2008

RECIPROCAL REGULATION OF PAR-4 AND CASPASE-8 IN THE TRAIL SIGNALING PATHWAY

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ABSTRACT OF DISSERTATION

Padhma Ranganathan

The Graduate School
University of Kentucky
2008
RECIPROCAL REGULATION OF PAR-4 AND CASPASE-8 IN THE TRAIL SIGNALING PATHWAY

Abstract of Dissertation

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Graduate School at the University of Kentucky

By
Padhma Ranganathan

Director: Dr. Vivek M. Rangnekar, Professor, Department of Radiation Medicine University of Kentucky, Lexington, Kentucky
2008
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Abstract of Dissertation

Reciprocal Regulation of Par-4 and Caspase-8 in the TRAIL signaling pathway

Par-4 is a pro-apoptotic tumor suppressor that is mutated, suppressed or inactivated in cancer. Par-4 exploits components of the extrinsic pathway to cause apoptosis selectively of cancer cells. This study identified Par-4 as an essential component of the apoptotic pathway induced by TRAIL, which selectively targets cancer cells. RNA interference-mediated knockdown of Par-4 rendered cancer cells unresponsive to TRAIL-induced apoptosis. Cells with knocked-down levels of Par-4 were deficient in the activation of the apoptosis-initiator caspase-8 and the apoptosis-effector caspase-3 in response to TRAIL. Par-4 was identified as a critical mediator of membrane translocation of caspase-8 and the adapter protein FADD. Surprisingly, Par-4 was also found to interact with caspase 8 in untreated cells, and was cleaved at the N-terminus at aspartic acid residue 123 in response to TRAIL. This, along with another cleavage by caspase-9 effectively generated a fragment containing the functional module of Par-4, the SAC domain, which is sufficient for apoptosis of cancer cells. Moreover, TRAIL activated caspase-8 was also found to be involved in nuclear translocation of Par-4, a crucial step during apoptosis induction by Par-4. Together, our findings suggest that Par-4 is an essential downstream target of caspase-8 that is activated by TRAIL signaling and that, in turn, activates caspase-8 and the downstream apoptotic pathway in response to TRAIL.

Keywords: Par-4, Caspase-8, TRAIL, Caspase activation, Caspase cleavage

Padhma Ranganathan
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October 2008
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RECIPROCAL REGULATION OF PAR-4 AND CASPASE-8 IN THE TRAIL SIGNALING PATHWAY

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To my Parents, Mythili and Ranganathan
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Chapter One

Introduction

Apoptosis or programmed cell death is a physiologically essential, conserved, energy dependent phenomenon necessary for proper maintenance of homeostasis in all metazoans. Cell death is indispensable for processes such as organogenesis, cell lineage differentiation, disposal of infected, damaged, aged or non-functional cells, and immune function, to name a few. Among the different forms of cell death, apoptosis is a very complicated process that has evolved to maintain order. Another commonly observed mode of death is necrosis that occurs as a result of trauma where cell swelling, bursting and leakage of contents into the surrounding tissue leads to inflammation. Apoptosis, in contrast, does not incite tissue injury or an inflammatory response, and can be caused by both physiological and pathological signals.

Apoptosis – the process

A cell undergoing apoptosis bears certain defining morphological features, such as shrinkage of the cytoplasm and nucleus, membrane blebbing, preservation of organelle structure, and containment of cell contents within membrane bound apoptotic bodies. In particular, chromatin condensation due to oligonucleosomal DNA fragmentation, which results in margination of chromatin along the nuclear membrane, and ‘Apoptotic DNA laddering’, is considered a hallmark of apoptosis, although recent studies indicate not all cells exhibit such an internucleosomal degradation pattern (Zakeri et al., 1993). Moreover, phosphatidylserine, a phospholipid that is normally present on the inner leaflet of the plasma membrane, is flipped over and exposed on the surface of apoptotic cells thus leading to recognition by the macrophages and prompt tissue clearance. Underlying such visible alterations is the activation of a series of enzymes, the caspases (Cysteine dependent ASPartic acid specific proteASE), which
are synthesized as inactive zymogens that lie dormant until awakened by apoptotic signals.

A cell is programmed to destroy itself when conditions of abnormality are sensed, either internally or in the extra-cellular environment. The cell has, as part of its apoptotic machinery, a variety of sensors. For example, cell surface receptors, such as EGFR (Epidermal Growth Factor Receptor), IGFR (Insulin like Growth Factor Receptor), certain GPCR (G-Protein Coupled Receptor), Integrins and other cell attachment molecules that signal for survival upon perception of surroundings conducive to growth, while death receptors, such as Fas, TNFR (Tumor Necrosis Factor Receptor) and DR4/DR5 (Death Receptor4 or Death receptor 5), trigger apoptosis upon recognition by their cognate ligands. Intracellular sentries initiate apoptosis in response to insults, such as DNA damage, hypoxia, energy deficiency, ionic imbalance and oncogene activation (Hanahan and Weinberg, 2000).

Among the intracellular triggers, when the primary elements of cell transformation, namely, oncogene activation and DNA damage, fail to activate apoptosis due to inactivation or defects in the apoptotic pathway, a significant road block to tumorigenesis is overcome. Typically, the receipt of pro-transforming signals in normal cells triggers one of the two pathways of apoptosis: the extrinsic or intrinsic pathway. Along the extrinsic pathway, binding of the cytokines FasL, TNF-α (Tumor Necrosis Factor), or TRAIL (TNF Related Apoptosis Inducing Ligand) to their respective oligomerized receptors at the cell surface allows formation of a DISC (Death Inducing Signaling Complex). DISC consists chiefly of the receptor, an adaptor protein FADD (Fas Associated Death Domain) and pro-caspase-8, where caspase-8 is activated. The activated caspase-8 then directly cleaves and activates the effector caspases, caspase-3, -6, and -7, which are responsible for dismantling the cell and also generating the classical morphological changes observed during apoptosis (Figure 1.1).
Figure 1.1: Extrinsic and Intrinsic pathways of Apoptosis

- FasL, TNFα, TRAIL
- Fas, TNFR, DR4/5
- FADD
- Pro-caspase-8/10
- Caspase-8
- Caspases -3,6,7
- Apoptosis
- Bax, Bak
- Bcl-2, Bcl-xl
- Cytochrome-c
- Apoptosome
- Growth Factor deprivation, DNA Damage, Oxidative stress

Growth factor deprivation, DNA damage, and oxidative stress are the extrinsic and intrinsic pathways that lead to apoptosis. FasL, TNFα, and TRAIL trigger the extrinsic pathway, while Fas, TNFR, and DR4/5 activate the intrinsic pathway. FADD, FasL, TNFα, and TRAIL interact, leading to the activation of pro-caspase-8/10, which is then cleaved into caspase-8. This activated caspase-8 cleaves Bid, leading to the release of Bax and Bak from the mitochondrial membrane, allowing cytochrome-c release. Cytochrome-c then activates caspase-9, which further activates caspases -3, 6, and 7, leading to apoptosis.
Figure 1: Extrinsic and Intrinsic pathways of cell death:

Death receptors such as Fas, TNFα and TRAIL initiate the extrinsic pathway of cell death when engaged by their respective ligands. Caspase-8 is activated by death receptor signaling with the aid of adapter protein FADD. The activated caspase-8 then activates caspase-3. In the cell intrinsic pathway, stress signals such as growth factor deprivation and DNA damage induce release of cytochrome-c from the mitochondria via pro-apoptotic proteins Bax and Bak. Additionally, cytochrome-c is also released by the action of caspase-8 substrate Bid, which activates Bax and Bak. This process is inhibited by the anti-apoptotic Bcl-2 family proteins. Cytochrome-c release from the mitochondria leads to activation of caspase-9 in the apoptosome complex. Caspase-3 is activated downstream of caspase-9 and results in apoptosis.
The mitochondria and the Bcl-2 family of proteins play a major role in the intrinsic pathway. The pro-apoptotic Bcl-2 family members, Bax and Bak, allow the release of cytochrome c from the mitochondria, which is inhibited by the anti-apoptotic Bcl-2 proteins such as Bcl-2, Bcl-xL, Mcl-1, and Bcl-W, and the balance between these two groups determines the fate of the cell. The cytochrome c that is released from the mitochondria enters into complex with an adaptor protein APAF-1 (Apoptotic Protease Activating Factor-1), pro-caspase-9 and ATP to activate caspase-9, which then activates the effector caspases (Figure 1.1) (Danial and Korsmeyer, 2004).

Apoptosis is exceedingly integral to the maintenance of proper health that, disorders in the regulation of apoptosis manifest as those of excess cell death such as chronic or acute degenerative diseases, immunodeficiency and infertility, or those of insufficient death such as cancer and autoimmunity (Danial and Korsmeyer, 2004).

**Apoptosis in cancer**

A cell becomes cancerous, or is transformed, when it taps into its potential for uncontrolled cell division. Such unrestricted division can result from the cell responding to promiscuous signals for proliferation, in addition to evading intrinsic defenses of growth arrest and apoptosis.

Appreciation of the contribution of apoptosis to transformation inhibition came with the recognition of Bcl-2 as the oncogene in B-cell follicular lymphomas (Danial and Korsmeyer, 2004). Bcl-2 is over-expressed in a wide variety of cancers, such as gastric, colorectal, renal, neuroblastomas, non-Hodgkin’s lymphoma, acute and chronic leukemias, prostate, breast and lung, and this dysregulation is associated with disease progression and development of chemo resistance (Reed, 1995). Likewise, the pro-apoptotic member of the Bcl-2 family, Bax, is inactivated by mutation in cancers of colon, stomach and hematopoietic origin (Reed, 1999). Among the components of the extrinsic pathway, FLIP (FLICE Like Inhibitory Protein), an inactive homolog and inhibitor
of caspase-8, is over-expressed in a broad range of tumors, thus inhibiting active apoptosis during transformation (Djerbi et al., 1999) and providing cancer cells with yet another strong mechanism of resistance to therapy (Longley et al., 2006; Zhang et al., 2004). Furthermore, inactivation of the components of the extrinsic pathway offers the cancer cells a means to escape tumor immune surveillance by the cytotoxic T cells, which activate death receptors on these recipient cells.

