by a number dictating chronological origin (ie. CASP6, caspase-6). Alternatively spliced gene isoforms originating from the same parent gene product are assigned greek letters based on chronological order of discovery (ie. CASP6α and CASP6β). Furthermore, each inactive zymogen is expressed as a full-length protein containing a pro-domain; therefore the pro-enzymes adopt the name pro-caspase (ie. proCasp-6). Processing of the pro-enzymes liberates two subunits, denoted as large p20 and small p10, which then reassemble with another large and small subunit pair to form an active heterotetrameric enzyme. These subunits are described as the enzyme name, followed by their particular molecular weight size (ie. Casp-6-p20 or Casp-6-p10). Lastly, splice variants of the caspase protein are assigned an English letter suffix by order of identification (ie. proCasp-6a, proCasp-6b) (Alnemri et al., 1996).

1.2.3 Fundamental Features of Caspases

1.2.3.1 Primary structure of caspases

Human caspase pro-zymogens are made up of the same basic organization. The N-terminal prodomain of varying length precedes the large subunit. The small subunit either directly follows the large subunit, or is sometimes separated by a short interdomain linker. Cleavage at target sequences ending with aspartate residues releases the prodomain and separates the large and small subunits. The active heterotetrameric caspase enzyme consists of two p20-p10 heterodimers. Each p20-p10 heterodimer requires the reassembly of one p20 and one small p10 subunit (**Fig. 1-1**).

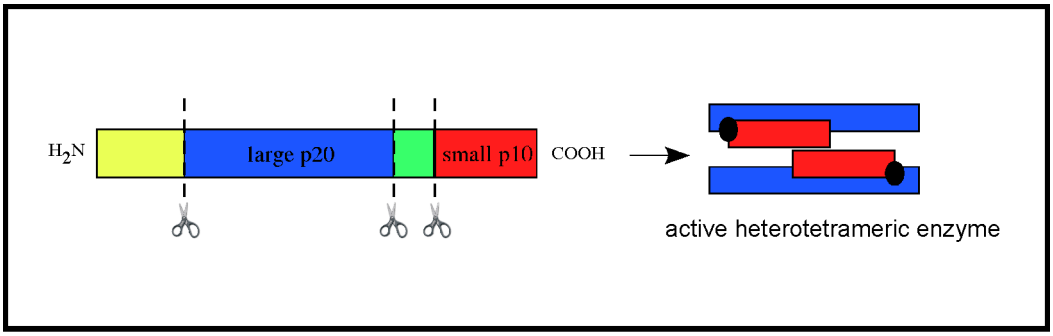

Figure 1-1. Prototype protein schematic of a caspase zymogen. The prodomain is shown in *yellow*, large subunits in *blue*, linker in *green*, and small subunits shown in *red*. Processing to activate the pro-zymogen results in a conserved active heterotetrameric enzyme as depicted, with active site cysteines marked with *black* circles.

Caspases share a number of distinct features at the protein level. All caspases share a homologous pentapeptide (active site) sequence, QACRG, which aids in substrate binding and enzyme catalysis (Cohen et al., 1997). There are two active sites at opposite ends of each active heterotetrameric enzyme, with the exception of a single functional active site in Casp-9 (Renatus et al., 2001). The organization

of residues forming the substrate-binding pocket (or active site) within each caspase heterotetramer confers specificity to binding target peptides. Specific four-peptide substrate target sequences are recognized and subjected to proteolysis by caspases. All caspases universally prefer an aspartate residue at the terminal position or fourth peptide. The global requirement for an aspartate residue is due to the strict conservation of side chain residues, Arg^{179}, Arg^{341} and Gln^{238} within caspases (denoted by Casp-1 numbering convention). The interaction between these residues creates a deep, highly basic pocket perfectly accommodating the aspartate side chain (Timmer et al., 2007).

1.2.3.2 Active site architecture: global fold, topology and quaternary structure

Each catalytic domain from a singular pro-caspase molecule is composed of a large α-subunit and small β-subunit, arranged in a twisted, mostly parallel central six stranded β-sheet, flanked by two layers of α-helices (Fuentes-Prior et al., 2004). Each α/β subunit heterodimer contains an active site cavity at the carboxy-terminal end of the parallel β-strands. The active sites formed by each monomer are situated on opposite ends of the $\alpha_2\beta_2$ tetrameric enzyme (**Fig. 1-1**) (Degterev et al., 2003). Based on several crystal structures, the overall backbone conformation of the caspase active site is highly conserved. Four surface loops, L1-L2-L3-L4, protrude from the central β-sheet to form the potential active site. Loop L1 and a segment of L2 that contains the catalytic site are encoded within the large subunit of the caspase enzyme. Subsequently, L3 and L4 loops are derived from the small subunit (Degterev et al., 2003). Structurally, L1 and L4 constitute the two walls of the substrate binding groove, L3 forms the base and the

L2 loop contains the active cysteine, which lies across the front of L1 and L4 (Shiozaki et al., 2004) (**Fig. 1-2**). Stability of the active site structure of one caspase molecule is supported by the amino-terminus of the L2' loop of the adjacent (dimer) molecule (Chai et al., 2001b).

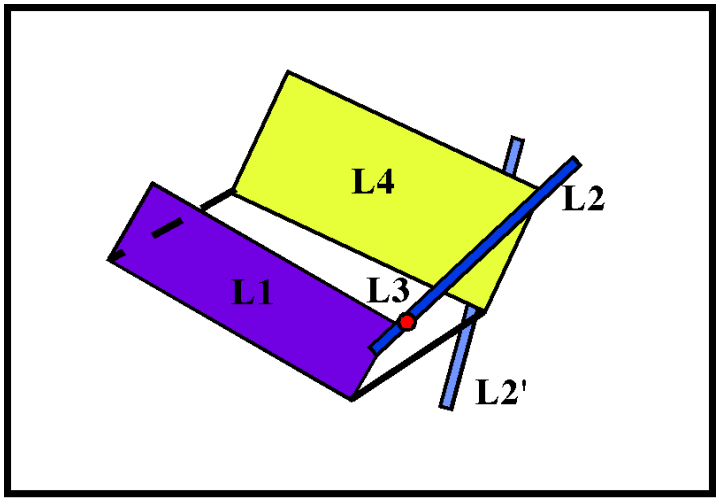

Figure 1-2. Representation of loop bundle organization forming a substrate-binding site. Organization of loops L1-L4 depicting the structure of the active site groove of a singular caspase molecule. The L2' loop of the adjacent caspase molecule is included as well as the position of the active site cysteine marked in *red*.

1.2.4 Classification of Caspases

The fervent identification of caspases following ICE characterization created a need for categorization of these enzymes. Caspases can be classified based on function, N-terminal domain length and structural organization (Eckhart et al., 2008), or preferred substrate motif denoted by recognition of a four peptide sequence P4-P3-P2-P1↓P1' (Margolin et al., 1997). Remarkably, common caspase groupings result from classification under these multiple criteria, which

strongly suggest that tight function-structure correlations exist within the caspase family. *See Table 1-1*.

Table 1-1. Given names of caspases and optimal substrate sequences

Caspase Name	Other Names	Substrate Preference	
Caspase-1	ICE	YVAD↓A[1] WEHD↓X	Inflammatory
Caspase-4	ICH-2 ICE_{rel}-II TX	LEVD↓X W/LEHD↓X	
Caspase-5	ICE_{rel}-III TY	W/LEHD↓X[3]	
Caspase-12	Casp-12P1	ATAD↓X	
Caspase-8	MACH FLICE Mch5	LETD↓X IETD↓X	Initiators
Caspase-9	ICE-LAP6 Mch6	LEHD↓X	
Caspase-10	Mch4 FLICE2	I/LEXD↓X[2]	
Caspase-2	ICH-1	VDVAD↓X[1] DEVD↓X	
Caspase-3	CPP32 YAMA Apopain	DEVD↓X VDVAD↓X[1] VEID↓N	Effectors
Caspase-7	Mch3 ICE-LAP3 CMH-1	DEVD↓X VDVAD↓X[1] VEID↓N	
Caspase-6	Mch2	VEID↓N	
Caspase-14	MICE	W/YEXD↓X[2]	Outliers

↓ denotes the position of caspase cleavage
ICE-LAP (ICE-like Apoptosis Protease)

[1] (Pereira et al., 2008)
[2] (Timmer et al., 2007), where X denotes Gly (G), Ala (A), Thr (T), Ser (S) and Asn (N)
[3] (Earnshaw et al., 1999)

1.3 Cytokine Activators: Inflammatory Caspases

Although Casp-1 was first identified as the mammalian homologue to the *C. elegans* death protease, later it was established that inflammatory caspases are not necessarily involved in apoptosis. Casp-1 is most renowned and well-studied as the sole enzyme that cleaves interleukin-1β (IL-1β), an key inflammatory cytokine involved in the innate immune response against pathogens, microbial invasions and tissue injury (Martinon et al., 2007). The other caspases of the inflammatory group are less studied, but it is postulated that Casp-4 and -5 act similarly to Casp-1 on account of strong sequence homology. Casp-1, -4, -5, and -12 are classified as inflammatory caspases (Martinon et al., 2004).

1.3.1 Primary Structure and Domains

Inflammatory caspases are synthesized as inactive pro-enzymes encompassing three domains. Beginning at the N-terminus is a long pro-domain containing a caspase-activating recruitment domain (CARD) followed by the stereotypic large and small subunits, which are separated by a short 2 kDa intersubunit linker (Cerretti et al., 1992; Thornberry et al., 1992).

1.3.2 Activation of Inflammatory Caspases

Activation of Casp-1 requires an activating complex called the inflammasome (Martinon et al., 2002). To date, there are four known inflammasome complexes

called NLRP1, NLRP3, IPAF and AIM2 (Schroder et al., 2010). The inflammasome is a multi-protein complex assembled by specific pattern-recognition receptors (PRRs) tailored to detect invariant microbial motifs (Schroder et al., 2010). Scaffold proteins assemble through CARD and pyrin domain interactions to form the inflammasome and recruit proCasp-1 molecules via CARD motifs. Oligomerization of Casp-1 into the inflammasome complex results in self-processing and the generation of active Casp-1 enzymes (Gu et al., 1995). Self-processing occurs by cleavage at preferred Casp-1 target sites (Thornberry et al., 1992). It is speculated that activation within some inflammasome complexes may also involve Casp-5, therefore suggesting co-operative roles in fostering a fully active immune response (Martinon et al., 2007).

1.3.3 Physiological Role and Substrates of Inflammatory Caspases

Some reported non-apoptotic functions of Casp-1 include pyroptosis induction (Franchi et al., 2006) and pro-domain-mediated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation (Lamkanfi et al., 2004). Furthermore, Casp-$1^{-/-}$ knockout mouse models are deficient in production and maturation of IL-1β, IL-1α, IL-18 and γ-interferon (Kuida et al., 1995). Lastly, other identified substrates of Casp-1 are nucleotide-binding oligomerization domain-leucine rich repeat protein, poly(ADP-ribose) polymerase-1 (PARP-1), and pyrin proteins (Chowdhury et al., 2008).

Casp-12 is phylogenetically linked to cytokine maturation and immunity by protein sequence homology to Casp-1 (Van de Craen et al., 1997), and in mice, known to be involved in endoplasmic reticulum (ER)-stress associated apoptosis (Nakagawa et al., 2000). Human Casp-12 is a pseudogene because it does not get expressed as a full-length protein except in humans of African descent (Fischer et al., 2002; Saleh et al., 2004). Physiologically, Casp-12 is hypothesized to hinder Casp-1 activation, thus increasing vulnerability to microbial and pathogenic host invasion and septic mortality (Martinon et al., 2007).

1.4 Initiator Caspases

Initiator caspases are signaling molecules (Alnemri et al., 1996; Pop et al., 2009) that are activated by auto-processing to cleave downstream effector caspases. The ultimate role of initiator caspases is to link a variety of upstream signals to downstream executive events that cause dismantling of cells by apoptosis. Casp-2, -8, -9, -10 and -12 are classified as initiator caspases.

1.4.1 Primary Structure and Domains

Initiator caspases exist mainly in the cytosol as monomeric inactive zymogens (Renatus et al., 2001). Initiator caspases possess a long pro-domain at the N-terminus, often containing one or more recruitment motifs such as a CARD domain, found in Casp-2, -9 and -12 or death effector domains (DED) in Casp-8 and -10. CARD and DED comprise six or seven antiparallel amphipathic α-helices that interact through hydrophobic or electrostatic interactions with other proteins (Earnshaw et al., 1999). Like the inflammatory caspases, the DEDs and

CARD are responsible for recruiting initiator caspases into activating complexes to generate active initiator enzymes.

1.4.2 Initiator Caspase Activation Models

The induced-proximity model dictates that clustering of initiator zymogens stimulates autoproteolytic activity to generate an active enzyme. Zymogenicity is defined as the ratio of active enzyme over unprocessed zymogen activity (Tachias et al., 1996). The zymogenicity of caspases varies amongst the caspase family, with Casp-8, -9 and -3 at 100, 10 and > 10,000, respectively (Salvesen et al., 2002). The induced-proximity model was proposed based on observations of autoprocessing by intrinsic enzymatic activity during *in vitro* protein purification in combination with *trans*-activation, or intermolecular processing via adaptor-mediated clustering of pro-enzymes (Salvesen et al., 1999). The increase in local net concentration was sufficient to stimulate proteolytic processing of all initiator caspases with the exception of Casp-9.

Induced-proximity dimerization occurs within non-specific regions of the monomeric enzymes, whereas specific targeted homodimerization at the dimer interface in proximity-driven dimerization seems more mechanistically relevant (Shi et al., 2004). Proximity-driven dimerization states that initiator pro-enzymes can auto-process when brought into close enough proximity by scaffold proteins to dimerize at the dimer interface. This requires specific protein-protein interactions to allow for correct positioning and self-activation (Shi et al., 2004), which are unaccounted for in the initial model. The refined model is more

relevant and physiologically supportive of initiator caspase activation, particularly to account for conformational changes arising from homodimerization that drive activation (Donepudi et al., 2003; Renatus et al., 2001). This refined model unifies the mechanistic drive behind activation of initiator caspases, including the paradoxical Casp-9. This particular pro-enzyme has very low zymogenicity, therefore, the enzymatic activity of Casp-9 is similar before and after processing and instead is entirely reliant on its scaffold cofactor, apoptotic protease activating factor-1 (Apaf-1) to mediate Casp-9 homodimerization (Rodriguez et al., 1999; Stennicke et al., 1999).

1.4.2.1 Scaffold-mediated activation triggered by cell surface receptors

Recruitment by specific adaptor molecules and assembly into particular macromolecular platforms drastically enhances the activation of initiator caspases (Fuentes-Prior et al., 2004; Shi et al., 2004) and allows for intramolecular auto-processing into mature enzymes (Chen et al., 2002). In humans (vertebrate cells), there are two recognized pathways, the extrinsic and intrinsic pathways that stimulate scaffold-mediated initiator caspase activation.

The extrinsic pathway is stimulated by an extracellular signal to receptors on the cell surface (**Fig. 1-3**). FasL and TNF-related apoptosis inducing ligand (TRAIL), bind pro-apoptotic death receptors, CD95/FS7-associated cell surface antigen (Fas) and death receptor-4/-5 (DR4/DR5), respectively (Ashkenazi et al., 2002; Blanchard et al., 1999). Ligand binding results in the trimerization of the death receptors at their death domains (DD) and recruitment of adaptor proteins, FADD

and/or TRADD, through the same motifs. This results in formation of the death-induced signaling complex (DISC) (Chinnaiyan et al., 1995; Hsu et al., 1995). Subsequent recruitment of Casp-8 or -10 by homotypic DED-DED interaction to the DISC sequesters the caspases, allowing autocatalytic processing of the initiator caspases by proximity-driven activation (Boatright et al., 2003; Salvesen et al., 1999).

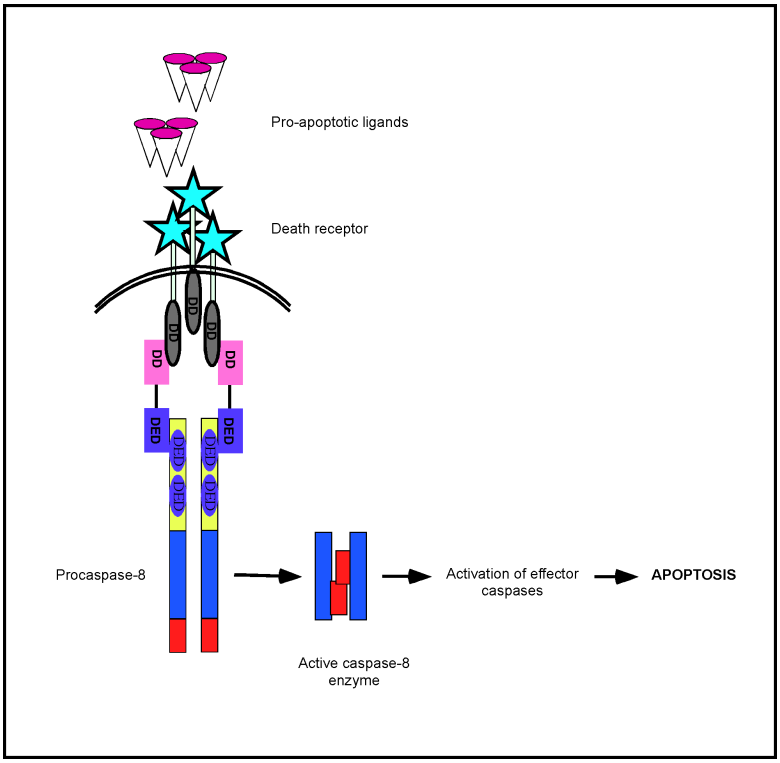

Figure 1-3. Receptor-mediated activation of initiator Casp-8 via DISC assembly

1.4.2.2 Scaffold-mediated activation triggered by external stimuli

External stressors cause DNA damage, oxidative and ER stress, which trigger the intrinsic cell death pathway (**Fig. 1-4**). Intracellular signaling triggers this mitochondria-dependent mechanism by insertion of oligomeric Bcl-2 associated X protein (Bax) and Bcl-2 antagonist or killer (Bak) into the outer mitochondrial

membrane and cytochrome c release into the cytosol. Cytochrome c binding to Apaf-1 in the presence of dATP or ATP, triggers a conformational change and oligomerization of Apaf-1 (Zou et al., 1997). ProCasp-9 is recruited to the reorganized Apaf-1 oligomer through an interaction of mutual CARD domains. The formation of the heptameric apoptosome mediates auto-activation of Casp-9 by proximity-induced dimerization (Li et al., 1997; Renatus et al., 2001).