Oncogene activation in a cell generates both proliferative and growth inhibitory signals in tandem. Growth inhibitory responses of arrest or apoptosis are overpowered by cytokine signaling and active suppression of cell death, which then leads to unrestrained proliferation and transformation of cells. Cells undergoing transformation encounter several cell cycle checkpoints and may experience a transient growth arrest; nevertheless, in most cases irreparable damage ultimately leads to cell death. Hence, apoptosis plays a critical role in inhibiting tumorigenesis. Unwarranted activation of both viral and cellular oncogenes stimulates an apoptotic response. For instance, papilloma virus oncoprotein E7 and adenoviral oncoprotein E1A are known to trigger the cell death program and therefore the viruses carry ancillary tools, such as E6 and E1B proteins, respectively, to quench the apoptotic reaction (Rao et al., 1992; Tommasino and Crawford, 1995). Similarly, deregulated expression of cellular oncogenes, such as c-myc and Ras, induces apoptosis instead of proliferation when coupled with growth factor deficiency (Downward, 1998; Harrington et al., 1994). In addition, inappropriate activation of components of the proliferative pathways such as the E2F transcription factors, also leads the cell towards suicide (Phillips et al., 1997).

The delicate balance between oncogenes and tumor suppressors in a cell translates into an intricate equation of tumorigenesis. Not surprisingly, as with most oncogene activation episodes, loss of a tumor suppressor leads to apoptosis as well, by the compensatory activation of another; this is exemplified by the inactivation of Rb (Retinoblastoma protein), which results in induction of p53 dependent apoptosis.
(Morgenbesser et al., 1994). Several tumor suppressor gene products such as p53, PTEN, APC, NF1, WT1, and BRCA1/BRCA2, function by inducing apoptosis; thus, the loss of these proteins’ function results in accelerated tumor development, and the cells exhibit a cell death resistant phenotype in most cases (Davies et al., 1999; Englert et al., 1995; Morin et al., 1996; Rao et al., 1996). A cell responds to numerous anomalies by initiating apoptosis that, the concerted loss of tumor suppressors and activation of oncogenes does not guarantee tumor development, unless defects in the cell death program are acquired. Moreover, developing deficiencies in the apoptotic pathways is critical for tumor progression and metastasis as cells must survive in the absence of anchorage and survival signals from the extracellular matrix.

Role of apoptosis in chemotherapy and therapeutic resistance

Transforming events are many, and each one can be countered individually to inhibit the process of transformation. Although strategies that negate transforming events may be therapeutically successful initially, tumor regression and complete therapy can be easily and efficiently achieved only if all of the cells are able to reinstate a functional apoptotic response. Consequently, most therapeutic options available currently utilize extrinsic stress signals to activate the cell death program in the cell. Cancer cells that retain certain functional components of the apoptotic machinery undergo spontaneous cell death and this is properly enhanced by therapeutic interventions in the form of irradiation, cytotoxic chemotherapy, hormone ablation, cytokine signaling and immunomodulation (Kerr et al., 1994).

One of the most important problems faced during cancer treatment is the development of multidrug resistant tumors, especially in patients with a previous treatment history. Although such resistance can be attributed to the upregulation of Mdr1, a plasma membrane transporter that actively transports drugs to the outside of the cell, it is seen in only a minority of the cases (Gottesman and Pastan, 1993). Besides, several cancers acquire resistance to radiation therapy, where transporters have no role
to play, which together indicate that a process common to a variety of stressors is responsible. Despite a widespread belief that therapies, being highly toxic, induce cell death by necrosis, the mechanism of cell death was found to be apoptosis (Lowe et al., 1993). As with tumorigenesis, defective regulation of apoptosis is a straightforward way to ensure cell survival following cancer therapy, as various toxic and stress signals converge on one single pathway to effect cell death. Not surprisingly, loss of p53 (Lowe et al., 1994) and over expression of Bcl-2 or Bcl-xL increase tumor resistance to antineoplastic agents, such as anthracyclines, topoisomerase inhibitors, DNA crosslinking agents, and mitotic spindle inhibitors (Fisher et al., 1993; Minn et al., 1995; Miyashita and Reed, 1992). While most of these treatment strategies highlight the requirement of an intact apoptotic response, the surrounding normal tissue offers abundant targets for highly toxic treatments. Hence, the issue of bystander toxicity has to be addressed in addition, while designing therapies.

Described here, in brief, are certain proteins that play very important roles in apoptosis, especially of cancer cells; this dissertation is devoted to the study of the interplay of these proteins under a few experimental conditions modeling certain physiological and pathological cellular states.

**Par-4 (Prostate Apoptosis Response – 4)**

**Identification and characterization of Par-4**

Prostate cancer, one of the most common forms of cancer in the United States, is usually treated by androgen-ablation, which results in tumor regression by apoptosis. The prostate tumor consists of a mixed population of androgen-dependent and -independent cells. Androgen withdrawal results in the initiation of an apoptotic response in the androgen-dependent cells, which rely on a steady supply of hormones for their survival.
In the androgen-dependent cells, the cell-death program is triggered by the induction of gene expression in response to intracellular calcium elevation on androgen withdrawal; this response is bypassed in the androgen-independent cells and thereby results in the survival of this population. Accordingly, this therapeutic approach, in some cases, generates an aggressive relapse because the few androgen-independent cells, which did not undergo apoptosis, are essentially selected for survival. The androgen-independent cells can be forced to undergo apoptosis in cell culture paradigms when intracellular calcium levels are increased with the treatment of the calcium ionophore, ionomycin. This apoptotic program can be abolished if transcription or translation inhibitors, such as actinomycin or cyclohexamide, are used, indicating that gene induction is necessary.

Par-4 was first cloned by inducing the rat androgen-independent prostate cells, AT-3, with ionomycin to undergo apoptosis. Using the differential hybridization strategy, Par-4 was identified as a member of a new set of genes that are transcribed in response to calcium elevation. Similarly, Par-4 expression has been observed during the involution of rat ventral prostate by apoptosis following castration, and this Par-4 induction was duly inhibited on pretreatment with the calcium-channel blocker, nifedepine (Sells et al., 1994). Par-4 expression was also inhibited by testosterone in the ventral prostate. Neither induction nor repression of Par-4 was observed in the liver, kidneys, or other organs that bear androgen receptors yet do not undergo apoptosis in response to castration. In addition, in the initial studies, Par-4 was induced in cell culture models exclusively by apoptotic signaling and not during necrosis, growth arrest, or serum stimulation.

Par-4 is evolutionarily conserved in vertebrates and is ubiquitously expressed in tissues of ectodermal, mesodermal, and endodermal origin. Par-4, however, is absent from specific cell types (such as lymphocytes, certain smooth muscle cells, the differentiated ductal cells of the prostate, the epithelial cells of the mammary gland,
and the terminally differentiated cells of the retina), thus indicating that Par-4 is
downregulated during differentiation (Boghaert et al., 1997). Aligned with the fact that
Par-4 is an apoptotic protein, its expression is increased during development in actively
dying cells, such as the interdigitating web cells of the mouse embryo and the involuting
tadpole tail. Par-4 also plays a key role in neuronal development, where an increase in
Par-4 levels results in apoptosis and serves to maintain the number of neurons in the
nervous system (El-Guendy and Rangnekar, 2003).

The Par-4 gene produces a 38-kDa protein consisting of 332 amino acids. This
protein has a variety of interesting structural domains that allude to its potential
mechanism of action. Par-4 has a 42-residue leucine zipper domain in its C-terminus,
along with a nuclear export sequence (NES). Two putative nuclear localization
sequences (NLS) are present in the amino-terminus, and both NLS are highly conserved
in the Par-4 protein among different species. In addition, Par-4 possesses a number of
conserved consensus sites for phosphorylation by kinases, such as PKA and PKC (Figure
1.2). Collectively, the presence of these motifs suggests that the function of Par-4 may
be tightly regulated by post-translational modification, localization, and dimerization
with partners of biological consequence.

**Pro-apoptotic role of Par-4**

The role of Par-4 in apoptosis has been well studied in cell culture models.
Overexpression studies have revealed that an increase in Par-4 level or activity in
normal or immortalized cells results in a lowered sensitivity threshold to death stimuli
(such as growth factor withdrawal, elevation of intracellular Ca2+, TNF-α, IFNγ, or UV, X-
ray, and gamma irradiation). In addition, Par-4 has been found to be an essential
downstream regulator of cell-death programs initiated by different agents such as
TRAIL, vincristine, doxorubicin, and radiation. In transformed cells, Par-4 is capable of
initiating the death program in the absence of a second stimulus. Interestingly, strong
oncogenic or growth-promoting factors render tumor cells susceptible to Par-4-induced
Figure 1.2: Domain Structure and Phosphorylation Sites of Par-4
Figure 1.2: Schematic representation of the Par-4 protein domain structure and phosphorylation sites:

The domains NLS1 (nuclear localization sequence), NLS2, SAC and leucine zipper (LZ) are represented by filled brackets underlining the region. Phosphorylation sites are indicated by bars – activating phosphorylation in red (PKA), inactivating modification in green (Akt) and phosphorylation sites of unknown function in black (PKC & CK2).
apoptosis. This ability of Par-4 to effect apoptosis exclusively in cancer cells is intriguing and indicates that Par-4 has merit as a potent therapeutic tool.

While most studies have focused on ectopic overexpression of Par-4, under specific conditions endogenous Par-4 expression is also powerful as an apoptotic agent. Beyond the aforementioned role of Par-4 in the tadpole tail and interdigitating web, Par-4 plays a role in neuronal apoptosis during embryonic development and serves to prevent hyperproliferation of nerve tissue (El-Guendy and Rangnekar, 2003). This is achieved by the asymmetric distribution of Par-4 protein during the mitosis of neuronal progenitor cells; the daughter cells lacking Par-4 differentiate into neurons, while those with high levels of Par-4 undergo apoptosis (Bieberich et al., 2003). A recent study demonstrated that this function of Par-4 can be exploited during neural transplant to beget selective apoptosis of pluripotent stem cells and enrich the neuronal precursors among this population of embryonic stem cells (Bieberich et al., 2004).

It is well established that normal physiological processes can become skewed during the onset of a disease and thereby augment the disease process. Several observations support the fact that Par-4 is also involved in the development of neurodegenerative disorders by increasing the apoptosis of healthy neurons. First, elevated levels of Par-4 mRNA and protein have been observed in the apoptotic neurons of patients, animal models, and cell culture models of diseases such as Alzheimer's, Parkinson's, Huntington's chorea, amyotrophic lateral sclerosis (ALS), and ischemic brain injury or stroke (El-Guendy and Rangnekar, 2003; Mattson et al., 1999). Second, Par-4 is also thought to facilitate the development of such degenerative conditions by inhibiting the uptake of choline at the cholinergic synapse (Xie and Guo, 2004). Finally, it was recently reported that Par-4 is involved in the regulation of the protease that cleaves the amyloid beta precursor protein to give Aβ peptide, the key pathogenic protein in Alzheimer's (Xie and Guo, 2005).
In addition to apoptosis, Par-4 has been identified as playing a non-apoptotic role in various cell types. One such example is the role of Par-4 in the modulation of immune response by keeping in check the secretion of IL-2 and other important immunomodulatory cytokines (Lafuente et al., 2003).

**Mechanism of apoptosis by Par-4**

Programmed cell death usually occurs through one of the two major signaling pathways, known as the extrinsic and the intrinsic pathway. The extrinsic pathway proceeds through death receptors while the intrinsic pathway is mediated by the mitochondria. The extrinsic pathway is initiated at the cell surface via the activation of transmembrane death receptors of the CD95 (Apo-1 or Fas)/TRAIL/tumor-necrosis factor (TNF) receptor 1 family. Engagement of these receptors with their cognate ligand triggers the assembly of a multiprotein complex that activates the upstream caspase 8. In the intrinsic pathway, reception of a stress/death signal is transduced to the mitochondria, which in turn release cytochrome c into the cytosol; this results in the formation of the apoptosome complex to activate caspase 9. Caspases 8 and 9 both activate downstream effector caspases that carry out the final steps of apoptosis, such as release of the endonuclease CAD (caspase-activated DNAase) from its inhibitor iCAD and cleavage of key cellular enzymes and proteins (Strasser et al., 2000). Although the intrinsic and extrinsic pathways ultimately converge at the level of the effector caspases, molecular cross talk between the two pathways is known to exist. A number of factors—both pro-apoptotic factors that drive the cell toward suicide and pro-survival proteins that protect the cell from death—are involved in regulating apoptosis at each level of this signaling cascade.

Since Par-4 is a pro-apoptotic protein, it sets the cell-death program in motion by acting at two levels: the activation of molecular components of the cell-death machinery and the inhibition of pro-survival factors. Par-4, as characterized to date, appears to act through the extrinsic pathway by enabling the trafficking of Fas and Fas ligand to the
plasma membrane. Fas/CD95 is a member of the TNF-R family and is activated by binding to Fas ligand. Such binding leads to the formation of a complex consisting of trimeric Fas, the adaptor protein FADD (Fas-associated death domain) and pro-caspase 8; this complex is called the death-inducing signaling complex, or DISC. The formation of DISC in turn leads to the cleavage and activation of the zymogen, pro-caspase 8, to its active caspase form. Interestingly, Par-4 translocates Fas and FasL to the plasma membrane in androgen-independent cancer cells, in which overexpression of Par-4 leads directly to death. However, the same effect is not observed in the androgen-dependent cancer cells, wherein Par-4 serves only to sensitize. The mechanism of apoptosis when Par-4 has a sensitizing effect may involve the intrinsic pathway or may vary depending on the second signal (Chakraborty et al., 2001).

As previously mentioned, in addition to its effect on the extrinsic pathway, Par-4 also plays a very important role in antagonizing the pro-survival/anti-apoptotic factors that present a major obstacle to the apoptosis cascade. One of the most important anti-apoptotic factors active in a cell is the transcription factor NF-κB. NF-κB is a heterodimer consisting of p65 (RelA) and p50 subunits. This dimer is held inactive in the cytoplasm as a result of being bound to the inhibitory protein IκB, which on the appropriate signal is phosphorylated by IκKβ, ubiquitinated, and ultimately degraded to release an active NF-κB. Following other phosphorylation events and translocation into the nucleus, the active NF-κB transcription factor binds to the promoter of its target genes and regulates transcription. One of the very important anti-apoptotic targets of NF-κB is the XIAP (X-linked inhibitor of apoptosis), which hinders the activity of both upstream and effector caspases (Baldwin, 1996; Barkett and Gilmore, 1999). Par-4 inhibits NF-κB activity and this Par-4-mediated inhibition of NF-κB is carried out without disrupting the DNA-binding capacity of the NF-κB complex. It is proposed that Par-4 acts in the nucleus to inhibit phosphorylation of NF-κB by IκKβ or PKCζ, which would otherwise confer maximal activity to NF-κB. In addition, interference with IκKβ phosphorylation of the IκB complex in the cytoplasm acts as another molecular
safeguard against the activation of NF-κB (Diaz-Meco et al., 1999). This ability of Par-4 to inhibit pro-survival factors might be essential to its apoptosis-sensitizing function.

Cancer Antagonism by Par-4

Consistent with its role in apoptosis, Par-4 has a tumor-suppressive function. Although the function of Par-4 in apoptosis is an important component of a cell's anti-transformation surveillance (Hanahan and Weinberg, 2000), Par-4 also exerts considerable influence in its tumor-suppressive role. In support of its role in tumor suppression, Par-4 is downregulated in a variety of cancers, such as renal-cell carcinomas, neuroblastoma, acute lymphoblastic leukemia, and chronic lymphocytic leukemia (Boehrer et al., 2001; Gurumurthy and Rangnekar, 2004). Moreover, Par-4 mRNA and protein are downregulated by oncogenic Ras in a variety of cell types through the MEK-ERK pathway (Barradas et al., 1999; Qiu et al., 1999b). This downregulation is necessary for Ras to initiate transformation because ectopic overexpression of Par-4 in Ras-transformed cells impedes formation of colonies in a soft agar assay (Qiu et al., 1999b). This anti-transformation effect may be attributed to the fact that Par-4 antagonizes the Ras-MAPK pathway by repressing ERK, an essential downstream kinase in this pathway (Qiu et al., 1999b). This function of Par-4 is independent of its pro-apoptotic ability although oncogenic Ras is known to sensitize cells to apoptosis by Par-4 (Nalca et al., 1999). Despite being down regulated by Ras in certain cell types, Par-4 is present at copious amounts in most cancer cells unlike a few tumor suppressors yet, is functionally inactivated by the cell survival kinase Akt. Binding and phosphorylation of Par-4 by Akt creates a binding site for the adapter protein 14-3-3θ. Par-4 is mislocalized to the cytoplasm and sequestered away from its site of action by interaction with 14-3-3θ during transformation for unchecked survival and proliferation (Goswami et al., 2005).

In addition to inhibiting transformation, Par-4 plays a role in tumor regression; hence, Par-4 is antagonistic to both tumor formation and tumor maintenance.
Subcutaneous tumors, which were generated in nude mice using PC-3 cells, regressed in less than 3 weeks after a single injection of Par-4 adenovirus (Chakraborty et al., 2001). In another study, Par-4 overexpression decreased tumor development in xenotransplants of A375-C6 melanoma cells in mice; tumor reduction was due to an increase in apoptosis (Lucas et al., 2001). This effect of Par-4 is due to its pro-apoptotic function because both activation of the Fas pathway and inhibition of NF-κB are essential for tumor regression (Chakraborty et al., 2001). Tumors expressing either dN-FADD (an inhibitor of the Fas pathway) or RelA (resulting in an active NF-κB) are resistant to the tumor-regressive effects of Par-4 (Chakraborty et al., 2001). Par-4 has been recently implicated in the suppression of tumors in hematopoietic stem cells by disrupting the BCR-Abl signaling pathway (Kukoc-Zivojnov et al., 2004). In addition, Par-4 may be a player in the control of metastasis as well because Par-4 inhibited the migration of B16F1 melanoma cells by acting as a negative regulator of PKC kinase activity (Sanz-Navares et al., 2001). Most importantly, the Par-4 knockout mice display a high predisposition to formation spontaneous and induced tumors, especially of the prostate, endometrium, lung and liver (Garcia-Cao et al., 2005). Moreover, Par-4 interacts with and regulates the function of Topoisomerase I in normal cells. Disruption of this function of Par-4, as during transformation by inactivation of Par-4, affords the cell unimpeded access to topoisomerase activity, which is thoroughly exploited to provide for the proliferative demands of the transformed state (Goswami et al., 2008). Collectively, the data discussed above validate Par-4 as a viable target for therapeutic intervention in cancer treatment.

**Regulation of Par-4 function**

Given the pro-apoptotic function of Par-4, it is essential for the cell to tightly regulate its activity, thereby keeping Par-4 in a dormant state and activating it only when necessary. Consequently, regulation is managed by localizing Par-4 to distinct cellular compartments. It was observed during Par-4 overexpression studies that Par-4 is translocated to the nucleus well before the cell undergoes apoptosis. Inhibition of
nuclear entry abrogated the apoptotic potential of Par-4. As mentioned earlier, Par-4 contains two putative NLS at its amino-terminus. A deletion mutant that lacks both of these nuclear localization sequences is incapable both of nuclear entry and promoting apoptosis. Additional deletion analysis indicated that the first NLS (NLS1) was not necessary for nuclear entry and that the second NLS (NLS2) plays an important role in nuclear translocation. Consistent with these results, it was observed that in androgen-dependent cancer cells (in which Par-4 is unable to induce apoptosis), Par-4 is predominantly localized in the cytoplasm. In contrast, nuclear localization of Par-4 is the norm in androgen-independent cancer cells, as well as many other cancer cells; in these cells, Par-4 has a lethal effect. The functional role of the nuclear export sequence present in the C-terminus has yet to be elucidated. Furthermore, there are substantial data indicating the presence of Par-4 in the PML bodies (promyelocytic leukemia bodies) of the nucleus. Although the significance of this localization remains to be clarified, it is possible that the Par-4 mechanism of apoptosis specifically resides in the PML bodies (Gurumurthy and Rangnekar, 2004). Control of Par-4 function cannot be ascribed to the regulation of its localization alone and, as explained below, there are additional mechanisms through which its activity is monitored.