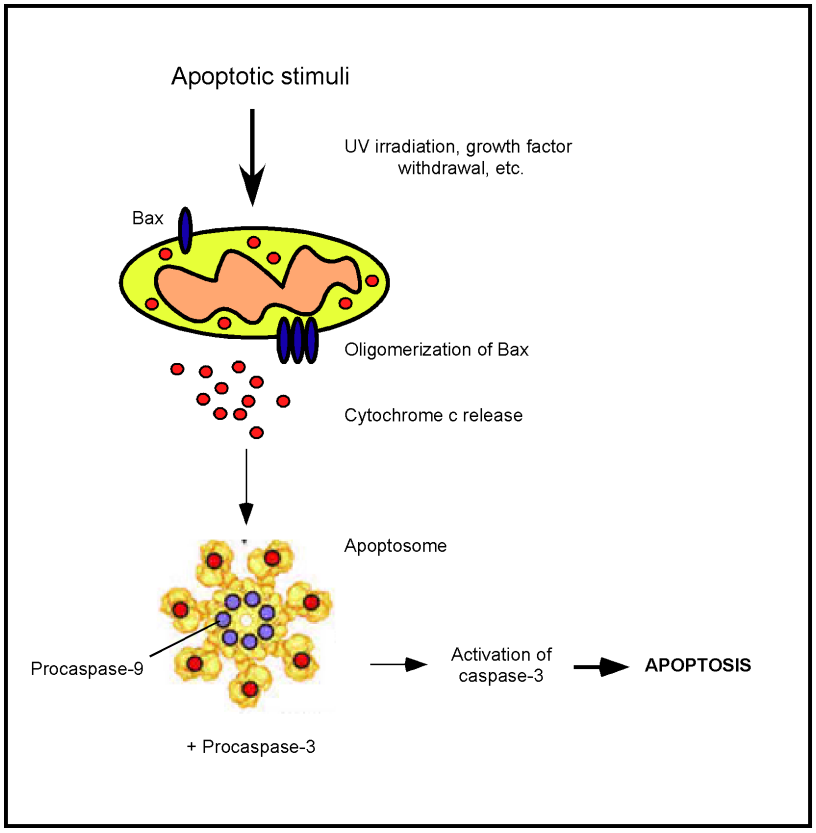

Figure 1-4. External stimuli mediated activation of initiator Casp-9 via apoptosome assembly

1.4.3 Physiological Role and Substrates of Initiator Caspases

The most striking feature is that initiator caspases are their own substrates. Initiator caspases auto-process, due to conservation of preferred target peptide

sites within its own protein (Degterev et al., 2003). Another critical feature of initiator caspases is the ability to recognize the effector caspases as specific substrate targets, a crucial aspect of apoptotic signaling.

1.4.3.1 Apoptotic function of initiator caspases

In the extrinsic pathway, proximity-driven dimerization can generate enough active Casp-8 enzymes to directly process effector caspases to execute cell death. Cells that induce apoptotic signaling through this direct (caspase-dependent), mitochondrial-independent manner are classified as Type I cells (Scaffidi et al., 1998). A second class of cells, Type II cells, producing minimal amounts of active Casp-8 at the DISC, thus requires mitochondrial-mediated amplification of the CD95 death signal (Scaffidi et al., 1998). More specifically, activated Casp-8 cleaves pro-apoptotic B-cell lymphoma 2 (Bcl-2) family member, Bid, generating a truncated form, tBid, which translocates to the mitochondria, where it interacts with two other pro-apoptotic proteins, Bax and Bak (Hockenbery et al., 1990; Korsmeyer et al., 1999). The interaction between tBid and Bax facilitates the oligomerization of Bax, a prerogative for outer mitochondrial membrane permeabilization. Lastly, a conductance channel resulting from the formation of a permeability transition pore (PTP) (Hunter et al., 1976) facilitates the release of cytochrome c (Yang et al., 1997) and other pro-apoptotic factors such as endoG (Li et al., 2001), HtrA2/Omi (Suzuki et al., 2001a), second mitochondrial activator of caspases/direct IAP binding protein with low pH (Smac/DIABLO) (Du et al., 2000) and apoptosis-inducing factor (Susin et al., 1996). At this point, the extrinsic pathway converges with the intrinsic apoptotic pathway, by the

release of cytochrome c from the mitochondria to activate executioner caspases to dismantle the dying cell.

1.4.3.2 Non-apoptotic functions of initiator caspases

Outside of the role of initial signaling for apoptotic cell death, several initiator caspases have other physiological functions. Casp-8 can regulate lymphocyte proliferation in a positive or negative manner, although long-term studies show a greater lymphoproliferative role rather than immunodeficiency in T-cell specific knockout murine models (Chun et al., 2002; Salmena et al., 2003). Casp-8 has also been implicated as a mediator of monocyte and placental villous trophoblasts differentiation (Black et al., 2004; Sordet et al., 2002).

Casp-10 appears to have different cleavage specificities implicating a role in other cellular processes in comparison to Casp-8 (Wang et al., 2001). More recently, an alternative isoform of Casp-10 has been implicated in the NF-κB signaling pathways (Wang et al., 2007). Loss of function of human Casp-10 is correlated with autoimmune lymphoproliferative syndrome (Wang et al., 1999), certain non-Hodgkin lymphomas (Shin et al., 2002) and gastric cancers (Park et al., 2002).

Active Casp-9 was reported to play a non-apoptotic role in muscle differentiation in a mitochondrial-dependent manner in the absence of cytochrome c and Smac/DIABLO release (Murray et al., 2008). Knockout mice with targeted disruptions to the Casp-9 gene often die perinatally, with the remaining survivors developing brain impairments with unusually large cerebrums due to ineffectual apoptosis during development (Hakem et al., 1998; Kuida et al., 1998).

1.5 Effector Caspases

1.5.1 Primary Structure and Domains

Apoptotic effectors or 'executioners' mediate the execution of apoptotic cell death, by cleavage of a large number of cellular proteins. The executioner caspases, Casp-3, -6 and -7 are cytosolic inactive dimeric zymogens (Boatright et al., 2003) that have short 23 amino acid pro-domains followed by a large p20 and a small p10 subunit separated by a small interdomain linker. Strong hydrophobic interactions exist at the dimer interface of effector caspases, which promote a three-fold tighter association as endogenous dimers (Bose et al., 2001).

1.5.2 Activation of Effector Caspases

Activation of effector caspases is an irreversible process, which is mediated by an exo-caspase (eg. initiator caspase) or by *cis/trans* auto-processing. Canonical cleavage-mediated activation occurs at three particular sites for Casp-3, -6 and -7: (1) between the pro-domain and large subunit, (2) between the large subunit and linker and (3) between the linker and small subunit (**Fig. 1-5**). The liberated large and small subunits reassemble into p20-p10 heterodimers, which dimerize to form the active tetramer. Here we discuss the doctrine of processing and remodeling of effector caspases during activation, and higlight new findings challenging this dogma.

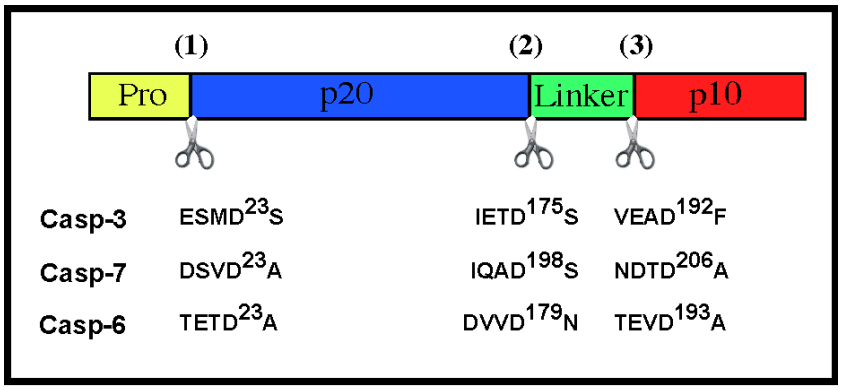

Figure 1-5. Schematic representation of the domains of an effector caspase. Shown are the three processing sites and sequences that are cleaved to generate catalytically active enzyme.

1.5.2.1 Cleavage-mediated activation (by exo-caspase processing)

1.5.2.1.1 Processing of effector caspases

Interestingly, the sequence of processing is the major difference between activation of Casp-3, -6 & -7. ProCasp-3 is known to be first processed between the intersubunit domain, followed by removal of the pro-peptide (Martin et al., 1996). Alternatively, proCasp-7 is activated by removal of the pro-domain, by Casp-3 or an initiator caspase, followed by removal of the linker region (Denault et al., 2003; Yang et al., 1998). The crystallized structures of active Casp-7 and proCasp-7 reveal that the pro-zymogen has a deformed active site cleft, occluded by the linker domain, which is removed during enzymatic activation (Donepudi et al., 2002). These aspartate-specific cleavage events can occur by intermolecular interactions between two effector caspase molecules. Or initiator caspases can process the effector caspase, also in a *trans-activating* manner (Chang et al., 2000).

In Casp-6, activation occurs by a unique mechanism involving both intermolecular (*trans*) and intramolecular (*cis*) self-processing. *In vivo,* pro-domain removal occurs (Cowling et al., 2002; Klaiman et al., 2009), yet the order of processing is inconsequential. More importantly is that full processing into the mature enzyme must first begin with intramolecular cleavage at the distal C-terminal site between the linker and small p10, which is then followed by processing to separate the large p20 subunit and linker (Klaiman et al., 2009; Wang et al., 2010).

1.5.2.1.2 Remodeling of effector caspases

Traditionally, cleavage to remove the interlinker subunit mediates activation, amplifying the enzymatic activity of the effector caspase by several orders of magnitude (Klaiman et al., 2009; Shiozaki et al., 2004). This crucial cleavage allows for the re-organization of loop bundles within the protein structure. Together, crystal structures of unliganded and ligand-bound Casp-3, -6 and -7 reveal that activation requires the translocation of three surface loops containing critical components of the catalytic site and specificity determinants. Cleavage releasing the linker domain has two essential functions: (1) removal of steric hindrance to allow for translocation of one of the loops of the zymogen structure, and (2) formation of new loop bundle interactions between the reassembled large and small subunits to stabilize the active conformation (Denault et al., 2006). A comparison of the crystal structures of the pro-enzyme with the active Casp-7 enzyme revealed that although the core structures are retained, there is a significant remodeling of the catalytic groove when activated (Chai et al., 2001b;

Riedl et al., 2001). Similarly, a study analyzing the CD spectra of inactive Casp-6 pro-enzyme to mature processed Casp-6 showed that the overall shape and intensities were spectrally similar. Furthermore, a quantitative measure of α-helical content revealed only a 5% difference of the total helical content, which represented approximately three amino acids of the sixty putative helical residues (Kang et al., 2002). The authors postulated that the slight differences were on account of local conformational changes to the substrate binding groove upon processing of Casp-6 to its mature form (Kang et al., 2002). Therefore, the pre-existing substrate binding site must undergo conformation changes to allow for substrate binding and enzymatic activity upon activation.

1.5.2.2 Challenging the fundamentals: processing and remodeling of caspase-6

The first report that Casp-6a was able to self-activate *in vivo* in HEK293T cells was surprising (Klaiman et al., 2009), since it challenged the dogma of exo-caspase-mediated activation of executioner caspases. Recently Wang et al, confirmed that processing of Casp-6a is unlike the other executioner proteases (Wang et al., 2010). This was sparked by the discovery of a unique longer L2 loop compared to Casp-3 or -7. The revelation of this self-processing mechanism is dependent on the length of the loop, and unrivaled by other caspases. Concurrent with this discovery was the evidence that Casp-6a undergoes intramolecular (*cis*) cleavage, where the active caspase cleaves itself. Crystal structure resolution showed that the TEVD[193] processing site forms a well-defined β-strand, which is capable of nestling in the substrate-binding groove within a single molecule of the Casp-6a enzyme. It was speculated that minor conformational changes would

facilitate cleavage at Asp^{193} of the β-strand by the catalytic cysteine at residue 163. The lengthy L2 loop allows for positioning over the active site. This is a critical prerequisite for self-activation, since removal of only four amino acids of the Casp-6a L2 loop abrogated self-processing. Furthermore, addition of active Casp-6, but not Casp-3 facilitated the processing of full-length proCasp-6aD179A zymogens, indicating that these sites are fully accessible and cleavable (Wang et al., 2010). Therefore, the activation of Casp-6a involves first the intra-cleavage at Asp^{193} to liberate the p10 subunit by its own catalytic cysteine, followed by intermolecular cleavage at Asp^{179} to free the large p20 subunit.

1.5.3 Physiological Role and Substrates of Effector Caspases

Apoptosis is a specific cellular process that occurs during normal tissue development, homeostasis and disease pathogenesis. A classic hallmark of apoptosis is the cleavage of chromosomal DNA, a process that is mediated by active effector caspases (Fischer et al., 2003). PARP, a DNA repair enzyme, is an excellent substrate of Casp-3 and -7, and minimally of Casp-6 (Duriez et al., 1997; Lazebnik et al., 1994). Degradation of structural intra-nuclear proteins such as lamins is mediated by active Casp-6 (Ruchaud et al., 2002). Furthermore, the fragmentation of DNA by caspase-activated DNase (CAD) occurs by dissociation from its inhibitor (ICAD) by Casp-3 cleavage (Wyllie et al., 1997).

Non-apoptotic functions of Casp-3 involve the differentiation of lens epithelial cells, erythroblasts, platelets, myoblasts, osteoblasts and neural stem cells (reviewed in (Lamkanfi et al., 2007). Recently, a role in inflammation has made

Casp-7 distinct from its role as a redundant partner to Casp-3. In macrophages stimulated with lipopolysaccharide or gram-negative pathogens, Casp-7 activation is dependent on Casp-1 inflammasome assembly instead of canonical initiator caspase -8 or -9 activation complexes (Lamkanfi et al., 2010).

In addition, Casp-6 has major functional roles in axonal degeneration (Nikolaev et al., 2009; Sivananthan et al., 2010), lymphocyte differentiation (Olson et al., 2003; Watanabe et al., 2008) and cytoskeletal remodeling (Klaiman et al., 2008). Casp-6 is known to specifically target neuronal proteins resulting in the overproduction of amyloid beta peptide (Aβ) (Gervais et al., 1999; LeBlanc et al., 1999; Pellegrini et al., 1999; Weidemann et al., 1999), tau protein (Guo et al., 2004; Horowitz et al., 2004) and Huntingtin (Htt) (Graham et al., 2006), which result in neuropathies of AD and Huntington disease (HD).

1.6 Outliers – the odd caspases

Casp-2 was the first cloned human apoptotic caspase. There is a dichotomy in the role of Casp-2, as it can function as both an initiator and executioner caspase (Degterev et al., 2003). Cellularly, Casp-2 is unique within the caspase family as a nuclear resident protein (Paroni et al., 2002). Classically known as an initiator caspase containing a CARD domain, this enzyme is activated by adaptor proteins in a complex called the PIDDosome (Chang et al., 2000). While the stoichiometry of the components are unclear, we know that the Casp-2 activating complex consists of adaptors, RIP associated Ich-1/CED homologous protein with death domain (RAIDD) and p53-induced protein with a death domain (PIDD) (Tinel et

al., 2004). In support of its role as an initiator, the Casp-2 sequence is most homologous to that of Casp-9 (Baliga et al., 2004). Casp-2 is activated early in response to DNA-damaging agents, and plays a role in the engagement of the mitochondrial apoptotic pathway (Guo et al., 2002). This interaction fueling direct cytochrome c release requires complete processing of the pro-enzyme, but does not require catalytic activity (Robertson et al., 2004). However, functionally active Casp-2 prefers typical substrates of effector caspases. Furthermore, Casp-2 is suggested to be an early responder to p53-mediated apoptotic signaling by auto-activation under particular circumstances like genotoxic stress (Lassus et al., 2002; Tinel et al., 2004) and function like an effector caspase to cleave cellular proteins. Polarized structural and functional roles of Casp-2 have created difficulty in assigning a particular classification. Physiologically, Casp-$2^{-/-}$ mice develop normally and retain the ability to undergo apoptosis in response to various stimuli depending upon cell lineage and developmental stage (Bergeron et al., 1998).

Unlike other caspases, the expression of Casp-14 is confined to epithelial cells, and is not associated with apoptosis or inflammation. Rather, this unique caspase plays a role in differentiation of epidermal keratinocytes, specifically in regulating skin homeostasis (Denecker et al., 2008; Eckhart et al., 2000) and a protective role against UVB-induced damage (Denecker et al., 2007). An important physiological substrate of Casp-14 is profilaggrin, a critical structural protein of the epidermis (Denecker et al., 2007). Interestingly, expression of this caspase is limited and thought to be transcriptionally regulated when required during terminal

differentiation (Denecker et al., 2008). Activation by processing has been observed only in cornifying epithelia such as the epidermis and rodent forestomach (Denecker et al., 2008). There are some reports that Casp-8 and -10 can activate Casp-14 *in vitro*. However, *in vivo*, Casp-14 is unlikely to be processed by exo-caspases since no exo-caspases are detectable during the process of epidermal differentiation. Furthermore, maturation by cleavage is unconventional as it occurs at Ile^{152} in human Casp-14 (Denecker et al., 2008).

1.7 Human Caspase Inhibitors

Since caspases are involved in a plethora of biological process, there is great interest in maintaining control over activation. Caspase activation occurs through tightly regulated mechanisms. There are numerous inhibitory pathways and proteins that actively intervene apoptotic signaling at various checkpoints. There are eight human inhibitor of apoptosis proteins (IAPs) identified: X-linked inhibitor of apoptosis protein (XIAP), cellular-IAP1 (c-IAP1), cellular-IAP2 (c-IAP2), neuronal apoptosis inhibiting protein (NAIP), Survivin, BRUCE/Apollon, IAP-like protein-2 (ILP-2), and ML-IAP (Salvensen et al., 2002) (**Fig. 1-6**). The first inhibitors of apoptosis described in baculoviral genomes led to the identification of orthologues in higher order species (Crook et al., 1993).

1.7.1 Primary Structure and Domains of IAPs

The IAP family has an evolutionarily conserved role in regulating apoptosis from insects to humans. IAPs are endogenously expressed proteins that share

structurally similar domains (Salvensen et al., 2002). Empirically, any protein of the IAP family must contain at least one ~70 residue zinc-binding baculovirus IAP repeat (BIR) domain (Miller et al., 1999). This particular structure includes a number of highly conserved hydrophobic and hydrophilic residues, capable of supporting protein-protein interactions. Two of the most common motifs of the IAPs are the BIR and RING domains. Other IAPS contain CARD, NACHT, leucine-rich repeat (LRR) and ubiquitin-conjugating (UBC) domains (**Fig. 1-6**).