Par-4 that is present in the cytoplasm is partnered with various proteins, and many of the functions of Par-4 can be attributed to the effect of these interactions. One of the very important and extensively studied partners of Par-4 is the kinase, PKCζ (Diaz-Meco et al., 1996). Par-4 binds to PKCζ and inhibits its major functions, not the least among them being activation of NF-κB. PKCζ-induced activation of NF-κB is lost in the presence of equal or excess amounts of Par-4. This effect on PKCζ is the key to NF-κB inhibition by Par-4, although this does not preclude the existence of other mechanisms. To add further complexity to this equation, another protein, p62, is sometimes bound to the Par-4/ PKCζ complex. When p62 interacts with the Par-4/ PKCζ complex, the effect of Par-4 on PKCζ is lost and, as a result, cell survival is promoted (Chang et al., 2002b).
Another important partner for Par-4 is the Wilms’ tumor protein, WT-1 (Johnstone et al., 1996). WT-1 is a transcription factor that possesses a zinc finger and acts as either an activator or repressor for many growth factor genes. WT-1 protein is expressed during development in the kidney, testis, and ovary and plays an important role in the development of Wilms’ tumor, as its name suggests. WT-1 induces the transcription of the anti-apoptotic protein Bcl2; this transcriptional activity is inhibited when Par-4 is bound to WT-1. Par-4, along with WT-1, binds to the promoter of Bcl2, inhibits transcription, and thereby promotes apoptosis (Qiu et al., 1999a). Par-4 regulates the function of WT-1 during the transcription of other genes as well; however, the significance of these regulations has yet to be studied.

Par-4 also binds to ZIP kinase (also known as ZIPK or DLK) in the PML bodies. This interaction is necessary for ZIPK-induced apoptosis because Par-4 promotes interactions between ZIPK and DAXX, which is a regulator of apoptosis (Page et al., 1999). In addition, Par-4 complexes with THAP-1, a pro-apoptotic protein that is also associated with the PML subnuclear bodies (Roussigne et al., 2003). These Par-4 interactions with ZIPK, DAXX, and THAP-1 all function to catalyze apoptosis, and it is likely that these associations are mediated by the C-terminal leucine zipper domain of Par-4, which is essential for the pro-apoptotic function of this protein (Sells et al., 1997). Beyond the binding interactions detailed here, there may be other binding partners of Par-4 for which identity and function are yet to be elucidated.

**Cancer Selective Apoptosis**

Since Par-4 is subject to regulation at multiple levels, a study was undertaken to identify the minimum region of Par-4 essential for apoptosis induction, and this search ultimately led to the discovery of Par-4’s remarkably unique property of cancer-selective apoptosis. Deletion analysis was used to identify the minimum essential domain that would facilitate apoptosis and yet be free of inhibitory interactions. Par-4, which
contains 332 amino acids, was progressively and selectively pared to make a variety of small mutants. In the course of this study, a mutant comprising the region spanning amino acids 137-195 was identified as the core domain that orchestrates the apoptotic function of Par-4. Upon further deletion up to the amino acid 187 (thus a fragment consisting of amino acids 137-187), the apoptotic function was lost. It was also observed that the small 137-195 domain was constitutively nuclear and caused selective apoptosis of cancer cells upon overexpression. Therefore, this 137—195 mutant is called SAC for selective apoptosis of cancer cells (El-Guendy et al., 2003). Moreover, recently, transgenic mice over-expressing the SAC module of Par-4 proved to be “cancer resistant” when crossed with the TRAMP (TRansgenic Adenocarinoma of Mouse Prostate) mice, with a staggering 80% reduction in tumor incidence. In addition, these mice were highly resistant to spontaneous tumors developed with age as well, further supporting the tumor inhibitory function of Par-4 (Zhao et al., 2007).

SAC, which localized to the nucleus both in normal and cancer cells, caused apoptosis only in cancer cells when overexpressed. This led to the conclusion that, in addition to nuclear entry, an activating event that is present only in cancer cells is necessary for Par-4 to attain its full apoptotic potential. The SAC region contains a few phosphorylation sites, and so the possibility of phosphorylation as a potential regulator was studied. Two amino acids, a serine at 154 and a threonine at 155 are consensus sites for PKA (protein kinase A). PKA is a universal kinase that phosphorylates a wide range of substrates that are involved in the regulation of metabolism, cell growth, and differentiation. Elevation of intracellular levels of cAMP (cyclic adenosine monophosphate) results in the activation of PKA and the transduction of a signal downstream via phosphorylation. PKA occurs in two isoforms, I and II, depending on the regulatory subunit that is associated with the kinase. Isoform I is overexpressed in human cancer cells and primary tumors and, consequently, the PKA enzymatic activity is higher in transformed cells than in normal or immortalized cells (Tortora and Ciardiello, 2002). This enhanced PKA activity may explain the cancer-specific activation of Par-4.
Indeed, further studies revealed that phosphorylation by PKA is a crucial event that triggers the apoptotic function of Par-4; in the absence of this phosphorylation, Par-4 completely lost its ability to effect apoptosis.

To further clarify the regulation of Par-4, the PKA consensus serine (154) and threonine (155) were individually mutated to alanine in order to eliminate phosphorylation at these sites. It was found that a mutation at the 155 residue abolished apoptosis by Par-4, while the Par-4-154A mutant retained its apoptotic ability. SAC with 155 mutated, although still nuclear in localization, was unable to kill transformed and androgen-independent cell types such as NIH3T3/Ras, lung cancer cells A549, H460, H157, and H838 and breast cancer cells MCF-7, MDAMB-231, and MDAMB-435. Phosphorylation of ectopically expressed Par-4 by PKA has been confirmed by metabolic labeling, while phosphorylation of endogenous Par-4 has been established using an antibody raised against the phospho-threonine residue at 155. These experiments reveal that Par-4 is phosphorylated in its active state and that this phosphorylation event is very common in cancer cells when compared to normal cells. Additional experiments, including the inhibition of phosphorylation of Par-4 (and thus apoptosis) with an inhibitory peptide and the provision of cAMP to normal cells in order to make them susceptible to apoptosis by Par-4, support the fact that Par-4 is controlled at several levels in the cell (Gurumurthy et al., 2005).

Par-4-inducible apoptosis can now be explained by the model that Par-4 requires two distinct events—specifically, nuclear entry and phosphorylation by PKA—for activation. Both regulating events are present and active in cells that display sensitivity to Par-4. In cells that are resistant to Par-4, such as hormone-dependent cancer cells and normal or immortalized cells, one or both of these activating events are absent, and, despite the presence of substantial amounts of Par-4, the cells remain resistant to apoptosis (Figure 1.3).
Figure 1.3: Cell type specific regulation of Par-4

Cancer cells

Par-4 sensitive

Par-4 resistant

Active PKA

Par-4

NF-κB

Apoptosis

Inactive PKA

Par-4 resistant

Normal cells

Par-4 resistant

Active PKA

Par-4

Akt-1

Cell survival

NF-κB

Apoptosis

Cell survival

?
**Figure 1.3 : Model for cell type specific regulation of Par-4 :**

Two independent events are necessary to activate Par-4—nuclear entry and phosphorylation by PKA. In normal or immortalized cells, owing to low levels of active PKA, Par-4 is not phosphorylated at the T155 residue; in addition, it is retained in the cytoplasm by an unknown mechanism, and hence Par-4 is inactive. In cancer cells that are resistant to apoptosis by Par-4, despite phosphorylation at the T155 residue, Par-4 is inhibited from entering the nucleus by Akt-1. In cancer cells sensitive to Par-4, it is phosphorylated by PKA at T155 and the inhibition by Akt-1 is absent. Par-4, which is thus activated by phosphorylation and nuclear entry, causes apoptosis of cancer cells.
Although studies so far have revealed much about the regulation and the mechanism of action of Par-4, it is likely that Par-4 acts in more complex ways than can be accounted for by the pathways detailed thus far. Par-4 is clearly emerging as a tumor suppressor that would be ideal for therapeutic intervention strategies, keeping in mind that each small effort, as is the case with all cancer research, takes us one step closer to understanding the genesis of cancer and the ultimate goal of developing a superior oncotherapy.

[Par-4 portion adapted with permission from (Ranganathan and Rangnekar, 2005)]

**Caspase-8**

Caspases, as mentioned earlier, are a family of cysteiny1 endopeptidases that are the primary executioners of programmed cell death. As an initiator caspase, caspase-8 is at the head of the extrinsic cascade hierarchy. The human caspase-8 gene is located on chromosome 2q33 (Earnshaw et al., 1999). Caspase-8 is synthesized as an inactive zymogen with a long amino terminal pro-domain that contains two Death Effector Domains (DED), and two catalytic subunits, which are approximately 20kd and 10kd in size. The DEDs mediate hydrophobic interactions with additional pro-caspase-8 molecules and other DED containing proteins such as the adaptor FADD.

Caspase-8 is activated primarily along the receptor mediated extrinsic pathway. Binding of death ligands to their respective receptors initiates the formation of the multi-protein DISC which provides a molecular platform for caspase-8 activation. The cell surface death receptors capable of activating caspase-8 include Fas/CD95/Apo-1, TNFR (Tumor Necrosis Factor α Receptor), DR-3/Apo-3, DR-4 and DR-5 (Death Receptor-4/ Death Receptor-5), and NGFR (Nerve Growth Factor Receptor) (Ashkenazi and Dixit, 1998). The functional DISC consists of a trimerized, ligand bound receptor, adapter proteins TRADD (TNFR Associated Death Domain) and/or FADD, and the pro-caspase-8 zymogen. Pro-caspase-8 is activated in the DISC by dimerization and the active enzyme is released into the cytosol following proteolytic auto-processing of the N terminal pro-
domain. The active caspase-8 enzyme is a heterotetrameric complex consisting of two large (20kd) and two small (10kd) subunits with two active sites for each complex. Caspase-8 may also be activated unconventionally by caspase-6 mediated processing in the final stages of the apoptotic program, contributing to the amplification loop (Cowling and Downward, 2002). The activated caspase-8 primarily cleaves and activates caspase-3, the effector caspase that is responsible for most of the phenotypic changes of apoptosis, in certain cell types, for instance, lymphocytes, which are termed as type I. In other types of cells, such as hepatocytes, the meager caspase-8 activated at the DISC cleaves Bid, which then induces cytochrome c release from the mitochondria (Ozoren et al., 2000). Cytosolic cytochrome c aids in activation of initiator caspase-9, which cleaves and activates caspase-3 (Li et al., 1997) (Figure1.1). Caspase-8 is also capable of activating caspases -1, -2, -6, -7, -9 and -11, in addition to caspase-3 (Van de Craen et al., 1999). Additionally, the activated caspase-8 cleaves certain non-caspase substrates such as PAK2 (p21 Activated Kinase), Wee1 kinase, FLIP$_L$ (Earnshaw et al., 1999), and plectin (Stegh et al., 2000), to carry out its apoptotic as well as non-apoptotic functions.