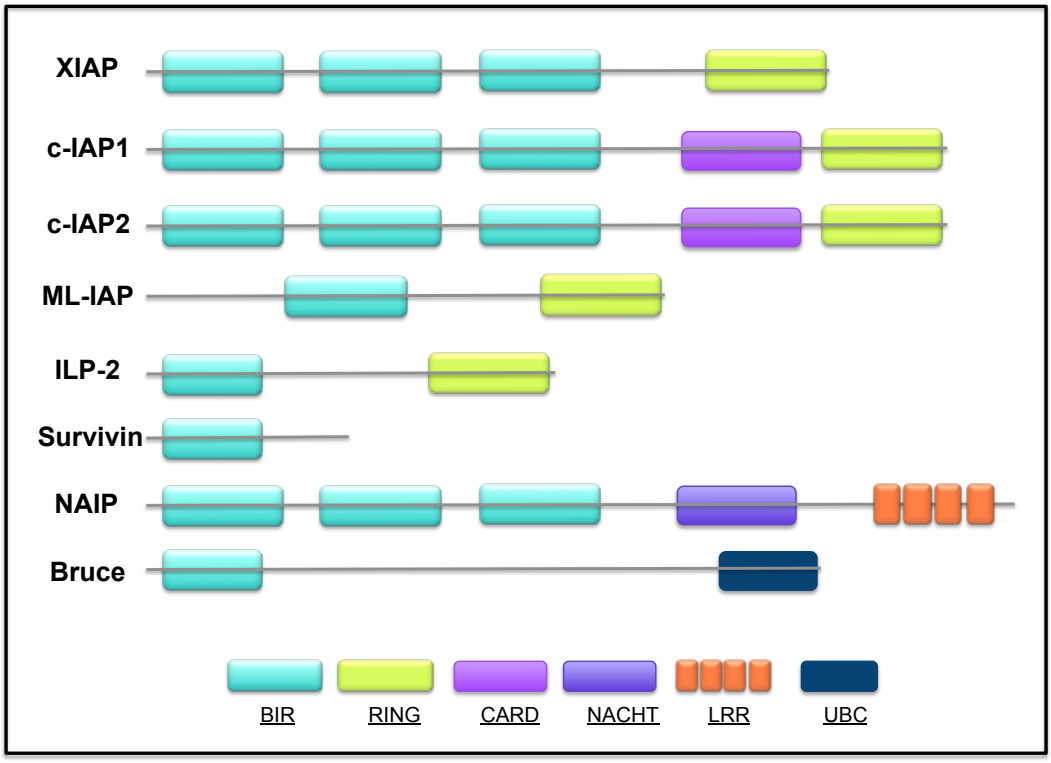

Figure 1-6. Domain organization of the eight IAP proteins

1.7.2 Mechanism of Inhibition

XIAP, c-IAP1, c-IAP2 and ML-IAP are direct binding interactors of caspases. XIAP is the most potent mammalian inhibitor of Casp-3, -7 and -9 (Deveraux et al., 1997). More specifically, XIAP contains three BIR domains and a C-terminal

RING domain. BIR 3 of XIAP specifically targets proCasp-9, whereas the flexible region preceding BIR2 is the critical region sufficient to inhibit active Casp-3 and -7 in the sub-nanomolar range (Suzuki et al., 2001b). Especially critical for caspase inhibition are the N-terminal linker of BIR2 and the C-terminal helix of BIR3, which drive BIR 2 and BIR 3 specificity for targeting proCasp-9 and Casp-3 & -7, respectively. The two-site interaction enhances affinity and selectivity for inhibition. As potent as XIAP is towards Casp-3, -7 and -9, it does not have any effect on Casp-1, -6 or -8 even at excess amounts of 50-fold (Deveraux et al., 1997). Similary, c-IAP1, c-IAP2 and ML-IAP, retain the typical fold and interacting-binding motifs like XIAP, however they lack the crucial inhibitory segments required for caspase inhibition (Eckelman et al., 2006; Salvensen et al., 2007). ILP-2 is similar to XIAP, except for a N-terminal truncation of its BIR domain, resulting in a partial unfolded conformation that ablates inhibitory ability (Salvensen et al., 2007). Indirectly, IAPs can also counteract caspase-mediated apoptosis by directing the proteasomal degradation of pro-apoptotic proteins through their RING finger domains, which act as E3 ubiquitin ligases (Deshaies et al., 2009; Morizane et al., 2005; Salvensen et al., 2007).

1.7.3 Other IAP Functions

The other three IAPs may not possess the necessary domains for caspase inhibition modeled under the XIAP effect, however they have other reported functions. Survivin is found to play a strong conserved role in cytokinesis, and is only minorly involved as an anti-apoptotic regulator (Li et al., 1998). Uniquely, BRUCE contains an additional C-terminal UBC domain and has anti-apoptotic

functions by BIR domain binding to Casp-9 and facilitating the proteasomal degradation of mature and active forms of Smac (Bartke et al., 2004; Qiu et al., 2005). Lastly, the anti-apoptotic function of NAIP, once thought to be limited to neuronal cells, is found in several human tissues and implicated in a pathogen-recognizing role in intestinal cells (Liston et al., 1996; Maier et al., 2007). More recently, NAIP is implicated in innate immunity through integration of the inflammatory response to intracellular pathogens (Salvensen et al., 2007).

1.8 Alternative Splicing (AS)

AS is the process of differential exonic usage of a primary gene transcript. AS is a ubiquitous phenomenon and responsible for generating 80% of gene heterogeneity and protein diversity within eukaryotes. A common mechanism for AS is exon skipping. In exon skipping, primary transcripts containing multiple non-coding intervening sequences (introns) must be removed to yield translatable mature messenger RNA (mRNA). Differential removal results in ligation of multiple combinations of exons to produce various protein isoforms (Black et al., 2003; Licatalosi et al., 2010).

1.8.1 Spliceosome

The fundamental process of AS is mediated by the assembly of the spliceosome consisting of five critical small nuclear ribonucleoproteins (snRNPs) termed U1, U2, U4, U5 & U6, whose role is to recognize specific *cis*-acting elements on the heterogeneous nuclear RNA substrate (Bindereif et al., 1987; Jamison et al.,

1992). The general splicing mechanism involves sequential assembly of the spliceosome to perform a two-step transesterification reaction. Initially, *trans*-acting factor U1 snRNP recognizes and binds to the 5' splice site of the nascent transcript, followed by binding of the U2 snRNP auxiliary factor to the branch region within the intronic sequence. This recruits U2 snRNP to the branchpoint adenosine (BPA) in an ATP-dependent manner. The initial reaction, where the nucleophilic 2' hydroxyl of the BPA attacks the 5' splice junction, dissociates the phosphodiester bond and is replaced with a 2'5'-phosphodiester linkage connecting the BPA with the terminal nucleotide at the 5' splice site (Moore et al., 1993). This forms an intronic lariat intermediate. The second step involves the recruitment of tri-snRNPs U4/U5/U6 to induce a conformational change in the spliceosome to catalyze the free 3' hydroxyl of the 5' exon attacking the 3' splice site junction (Moore et al., 1993). Thus creating a newly formed phosphodiester bond to ligate the 5' exon and 3' exon and the release of the intron lariat to be degraded (**Fig. 1-7**).

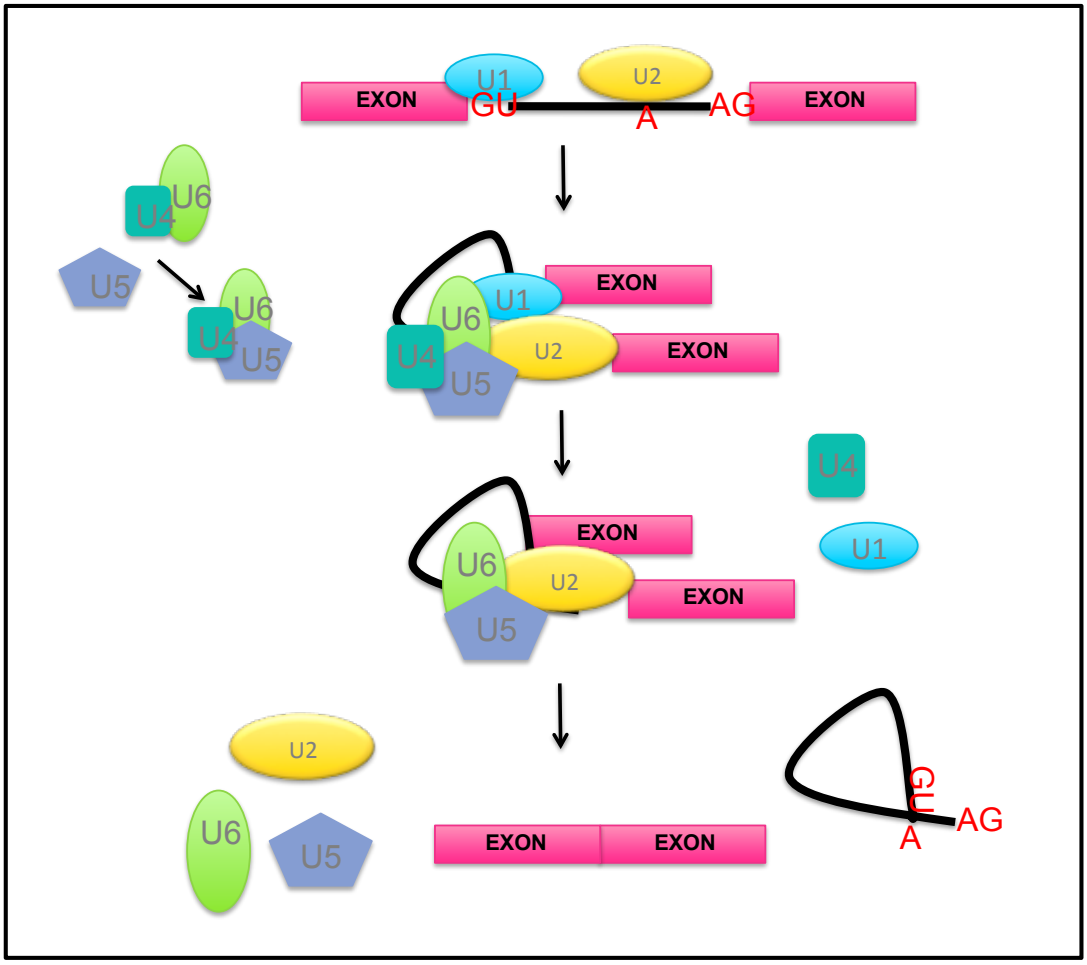

Figure 1-7. Spliceosome-mediated splicing of pre-mRNA

1.8.2 Components and Regulatory Modulators

In addition to the snRNPs, a plethora of *trans*-acting RNA binding proteins have corresponding auxiliary *cis*-acting RNA sequence elements that coordinate various RNA-RNA, RNA-protein and protein-protein interactions to regulate this highly intricate process. An important function of these secondary elements is ensuring high fidelity of exon recognition by the snRNPs. Through mass spectrometry, tremendous advances have been made in the discovery of over 300 proteins that are involved in AS (Jurica et al., 2003; Neubauer et al., 1998). *Trans*-

acting proteins such as intronic and exonic splicing activators and repressors influence splice site recognition by binding to *cis*-acting regulatory sites; enhancers and silencers. Typically the proteins involved in promoting splicing are members of the serine/arginine (SR) protein family (Zahler et al., 1992), whereas the repressors are a family of heterogeneous nuclear ribonucleoproteins (hnRNPs). These SR proteins often contain RNA recognition motifs (RRM) and serine-rich (RS) regions. They function as mediators of cross-exon and cross-intron interactions between snRNPs (Hastings et al., 2001). Increasing the concentration of specific SR proteins influences the use of proximal versus distal splice sites on several targets (Mayeda et al., 1992).

Other auxiliary factors link the splicing machinery to other processes such as transcription, capping and 3'-UTR formation (Proudfoot et al., 2002). Components of the polyadenylation machinery, as well as the transcription export complex and exon junction complex have been identified as intermediaries between AS and mRNA metabolism (Hastings et al., 2001). There seems to be mounting evidence of functional coupling between many pre-mRNA and mRNA machinery/metabolic processes.

1.8.3 Alternative Splicing in Caspases

Caspases are commonly alternatively spliced to generate splice variants (**Table 1-2**). Among them, Casp-1 has six characterized variants, some of which play a pro-apoptotic role while others are inactive or anti-apoptotic. Interestingly, the most

recent isoform Casp-1ζ, does not share the same start codon with the other five (Feng et al., 2004). The Casp-2 gene can generate two splice variants, the short and long forms, which results in anti- or pro-apoptotic properties, respectively (Iwanaga et al., 2005). Competitive binding between SR protein ASF/SF2 and hnRNP/A1 onto the pre-mRNA dictates exon 9 skipping or inclusion to alter the ratio of Casp2L: Casp2S production. Similarly for the Casp-3 gene, AS generates a full-length pro-apoptotic protein, Casp-3L, and the exon 6-lacking anti-apoptotic Casp-3S (Huang et al., 2001). The Casp-4 gene can be spliced in two different forms, however they have yet to be fully characterized (Mao et al., 2010). A total of six Casp-5 splice variants have been identified. Five variants arose from differential exon skipping (Casp-5a/e) and one generated from a cryptic splice acceptor site in intron 1 (Eckhart et al., 2006). Two splice variants have been identified for Casp-6; Casp-6a and Casp-6b (Fernandes-Alnemri et al., 1995a). The shorter Casp-6b isoform retains the catalytic site and plays an inhibitory role against the activation of Casp-6a (Lee et al., 2010). This Casp-6b splice variant will be the focus in the following chapter of the thesis. Splicing of the Casp-7 gene derives two spliced isoforms, a full-length Casp-7a protein and truncated Casp-7b inactive protein. Two simultaneous AS events generate the catalytically inactive β-isoform whose dominant-negative role of inhibition is speculated (Fernandes-Alnemri et al., 1995b). As a result of AS, at least seven spliced isoforms of Casp-8 have been identified, the first two are full-length pro-apoptotic enzymes, whereas the β-types are inactive and thought to be modulators of the α forms (Horiuchi et al., 2000). Classically known to be a pro-apoptotic protease,

inactive Casp-8 isoforms seem to function in T- and B-lymphocyte proliferation and macrophage differentiation (Kang et al., 2008). An endogenous splice variant of Casp-9 that lacks the central large subunit including the active site was identified and named Casp-9b. This AS variant was characterized to act in a DN manner to inhibit the apoptotic properties of Casp-9a by interfering with its interaction with Apaf-1 (Srinivasula et al., 1999). There are seven reported spliced transcripts of the Casp-10 gene. Casp-10a, -10b and -10d function as apoptotic proteases (Wang et al., 2001), whereas others are truncated and lack the catalytic sites or C-terminal domains (Ng et al., 1999). The most recent variant, Casp-10g is a pro-domain only protein containing two DEDs, which functions in an anti-apoptotic manner through the NF-κB signaling pathway (Wang et al., 2007). Molecular and biochemical analysis of the Casp-12 gene revealed nine alternatively spliced transcripts, however due to a deleterious mutation, none are expressed as full-length protein except in humans of African descent. A recently characterized tenth variant, Casp-12S encodes only the CARD portion of the pro-domain and has yet to be defined functionally (Fischer et al., 2002). Intron inclusion at the 5'end of exon 6 generates the longer Casp-14/b, which translates to a unique C-terminal end due to a frame-shift. The predominant form is the full-length, Casp-14/a (Eckhart et al., 2000). The antagonistic variant often plays an inhibitory role in a dominant-negative fashion as observed in Casp-1, -2, -3, -8, -9 and -10, which is speculated to occur for Casp -6 and -7 (Fernandes-Alnemri et al., 1995a, et al., 1995b; Friedlander et al., 1997; Horiuchi et al., 2000; Srinivasula et al., 1999). Alternatively spliced variants may likely be an evolutionarily

retained process to regulate the lethal proteolytic activity of many of these caspases.

Table 1-2. Splice variants of the caspases and reported function

Caspase Name	Alternative spliced forms	Function
Caspase-1	Casp-1α[4]	Pro-apoptotic
	Casp-1β	Pro-apoptotic
	Casp-1γ	Pro-apoptotic
	Casp-1δ	No effect
	Casp-1ε	Anti-apoptotic (DN inhibitor)
	Casp-1ζ[5]	Pro-apoptotic
Caspase-2	Casp-2L[6]	Pro-apoptotic
	Casp-2S	Anti-apoptotic
Caspase-3	Casp-3L[7]	Pro-apoptotic
	Casp-3S	Anti-apoptotic
Caspase-4	CASP4α[8]	Pro-apoptosis
	CASP4γ	Undefined
Caspase-5	Casp-5/a[9]	Undefined
	Casp-5/b	
	Casp-5/c	
	Casp-5/d	
	Casp-5/e	
	Casp-5/f	
Caspase-6	Casp-6a[10]	Pro-apoptotic/ various
	Casp-6b	Anti-apoptotic (DN inhibitor)
Caspase-7	Mch3a[11]	Pro-apoptotic
	Mch3b	Anti-apoptotic (DN inhibitor)
Caspase-8	Casp-8a (MACH α1)[12]	Pro-apoptotic
	Casp-8b (MACH α2)	Pro-apoptotic
	Casp-8c (MACH α3)	Anti-apoptotic
	Casp-8d (MACH β1)	Inactive
	MACH β2	Inactive
	MACH β3	Inactive
	MACH β4	Inactive
	Casp-8L[13]	Anti-apoptotic (DN inhibitor)
Caspase-9	Casp-9a[14, 15]	Pro-apoptotic
	Casp-9b	Anti-apoptotic (DN inhibitor)
Caspase-10	Casp-10a[16]	Pro-apoptotic
	Casp-10b	Pro-apoptotic

	Casp-10c Casp-10d Casp-10e Casp-10f Casp-10g	Non-translated Pro-apoptotic Undefined Undefined Anti-apoptotic, regulator
Caspase-12	Casp-12L[17] Casp-12S (CARD-only)	Non-translated, inflammatory? Inactive
Caspase-14	Casp-14/a[18] Casp-14/b	Keratinocyte differentiation Undefined

[4] (Alnemri et al., 1995) [5] (Feng et al., 2004)
[6] (Wang et al., 1994)
[7] (Huang et al., 2001)
[8] (Mao et al., 2010)
[9] (Eckhart et al., 2006)
[10] (Fernandes-Alnemri et al., 1995a)
[11] (Fernandes-Alnemri et al., 1995b)
[12] (Boldin et al., 1996) [13] (Horiuchi et al., 2000)
[14] (Seol et al., 1999) [15] (Srinivasula et al., 1999)
[16] (Wang et al., 2007)
[17] (Fischer et al., 2002)
[18] (Eckhart et al., 2000)

1.9 The Role of Caspases in Human Disease

1.9.1 Proliferative: Caspases and Cancer

Apoptosis is an important mechanism to eliminate toxic and mutant cells from the body. The primary hallmark of cancer is acquired resistance to apoptotic programmed cell death. Dysregulation of this process can lead to fatal pro-survival of tumorigenic/mutant cells which fuel cancer progression. Furthermore, mutant cells can confer resistance to apoptosis as a mechanism for survival and proliferation, of which the most common is an acquired mutation of the p53 tumour suppressor gene, which is observed in over 50% of all cancers (Harris et al., 1996). This results in the removal of a key sentinel in caspase-mediated apoptotic signaling. Secondly, downregulation of caspase expression or mutations

of genes contribute heavily to the carcinogenesis of various cancers (Philchenkov et al., 2004). Additionally, the anti-apoptotic PI3K-Akt/PKB pathway is likely to mitigate apoptosis in a substantial fraction of human cancers. Decreases in expression of Casp-1, -2, -3, -6, -7, -8, -9 & -10 have been reported in a number of well-characterized disorders and cancerous tissues compared to control specimens and normal tissue samples (reviewed in (Philchenkov et al., 2004).

1.9.2 Degenerative: Caspases and Neurological Diseases

Neurodegenerative diseases such as stroke, brain trauma, ALS and HD show signs of neuronal cell death, either by necrosis or apoptosis (Friedlander et al., 2003; Kermer et al., 2004). The largest difference between the two pathways to cell death is in the magnitude of the stimulus. Acute neurological diseases often result from both necrosis and caspase-mediated apoptosis, whereas chronic neurodegeneration stems predominantly from caspase-mediated apoptotic stimuli (Friedlander et al., 2003). Since there is little to no regenerative capacity of most cells of the central nervous system (CNS), any unnecessary damage should be avoided at all costs.

1.9.2.1 Acute neurological diseases involving caspases other than Casp-6

Various reports have shown that cleaved caspases and specific caspase substrates have been detected in the neuropathology of diseases such as ischemia, stroke and spinal/head traumas. Activation of Casp-1 was first documented in ischemic stroke and is responsible for pronounced tissue damage (Friedlander et al., 1997).

It is now well-established that following cerebral ischemia, Casp-1, -3, -8 and -9 are activated (Benchoua et al., 2001).

Acute cerebral inflammation from bacterial infections of the CNS can cause a large degree of neuronal damage mediated by Casp-3 activation. Furthermore, viral infections from AIDS, encephalopathy, poliomyelitis or rabies can cause severe neurological defects (Jackson et al., 1997; Shi et al., 1996). Many of these viruses can activate several caspases, including Casp-3 to cause neuronal cell death (Allsopp et al., 1998). More specifically, HIV infected individuals can develop HIV-associated dementia, which is associated with active Casp-3 immunoreactivity in the soma and dendrites of neurons within the diseased areas of the brain (Garden et al., 2002).

Lastly, traumatic brain injuries (TBI) commonly result in the upregulation of Bcl-2 and Casp-3, and cleavage of Casp-1 and -3. This is reported in Clark *et al*, in human brain samples removed during surgical decompression for intracranial hypertension (Clark et al., 1999).