Caspase-8 activation can be regulated by several proteins such as FLIP, BAR, ARC, p35 (baculoviral origin), and CrmA (cowpox virus protein). FLIP exists in two forms, a short one (FLIP$_S$) and a long one (FLIP$_L$). FLIP$_S$ contains two DEDs, while the longer FLIP has a non-functional caspase like domain in addition to the DEDs. FLIP$_S$ inhibits caspase-8 activation by virtue of the death effector domains that bind with high affinity to the DISC in place of pro-caspase-8. FLIP$_L$, on the other hand, is known to both activate caspase-8, by perhaps assisting dimerization of pro-caspase-8 molecules, and at times, inhibit, depending on the prevailing conditions (Chang et al., 2002a). BAR (Bifunctional Apoptosis Regulator) contains a DED and impedes caspase-8 activation by competing with FADD for binding to pro-caspase-8 (Zhang et al., 2000). ARC (Apoptosis Repressor with CARD) interacts with caspase-8 and inhibits its activation in a manner similar to BAR, however, owing to the fact that the protein has a CARD instead of a DED, the mechanism of action is not fully understood (Koseki et al., 1998).
Caspase-8 mediated apoptosis is essential for embryonic development as demonstrated by the knockout, which exhibited embryonic lethal phenotype owing to defects in extra-embryonic yolk sac vasculature, cardiac development and endothelial cell homeostasis (Varfolomeev et al., 1998). In addition to these well recognized apoptotic functions, caspase-8 also plays a role in the expansion of hematopoietic progenitors in the bone marrow, T cell activation and development (Kang et al., 2004; Salmena et al., 2003). Furthermore, it plays a role in cell motility by regulating Rac and Calpain activation for lamellipodial assembly (Helfer et al., 2006).

Caspase-8 is vital to the initiation of a rapid death cascade that it is predictably inactivated in a number of cancers. Caspase-8 protein expression is decreased due to various causes in childhood neuroblastomas, renal cell carcinoma, Small Cell Lung Carcinoma (SCLC) and certain Non-Small Cell Lung Carcinomas (NSCLC) (Shivapurkar et al., 2002; Teitz et al., 2000). Caspase-8 is mutated in 5% of all invasive colorectal carcinomas (Kim et al., 2003), and is mutated in cell lines of head and neck cancer (nonsense), neuroblastoma (missense) and vulvar squamous carcinoma (deletion) (Philchenkov et al., 2004). In general, the level of expression of this protein is decreased in cancer cell lines and neoplastic tissues when compared to control or surrounding normal tissue. In addition to such inactivations, the caspase-8 promoter is hypermethylated in several neuroblastomas, rhabdomyosarcomas, meduloblastomas, retinoblastomas, and human hepatocellular carcinomas (Harada et al., 2002; Yu et al., 2002). Moreover, in neuroblastomas, metastasis is permitted by loss of caspase-8, owing to its function in cell motility (Stupack et al., 2006).

Caspase-8 is an emerging target of cancer gene therapy and both in vitro and in vivo studies have demonstrated its tumoricidal efficacy. Adenoviral vector based expression of caspase-8 potentiated both x-ray and 5-fluorouracil induced apoptosis in colon cancer cells (Uchida et al., 2003). Growth inhibition of subcutaneously established
tumor of glioblastoma in mice by augmented expression of caspase-8 from the hTERT promoter further underscores the value of caspase-8 in apoptosis based tumor suppression. Hence, characterization of realistic, non-toxic means of inducing intrinsic caspase-8 activity in the cancer cell is an important goal in the development of cancer therapies.

TRAIL

TNF Related Apoptosis Inducing Ligand (TRAIL/Apo2L), a member of the TNF family of cytokines, induces apoptosis specifically in the cancer cells while remaining relatively non-toxic to normal cells. Recombinant TRAIL causes tumor regression and reduces tumor incidence in murine cancer models (Ashkenazi et al., 1999; Walczak et al., 1999) and is currently in phase I clinical trial (Ashkenazi and Herbst, 2008), (www.cancer.gov). Despite being such a promising candidate for therapeutic usage, mechanistic details of the signal transduction pathways that lead to TRAIL resistance and sensitivity have not been studied extensively.

TRAIL is expressed as a type II transmembrane protein that can signal as an intact protein in situ, or generate by proteolytic cleavage, an extracellular domain to be utilized in signaling to surrounding cells. This ligand forms a homotrimer that can bind to any of its five designated receptors, two of which are apoptotic. DR4/TRAIL-R1 and DR5/TRAIL-R2, with a functional death domain (DD) in the intracellular region, transduce the apoptotic signal upon ligand binding. DcR1/TRAIL-R3, DcR2/TRAIL-R4, and the soluble receptor OPG (Osteoprotegerin), lack functional death domains and serve as decoy receptors to inhibit apoptosis (Almasan and Ashkenazi, 2003). Binding of TRAIL to either DR4 or DR5 permits recruitment of the adaptor protein FADD and initiator caspase-8 to the receptor. FADD binds to the receptor through its DD (Death Domain) and to caspase-8 through DED (Death Effector Domain) to form the DISC (Death Inducing Signaling Complex) (Kischkel et al., 2000; Sprick et al., 2000). Caspase-8 is activated in the DISC by dimerization (Boatright et al., 2003) and the active dimers are
released into the cytosol by self cleavage at the N-terminus. Caspase-8 then directly cleaves and activates caspase-3 in the Type I cells. Whereas, in the Type II cells, the minimal caspase-8 that is activated cleaves Bid, which then induces cytochrome c release from the mitochondria (Ozoren et al., 2000). Cytosolic cytochrome c aids in activation of initiator caspase-9, which cleaves and activates caspase-3 (Li et al., 1997). The effector caspase-3 cleaves death substrates such as CAD (Caspase Activated DNAse), PARP (Poly ADP Ribose Polymerase), fodrin, actin and lamin to complete apoptosis (Kidd, 1998).

High levels of decoy receptor expression are found in most normal adult tissues, possibly accounting for the resistance of normal cells to TRAIL. However, a strong correlation between decoy receptor expression and resistance in cancers is not seen. Similarly, FLIP (FLICE Inhibitory Protein), the non-catalytic homolog of caspase-8, is expressed at high levels in certain TRAIL resistant cancer lines, which can be sensitized by inhibiting protein synthesis or by activating proteosome based degradation of FLIP. Additionally, in the Type II cells, high levels of IAP (Inhibitor of Apoptosis Protein) can inhibit the caspase cascade by interfering with the activation and activity of caspases-9 and -3. In terms of inhibiting apoptosis, NF-κB pathway can also be implicated as pro-survival proteins, such as FLIP, XIAP and Bcl-XI are induced in response to NF-κB activation. However, NF-κB is not significantly stimulated in response to TRAIL even if only in response to high concentrations of the ligand (Almasan and Ashkenazi, 2003).

**Research objectives**

Par-4 is a protein that specifically targets cancer cells for apoptosis upon over expression. Prior to this study, the data demonstrated only of the effects of Par-4 under conditions of artificial over expression and the aim of this work is to characterize the function, if any, of the abundant endogenous Par-4 with regard to apoptosis. The TRAIL pathway was used as a model for this study due to the fact that TRAIL shares the
features of cancer specific toxicity with Par-4. In specific, this study has addressed the following issues in two chapters:

Chapter 2 – Regulation of Caspase-8 by Par-4

1. Whether Par-4 is involved in and is an integral component of the apoptotic cascade initiated by TRAIL
2. Whether Par-4 plays a role in activation of any of the caspases in response to TRAIL
3. The mechanism of Par-4 mediated caspase-8 activation

Chapter 3 – Regulation of Par-4 by Caspase-8

1. The impact TRAIL signaling has on Par-4.
2. Whether Par-4 is a substrate of caspases activated by TRAIL and the identity of the specific caspases involved.
3. The sites of cleavage in Par-4 and the regulation of cleavage by phosphorylation.
4. Whether the caspases involved co-operate to naturally generate the highly potent functional core of Par-4.