Success has been reported with various specific and broad-spectrum caspase inhibitors administered for treatment of mild and severe ischemia, viral infections and TBI in mice (Braun et al., 1999; Fink et al., 1998). However, since then there have not been any recent developments with this type of treatment. Furthermore, caspase knockout models or dominant negative (DN) expression models have proven that symptomatic decline in these afflictions is at the least, partially due to active caspases (Friedlander et al., 1997; Schielke et al., 1998). This implicates a

serious role in caspase-mediated neuronal damage in these acute neurological conditions.

1.9.2.2 Chronic neurological diseases involving caspases othen than Casp-6

Transcriptional upregulation of caspases is commonly observed in chronic neurological diseases such as amyotrophic lateral sclerosis (ALS), HD and AD (Friedlander et al., 2003).

ALS is a progressive and degenerative disease of the motor neurons in the CNS. Familial cases of ALS are associated with an inherited mutation of the superoxide dismutase-1 (SOD1) gene. A functional role of Casp-1 and -3 is implicated by ameliorating symptoms of a mouse model of ALS by the broad caspase inhibitor, z-VAD-FMK (Li et al., 2000). Furthermore, Casp-9 activation has been detected in transgenic ALS mouse models (Guegan et al., 2001) and suggested to play a crucial role in the pathogenesis of the disease (Inoue et al., 2003). A later study linked caspase-mediated apoptosis to the cytotoxic properties of SOD1 in a familial ALS rodent model. Tokuda *et al*, reported that Casp-3, -8 and -9 were upregulated and activated in the spinal cord of the transgenic rodents at 8 and 16 weeks, corresponding to early and advanced stages of the disease, respectively (Tokuda et al., 2007). The collective finding from murine models is confirmed by Casp-1 and Casp-3 activation in human spinal cord samples (Friedlander et al., 2003).

HD is a chronic neurodegenerative disease characterized by specific cell death in the neostriatum and cortex. It is characterized by an abnormal expansion of CAG-

encoded polyglutamine repeats on the mutated gene. Positive feedback mediated by caspase activity and upregulation causes the aggregation of cleaved mutant huntingtin protein (Htt). This results in the formation of intranuclear inclusions of mutant Htt, followed by nuclear translocation, which stimulates transcription of Casp-1 and -3. Evidence of Casp-1, -3, -8 and -9 have been found in human striatal brain tissue (reviewed in (Friedlander et al., 2003). Furthermore, crossing a DN mutant Casp-1 mouse model with another expressing abnormal human Htt showing a typical HD neurological phenotype resulted in a dramatic delay of symptomatic progression, suggesting a critical role in the onset and development of the disease (Braun et al., 1999).

Furthermore, Casp-3, -6 and -8 are implicated in AD since they cleave amyloid precursor protein (APP) and tau proteins, which accumulate in plaques and tangles (Gamblin et al., 2003; Gervais et al., 1999; LeBlanc et al., 1999). The detection of caspases and subsequent elevation of their enzymatic activity in AD brains suggests an increasingly critical role in the pathogenesis of the disease (Guo et al., 2004; LeBlanc et al., 1999; Masliah et al., 1998; Pellegrini et al., 1999; Rohn et al., 2002; Weidemann et al., 1999). More recently, cytoskeletal proteins of the neuronal network are found to be targets of Casp-6, indicating a role of this caspase in the disruption of the neuronal structure in the absence of cell death (Klaiman et al., 2008).

1.10 Caspase-6 and Neurodegeneration

Caspase-6 (Casp-6) was first identified in 1995 (Fernandes-Alnemri et al., 1995a), and its activated form is emerging as a candidate for causing neurodegeneration in AD (Guo et al., 2004; LeBlanc et al., 1999; Zhang et al., 2000). Casp-6 activity is abundant in the neuropil threads (NPTs), senile plaques (SPs) and neurofibrillary tangles (NFTs) of AD brains and in the neurites and nuclei of apoptotic neurons in human fetal and adult ischemic brains (Albrecht et al., 2007; Guo et al., 2004). In addition, Casp-6 activity has been shown to correlate negatively with the global cognitive score of aged individuals (Albrecht et al., 2007). Casp-6 cleaves several proteins of the cytoskeleton and synapse in human neurons and in AD (Albrecht et al., 2007; Klaiman et al., 2008). Thus, the activity of Casp-6 or detection of proteolytic substrate processing may be an excellent predictor of cognitive decline. Full-length proCasp-6 protein is present in human brain tissue and furthermore, Casp-6 is the only effector protease activated during serum-deprivation of primary human neurons, a biologically relevant model for AD (LeBlanc et al., 1999). However, in contrast to Casp-3 and Casp-7, activation of Casp-6 in mammalian cells does not induce apoptosis (Klaiman et al., 2009), instead Casp-6a initiates a protracted type of cell death in primary human neurons (LeBlanc et al., 1999; Zhang et al., 2000). More recently, Casp-6a activity has been shown to be responsible for axonal pruning and degeneration in mouse neurons (Nikolaev et al., 2009). Furthermore, Casp-6 dependent neuritic degeneration was reported in models of familial AD with the overexpression of known APP mutations (Sivananthan et al., 2010). Collectively, there is an

implication that the activity of Casp-6 may be of paramount importance in neurodegeneration rather than in cell death.

Therefore, the regulation of Casp-6 needs to be uncovered to facilitate manipulation and control of its aberrant activity in diseases such as AD. As mentioned previously, one popular mechanism of regulation is harnessed in AS caspase variants acting as DN inhibitors of the wildtype enzyme. The identification of the sixth member of the cysteine protease gene family, CASP*6* (Casp-6), resulted in the discovery of two isoforms (Fernandes-Alnemri et al., 1995a). The CASP6b transcript is an alternatively spliced form of the CASP6 transcript. It encodes only part of the pro-domain and Casp-6 p20 subunit but retains the catalytic site encoding region and the p10 subunit (Fernandes-Alnemri et al., 1995a). The alternatively spliced CASP6*β* product, proCasp-6b, may have catalytic activity or act as a DN inhibitor of Casp-6a. A DN alternative form of Casp-6a could translate into a promising facet of therapeutics for treatment or prevention of early AD.

1.11 Hypothesis and Thesis Objectives

We propose to investigate if the protein product of CASP6β, proCasp-6b, generates Casp-6 activity or acts as an inhibitor of the pro-enzyme Casp-6a.

We have conducted experiments to first validate the expression pattern of endogenous Casp-6 isoforms in various human cell lines and tissues by RT-PCR and immunoblotting. Then we assessed the functional role of proCasp-6b. We

investigated whether proCasp-6b possesses Casp-6 enzymatic activity like its wildtype partner or has a dominant negative role on Casp-6a using fluorogenic enzymatic activity assays for Casp-6 activity and western blotting. Lastly, based on the results obtained, we were able to eliminate several mechanisms of inhibition and propose one mechanism of proCasp-6b DN function.

CHAPTER 2: IDENTIFYING THE ROLE OF SPLICE VARIANT, CASPASE-6B ON THE ACTIVATION OF CASPASE-6A

2.1 Preface

The following chapter summarizes the work conducted to examine the role of proCasp-6b, an alternatively spliced variant of the Caspase-6 protein, and its mechanism as a direct inhibitor of proCasp-6a activation. This work is published in the *Journal of Biological Chemistry*. (2010). 285(42): 31974-31984.

Title:

Alternatively spliced caspase-6B isoform inhibits the activation of caspase-6A

Andrea W. Lee[1,2], Nathalie Champagne[1], Xiaojun Wang[3], Xiao-Dong Su[3], Cynthia Goodyer[4], Andrea C. LeBlanc[1,2]

From The Bloomfield Center for Research in Aging, Lady Davis Institute for Medical Research[1], College of Life Sciences, Peking U.[3], Dept of Neurology and Neurosurgery[2], and the Dept of Pediatrics[4], McGill University, 2300 Tupper Street, Montreal, Quebec, Canada H3A 2T5.

Contributions of the Authors and Acknowledgements

The work presented in this chapter was performed mainly by me, *Andrea W. Lee*. I wrote the manuscript under the critical evaluation of *Dr. Andrea C. LeBlanc*. The first purification and characterization of *in vitro* proCasp-6b in figure 2-2 was carried out by *Nathalie Champagne*. *Xiaojun Wang* helped with the purification of proCasp-6b protein. *Dr. Xiao-Dong Su* helped with the intepretation of the data. We acknowledge the help of *Dr. Cynthia G. Goodyer* for providing the fetal brain tissues for critical primary human neuron experiments. Lastly, *Dr. Andrea C. LeBlanc* provided the conceptual and critical evaluation of the project and writing of the manuscript.

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2.2 ABSTRACT

Caspase-6 (Casp-6) is activated early in Alzheimer disease and involved in axonal degeneration but the regulation of Casp-6 activity has not been explored. Several alternatively spliced forms of caspases act as inhibitors of caspase activation. The CASP6 gene generates an alternatively spliced transcript known as CASP6β in addition to the CASP6α that encodes proCasp-6a. Here, we show that the CASP6β transcript and the proCasp-6b protein are present in many cell lines, in primary human neurons, and in human brains. Unlike most other alternatively spliced caspase transcripts, proCasp-6b contains a catalytic site. However, purified proCasp-6b did not have caspase activity nor did it inhibit already activated Casp-6a. ProCasp-6b prevented the proteolytic activation of proCasp-6a *in vitro* and in cells. ProCasp-6b interacts directly with proCasp-6a. This work shows that proCasp-6b is an inhibitor of proCasp-6a activation. These results imply that proCasp-6b could negatively regulate proCasp-6a activation in neurons and prevent Casp6a-mediated axonal degeneration.

2.3 Introduction

Caspases are well-known cysteinyl proteases that regulate tissue homeostasis and whose deregulation can often lead to disease (Kumar et al., 2007b). One mode of regulation of caspases is through alternatively spliced isoforms that act as dominant negative inhibitors of caspase activation. All caspases are constituted of a pro-domain, a large and a small subunit often separated by a linker domain. Caspases are activated through proteolytic processing and reassembly of the large and small subunits into a tetrameric active enzyme. The catalytic site is contained within the large subunit. Most of the alternatively spliced dominant negative forms have lost their catalytic sites and are proposed to associate with the subunits of the active form to inhibit activation of the caspase. The RCasp-1ε lacking part of the pro-peptide and the p20 subunit interacts with the RCasp-1a-p20 subunit and increases cellular survival (Alnemri et al., 1995). The Casp-3s isoform, which lacks the catalytic site and p12 subunit of Casp-3, prevents staurosporin-activated Casp-3-dependent poly-ADP-ribose polymerase cleavage and apoptosis (Huang et al., 2001). Casp-8L lacks the catalytic site and part of the C-terminus of Casp-8 and interferes with the interaction of Casp-8 with Fas-associated death domain, which is necessary for Casp-8-mediated cell death (Himeji et al., 2002; Mohr et al., 2005). Casp-9s lacks the large subunit of normal Casp-9 and interacts with Apaf-1 to block recruitment and activation of Casp-9 to the apoptosome (Seol et al., 1999). Yet, a few alternatively spliced forms retain the catalytic site and can prevent activation of caspases by interfering with regulatory proteins. For

example, the Casp-2s isoform prevents activation of Casp-2 through interaction with the Ich-1S (caspase-2S)-binding protein (Ito et al., 2000; Wang et al., 1994).

The CASP6β transcript is an alternatively spliced form of the CASP6 nascent transcript. The CASP6β mRNA lacks amino acids 15 to 103, encoding a portion of the pro-domain and the Casp-6 p20 subunit but retains the catalytic site (QACRG) and the p10 subunit (Fernandes-Alnemri et al., 1995a). It is not known if the alternatively spliced CASP6β product, proCasp-6b, has any catalytic activity or acts as a dominant negative inhibitor of Casp-6a. A dominant negative alternative form of Casp-6a would be important because increasing evidence suggests that Casp-6a has essential physiological functions and can be activated in pathological situations. Casp-6a activity precedes Casp-3 and Casp-7 activity in isolated intestinal epithelial cells submitted to detachment (anoikis) (Grossmann et al., 1998). Casp-6a is involved in lymphocyte differentiation and proliferation (Olson et al., 2003; Watanabe et al., 2008). Although debated, Casp-6a is thought to be activated during the dissolution of organelles in the lens of the eye (Foley et al., 2004; Morozov et al., 2006; Zandy et al., 2007, et al., 2005). Casp-6a is activated and induces a protracted type of cell death in primary cultures of human neurons in the absence of Casp-3 and Casp-7 activity (LeBlanc et al., 1999; Zhang et al., 2000). Active caspase-1 (RCasp-1) activates Casp-6a *in vitro* and in human neurons in culture (Guo et al., 2006). Casp6a activity is abundant in the neuropil threads, neuritic plaques and neurofibrillary tangles of sporadic and familial forms of Alzheimer disease (AD) brains and in the neurites and nuclei of apoptotic neurons in human fetal and adult ischemic brains (Albrecht et al., 2009; Guo et

al., 2004). Furthermore, active Casp-6a is present in some aging brains and correlates negatively with the global cognitive score of aged individuals (Albrecht et al., 2007). Casp-6a cleaves several proteins of the cytoskeleton and synapses in human neurons and in AD (Albrecht et al., 2007; Guo et al., 2004; Klaiman et al., 2008). However, in contrast to Casp-3 and Casp-7, activation of Casp-6a in mammalian cells does not induce cell death (Klaiman et al., 2009). More recently, Casp-6a activity has been shown to be responsible for axonal pruning and degeneration in mouse neurons (Nikolaev et al., 2009). Abnormal Casp-6a cleavage of the DJ-1 and Huntingtin proteins may also be implicated in Parkinson and Huntington diseases, respectively (Giaime et al., 2010; Graham et al., 2006). Together, these results indicate that active Casp-6a may be predominantly responsible for neurodegeneration rather than cell death.

The regulation of Casp-6 activity is not well known. Casp-6a is self-activated *in vitro* and in cells and this is regulated by the pro-domain (Klaiman et al., 2009). Unlike the other two effector caspases, Casp-3 and Casp-7, Casp6 is not inhibited by inhibitor of apoptosis proteins (IAPs) (Vaux et al., 2005). Estrogen induces an inhibitor of the active form of Casp-6a in human primary neurons (Zhang et al., 2001). Here, we investigate if the protein product of CASP6β, proCasp-6b, generates Casp-6 activity or acts as an inhibitor towards proCasp-6a.

2.4 Results

CASP6β mRNA and its protein product, proCasp-6b, are expressed in various cell lines, primary human neurons, and adult human brains. The CASP6 gene has been shown to yield two alternatively spliced mRNAs called Mch2a (CASP6α) and Mch2b (CASP6β) (Fernandes-Alnemri et al., 1995a) (**Fig. 2-1A**). ProCasp-6a, translated from the CASP6α mRNA generates the pro-enzyme form that is proteolytically processed into a fully active caspase, while the proCasp-6b results from the translation of the shorter CASP6β mRNA. To assess if the CASP6β mRNA is generated with the CASP6α mRNA, we performed RT-PCR on total RNA extracted from various cell types with CASP6 specific primers that would amplify both cDNAs (indicated by arrows in Fig. 1A). The expected 564 bp PCR product of CASP6α was detected in human colon carcinoma HCT116, embryonic kidney 293 (HEK293T), breast carcinoma MCF7, and neuroblastoma MC-IXC and BE(2)M17 cells (**Fig. 2-1B**). In addition, a second PCR amplified DNA band of 325 bp corresponding to the CASP6β transcript was also detected. The 564 bp and 325 bp were strongly amplified from CASP6α and CASP6β-transfected MCF7 cells. The ratio of CASP6α over CASP6β transcripts was consistently greater than 1 in HCT116, HEK293T, MCF7 and MC-IXC, and less than 1 in the BE(2)M17 cell line. In cultures of human primary neurons, CASP6β mRNA levels slightly exceeded those of CASP6α. CASP6 siRNA strongly reduced the levels of PCR-amplified CASP6α, but had only slight effects on the levels of CASP6β (**Fig. 2-1C**). The lack of down-regulation of CASP6β by siRNA was surprising since the four pooled CASP6 siRNAs target sequences in

exons 5, 6 and 7 shared by CASP6α and CASP6β. To confirm the identity of the two CASP6 transcripts, we subjected purified CASP6β or unpurified PCR amplified CASP6α and CASP6β to enzymatic digestion with *Nco*I (**Fig. 2-1D**). Digestion of the 325 bp band generated the expected 219 bp product indicative of a unique *Nco*I site cleavage at 93 bp. Digestion of both transcripts generated a digested product of CASP6α at 360 bp and the same 219 bp CASP6β band. As attempt to generate a custom-made siRNA to the exon1/exon5 junction of CASP6β also failed to down-regulate CASP6β (results not shown). We concluded that the siRNA may not effectively downregulate CASP6β because of a low turnover of the CASP6β mRNA or its interaction with RNA binding proteins (Dorsett et al., 2004).

The proCasp-6b protein resulting from CASP6β translation lacks part of the pro-domain and part of the large p20 subunit of proCasp-6a (**Fig. 2-1A).** Therefore, the expression of proCasp-6a can be differentiated from that of proCasp-6b with the α-Casp6a antiserum, but both proCasp-6a and proCasp-6b should be recognized with the α-p10Casp6 antibody (**Fig. 2-1A).** Western blot analysis of total proteins showed the presence of two α-p10Casp6 immunoreactive proteins in most cell lines and primary human neurons (**Fig. 2-1E).** The 34 kDa and 23 kDa proteins corresponded to the expected size for proCasp-6a and proCasp-6b, respectively. The level of proCasp-6a relative to proCasp-6b protein was consistent with the observed levels of transcripts in Fig. 1B**.** CASP6 siRNA reduced the levels of proCasp-6a significantly but only minimally reduced the

levels of proCasp-6b (**Fig. 2-1F**), consistent with the observed effect of CASP6 siRNA on the mRNA levels. To confirm that the 23 kDa protein detected with α-p10Casp6 antibody was indeed proCasp6b, we also performed western blot analyses with the Neomarker and Upstate α-Casp6a antisera. While immunoblot analysis revealed the proCasp-6a and the p20 subunit of recombinant Casp6, they did not detect any protein at 23 kDa, consistent with the lack of this epitope in proCasp-6b (**Fig. 2-1G**). Western blot analysis of proteins extracted from human temporal cortex, frontal cortex, and cerebellar tissues with α-p10Casp6 did not reveal a 32 kDa protein expected for proCasp-6a but showed a 23 kDa protein consistent with proCasp6b (**Fig. 2-1H**). The 23 kDa immunoreactive protein in human brains migrated exactly as that observed in MCF7 cells (not shown). This protein varied in intensity in different cases but was consistently present in the three areas of the brain investigated. The identity of an additional lower MW protein was not pursued but could represent a post-translationally modified form of proCasp-6b or a non-specific cross-reacting protein. Together, these results confirmed that the CASP6β transcript, as shown by Northern blot analysis previously (Fernandes-Alnemri et al., 1995a), and the proCasp-6b protein were expressed in various cell lines, primary human neurons, and in normal human brain tissue.

Figure 2-1.