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Chapter Two

Regulation of Caspase-8 activation by Par-4 in response to TRAIL

Introduction

TNF Related Apoptosis Inducing Ligand (TRAIL / Apo2L), a member of the TNF family of cytokines, induces apoptosis specifically in cancer cells while remaining relatively non-toxic to normal cells. Recombinant TRAIL causes tumor regression and reduces tumor incidence in murine cancer models (Walczak et al., 1999), and is currently in Phase I clinical trials (www.cancer.gov). TRAIL initiates death signaling by binding to either of its two functional receptors, DR4 or DR5; binding is followed by activation of the initiator caspase-8, at the receptor-associated DISC (death inducing signaling complex), followed by effector caspase-3 activation to induce apoptosis (Plati et al., 2008). Cells are categorized as type I or type II, depending on the initiator caspase (i.e., either caspase-8 or caspase-9) that activates caspase-3. In the type I cells, profuse activation of caspase-8 at the membrane results in caspase-3 activation; on the other hand, in type II cells, the insufficiently activated caspase-8 induces activation of caspase-9, via t-Bid-mediated mitochondrial cytochrome c release (Ozoren and El-Deiry, 2002). Although TRAIL can be a very valuable therapeutic tool owing to its selective toxicity to cancer cells, a number of tumors and cancer cell types acquire resistance to TRAIL, limiting its clinical use. Sensitivity to TRAIL is modulated by a number of factors, such as increased levels of decoy receptors, FLIP (FLICE inhibitory protein), expression of anti-apoptotic Bcl-2 family members and IAP, and enhanced activity of NF-κB; each of these proteins targets a distinct stage of the apoptotic pathway (Almasan and Ashkenazi, 2003). I report here that prostate apoptosis response-4 (Par-4) is crucial for apoptosis downstream of TRAIL, as activation of caspase-8 following receptor engagement is regulated by Par-4.
Par-4 is a unique pro-apoptotic protein, which, upon ectopic over-expression, selectively induces apoptosis in transformed cells, but not in normal or immortalized cells (Ranganathan and Rangnekar, 2005). Two activating events responsible for susceptibility to Par-4-induced apoptosis are nuclear translocation of Par-4 and phosphorylation by PKA (Protein Kinase A). Indeed, phosphorylation of Par-4 at a critical residue by PKA accounts for the cancer selective apoptotic property of Par-4 (Gurumurthy et al., 2005). Par-4 is inactivated during tumorigenic transformation by a number of mechanisms, which include down-regulation by oncogenes, mislocalization and functional inactivation by the pro-survival kinase Akt (Goswami et al., 2005), deletion of the Par-4 chromosomal region, Par-4 promoter methylation, and nonsense mutation (Ranganathan and Rangnekar, 2005). Here, I report Par-4 is an essential component of the apoptotic cascade induced by TRAIL as Par-4 is intimately involved in activation of caspase-8 at the DISC during death receptor signaling.

Materials and Methods

Cell lines and reagents

Non small cell lung carcinoma cell H460 and, colon carcinoma cells, SW480 and HCT116, were purchased from ATCC, and cultured as per instructions. PC-3 cells have been previously described (Gurumurthy et al., 2005). TRAIL was purchased from Calbiochem.

Plasmids, small interfering RNA (siRNA) and transfection

Expression constructs for GFP, Par-4-GFP, and Par-4/155A-GFP, which acts as a dominant negative mutant of Par-4, ΔZip-GFP and 1-204-GFP were previously described (El-Guendy et al., 2003). Another dominant negative Par-4 mutant (DN-Par-4) was generated by PCR amplification of the region encoding amino acids 240-332 using rat Par-4 cDNA as the template and the primers (forward)
The primers included built-in EcoR I (forward) and Kpn I (reverse) sites (underlined). The amplified DNA fragment was cloned into pEGFP-N1 (EGFP, enhanced green fluorescent protein; Clontech) using the restriction enzymes mentioned above to generate DN-Par-4-GFP, with the EGFP tag at the C-terminus. Duplexes representing siRNA for Par-4 (L-004434-00) and a non-targeting control (D-001210-01) were from Dharmacon, Inc. Cells were transiently transfected with the indicated plasmids using lipofectamine reagent (Invitrogen Life Technologies, CA) as per manufacturer’s instructions, and appropriately processed 24 hours later. For RNA-interference, siRNA duplexes were transfected using Lipofectamine 2000 (Invitrogen Life Technologies, CA) according to manufacturer’s instructions for 72 hours before the indicated treatment.

**Western blot and antibodies**

Cells were harvested and subjected to SDS-PAGE followed by Western blot analysis using appropriate antibodies (Vasudevan et al., 2006). Antibodies for Par-4 (sc-1807), FADD (sc-5559), caspase-8 (sc-7890 and sc-5263), DR5 (sc-65314), Sodium Pottasium (Na⁺/K⁺) ATPase (sc-28800) and caveolin-1 (sc-894) were from Santa Cruz Biotechnology, Inc. Antibodies for cleaved caspase-8 (9496), cleaved caspase-3 (9664), phospho-Par-4/T163 (2329), and DR5 (3696) were from Cell Signaling, Beverly, MA. The antibody for actin was from Sigma-Aldrich, St. Louis, MO. HRP conjugated secondary mouse (NA931) and rabbit (NA934) antibodies were from Amersham/GE Healthcare, Buckinghamshire, UK.

**Apoptosis assay and immunocytochemistry**

Cells plated in chamber slides, were transfected with siRNA duplexes (for control or Par-4) or plasmids (expressing GFP control, Par-4-155A-GFP, or DN-Par-4-GFP), treated with TRAIL, and formalin fixed after the indicated times. The cells were
subjected to immunocytochemistry for Par-4, caspase-8, FADD, caveolin-1, ATPase or DR5 (sc-65314) with the respective antibodies, followed by secondary antibody conjugated to Alexa Fluor 488 (green fluorescence) or Alexa Fluor 594 (red fluorescence) from Molecular Probes, Invitrogen. Nuclei were stained with DAPI (4, 6, diamidino-2-phenyl indole for cyan fluorescence) from Vector Laboratories, Inc., Burlingame, CA. The cells were visualized via confocal, fluorescent microscopy to examine either co-localization, or nuclear condensation (a marker for apoptosis). A minimum of 200 cells were scored for apoptosis in each experiment, which was performed at least three times (Gurumurthy et al., 2005).

**Caspase activity assay**

Caspase-8 activity was assayed using CaspGLOW Red Active caspase-8 staining kit (K198-25) from Biovision, Inc., according to manufacturer’s instructions. Cells were assayed in a 96-well plate, and fluorescence read at 570 nm.

**Co-immunoprecipitation**

Cells were grown in 100-mm plates and following treatment, the cells were lysed for 20 mins in RIPA (Radio Immuno Precipitation Assay) buffer containing 10mM TRIS, pH 7.5, 150mM Sodium Chloride, 1% Sodium deoxycholate, 1% Triton X-100, 0.1% SDS and appropriate amount of protease inhibitor cocktail, on ice. The cell lysates were then centrifuged at high speed and the supernatant protein fraction was used for further processing. The proteins of interest were immuno precipitated for with 2 micrograms of the respective antibodies per milligram of total protein for 3 hours, followed by incubation with 75 microliters each of sepharose G beads (Amersham/GE healthcare, Buckinghamshire, UK) for 1 hour. The immuno precipitates were then washed thrice with RIPA buffer, boiled on SDS-PAGE sample loading buffer and run on SDS-PAGE for western blotting.
Results

Par-4 is a necessary component of TRAIL inducible apoptosis

I studied the requirement of Par-4 for TRAIL signaling in various cell lines: PC-3, SW480, H460 and HCT116. Endogenous Par-4 was silenced in these cells using siRNA (Fig. 2.1 A & B), and the cells were incubated with TRAIL and assayed for apoptosis. TRAIL induced apoptosis in each cell line tested, and this effect was attenuated by Par-4 knockdown (Fig. 2.1 A & B). PC-3 and SW480 are type I cells, while H460 and HCT116 generate a type II response to TRAIL, yet the effect of Par-4 was not restricted to either cell type. To further confirm that the loss of efficacy of TRAIL was a consequence of loss of Par-4 function, I transfected PC-3 and SW480 cells with two distinct dominant negative mutants of Par-4: DN-Par-4-GFP and Par-4/155A-GFP (Gurumurthy et al., 2005) Following TRAIL treatment, I measured apoptosis, and noted it was significantly reduced ($P < 0.001$) in the cells transfected with either one of the two dominant negative Par-4 mutants (Fig. 2.1 C).

Par-4 is necessary for activation of initiator caspase-8

As Par-4 is indispensable for apoptosis by TRAIL in both type I and type II cells, I hypothesized Par-4 must regulate a generic step common to both pathways. To identify precisely the stage at which Par-4 acts, I examined regulation of the downstream effector, caspase-3. I transfected PC-3 cells with non-specific siRNA or siRNA for either Par-4 or PKA and the cells were then incubated with TRAIL. I confirmed knock down of both proteins by either Par-4 expression or by PKA-dependent phosphorylation of the T163 residue on Par-4 by Western blot analysis, since PKA activates Par-4 by phosphorylation (Gurumurthy et al., 2005) (Fig. 2.2). As seen in Fig. 2.2, caspase-3 was activated following TRAIL treatment in the non-specific siRNA control cells, but was inactive when either Par-4 or PKA was silenced.
Figure 2.1: Loss of TRAIL apoptotic potential in the absence of Par-4

A

PC-3

% Apoptosis

Control Par-4 siRNA

UT TRAIL

siRNA: Control Par-4
Par-4
Actin

SW480

% Apoptosis

Control Par-4 siRNA

UT TRAIL

siRNA: Control Par-4
Par-4
Actin
Figure 2.1: Loss of TRAIL apoptotic potential in the absence of Par-4

**H460**

![H460 graph showing % Apoptosis for Control and Par-4 siRNA with UT and TRAIL bars]

**HCT116**

![HCT116 graph showing % Apoptosis for Control siRNA and Par-4 siRNA with UT and TRAIL bars]

**siRNA:** Control Par-4

![Par-4 and Actin Western blots for H460 and HCT116]
Figure 2.1: Loss of TRAIL apoptotic potential in the absence of Par-4.

A & B: Silencing of Par-4 using siRNA inhibits TRAIL inducible apoptosis: Type I PC-3 and SW-480 cells (A), or type II H460 and HCT116 cells were transfected with a non specific control or Par-4 siRNA and 48hrs later treated with TRAIL for an additional 24hrs. Apoptosis was analysed by nuclear condensation using DAPI. The results shown are a mean of three independent experiments and a representative western blot is shown for each cell line showing Par-4 knockdown with siRNA. The apoptosis results were statistically analyzed using two way anova and significance when compared to control is denoted by a * (p<0.001).
Figure 2.1: Loss of TRAIL apoptotic potential in the absence of Par-4
Figure 2.1: Loss of TRAIL apoptotic potential in the absence of Par-4.