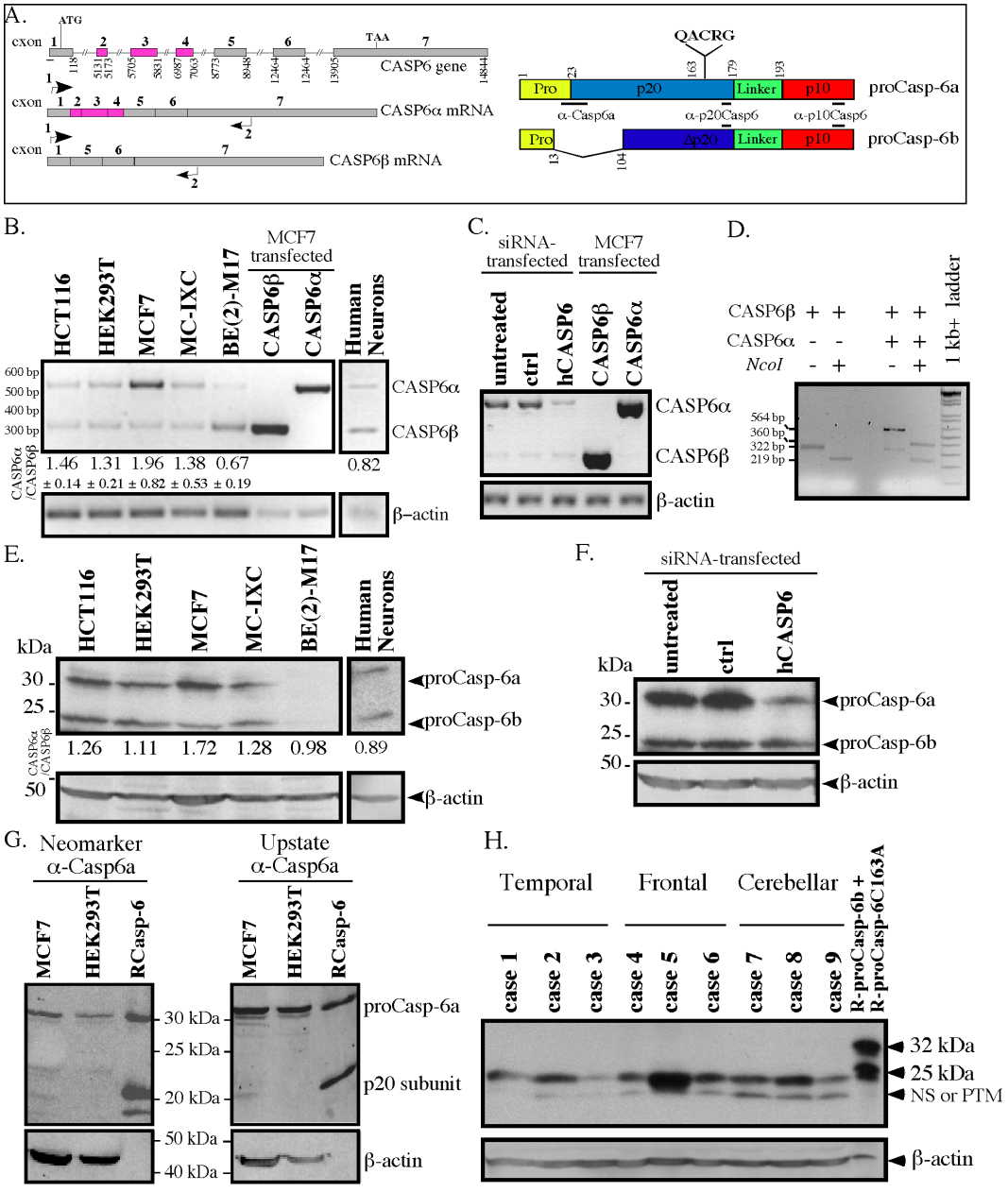

A.
ATG
TAA
exon
CASP6 gene
CASP6α mRNA
CASP6β mRNA
QACRG
Pro
p20
Linker
p10
proCasp-6a
α-Casp6a
α-p20Casp6
α-p10Casp6
Δp20
proCasp-6b
B.
MCF7 transfected
HCT116
HEK293T
MCF7
MC-IXC
BE(2)-M17
CASP6β
CASP6α
Human Neurons
CASP6α
CASP6β
β-actin
C.
siRNA-transfected
MCF7 transfected
untreated
ctrl
hCASP6
D.
CASP6β
CASP6α
NcoI
1 kb+ ladder
E.
kDa
proCasp-6a
proCasp-6b
β-actin
F.
siRNA-transfected
G.
Neomarker α-Casp6a
Upstate α-Casp6a
RCasp-6
proCasp-6a
p20 subunit
H.
Temporal
Frontal
Cerebellar
R-proCasp-6b + R-proCasp-6C163A
32 kDa
25 kDa
NS or PTM

Figure 2-1: Expression of CASP6α and CASP6β mRNA and proteins. A. Left panel: Schematic diagram of the exons and introns in the CASP6 gene and the resulting CASP6α and CASP6β transcripts. Numbering starts at the first nucleotide of the first exon. The arrows represent primers used for the RT-PCR. Right panel: Schematic diagram showing the main domains of proCasp-6a and proCasp-6b and the position of the epitopes for the antibodies that were used in the study. Pro: pro-domain. **B**. Ethidium bromide stained agarose gel of CASP6α and CASP6β PCR products with primer pairs indicated in A and with β-actin primers. The marker is a 100 bp DNA ladder. Densitometry of CASP6 transcripts was analyzed by ImageQuant software and expressed as a ratio of CASP6α/CASP6β. **C.** Ethidium bromide stained agarose gel of CASP6α and CASP6β PCR products from CASP6 siRNA-transfected MCF7 cells and control CASP6α and CASP6β-transfected MCF7 cells. **D.** Ethidium bromide stained agarose gel showing the *Nco*I restriction digestion of purified PCR-amplified CASP6β and of non-purified PCR amplified CASP6α and CASP6β. **E.** Western blot analysis with anti-p10Casp6 antibody on total protein extracts from cell lines and human primary neurons. **F.** Western blot analysis with anti-p10Casp6 antibody on total protein extracts from MCF7 cells transfected with CASP6 siRNA. **G.** Western blot analysis with Neomarkers or Upstate α-Casp6a antisera of total protein extracts from MCG7 and HEK293T cells. **H.** Western blot analysis with anti-p10Casp6 or β-actin antibodies of total protein extracts from human temporal cortex, frontal cortex, or cerebellum.

Purified bacterially expressed recombinant proCasp6b does not possess catalytic activity and does not inhibit the active form of Casp6a. To assess the function of proCasp-6b, C-terminally His-tagged bacterially expressed proCasp-6b was purified. With the addition of the His tag to proCasp-6b, the protein had the expected mass of 27 kDa. We isolated the IPTG-induced 27 kDa proCasp-6b protein to over 90% purity (not shown). The proCasp-6b protein was immunoreactive with α-p10Casp6 (**Fig. 2-2A**). The α-p10Casp6 antibody also recognized the catalytic mutant proCasp-6aC163A, proCasp-6a and the p10 subunit of active Casp-6a. As expected, the α-Casp6a antiserum (**Fig. 2-1C**) recognized the proCasp-6a and the proCasp-6aC163A, but not proCasp-6b. The neoepitope α-p20Casp6 antiserum recognized only the p20 subunit in active Casp-6a indicating that the D179 site was not cleaved in bacterially expressed proCasp-6b. In contrast, bacterially expressed proCasp-6a was always self-proteolytically cleaved into its p20 and p10 subunits. Nevertheless, proCasp-6b contains the catalytic QACRG site of caspases. To determine if proCasp-6b might retain some catalytic activity, pure proCasp-6b was incubated at 37ºC for 1 hr (**Fig. 2-2B**). This did not result in any proteolytic processing indicating that proCasp-6b did not have self-processing activity *in vitro*. Furthermore, even at very high concentrations, proCasp-6b did not proteolytically process Ac-VEID-AFC substrate (**Fig. 2-2C).** To determine if proCasp-6b inhibited active RCasp-6a, we assessed the VEIDase activity of active Casp-6a in the absence or presence of increasing amounts of purified proCasp-6b (**Fig. 2-2D**). In this experiment, the RCasp-6a was incubated with different concentrations of the substrate Ac-VEID-

AFC and proCasp-6b. We did not observe any variation in the specific activity of 46 nM of active RCasp-6a with up to 100 nM of proCasp-6b. These results indicated that proCasp-6b was not catalytically active and could not directly inhibit the already activated RCasp-6a.

Figure 2-2.

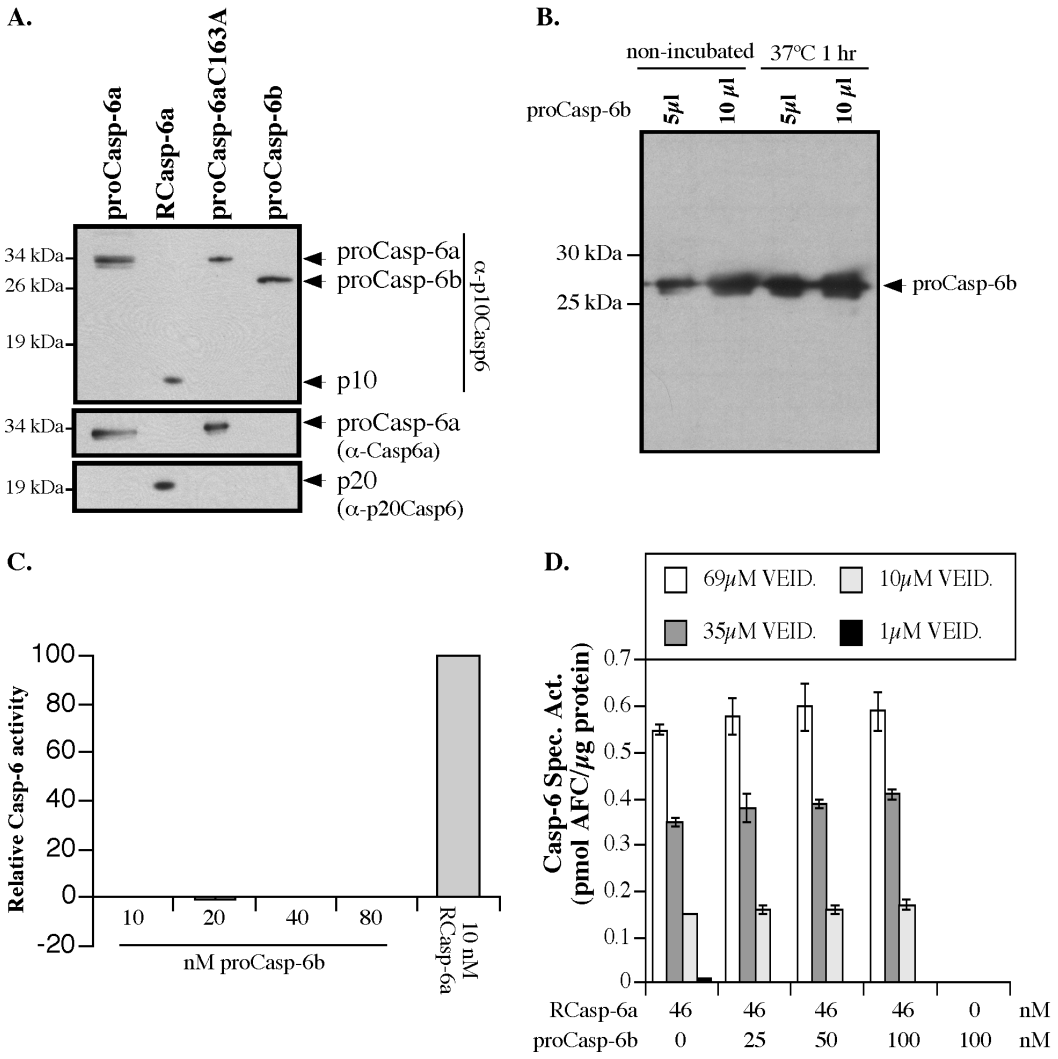


Figure 2-2: Purification of proCasp-6b from *E. Coli* lysate. **A**. Western blots of proCasp-6a, active RCasp-6a, inactive proCasp-6aC163A, and proCasp-6b with anti-p10Casp6 (top panel), Upstate anti-proCasp6a (middle panel) and neoepitope anti-p20Casp6 (lower panel). **B.** Western blot with anti-p10Casp6 of purified proCasp-6b incubated for 1 hr at 37ºC. **C.** Relative VEIDase activity with increasing amounts of proCasp-6b relative to active RCasp-6a. **D.** Specific VEIDase Casp-6 activity for purified recombinant active Casp-6a in the absence or presence of proCasp-6b at the indicated concentrations.

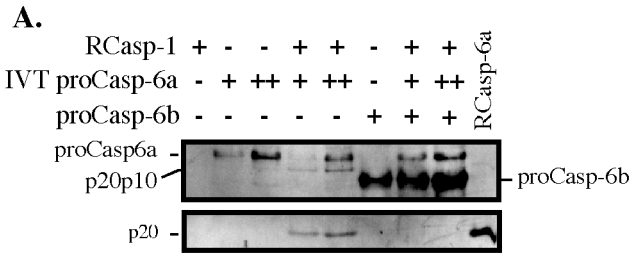
ProCasp-6b inhibits RCasp-1-mediated processing of proCasp-6a in vitro. To determine if proCasp-6b can inhibit the activation of proCasp-6a, we activated proCasp-6a by adding active RCasp-1 (Guo et al., 2006). The addition of active RCasp-1 to IVT proCasp-6a resulted in processing of proCasp-6a at D23 and D179, thereby producing the p20p10Casp-6a and the p20 subunit detected with the anti-p20 neoepitope antiserum, respectively (**Fig. 2-3A**). Consequently, as p20p10Casp-6a and p20 subunits were generated, the full-length proCasp-6a decreased (**Fig. 2-3A).** ProCasp-6b completely inhibited the processing of IVT proCasp-6a at D23 and D179 as evident by the lack of p20p10Casp-6a and p20 subunits. In contrast, proCasp-6a levels were maintained in the presence of proCasp-6b indicating a complete inhibition of proCasp-6a processing. These results showed that proCasp-6b had robust inhibitory activity.

To assess proCasp-6a activation in these conditions, we measured IVT Casp-6a VEIDase activity in three independent experiments. The molar amount of purified proCasp-6b was assessed by spectrophotometry, while IVT proCasp-6a in the *E. coli* lysate was assessed relative to proCasp-6b by immunoblotting with α-p10Casp6 antibody. Variations in specific activities were observed in each experiment and depended on the number of freeze-thaw cycles of RCasp-1, proCasp-6b and IVT proCasp-6a. ProCasp-6b and active RCasp-1 had no VEIDase activity but IVT proCasp-6a displayed a small amount of activity consistent with a certain amount of self-activation during synthesis in *E. coli* lysates. The addition of RCasp-1 to IVT proCasp-6a resulted in increased VEIDase activity consistent with the RCasp-1-mediated increased processing of

proCasp-6a into its active subunits (**Fig. 2-3B)**. Addition of an equimolar amount of proCasp-6b to IVT proCasp-6a inhibited RCasp-1-mediated activation of proCasp-6a by 50 to 90% in three independent experiments. Adding twice the amount of proCasp-6b exhibited 90 to 100 % inhibition. ProCasp-6b had no effect on RCasp-1 YVADase activity in the absence or presence of IVT proCasp-6a (**Fig. 2-3C).**

These results showed that proCasp-6b inhibited RCasp-1-mediated activation of proCasp-6a. The results excluded the possibility that proCasp-6b directly inhibited RCasp-1 activity. From this, we deduced only two other possibilities. ProCasp-6b could interact with proCasp-6a and block the access of RCasp-1 to the processing sites of proCasp-6a. Alternatively, proCasp-6b could inhibit the self-processing activity of p20p10Casp-6a generated by RCasp-1 cleavage at the D23 site of the pro-domain (Klaiman et al., 2009).

Figure 2-3.



B.

	Ratio (6a:6b)	VEIDase Activity (pmol/min) IVT proCasp-6a	IVT proCasp-6a + RCasp-1 -proCasp6b	IVT proCasp-6a + RCasp-1 +proCasp6b	% Inhibition
Exp 1	1:1	2.71	5.04	0.53	89.5
	1:2	2.71	5.04	0	100
Exp 2	1:1	3.24	10.55	1.09	89.7
	1:2	3.24	10.55	0	100
Exp 3	1:1	0	1.07	0.51	52.3
	1:2	0	1.07	0.11	89.7

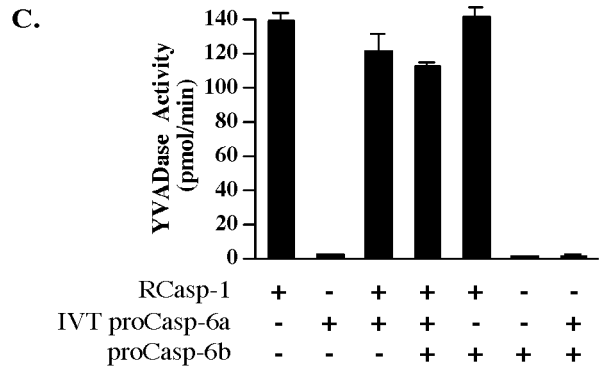


Figure 2-3. Effect of proCasp-6b on RCasp-1-mediated processing of IVT proCasp-6a. A. Western blot with anti-p10Casp6 or the neoepitope anti-p20Casp6 antiserum showing the effect of proCasp-6b on RCasp-1-mediated processing of the catalytically competent proCasp-6a. **B.** VEIDase activity of IVT proCasp-6a treated with or without active RCasp-1 in the absence or presence of proCasp-6b in three independent experiments. The results from the third experiment show that with freeze-thaw cycles and prolonged storage time in freezer, the purified caspases and proCasp-6b are unstable. **C.** RCasp-1 activity in the experiments shown in A on Ac-YVAD-AFC substrate.

Effect of proCasp-6b on RCasp-1-mediated processing of proCasp-6aC163A in vitro. To determine if proCasp-6b inhibits RCasp-1-mediated p20p10Casp-6a self-activation, we repeated the above experiments with catalytically incompetent proCasp-6aC163A. Active RCasp-1 was incubated with the catalytically inactive proCasp-6aC163A in the absence and presence of an equal amount of proCasp-6b (**Fig. 2-4A**). RCasp-1 only minimally processed proCasp-6aC163A at D23 resulting in a small amount of p20p10Casp-6aC163A. ProCasp-6b did not inhibit RCasp-1-mediated cleavage of proCasp-6aC163A at D23. We excluded the possibility that RCasp-1 was inhibited by proCasp-6b or proCasp-6aC163A by measuring RCasp-1 YVADase activity in each of the samples shown in the western blot (**Fig. 2-4B)**. Neither proCasp-6aC163A nor proCasp-6b had any YVADase activity and RCasp-1 YVADase activity was unaffected in the presence of proCasp-6aC163A, proCasp-6b, or both. From these results, we concluded that proCasp-6b is inhibiting p20p10Casp-6a self-processing.

Furthermore, RCasp-1 did not process the proCasp-6b (**Fig. 2-4A)** excluding the possibility that a truncated Casp-6b p20 subunit associated with p20 subunits of Casp-6a to act in a dominant negative manner.

Surprisingly, when proCasp-6b was added to proCasp-6aC163A, a protein fragment migrated at 26 kDa, slightly below the proCasp-6b, and a very faint amount of p10 subunit appeared despite the absence of RCasp-1. Both these fragments were detected with the anti-p10Casp6 antibody. Since the proCasp-6aC163A is catalytically inactive, these results indicated that under some

circumstances, the proCasp-6b can have minor catalytic activity at D193 of proCasp-6a to generate the p10 subunit and additionally cleaved within the p20 subunit to yield a 26 kDa fragment containing the p10 subunit. Processing at this site was partially inhibited when active RCasp-1 was added to proCasp-6aC163A and proCasp-6b, suggesting that RCasp-1-mediated cleavage at the D23 site decreased the accessibility of this additional processing site to proCasp-6b.

These results showed that the proCasp-6b must inhibit p20p10Casp-6a self-activation rather than inhibit RCasp-1-mediated activation.

Figure 2-4.