C : Interfering mutants of Par-4 inhibit apoptosis by TRAIL: PC-3 and SW-480 cells were transfected with GFP, Dominant negative Par-4 or Par-4 155A constructs and treated with TRAIL 24hrs later. Apoptosis was analyzed by nuclear condensation using DAPI and the results were statistically analyzed using two way anova. The results shown are a mean of 3 independent experiments and statistical significance is denoted by * (p<0.001)
I next examined the impact of Par-4 on caspase-8 activation in response to TRAIL. Par-4 was knocked-down with siRNA in the type I cells, PC-3 and SW480, and the cells were then treated with TRAIL. Activation of caspase-8 was studied by Western blot analysis using a cleaved caspase-8 specific antibody, and by measuring the amount of active caspase-8 using a fluorescent substrate. The activation of caspase-8 that follows TRAIL treatment was compromised when Par-4 was knocked-down (Fig. 2.3 A & B). Abundant activation of caspase-8 does not occur in type II cells, as the very low levels of active caspase-8 formed at the DISC is insufficient to directly activate caspase-3. Instead, the active caspase-8 is adequate to trigger initiator caspase-9, thus the cascade proceeds via the mitochondrial amplification loop. Since apoptosis in type II cells requires Par-4, I examined whether activation of caspase-8 and caspase-9 is compromised in the absence of Par-4. The Par-4 knock-down and control H460 cells were exposed to TRAIL and initiator caspases-8 and -9 were checked for activation. Negligible activation of caspase-8 was observed in the Par-4 knock-down cells relative to the control cells, and this low activity was inadequate to trigger the mitochondrial amplification loop, as judged by the complete loss of caspase-9 activation in the absence of Par-4 (Fig. 2.3 C).

**Par-4 regulates membrane translocation of caspase-8 and DISC formation**

I analyzed specific early events in the TRAIL signaling pathway to evaluate the mechanism by which Par-4 regulates activation of caspase-8. Pro-caspase-8, which normally resides in the mitochondria, is recruited to the DISC during death ligand signaling (Qin et al., 2001). Pro-caspase-8 translocates to the cell membrane upon death receptor activation, and is recruited to this receptor by FADD for formation of the DISC, where pro-caspase-8 converts to active caspase-8. Since several lines of evidence, such as regulation of Fas trafficking by Par-4 (Ranganathan and Rangnekar, 2005), interactions of Par-4 with actin (Vetterkind et al., 2005), and Par-4 regulation of myosin light chain phosphorylation (Vetterkind and Morgan, 2008), indicate a role for Par-4 in controlling intracellular protein traffic, I investigated whether Par-4 is involved in the
Figure 2.2: Par-4 is necessary for activation of caspase-3

Par-4 inhibition inhibits caspase-3 activation by TRAIL: PC-3 cells were transfected with control, Par-4, or PKA siRNA, and 72hrs later treated with TRAIL for 6hrs. The cells were lysed, and Western blot analysis was done for Par-4, phospho Par-4, and active caspase-3. Actin was used as a loading control.
Figure 2.3: Par-4 is necessary for caspase-8 activation by TRAIL
Figure 2.3: Par-4 is necessary for caspase-8 activation by TRAIL

![Image of Western Blot](image)

siRNA: Control | Par-4

**Caspase 8 Activation - SW480**

- **UT**
- **15**
- **60**

![Bar Chart](image)

- Control
- Par-4 KD
Figure 2.3: Par-4 is necessary for activation of caspase-8 by TRAIL.

PC-3 (A) and SW-480 (B) cells were transfected with control or Par-4 siRNA, and, 72hrs later, treated with TRAIL for 1 or 3 hours (h). The cells were analyzed for caspase-8 activation by a fluorometric caspase activation assay and western blot. The blot was probed with antibodies specific for cleaved caspase-8, Par-4, and actin. The graph in the side panel shows the results of the caspase activation assay. The results are a mean from at least 3 independent experiments and statistical analysis was done by two way ANOVA. Statistical significance is denoted by ** (p<0.001) or * (p<0.01).
Figure 2.3: Par-4 is necessary for activation of caspase-8 by TRAIL

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C: Caspase-8 activation in the type II cells is inhibited in the absence of Par-4: H-460 cells were transfected with control or Par-4 siRNA, and, 72hrs later, treated with TRAIL for 1 or 3 hours (h). The cells were analyzed for Par-4 knock-down and caspase-8 activation by Western blot. Actin was used as loading control.
Figure 2.4: Par-4 facilitates membrane translocation of caspase-8 and FADD

A

TRAIL: UT 5mins 15mins

siRNA Control

siRNA Par-4

B

UT TRAIL UT TRAIL

FADD DR5 Par-4 Caveolin-1

siRNA: Control Par-4

Percentage of cells
**Figure 2.4 : Par-4 facilitates membrane translocation of caspase-8 and FADD**

A : PC-3 cells were transfected with control or Par-4 siRNA for 72hrs, and subsequently treated with TRAIL for the indicated time points. The cells were fixed, and immunostained for caspase-8 (shown in green) and Na+/K+ ATPase (Red). Representative confocal microscope images are shown for each time point and co-localization of caspase-8 with the marker at the membrane is indicated with arrows. The Western blot shows Par-4 knock-down with siRNA. The bar graph indicates the mean number of cells showing caspase-8 in the membrane of control and Par-4 knock-down cells. The results are a mean of 3 independent experiments and statistical significance as measured by two way ANOVA is denoted by * (p<0.001).

B : Fractionation analysis - The PC-3 cells were transfected with siRNA for Par-4 or a non-specific control, and, after 72hrs, treated with TRAIL for 15mins. The membrane was fractionated from the whole cell lysate, and analyzed by Western blot for FADD, DR-5, Par-4, and caveolin-1.
trafficking of DISC components to the membrane. I immunostained untreated or TRAIL-treated cells (with or without Par-4 knock-down) for pro-caspase-8 and Na⁺/K⁺ ATPase, a plasma membrane marker, to detect changes in caspase-8 localization. Caspase-8 translocated to the membrane upon TRAIL signaling only in cells with basal levels of Par-4, as apparent from co-localization with the membrane marker protein, and was reduced by about 50% at 15 minutes in the Par-4 knock-down cells (Fig. 2.4 A). I also looked at trafficking of FADD and DR5 to the cell membrane by Western blot analysis of membrane fractions. FADD recruitment to the membrane following TRAIL treatment was blocked in the Par-4 knock-down cells, while no change in membrane localization of DR5 was observed in the presence or absence of Par-4 (Fig. 2.4 B). As Par-4 is known to functionally interact with several proteins, I scrutinized Par-4 interaction with the caspase(s) that Par-4 regulates. Using PC-3 cell extracts, immunoprecipitations for Par-4, pro-caspase-8, FADD, and caspase-3 revealed Par-4 was easily detectable with both caspase-8 and FADD, but not with caspase-3 (Fig. 2.5 A), further substantiating Par-4 as a regulator of caspase-8 and FADD membrane trafficking. In order to evaluate which domain of Par-4 was responsible for this interaction, I transfected the PC-3 cells with two different mutants of Par-4, a ΔZIP-GFP mutant and another mutant containing the region of amino acids from 1-204-GFP, both of which lack the c-terminal leucine zipper motif, known for mediating protein-protein interactions and performed co-immunoprecipitations with caspase-8. Not surprisingly, I discovered that the leucine zipper domain was central to the interaction of Par-4 and caspase-8 as the mutants lacking the leucine zipper failed to co-immunoprecipitate with caspase-8 (Fig 2.5 B).

DR5 and DR4 have been reported to concentrate in the caveolin-rich lipid raft microdomains in the cell membrane during apoptotic signaling by TRAIL, while activation of NF-κB occurs from the non-raft localized death receptors in response to TRAIL (Song et al., 2007). Although DR5 continued to be localized to the membrane upon Par-4 silencing, I assessed whether Par-4 regulates passage of DR5 into the rafts. PC-3 cells were treated with control or Par-4 siRNA, exposed to TRAIL for the indicated
Figure 2.5: Interaction of Par-4 with caspase-8 and FADD

**A**

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**B**

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<table>
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<td>Δ-ZIP GFP</td>
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<tr>
<td>W.B. : Par-4</td>
</tr>
</tbody>
</table>
Figure 2.5: Interaction of Par-4 with caspase-8 and FADD

A: Par-4 interacts with pro-caspase-8 and FADD in PC-3 cells: PC-3 cells were immunoprecipitated with control, Par-4, FADD, and pro-caspase-8 antibodies, and the immunoprecipitates were analyzed for Par-4 by Western blot. Ten percent of the whole cell lysate used for each immunoprecipitation was used as input control. Additionally, in a similar experiment, the PC-3 cells were immunoprecipitated using control, Par-4, caspase-8, or caspase-3 antibodies, and analyzed for Par-4 by Western blot.

B: Identification of the domain of Par-4 binding to caspase-8: PC-3 cells were transfected with Par-4-GFP, ΔZIP-GFP or 1-204-GFP for 48h and then the cell lysates were immunoprecipitated with control, Par-4 and pro-caspase-8 antibodies. The immunoprecipitates were analyzed for Par-4 using Western blot.
time points, and immunostained for DR5 or caspase-8 and caveolin-1, a marker for lipid rafts. When cells with siRNA-mediated knock-down of endogenous Par-4 were treated with TRAIL, DR5 did not co-localize with caveolin-1. In contrast, in cells with control siRNA, TRAIL treatment stimulated movement (as early as 15 mins) of DR5 into the rafts, as seen from co-localization of DR5 with caveolin-1 (Fig. 2.6 A). Similarly, in the control cells caspase-8 was found to traffic to the caveolin-1-rich compartment (within 15 mins after TRAIL treatment), but not in the Par-4 knock-down cells. Interestingly, the number of cells with caspase-8 in the lipid rafts decreased gradually with time in the cells with basal levels of Par-4, coincident with the activation of caspase-8 (Fig. 2.6 B).

Stabilization of receptor aggregates following DISC formation is an essential mechanism among death receptors to ensure complete and profuse activation of the initiator caspase (Feig et al., 2007). The presence of endogenous Par-4 in the control cells allowed stabilization of the receptor aggregates for completion of the pathway, but formation of aggregates (within 5 mins) and quick dissolution of the complex (as early as 15 mins) was observed in the Par-4 knock-down cells (Fig. 2.7). All these observations together suggest a crucial role for Par-4 in the activation of initiator caspase-8 following TRAIL signaling.