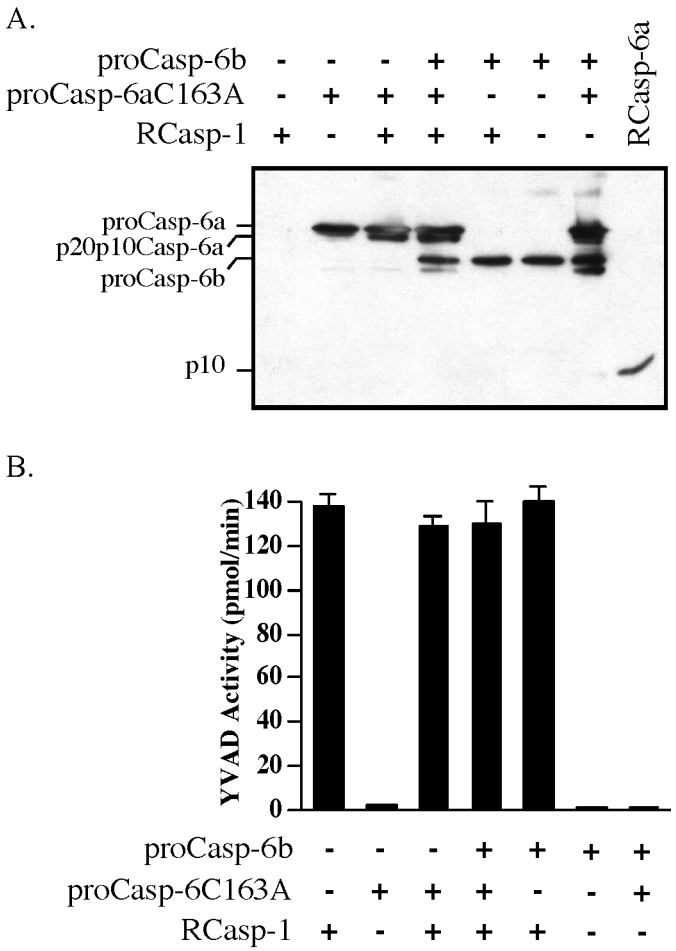


Figure 2-4. Effect of proCasp-6b on RCasp-1-mediated processing of proCasp-6aC163A. A. Western blot with anti-p10Casp6 showing the effect of proCasp-6b on RCasp-1-mediated processing of the catalytically inactive proCasp-6aC163A mutant. **B.** RCasp-1 activity in the experiments shown in A on Ac-YVAD-AFC substrate.

ProCasp-6b inhibits proCasp-6a activation in mammalian cells. To test if proCasp-6b inhibits proCasp-6a activation in cells, we used a model of serum-deprived human primary neurons where proCasp-6a is activated via RCasp-1 (Guo et al., 2006). Unfortunately, the low efficiency of transfection of these primary neurons and the lack of a specific proCasp-6b antibody does not allow biochemical analysis of the expression of the exogenously expressed proCasp-6b. However, we confirmed expression of proCasp-6b from the pCep4β eukaryotic construct in the SK-N-SH cell line (**Fig. 2-5A)**. In mock and vector-transfected cells, there were low levels of 23 kDa proCasp-6b. This protein increased significantly in proCasp-6b cDNA transfected cells. In human neurons, we found that serum-deprived non-transfected or pCep4βEGFP-transfected neurons underwent 50% cell death with serum deprivation (**Fig. 2-5B**). ProCasp-6b cDNA-transfected neurons showed significantly less cell death. These results suggested that proCasp-6b prevented cell death in primary human neurons.

Figure 2-5.

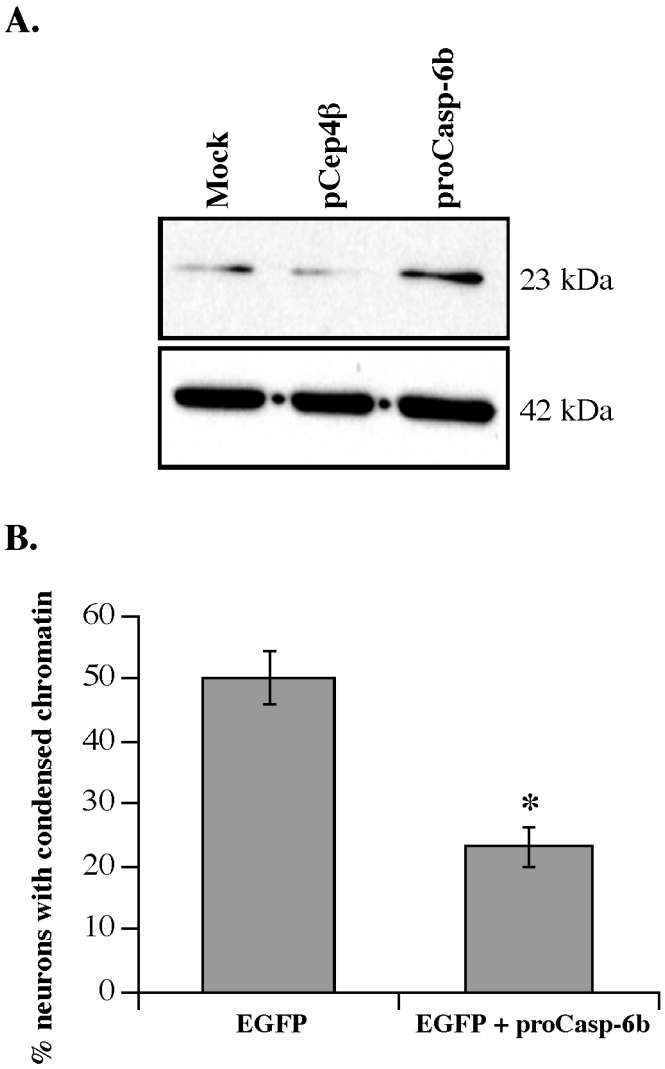


Figure 2-5. ProCasp-6b inhibits Casp-6a-mediated cell death in primary cultures of human neurons. **A**. Western blot of protein extracts of mock, pCep4β, or pCep4β-proCasp-6b cDNA-transfected cells with anti-p10Casp6 and β-actin antibodies. **B**. Neuronal cell death of neurons transfected with pCep4β/EGFP alone or pCep4β/EGFP and pCep4b/proCasp-6b cDNA (ratio 1:3) and serum-deprived. The baseline of cell death in non-transfected and non-serum deprived neurons was 5%. Non-transfected neurons had approximately 50% cell death due to serum deprivation indicating that EGFP is not additionally toxic to these neurons. * $p<0.0005$ by ANOVA and Scheffé's post-hoc analysis.

To confirm that proCasp-6b inhibited proCasp-6a activation in cells, we used an alternate cellular model that allows verification of the expression and activity of Casp-6a. HCT116 cells were transfected with increasing concentrations of CASP6β cDNAs and the cellular extracts tested for VEIDase activity 24 hrs after the transfection (**Fig. 2-6A**). As observed *in vitro*, the expression of proCasp-6b, detected by western blotting with either the α-p10Casp6 or α-His antibodies, did not generate VEIDase activity. Similar to previous observations in HEK293 cells (Klaiman et al., 2009), the p20p10Casp-6a cDNA-transfected HCT116 cells exhibited significant VEIDase activity compared to control cDNA-transfected cells (**Fig. 2-6A**). Co-transfection of proCasp-6b cDNA with the p20p10Casp-6a cDNA resulted in a four-fold inhibition of the VEIDase activity generated by p20p10Casp-6a (**Fig. 2-6B**).

However, the co-expression of proCasp-6b with p20p10Casp-6a also considerably decreased the abundance of the p20p10Casp-6a and proCasp-6b proteins (**Fig. 2-6B**). To investigate if co-transfections altered the mRNA levels of p20p10Casp-6a and proCasp-6b, we performed RT-PCR on RNA extracted from HCT116-transfected cells. Two different primer pairs had to be specifically designed for proCasp-6b and p20p10Casp-6a cDNAs because the p20p10Casp-6a lacks the pro-domain sequence that is shared by proCasp-6b and proCasp-6a mRNA. The p20p10Casp-6a primers amplified the expected 562 bp CASP6α DNA from pCep4β vector but not in non-transfected cells indicating an increase in endogenously expressed CASP6α mRNA during transfection. CASP6α mRNA was slightly increased in cells transfected with the p20p10Casp-6a cDNA (**Fig. 2-**

6C). The proCasp-6b primers amplified the expected 325 bp DNA from proCasp-6b-transfected cells. Co-transfection of proCasp-6b and p20p10Casp-6a cDNAs did not alter the levels of proCasp-6b mRNA. Reduced mRNA levels were observed in vector and non-transfected cells. However, in p20p10Casp-6a-transfected cells, there was an increased level of proCasp-6b PCR product. These results indicated that proCasp-6b and p20p10Casp-6a expression affect endogenously expressed CASP6αβ or CASP6β mRNA levels. More work will be required for a thorough understanding of this phenomenon. However, the detection of CASP6α and CASP6β mRNAs in the p20p10Casp-6a and proCasp-6b co-transfected cells indicated that the reduction of protein levels observed in Fig. 2-5B was not the result of attenuated mRNA. These results suggest that the decreased levels of p20p10Casp-6a and proCasp-6b proteins in co-transfected cells may be due to either increased protein degradation or decreased translation.

We have previously demonstrated that the p20 and p10 subunits of Casp-6a are degraded by the proteasome (Tounekti et al., 2004). To determine if proteasomal degradation is responsible for the reduction of p20p10Casp-6a and proCasp-6b protein levels in the co-transfected HCT116 cells, we compared protein extracts from transfected cells treated with or without the irreversible proteasomal inhibitor, epoxomicin (**Fig. 2-6D).** The p10 subunit became detectable in the epoxomicin-treated p20p10Casp-6a-transfected HCT116 cells and the p20 subunit increased in both the p20p10Casp-6a and co-transfected cells. However, the levels in co-transfected cells were still lower than in the p20p10Casp-6a-transfected

cells. Furthermore, the p20p10Casp-6a protein did not increase in epoxomicin-treated cells. These results indicated that the p20p10Casp-6a was still converted into the p20 subunit when co-expressed with proCasp-6b and that there was proteasomal degradation of p20 and p10 subunits in these cells. However, the lower levels of p20p10Casp-6a in co-transfected cells also could indicate that the co-transfection inhibited the translation of these proteins or increased degradation of p20p10Casp-6a and its proteolytic subunits through a proteasomal-independent pathway.

Figure 2-6.

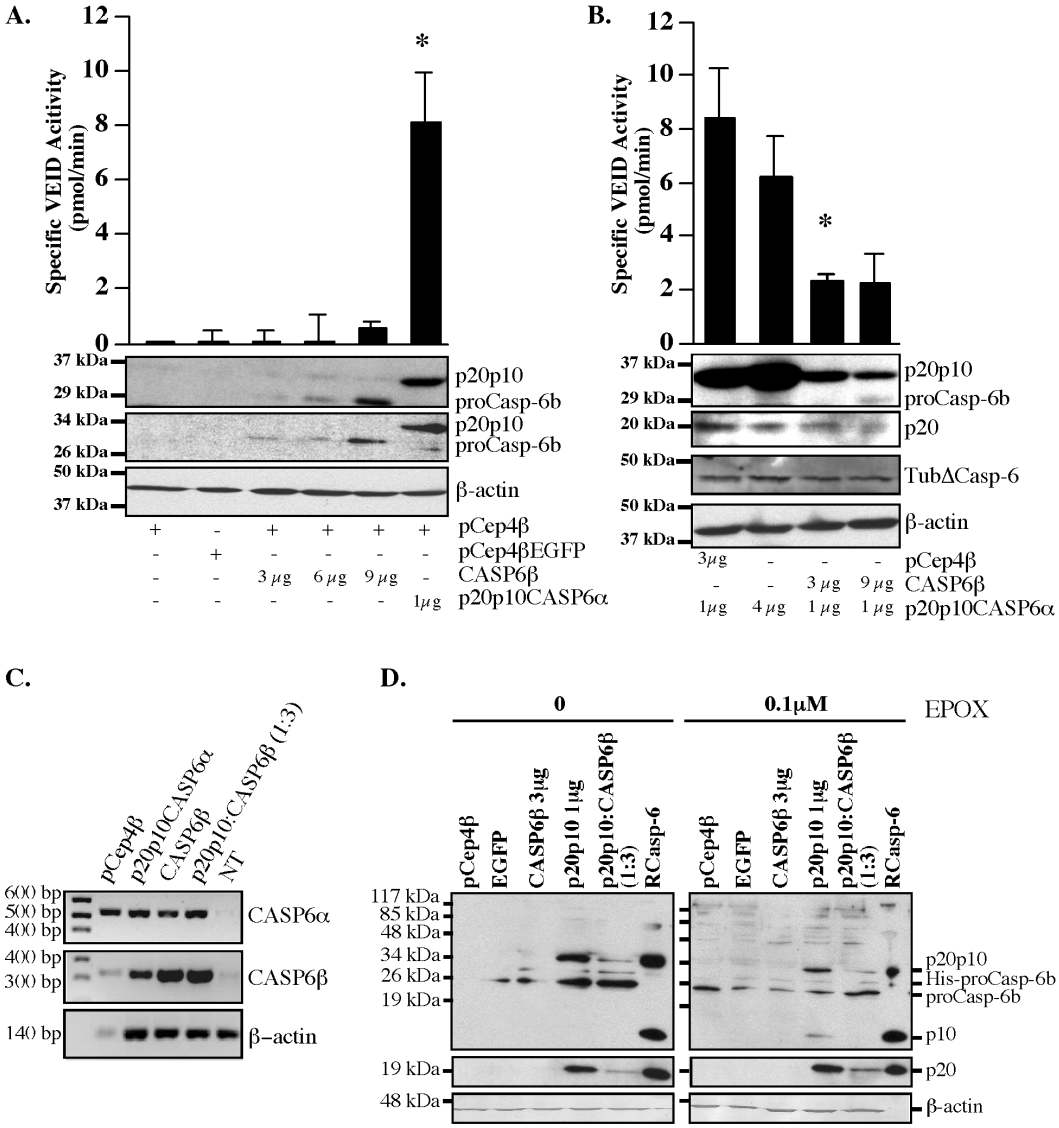


Figure 2-6. ProCasp-6b inhibits p20p10Casp-6a-mediated processing and activation in HCT116 cells. A. VEIDase activity and western blot analysis with anti-p10Casp6 and anti-His antibodies of protein extracts from HCT116 cells transfected with vector alone (pCep4β), pCep4βEGFP, pCep4βproCasp-6b or pCep4βp20p10Casp-6a. **B.** VEIDase activity and western blot analysis with anti-p10Casp6 and anti-p20 neoepitope antiserum of protein extracts from HCT116

cells transfected with vector alone (pCep4β), pCep4βp20p10, pCep4βp20p10:pCep4βproCasp-6b (1:3 cDNA ratio) or pCep4βp20p10:pCep4βproCasp-6b (1:9 cDNA ratio)-transfected HCT116 cells. **C.** Ethidium bromide stained agarose gel of RT-PCR products from HCT116-transfected cells. **D.** Western blot analysis with anti-p10Casp6 antibody (top panels), anti-neoepitope p20 antiserum (middle panels), and anti-β-actin antibodies of proteins extracted from transfected HCT116 cells treated in the absence or presence of epoxomicin (EPOX).

Co-immunoprecipitation of proCasp-6a and proCasp-6b. To investigate the underlying mechanism of proCasp-6b inhibition of proCasp-6a activation, we performed an immunoprecipitation with pre-immune serum and α-Casp6a from a mixture of purified recombinant proCasp-6a and proCasp-6b and western blotted with α-p10Casp6. The western blot revealed that α-Casp6a antiserum co-immunoprecipitated proCasp-6b with proCasp-6a and Casp-6a lacking the pro-domain **(Fig. 2-7)**. Similarly, α-Casp6a antiserum co-imunoprecipitated the catalytically inactive proCasp-6C163A and proCasp-6b. In contrast, neither proCasp-6a nor proCasp-6b immunoprecipitated with the pre-immune serum.

Figure 2-7.

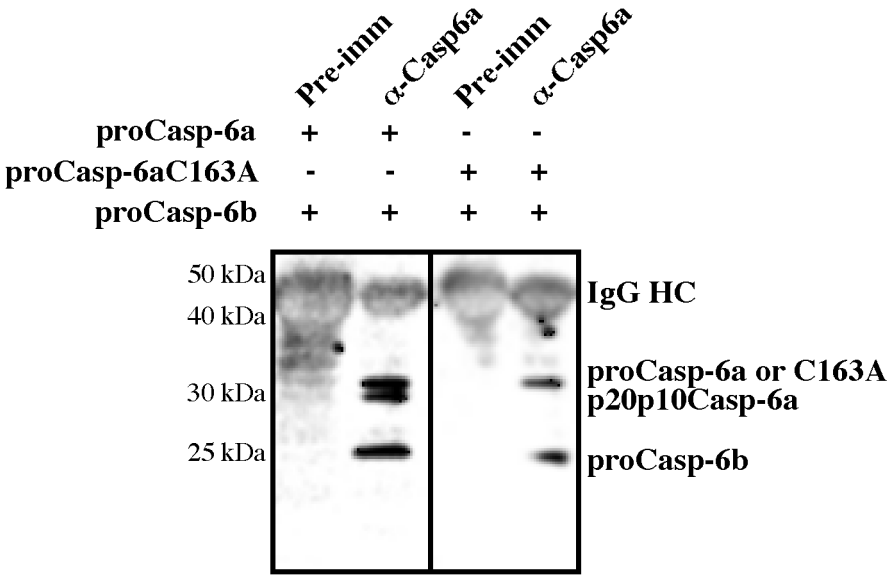


Figure 2-7. Co-immunoprecipitation of purified recombinant proCasp-6a and proCasp-6b. Western blot analysis with α-p10Casp6 of a mixture of purified recombinant proCasp-6a and proCasp-6b immunoprecipitated with α-Casp6 or pre-immune antisera.

2.5 Discussion

Understanding the regulation of Casp-6a activity could provide a viable treatment to inhibit and possibly reverse neuronal degeneration in several pathological conditions. Indeed, Casp-6a is highly activated in mild cognitive impairment and at all stages of sporadic AD and in familial AD (Albrecht et al., 2009, et al., 2007; Guo et al., 2004). Furthermore, Casp-6 activity may be involved in the clinical manifestation of Huntington and Parkinson diseases (Giaime et al., 2010; Graham et al., 2006). In addition, the role of Casp-6 in neurodegeneration rather than cell death has been recently well documented (Guo et al., 2004; Klaiman et al., 2009; Nikolaev et al., 2009). Therefore, finding a role for proCasp-6b as a potential inhibitor of proCasp-6a activation provides a novel therapeutic target against neurodegeneration.

We investigated the proCasp-6b protein product of the alternatively spliced mRNA of the CASP6 gene because several alternatively spliced isoforms of caspases regulate their cognate caspase activity. We confirmed that the proCasp-6b is expressed as mRNA and protein in several cell lines. The existence of the proCasp-6b mRNA has been previously demonstrated in Jurkat cells (Fernandes-Alnemri et al., 1995a); our results further indicated that proCasp-6b expression is ubiquitous in different cell types including human colon carcinoma, breast carcinoma, neuroblastoma cells, primary human neurons, and human adult normal brains.

ProCasp-6b did not have significant catalytic activity despite retaining its catalytic site. We cloned and purified the recombinant proCasp-6b and investigated if it demonstrated either self-processing or VEIDase activity as does bacterially expressed and purified recombinant proCasp-6a. We did not find any evidence of proCasp-6b self-processing by western blotting and the proCasp-6b did not display any processing activity on the Ac-VEID-AFC substrate that is well cleaved by Casp-6a. However, we did observe the production of a 26 kDa protein when proCasp-6b was added to proCasp-6aC163A. The processing occurred at an unexpected site within the p20 subunit domain. Since this processing occurred with the catalytically inactive proCasp-6aC163A, the activity must arise from the proCasp-6b catalytic site. Which of the two proteins is cleaved is not clear at this time but this result hints at the ability of proCasp-6b to carry minor catalytic activity in some situations.

ProCasp-6b cannot inhibit Casp-6a once it is activated. *In vitro*, the addition of proCasp-6b to recombinant purified active Casp-6a did not alter its VEIDase activity. However, the proCasp-6b inhibited the self-activation of proCasp-6a. This was deduced from the fact that proCasp-6b inhibited RCasp-1-mediated activation of IVT proCasp-6a but had no effect on the RCasp-1-mediated processing of the catalytically incompetent proCasp-6aC163A protein. This type of inhibition is consistent with the mechanism by which other alternatively spliced forms of caspases inhibit their cognate caspases (Alnemri et al., 1995; Fernandes-Alnemri et al., 1995a; Himeji et al., 2002; Ito et al., 2000).

ProCasp-6b also inhibited Casp-6a activation in transfected human neurons and in HCT116 cells. We confirmed inhibition in serum-deprived human neurons, a condition known to induce RCasp-1-mediated activation of Casp-6a (Guo et al., 2006). However, because of the inherent difficulty of transfecting human neurons with viral vectors or liposome-based products, we were limited to performing single cell analyses on proCasp-6b Genegun-transfected neurons. Therefore, to confirm the ability of proCasp-6b to inhibit proCasp-6a activation in cells, we took advantage of a recently developed system where the pro-domain lacking p20p10Casp-6a induced self-processing and VEIDase activity when transfected in mammalian cells (Klaiman et al., 2009). In HCT116 cells, proCasp-6b did not demonstrate any VEIDase catalytic activity but p20p10Casp-6a did. In cells co-transfected with proCasp-6b and p20p10Casp-6a cDNAs, the p20p10Casp-6a-mediated VEIDase activity was significantly inhibited.

We could exclude two potential mechanisms to explain proCasp-6b inhibitory activity. First, proCasp-6b did not inhibit RCasp-1 activity **(Fig. 2-8A)**. Second, since proCasp-6b was not processed by either RCasp-1 or activated Casp-6a, we can exclude the possibility that the truncated p20 subunit of proCasp-6b might have formed a complex with Casp-6a subunits and resulted in the dominant negative inhibition of the active enzyme **(Fig. 2-8B)**. Our results showed that proCasp-6b inhibited the self-processing of proCasp-6a after the removal of the pro-domain **(Fig. 2-8C)**. A likely mechanism by which proCasp-6b inhibited RCasp-1-activated Casp-6a self-processing is via an association between the p20p10Casp-6a and proCasp-6b. Both the proenzyme and the active form of

Casp-6a exist naturally as symmetrical dimers, which form through an interaction of the p10 subunits. Since the p10 region of proCasp-6b is intact, it is possible that it interacts with proCasp-6a to form an asymmetrical dimer. Indeed, a direct interaction of proCasp-6a and proCasp-6b was observed by co-immunoprecipitation. This interaction possibly disrupts the catalytic activity of Casp-6a.

Figure 2-8.

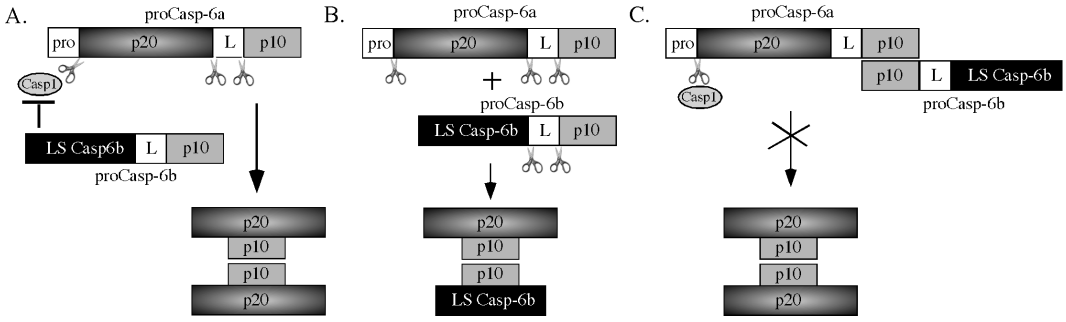


Figure 2-8. Schematic diagram of three possible mechanisms for proCasp-6b inhibition of proCasp-6a activation. A. Mechanism by which proCasp-6b inhibits the active Casp-1. Pro: pro-domain, L: linker, LSCasp-6b: proCasp-6b large subunit. **B.** Dominant negative inhibition of proCasp-6a by proCasp-6b because the large subunit of proCasp-6b could replace one p20 subunit of Casp-6a in the tetrameric enzyme. **C.** ProCasp-6b inhibition of proCasp-6a self-processing at D179 and D193.

This model predicts that proCasp-6b should inhibit proCasp-6a activation at equimolar concentrations. Accordingly, we observed that equimolar concentrations of proCasp-6b to proCasp-6a resulted in 50-90% inhibition of RCasp-1-activation of proCasp-6a *in vitro*. In cell lines and in human primary neurons, the levels of proCasp-6a and proCasp-6b were mostly equivalent, suggesting that transient activation of proCasp-6a could be kept in check by proCasp-6b. In the adult human brain tissue, the levels of proCasp-6b largely exceeded those of proCasp-6a suggesting that proCasp-6b could act as a mechanism to prevent Casp-6a activation *in vivo*. This may be particularly important given the role of active Casp-6 in neurodegeneration (Nikolaev et al., 2009).

The role of proCasp-6b as an inhibitor of proCasp-6a activation could be very important physiologically. Given the recent finding that active Casp-6a induces neurodegeneration and not cell death (Nikolaev et al., 2009), regulation of proCasp-6b expression could prevent Casp-6a-mediated damage to neurons. Not much is known about the regulation of proCasp-6a activity. The inhibitor of apoptosis proteins (IAPs) do not inhibit Casp-6a despite being strong inhibitors of the other two effector caspases, Casp-3 and Casp-7 (Vaux et al., 2005). Estrogen induces an inhibitor of active Casp-6a in human primary neurons (Zhang et al., 2001). Our present findings thus provide a novel type of regulation of Casp-6a activity.

2.6 Materials and Methods

DNA constructs and siRNA. Control siRNA (sc-3007) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and ON-TARGETplus SMARTpool human CASP6 siRNA (L-004406-00) was purchased from Dharmacon (Lafayette, CO). Custom designed ON-TARGETplus siRNA sequence to the exon1/exon5 junction of CASP6β was synthesized by Dharmacon to recognize the target sequence 5' CACCCGGCAGUGUCAACUGUUUU 3'.

Reagents and antibodies. Purified recombinant active Casp1 (RCasp1), Ac-VEID-AFC and Ac-YVAD-AFC were purchased from BioMol (Philadelphia, PA). Anti-Casp6a polyclonal antiserum from Upstate (α-Casp6a$^{16-32}$) and NeoMarkers Ab-4 (Fremont, CA) recognize the N-terminal region of human Casp-6, a region that is lacking in proCasp-6b. The α-p10Casp6 (Pharmingen) monoclonal antibody (α-Casp6$^{271-285}$) recognizes the p10 subunit and full length of proCasp-6a or proCasp-6b. The α-p20Casp6 10630 neoepitope antiserum to the cleaved p20 subunit of active Casp-6a (amino acid 179 of human proCasp6a) was made in our laboratoy (Guo et al., 2004). The monoclonal anti-β-actin antibody (Sigma, St Louis, MO) was raised against the N-terminal 16 amino acids of the protein. The α-His antibody is a monoclonal from Novagen (VWR, Mississauga, ON).

Cell lines and primary human neuronal culture. The HCT116, MCF7, MC-IXC and BE(2)M17 cell lines were obtained from ATCC (Manassas, VA). The HEK293T cell line was obtained from colleagues at the Lady Davis Institute.

Primary human neurons were cultured from fetal brains obtained under ethical approval from McGill University's Institutional Review Board as described previously (LeBlanc et al., 1995).

RT-PCR analysis. Total RNA was isolated from HCT116, HEK293T, MCF7, MC-IXC and BE(2)M17 cell lines and from primary human neurons with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) to determine endogenous levels of CASP6α and CASP6β. RNA was extracted 24 hrs post-transfection from MCF7 cells transfected with CASP6α and CASP6β expressing constructs, and ran as markers. The first strand cDNA synthesis reaction (20 µl) was performed with 1 µg total RNA, 1 mM dNTP, 0.5 µl RNAsin (Roche, Indianapolis, IN, USA), 5 µM Oligo dT (Amersham, Mississauga, ON) and 40 units of avian myeloblastosis virus (AMV) reverse transcriptase (Roche Diagnostics, Laval, QC) for 10 min at 25°C, 60 min at 42°C and followed by 10 min at 70°C. The cDNA was used as template to amplify CASP6α and CASP6β, using the primers 5'-CGC GGA TCC ACC ATG AGC TCG GCC TCG-3' (primer 2 on Fig. 1A) and 5'-CGG AGG CTG CAG CCA CCT CAG TTA TG-3' (primer 2 on Fig. 1A). The PCR reactions (25 μl) were performed with 0.025 units/μl of FideliTaq DNA Polymerase (USB, Cleveland, Ohio, USA), 0.2 mM each dNTP, 1 μM each primer and 2 μl cDNA. The reactions were incubated for 5 min at 95°C, followed by 25 cycles of 30 s at 95°C, 1 min at 55°C, and 1 min at 68°C, followed by 5 min at 68°C. To amplify the p20p10 CASP6α product, PCR was conducted using forward primer 5' – CGG GGT ACC ATG GCC TTC TAT AAA AGA GAA ATG – 3' and primer 2. The β-actin amplification was performed

using forward primer 5' CTG GAA CGG TGA AGG TGACA 3' and reverse primer 5' AAG GGA CTT CCT GTA ACA ATG CA 3'. The products were analyzed on 1.5-2.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

CASP6 silencing. MCF7 cells were transfected with 40 nM scrambled or CASP6 siRNA (Dharmacon ThermoScientific) and 5 μl of Lipofectamine^{2000} reagent (Invitrogen, Carlsbad, CA). Cells were given a second treatment of 10 nM siRNA at 48 hrs, and harvested at 72 hr for total RNA and protein.

Enzymatic digestion. To confirm the identity of the PCR amplificed CASP6α and CASP6β bands, amplicons were digested with 50 units of *Nco*I enzyme (New England BioLabs, Pickering, ON), in a 25 μl reaction containing NEB buffer 3 for 2 hrs at 37°C. Digested products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

Protein extractions. Total proteins were extracted from cell cultures in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 mM Tris, pH 8.0) supplemented with protease inhibitors (0.1 μg/ml TLCK, 0.5 μg/ml leupeptin, 38 mg/ml AEBSF and 0.1 μg/ml pepstatin A). Frozen tissues of 9 non-cognitively impaired (NCI) brains were obtained from Dr. Catherine Bergeron (University of Toronto, ON). Tissues were homogenized mechanically with a Polytron Homogenizer in 5x radio-immunoprecipitation assay buffer (RIPA), containing 5% NP-40, 2.5% sodium deoxycholate, 0.5% SDS, 500 mM Tris, pH 8.0) supplemented with protease inhibitors (0.1 μg/ml TLCK, 0.5 μg/ml leupeptin, 38

mg/ml AEBSF and 0.1 μg/ml pepstatin A). Temporal, frontal and cerebellar cortices were analyzed. Protein extracts lysed in RIPA buffer were cleared by centrifugation at 13 000 g for 20 mins. For Casp-6a activity assays, transfected HCT116 cells were lysed in CHAPS buffer (50 mM HEPES, 0.1% CHAPS, 0.1 mM EDTA, 1mM DTT) containing the same set of protease inhibitors as above. Protein extracts were quantified with the BCA protein assay (ThermoScientific, Rockford, IL).

Cloning of CASP6β. The proCasp-6b cDNA was obtained by targeted PCR reactions from the pET23b Casp6a-His tagged expressing vector (kind gift from Dr. Guy Salvesen, Burnham Institute, LaJolla, CA). The oligonucleotides 5'-CTA ACC AGT AAG GCA ACC CC-3' and 5'-CAG TTG ACA CTG CCG GGT GCC CCC TGC GG-3' amplified the 5' end generating a 0.23 kb fragment, and 5'-CAC CCG GCA GTG TCA ACT GTT AGC CAC GCA G-3' and 5'-CCG GAA TTC GCA GCC GGA TCT CAG TGG-3' amplified the 3' end generating a 0.61 kb fragment. The two fragments were ligated and served as a template for a second PCR using the oligonucleotides 5'-CGC GGA TCC ATG AGC TCG GAA TCG-3' and 5'-CCG GAA TTC GCA GCC GGA TCT CAG TGG-3'. The PCR product was digested with *Bam*HI and *Xho*I, and cloned into the pET23b (Novagen, MA, WI, USA) prokaryotic expression vector. The proCasp-6a was sub-cloned into pCep4β eukaryotic and pIVEX prokaryotic vectors via the XhoI/NotI and SpeI/XhoI restriction sites, respectively.

Recombinant protein expression and purification. Catalytic mutant proCasp-

6aC163A (Guo et al., 2004) was expressed in BL21 (DE3) *E. Coli* (Novagen, MA, Wisconsin, USA) and purified as previously described (Denault02)**.** His-tagged proCasp-6b was expressed in the BL21 (DE3) *E. Coli* strain. Overnight starter culture was diluted 50x in 2L of 2x YT media supplemented with 100μg/ml ampicillin, and grown at 37°C until an OD_{600} of 0.6 was reached. Protein expression was induced with 500 μM IPTG (Fischer Scientific, Fair Lawn, NJ) for 4 hrs at 28°C. Cells were collected by centrifugation at 6000 g for 15 mins and lysed by sonication in resuspension buffer (50 mM Tris HCl pH 8, 300 mM NaCl), with 1 mg/ml lysozyme (Sigma Aldrich, St Louis, MO). The lysate was cleared by centrifugation at 26 000 g for 1 hr at 4°C. The supernatant was then loaded on a 3 mL Ni-Sepharose-6 Fast Flow column (GE Healthcare Bio-sciences, Uppsala, Sweden) pre-equilibrated in buffer (50 mM Tris HCl pH 8, 300 mM NaCl, 10 mM imidazole). Bound proteins were washed with 10x column volume of wash buffer (50 mM Tris HCl pH 8, 300 mM NaCl, 20 mM imidazole). The proteins were eluted with 50 mM Tris HCl pH 8, 300 mM NaCl, 500 mM imidazole elution buffer over 14 X 1mL fractions. Proteins from each fraction were separated on a 15% PAGE and immunoblotted with anti-p10Casp6 antibody or the gel was directly stained with 2.5 g/L Coomassie Brilliant Blue R250 in 45% methanol and 10% acetic acid. Fractions predominately containing proCasp-6b were pooled, dialyzed against caspase buffer (50 mM Tris HCl pH 8, 100 mM NaCl) in 10 kDa MW cut-off dialysis cassettes (Thermo Scientific, Rockland, IL) for 8 hrs at 4°C, and concentrated on a 15 kDa MW cut-off Amicon Ultra column. ProCasp-6b was detected as described above.

Effect of proCasp-6b on RCasp-6a and RCasp-1 activity. Recombinant Casp-6 activity was detected by measuring the release of AFC from Ac-VEID-AFC (Sigma, St-Louis, MO) at 37°C using a Bio-Rad Fluoromark fluorometer (Hercules, CA) at an excitation wavelength of 390 nM and an emission wavelength of 538 nM. Measurements were read every 2 mins for 1 hour and the amount of released moles of AFC was calculated from a standard curve of 0-2500 pmoles free AFC. The reactions were performed in a 50 µl total volume with Stennicke's buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% sucrose) and the following conditions: (1) for measurement of Casp-6 activity, 69 µM Ac-VEID-AFC, 46 nM RCasp-6a, and the indicated concentrations of proCasp-6b were used with the final total protein concentration adjusted to 200 ng/µl with BSA, (2) for measurement of RCasp-1 activity, 5 µM Ac-YVAD-AFC, 56 nM proCasp-6aC163A, 56 nM proCasp-6b, and 2.2 nM RCasp-1 were used as indicated. For the latter, proCasp-6aC163A was pre-incubated with proCasp-6b for 1 hr at 37°C prior to the addition of RCasp-1 and the substrate. The reaction mix was then boiled in Laemmli buffer and 50 μl was loaded on a 15% SDS-PAGE gel and analyzed by Western blotting with α-p10Casp6 antibodies and α-p20Casp6 antiserum.

In vitro translation (IVT) of proCasp-6a. The proCasp-6a cDNA was subcloned into the pIVEX vector and proCasp-6a translated in *E. Coli* with the Rapid Translation System (Roche Diagnostics Canada, Laval, QC).

Effect of proCasp-6b on caspase-mediated activation and processing of IVT proCasp-6a. For measurement of RCasp-6a and RCasp-1 activity, proCasp-6b and IVT proCasp-6a were mixed with Stennicke's buffer containing 5 μM Ac-VEID-AFC or Ac-YVAD-AFC, and 2.2 nM RCasp-1 added immediately prior to the start of the activity assay. The activity assays were performed as described above followed by Western blot analysis using anti-p10 and anti-p20 Casp6 antibodies.

Effect of proCasp-6b on the activation of p20p10Casp-6a in vivo. Human neurons. The expression of proCasp-6b from the pCep4β construct was verified by transfecting SK-N-SH cells (ATTC, Manassas, Vermont) with 3μg DNA/35 mm well using ExGen500 (Fermentas, Burlington, ON) according to the manufacturer's instructions. Proteins were extracted and western blotted against the anti-p10Casp6 antibody. Primary human neurons prepared as previously described (LeBlanc et al., 1995) were transfected with the Helios Gene Gun system (BioRad, Mississauga, ON) at a shooting pressure of 100 psi according to the manufacturer's protocol. Transfection cartridges were prepared with a 1:3 ratio of pCep4β or pCep4β-proCasp-6b DNA to pCep4β-EGFP, 4.2 mg of gold microcarrier beads in 0.1 ml 1 M calcium chloride and 0.1 ml 0.05 M spermidine, as described previously (Roucou et al., 2005). Forty-eight hrs post-transfection, the primary human neurons were serum-deprived for 18 hrs. Cells were stained with Hoechst 33342 and condensed chromatin EGFP-positive neurons counted as a percentage of all EGFP-positive neurons to give a % cell death. Neurons were counted blindly. ***HCT116 cells.*** HCT116 cells were plated overnight in a 6-well

plate at a density of 350,000 cells per well in McCoy's media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Fischer, Ottawa, ON, Canada). Transfections were performed on the following day using a DNA to polyethylenimine (PEI: Polysciences Inc., Warrington, PA, USA) at a ratio of 1:5. The transfection complex was prepared in 100 μL of serum-free media. Six hrs following transfection, complete media was replaced and cells were incubated for an additional 18 hrs to allow for expression. Cells were harvested in CHAPS buffer containing protease inhibitors. Caspase activity assays were performed using 10 μg of total protein lysate and 10 μM Ac-VEID-AFC. The caspase assay reaction mix was analyzed by western blotting using α-p10Casp6, α-p20Casp6, and anti-β-actin antibodies.

Effect of proteosomal inhibition on proCasp-6b protein stability. HCT116 cells were transfected as described above with 4 μg total cDNA and 20 μg of PEI. Epoxomicin was added at a final concentration of 0.1 μM immediately following transfection for 24 hrs.

Co-immunoprecipitation. Recombinant proteins were incubated for 4 hrs at 4°C with protein A-sepharose beads and Upstate α-Casp6a antibody at a dilution of 1:100 in 50 mM Tris pH 8.0, 100 mM NaCl. The beads were preblocked for 1 hr at 4°C with 1% BSA solution. Beads were vigorously washed 3x with the buffer and then resuspended in Laemmli SDS buffer. Samples were boiled and loaded on a SDS-PAGE gels and immunoblotted with the α-p10Casp6 antibody.

Statistical evaluations. Statistical evaluations of the data were performed by ANOVA and post-hoc analysis as indicated in the legend of each figure.

CHAPTER 3. GENERAL DISCUSSIONS AND CONCLUSION

3.1 GENERAL DISCUSSIONS

Primarily studied in this thesis under the context of AD, Casp-6 has been found in NFTs, SP and NPTs, pathological hallmarks of AD (Albrecht et al., 2009, et al., 2007; Guo et al., 2004). Furthermore, the detection of active Casp-6 in the ERC, the earliest area of the brain to be affected in humans (Albrecht et al., 2007), implicates this protease as a major instigator to the disease onset and progression. Since IAPs, well-known caspase inhibitors, do not have any effect on Casp-6 (Vaux et al., 2005), it is of vital importance to characterize the role of the splice variant, proCasp-6b, as a regulator of Casp-6a enzymatic activity. This is a common mechanism in the regulation of other caspases (discussed in section *1.8.3*). This study presents the first evidence that the splice variant, proCasp-6b, inhibits the self-processing of full-length proCasp-6a protein, likely in a dominant-negative fashion through dimerization at the dimer interface.

3.1.1 ProCasp-6b inhibits the activation of proCasp-6a

We report to our knowledge the first evidence that the alternatively spliced variant, proCasp-6b, directly inhibits the activation of proCasp-6a. This finding is novel since there are no reported human inhibitors of Casp-6. ProCasp-6b acts as a dominant-negative inhibitor of proCasp-6a. A dominant-negative is defined as a mutation/alteration whose gene product adversely affects the wild-type protein within the same cell, usually through an interaction with the same elements, but block/restrict some aspect of function (Michaels et al., 1996).

The interaction between the Casp-6 isoforms is distinct since other spliced caspase isoforms often require an interaction with adaptor molecules, particularly common in initiator caspases where a recruitment complex is needed for activation. For example, a shorter splice variant of Casp-2, Casp-2s, inhibits the apoptotic function of wildtype Casp-2 by blocking the interaction with adaptor protein RAIDD, for recruitment to form the PIDDosome (Wang et al., 1994). Similarly, the Casp-8L variant retains the two DED motifs in the prodomain and inhibits Casp-8 auto-activation by competitively binding to FADD (Himeji et al., 2002). Both splice variants, Casp-2s and Casp-8L, interact with their particular adaptor protein through their mutual CARD and DED domains, respectively. Moreover, the Casp-9b splice variant acts as a DN decoy and blocks the interaction between wildtype Casp-9 and adaptor Apaf-1, which is critical for Casp-9 activation (Srinivasula et al., 1999). However, splice variants of effectors Casp-3 and Casp-7, called Casp-3s and Casp-7b, are shown to counteract the apoptotic activity of their wildtype counterparts, although the mechanism is unknown it could be through an interaction (Fernandes-Alnemri et al., 1995b; Huang et al., 2001). Lastly, an AS variant Casp-1ε, which exist only as the small p10 unit, interacts with the large p20 subunit of wildtype Casp-1, impairing assembly into a fully active heterotetrameric enzyme (Alnemri et al., 1995).

In our study, we also found that proCasp-6b strongly inhibits proCasp-6a at an equal molar ratio *in vitro*. Several *in vitro* binding assays with caspase splice variants show a wide range in efficacy. For instance, Casp-8L was reported to inhibit Casp-8 activation even at one eighth of the molar amount of the parent

enzyme (Horiuchi et al., 2000). The differences in inhibitory potency and efficacy of the spliced inhibitor may reflect a distinction between caspases that require scaffold-mediated recruitment to activate, from those that activate by direct cleavage. In addition, the intracellular ratio required for complete inhibition of its wildtype caspase may be an important intrinsic factor determining the susceptibility of the cell to caspase activation.

The greatest exception is the ability of proCasp-6b to act as a DN inhibitor even though it retains its catalytic site. This is unique amongst the caspase family since no splice variants contain the conserved pentapeptide active sequence (Fernandes-Alnemri et al., 1995b; Horiuchi et al., 2000; Seol et al., 1999), with the exception of the Casp-2s isoform (Wang et al., 1994). Structure-altering splicing events in which entire domains are removed or relocated occur more frequently than expected by random chance (Resch et al., 2004), therefore, the catalytic site within the truncated p20 domain might be retained to maintain a structural feature (ie. beta sheet or helical fold) within the central structure of proCasp-6b. However, at this time we could not detect any Casp-6 enzymatic activity, although the catalytic site could still likely represent an additional function of proCasp-6b, which may need further characterization. Interestingly, we observed a cleavage product by western blotting that could only be due to activity of proCasp-6b since it is the only catalytically active enzyme present (**Fig. 2-4,** *lanes 4 & 8*). Furthermore, considering the strong sequence similarity and homology between Casp-6 and Casp-3, -7 and -2, there may be potential cross-talk for other enzymatic activities. The lack of the exon 2, 3 and 4 cassette in proCasp-6b may

cause structural changes to the protein, potentially to the critical residues of the substrate-binding site. Thus, these changes could disrupt the specificity for preferred residues, and proCasp-6b may instead cleave other caspase substrates than VEID with better affinity.

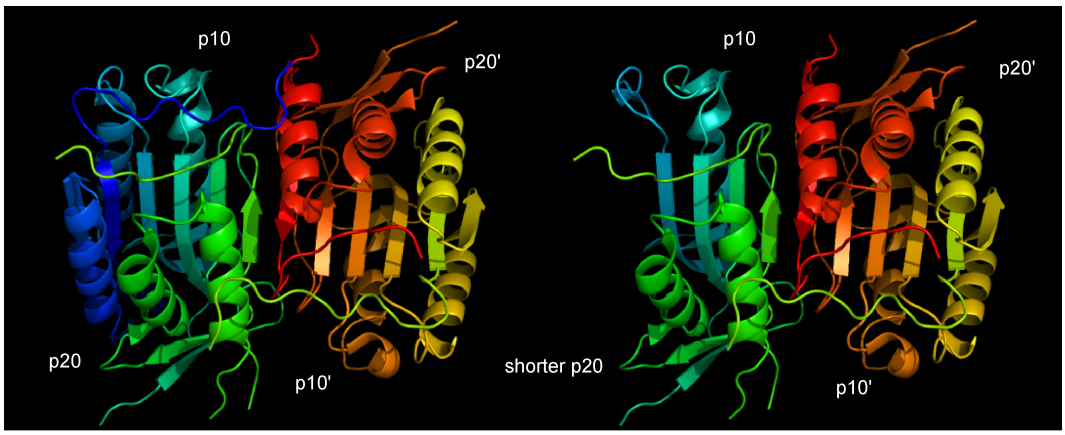
The most prominent similarity observed in all spliced variant caspase regulators like proCasp-6b, is the inhibition of activation (or processing) of the wildtype isoform, and not inhibition of activity of the active enzyme. Simlarly, we observed that proCasp-6b inhibits proCasp-6a activation by 50-90% *in vitro* and 75% in HCT116 cells. Another commonality amongst the other reported splice variants is the tendency for the shorter isoform to play the antagonistic role compared to the longer wildtype enzyme. With the exception of Casp-8L, all other isoforms were spliced in a manner to produce a truncated protein. This is potentially an evolutionarily conserved feature since exon skipping is a common AS mechanism (Black et al., 2003). Lee *et al*, reports that AS events tend to remove or insert whole units of protein that correlate to an entire domain (Lee et al., 2005). Furthermore, another study shows that splicing events favour the deletion or insertion of complete protein domains more frequently than expected by chance, than splicing events resulting in a disruption of domains or structural motifs (Kriventseva et al., 2003). Overall, these results suggest that AS has been under some selection pressure to avoid structurally unsound changes that are inconsistent with protein domain structure. This may explain why AS tends to generate a majority of inactive and consequently shorter caspase proteins that lack the entire large subunit containing the catalytic site.

The work in this thesis verifies endogenous expression of both Casp-6 isoforms and thus proCasp-6b could potentially be upregulated or promoted under certain environments to therapeutically obstruct Casp-6a mediated neurodegeneration. As proCasp-6b is already naturally present, modulating the expression would avoid any pleiotropic effects of introducing artificial proteins into the CNS. Furthermore, an option would be to specifically target and degrade CASP6α mRNA by a RNA interference (RNAi)-mediated modality. The key challenge would be transporting these RNAi treatments across the blood-brain barrier (BBB), however several new reports have demonstrated success in novel small interfering RNA/ short hairpin RNA (siRNA)/(shRNA) delivery methods. Kumar and coworkers reported nicotinic acetylcholine receptor-mediated transcytosis of siRNA across the BBB, by strategically synthesizing a rabies virus glycoprotein to enter neuronal cells and spread through the brain (Kumar et al., 2007a). Furthermore, evidence of shRNA delivery coupled with drug-targeting technology was reported in mice with intra-cranial brain cancer (Pardridge et al., 2007). Given the recent success with crossing the BBB and the efficacious delivery of targeted drugs or mimetics, there is great promise in the future for potential remediation of Casp-6a mediated neurodegeneration and disease-modifying therapeutics.

3.1.2 ProCasp-6b interacts directly with proCasp-6a, likely by dimerization

We suggest a unique mechanism of inhibition between the full-length isoforms by direct interaction through asymmetric dimerization (**Fig. 3-1,** *right*). As previously

mentioned, strong hydrophobic interactions between residues of the small subunits is crucial for homodimer stability of effector caspase zymogens (**Fig. 3-1,** *left*) (Boatright et al., 2003). Hence, we speculate that hydrophobic interactions occur at the heterodimer interface between the conserved small p10 subunits of proCasp-6a and proCasp-6b. We conclude this finding based on (1) evidence that proCasp-6b remains intact and unprocessed by an exo-caspase or itself, and (2) co-immunoprecipitation of both isoforms, which suggest a direct interaction. In addition, as described in Chapter 1, conformation changes are crucial for proper active site formation and proteolytic activity of effector caspases. Physiologically, we suggest the possibility of *in vivo* heterodimerization of endogenous proCasp-6a and proCasp-6b.



Caspase-6a homodimer Caspase-6a/Caspase-6b heterodimer

Figure 3-1. Helical representation of effector caspase dimerization. Two Casp-6a homodimers interact through their p10 subunits (*left*). One molecule of Casp-6a and one molecule of Casp-6b dimerize at the p10 dimer interface to form an asymmetric heterodimer (*right*). Images were constructed on the Pymol program with crystal structure of Casp-6 PDB# 3NR2 (Wang et al., 2010)**.**

Structurally, proCasp-6b hinders the ability for remodeling and processing of proCasp-6a into an active enzymatic conformation. We hypothesize that the binding of proCasp-6b at the dimer interface with proCasp-6a interferes with proper loop bundle formation critical for self-activation. We propose that this is the DN mechanism of function of proCasp-6b.

Our model of dimerization is supported by reports that proCasp-3 and proCasp-7 could potentially heterodimerize (Fernandes-Alnemri et al., 1995b), a convincing argument due to 53% sequence identity, high structural similarity and 100% identity of peripheral residues with side chains facing the central dimer cavity (Chai et al., 2001a; Hardy et al., 2004). Given the fact that the protein sequence of proCasp-6b is almost identical to that of proCasp-6a indicates that this interaction is physiologically feasible. Our findings provide further evidence for a functional role of proCasp-6b in the regulation of proCasp-6a activation. Furthermore, the collective results from this study allowed us to eliminate several models of inhibition and provide support for the asymmetric dimerization model that remains. More detailed biochemical work must be performed to fully establish that this interaction occurs at the dimer interface.

According to our suggested mechanism, this novel interaction could be applicable for other caspase splice variants. In particular, there may be a shared DN mechanism for the shorter AS isoforms of Casp-3 and -7, which have not been fully investigated. Furthermore, knowledge of this regulation on proenzymes could be extrapolated for drug-treatment purposes. Often times, caspases have

been found to be upregulated but not activated during early stages of many neurological diseases and cancers. This could present a novel therapeutic window, during which an AS variant could be a treatment modality to prevent the activation of the wildtype caspase prior to damaging effects by the active enzyme.

3.1.3 Differential expression of Casp-6 mRNA and protein isoforms across samples

The findings from the work described in this thesis indicate that splice variant proCasp-6b is naturally co-expressed with wildtype proCasp-6a. However, we observed that the mRNA and protein expression levels differ across several human cell lines, primary human neurons and adult brain regions. This phenomenon is supported by the observation of two mRNAs of CASP*6* in human B-lymphocytes and Jurkat T-lymphocytes by Northern blot analysis. The expression levels of the two mRNAs fluctuate between the samples tested, but confirm the presence of CASP6α and CASP6β mRNAs (Fernandes-Alnemri et al., 1995a). Similarly, there is co-expression amongst wildtype Casp-1, -2, -3, -6, -7, -8, -9 and -10 and their associated splice variants, although expression is to varying relative levels. Furthermore, there are tissue-specific differences in expression levels of the wildtype and the AS variants, where abundance in particular types of tissues or localizations may be inherent features of susceptibility and tighter stringency required for regulation (Alnemri et al., 1995; Fernandes-Alnemri et al., 1994, et al., 1995a, et al., 1995b; Himeji et al., 2002; Seol et al., 1999; Wang et al., 2001; Wang et al., 1994). Interestingly, the mammalian brain is reported to be the tissue with the most AS and diversity,

likely due to the plethora of different cell types that make up the entire neuronal population (Licatalosi et al., 2010).

There are additional modes of regulating gene expression. Similarly to Casp-9s, proCasp-6b contains a longer 3'UTR region than their wildtype counterparts (Fernandes-Alnemri et al., 1995a). It is suggested that the differences in 3'UTR regions, even between two closely related mRNAs, can influence mRNA stability, translation efficiency and localization of tissue-specific expression (Edwalds-Gilbert et al., 1997; Licatalosi et al., 2010). The authors report that polyadenyation at a promoter-proximal site, by a series of poly(A) signals within a 3'UTR region, influences the expression of several mRNAs (Edwalds-Gilbert et al., 1997). In particular, the brain as a non-proliferative tissue seems to regulate polyadenylation by generating larger than average 3' UTRs (Licatalosi et al., 2010). This could also potentially explain the lack of a significant knockdown effect of CASP6β mRNA and protein in the siRNA experiments, although an effect was observed with CASP6α. Furthermore, Lewis *et al* have proposed a novel mechanism termed regulated unproductive splicing and translation (RUST), based on AS and nonsense-mediated decay (NMD) for regulation of gene expression (Lewis et al., 2003). Essentially AS can regulate gene expression by generating spliced transcripts that are unproductive and degraded. They report that one-third of AS transcripts examined are potential targets for NMD. It is plausible that in tissues and cell lines where we observe low proCasp-6b expression, the expression of the CASP6β transcript is regulated and most of the transcript may be targeted for decay.

In addition, multiple methods are known for regulating AS. Traditionally, this includes transcriptional regulation and post-translational modifications such as phosphorylation, ubiquitination and nitrosylation. Several factors such as environment, auxiliary splicing factors and gene modifications can affect alternative splicing of the parent gene. More specifically, AS and the ratio of expressed isoforms can be manipulated by apoptotic stimulus, splicing enhancers, splicing silencers and post-translational modifications. For example, differential Bcl-x gene expression can produce proteins involved in both positive and negative regulation of apoptosis (Boise et al., 1993). Several papers have reported differential isoform expression under various apoptotic stimuli.

Furthermore, AS is also implicated in disease, where a large number of genetic disorders stem from aberrant splicing. It has been reported that there is a global reduction in AS in cancerous cells compared to healthy ones. In addition, there seems to be an increase in intron retention and concomitant reduction in exon skipping than in normal cells (Kim et al., 2008). More specifically, reversible phosphorylation has been recently implicated as an AS regulator (Stamm et al., 2008). Recently, two groups published findings of phosphorylation mediating the functions of splicing enhancing and silencing proteins in determining Casp-9 isoform expression. Within a context of cancer, Casp-9, a pro-apoptotic protein is generated by the inclusion of exons 3, 4, 5 and 6, which can be promoted by binding of splice enhancer SRp30a (Shultz et al., 2010). A parallel study showed that phosphorylation at serine residue 52 is the critical modulator of the Casp-

9/Casp-9b ratio. Heterogeneous nuclear ribonucleoprotein-L (hnRNP L) binds to an exonic splicing silencer (ESS), which promotes the exclusion of the four-exon cassette, to generate the short anti-apoptotic Casp-9b (Goehe et al., 2010). Dysregulation of AS in Casp-9 is implicated in non-small cell lung carcinoma, with an increase of the Casp-9b isoform promoting pro-oncogenesis and cancer progression (Shultz et al., 2010). Furthermore, specific splicing proteins can enhance or interfere with snRNP binding and ultimately affect the 5' and 3' splice site recognition. Eperon *et al* reports that SF/ASF, an essential SR-protein splicing factor promotes a dose-dependent shift of U1snRNP binding to downstream 5' splice sites. Meanwhile hnRNP A1 shifts the binding to upstream splice sites, and globally down-regulates the binding of U1snRNP to both splice sites (Eperon et al., 2000). Further research must be carried out to identify the putative enhancing and silencing features related to Casp-6 splicing. Targeting analogous splice altering sites for generation of particular Casp-6 isoforms could provide a therapeutic application to alter the ratio of isoforms by promoting the abundance of Casp-6b.

Furthermore, specific caspases are implicated in certain cancers and diseases and in particular the abundance of one isoform over another is often a predictor of clinical outcome or prognosis. For example, the overexpression of splice variant Casp-3s is associated with chemoresistance and a poor outcome to neoadjuvant chemotherapy (Vegran et al., 2006). Similarly, the increase in apoptosis of human peripheral blood lymphocytes in individuals with systemic lupus erythematosus was in part, a result of decreased Casp-8L expression (Horiuchi et al., 2000).

Conserved allosteric sites in inflammatory and apoptotic caspase zymogens have recently been a new facet of caspase regulation being explored (Scheer et al., 2006). For example, binding of small molecules to the allosteric Casp-7 site abrogates the critical translocation of the L2' loop to stabilize the active enzyme conformation. Binding of the allosteric site pins the L2' loop, which locks the enzyme in a catalytically inactive state (Hardy et al., 2004). The deadly nature of caspases has necessitated these complex levels of regulation. More recently, small molecules have been synthesized to trap proCasp-3 and -6 in an active state (Wolan et al., 2009). The converse of this model could be exploited for trapping Casp-6a in an inactive state. Allosteric regulation may be an additional mechanism to ensure that activity occurs only when required.

3.2 CONCLUSION

Globally, the work of this thesis highlights the importance of elucidating and characterizing the splice variants of the canonical caspases. The fact that several alternatively spliced isoforms of other caspases act in a DN manner strongly implies a conserved role in regulating these potent caspases. The primary reason for conducting the work described in this thesis is two-fold; the increasingly detrimental role of Casp-6 activation and activity in biological models and the lack of a suitable and potent inhibitor or regulator of this particular effector caspase. We show that the expression of an alternatively spliced gene variant named proCasp-6b can antagonize wild-type proCasp-6a activation.

Implications for the findings in this thesis could translate to help as a treatment modality using endogenous proCasp-6b to alleviate Casp-6a-mediated neurodegeneration in AD. Furthermore, biochemically we have provided more insight into the interaction between effector dimers, in agreement with other groups that have postulated heterodimeric interactions. We suggest that these proCasp-6a and proCasp-6b interact at the dimer interface to form an asymmetric dimer as a modulatory interaction mechanism. Pending a detailed investigation into regulatory features (ie SR and hnRNP proteins), this work could also provide an approach to dysregulate isoform expression for a therapeutic advantage. Collectively, this inhibitor could be exploited as a clinical modality for the treatment of Casp-6 mediated disorders like AD.

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