Discussion

In this study I have identified a unique regulatory role for Par-4: facilitating activation of caspase-8 in the TRAIL apoptotic pathway. Par-4 mobilizes caspase-8 and FADD to the cell membrane, and is thus involved in the TRAIL signaling pathway at a very early stage. Since a basic, generic step such as activation of one of the most initial caspases, caspase-8 is regulated by Par-4, apoptosis in both the type I and type II cells depend on Par-4 function. Consequently, Par-4 also contributes to the major downstream molecular events associated with DISC formation, such as receptor movement to the rafts and receptor aggregation.
Figure 2.6: Par-4 regulates movement of DR5 into lipid rafts with TRAIL.
Figure 2.6: Par-4 regulates movement of caspase-8 into lipid rafts
**Figure 2.6**: Par-4 regulates trafficking of DR5 and caspase-8 to the lipid rafts:

A: PC-3 cells were treated with Par-4 or control siRNA, followed by TRAIL treatment for the indicated times. The cells were then fixed and immunostained for either DR5 (Green) along with Caveolin-1 (red), and visualized via confocal microscopy.

B: PC-3 cells were treated with control or Par-4 siRNA, followed by TRAIL treatment for the indicated times. The cells were then fixed and immunostained for either Caspase-8 (Green) along with Caveolin-1 (red), and visualized via confocal microscopy.
Figure 2.7: Destabilization of Death receptor aggregates in the absence of Par-4

PC-3 cells were transfected with control or Par-4 siRNA, and, after 72hrs, treated with TRAIL for the indicated time points. The cells were then fixed, and immunocytochemistry was performed for DR-5 (green) and Par-4 (red). In the representative fluorescent images shown for each time point, receptor clusters are indicated with arrows.
The TNF family of receptors can be classified as TNF like or CD95 like based on whether or not NF-κB activation is the predominant downstream event of receptor signaling. CD95/Fas, DR4 and DR5 preferentially activate caspase-8 subsequent to receptor activation (Peter, 2000) and fall into the same category. Several molecular events associated with DISC formation have been reported for Fas, as follows. As a first step, a number of ligand bound death receptor trimers cluster together to form SDS stable microaggregates where a low level DISC is formed by recruitment of FADD and caspase-8. This complex is relocated to the lipid rafts where, the receptor aggregates form large platforms for efficient activation of a large number of caspase-8 molecules. The caspase-8 that is activated by conformational change undergoes auto catalytic cleavage and is released into the cytoplasm (Boatright et al., 2003). Since TRAIL receptors and Fas display similar downstream signaling, it is possible that the associated minor molecular events are also comparable. Not surprisingly, I found formation DR5 clusters upon TRAIL signaling and a remarkable dissolution of the gathered clusters in the absence of Par-4. Par-4 may, however, enable stabilization of clusters consequent to caspase-8 activation, as reported for Fas (Algeciras-Schimnich et al., 2002) and not have a direct role in aggregation.

Soluble TRAIL is a popular cancer selective apoptotic agent; however, aggregated or antibody linked forms of TRAIL that are insoluble do cause apoptosis of certain normal cells owing to increased receptor aggregation by these forms of TRAIL (Almasan and Ashkenazi, 2003). The receptor Fas, on the other hand, is normally found as pre-associated micro aggregates. Such a discrepancy in receptor aggregation could be one of the underlying mechanisms of cancer selective apoptotic potential of TRAIL, which is again redolent of Par-4, given the effect of Par-4 on receptor aggregation in TRAIL treated cells (Fig. 2.7).

Although I detected some similarity in the downstream molecular events of both DR5 and Fas, TRAIL signaling, unlike FasL, selectively targets transformed cells. Par-4 is
actively involved in the Fas pathway as well; however, it is not known whether Par-4 has additional roles in the Fas pathway besides trafficking Fas to the cell membrane. Akt, a known inhibitor (Goswami et al., 2005) and substrate (Joshi et al., 2008) of Par-4, inhibits DISC assembly and caspase-8 recruitment during Fas mediated apoptosis in CD28 activated cells (Jones et al., 2002), pointing to a role for Par-4 in the process. Moreover, PI3K, the activator of Akt signaling inhibits CD95 receptor aggregation and caspase-8 cleavage at the DISC by modulating lateral diffusion of Fas (Varadhachary et al., 2001), and contributing to the resistance of Th2 lymphocytes to Fas mediated apoptosis (Varadhachary et al., 1999). Remarkably, loss of Par-4 favors Th2 type differentiation over Th1, in the CD4+ T helper lymphocytes (Lafuente et al., 2003); it would be interesting to determine whether all the effects of PI3K and Akt on the Fas pathway are attributable to Par-4. Nevertheless, it is tempting to speculate that Par-4 confers cancer selective apoptotic potential to TRAIL while being an obligatory member of the Fas pathway, at least in the cancer cells.

Par-4 is found in complex with caspase-8 and FADD in untreated PC-3 cells. A readily available complex would facilitate swift recruitment to the membrane upon signaling. However, it is not clear whether Par-4, FADD and caspase-8 are present in a single complex or discrete pools of Par-4 are associated with FADD and caspase-8 independently. It is also possible that the Par-4 – caspase-8 complex detected in the untreated PC-3 cells is the source of the basal level of active caspase-8 observed in these cells (Figure 2.3 A) and such a complex is not formed in other normal cell types and in vivo under normal physiological conditions.

Previous studies suggest a requirement for adaptors (e.g., FADD and TRADD), additional caspases (e.g., caspase-2 and -10) (Shin et al., 2005), or kinases (e.g., RIP, CK-1, and CK-2) for the activation of caspase-8 in response to death ligands, particularly TRAIL (Almasan and Ashkenazi, 2003; Wang et al., 2006). However, most of the work stipulating conditions of initial caspase activation in response to death ligands such as
TRAIL were performed in cells with ample Par-4, and, therefore, were not predisposed to discover a role for Par-4 in caspase-8 activation. Besides, very early studies of caspase-8 activation were done in vitro, where pro-caspase-8 molecules would have been readily available for activation and not lodged in a spatially distinct compartment (the mitochondria) that necessitates caspase-8 transport to its site of activation. In view of these circumstances, it is reasonable that the role of Par-4 as a vital component of the caspase-8 activation complex has remained undiscovered until now. In addition to regulating caspase-8 activation, Par-4 may also be required for completion of TRAIL apoptotic signaling, as Par-4 regulates many of inhibitors of apoptosis, such as c-FLIP (Gao et al., 2006), Bcl-2, and NF-κB (Ranganathan and Rangnekar, 2005). Certain epithelial tumors acquire resistance to TRAIL as a result of over-production of the cytokine IL-4 (Todaro et al., 2008), which is yet another target down-regulated by Par-4 (Lafuente et al., 2003). This implies Par-4 may be a crucial regulator of various facets of TRAIL signaling. Moreover, Par-4 is inactivated by Akt1 in certain cancer cells (Goswami et al., 2005) that are resistant to TRAIL, and Par-4 is activated by microtubule- and Akt-inhibitors, as well as doxorubicin (Goswami et al., 2005; Gurumurthy et al., 2005), all of which are known to sensitize resistant cancer cells to apoptosis by TRAIL (Wu et al., 2004). Hence it is highly probable that Par-4 is yet another novel entity that contributes to TRAIL resistance among some tumors.

With this newly discovered Par-4 requirement in TRAIL signaling, therapeutic strategies can be revisited, taking into consideration that Par-4 is frequently inactivated by kinases and oncogenes in cancer. As Par-4 induces apoptosis selectively in cancer cells, specifically activating Par-4 would be a prudent way to sensitize tumors to TRAIL, thereby making the combination treatment a relatively non-toxic, therapeutic option.

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Chapter Three

Regulation of Par-4 by Caspases

Introduction

Apoptosis by TRAIL involves activation of both initiator and effector caspases, which target various death substrates to complete the apoptotic program. Effector caspases, such as caspases -3, -6 and -7, are principal proteases that structurally and functionally dismantle the cell by breaking down death substrates, which include cytoskeletal proteins, nuclear matrix components, and other proteins essential for the survival. Additionally, certain proteins, such as CAD (Caspase Activated DNase), Acinus, DEDD (an apoptosis inducer that translocates from cytoplasm to the nucleolus), that participate actively in apoptosis, as well as kinases such as PAK (p21 Activated Kinase), FAK (Focal Adhesion Kinase) and PKC are activated by caspases for apoptotic or non-apoptotic purposes (Earnshaw et al., 1999; Lee et al., 2002; Sahara et al., 1999).

Numerous substrates have been identified for effector caspases, yet the initiator caspases, especially caspase-8, have only a limited substrate repertoire. Among those reported, caspase-3, Bid, RIP (Receptor Interacting Protein), and plectin are the prime substrates of caspase-8 (Stegh et al., 2000). Caspase-9, on the other hand, acts on Gelsolin, Vimentin (Nakanishi et al., 2001), Raf-1 (Cornelis et al., 2005) and Rb (Lemaire et al., 2005), in addition to its proverbial substrates, the effector caspases. Thus, the initiator caspases take on dual roles, that of an initiator by activating effector caspases and that of an effector by acting on non-caspase substrates.

Par-4 is a regulator of apoptosis in that it promotes Fas trafficking to the cell membrane, and inhibits the anti-apoptotic NF-κB activity. In addition, Par-4 helps augment the apoptotic signal via the mitochondrial amplification loop by down-regulating Bcl2 (Cheema et al., 2003). The functional core of Par-4, the SAC domain causes selective apoptosis of cancer cells and confers tumor resistance in transgenic mice. Here I report that Par-4 is actively regulated along the TRAIL pathway in two different ways: (1) Enhanced nuclear translocation, and (2) Endogenous generation of a
SAC-like domain by caspase mediated cleavage. Incidentally, Par-4 activation serves as a molecular nexus for the extrinsic and intrinsic apoptotic pathways in that it allows unhindered progress of cell death.

**Materials and Methods**

**Cell lines and Reagents**

Androgen independent prostate cancer cells PC-3 were previously described (El-Guendy et al., 2003). Human lung cancer cells H460 were bought from ATCC and maintained as per instructions. TRAIL was purchased from Calbiochem. Active recombinant human caspase-8 and specific caspase inhibitors were purchased from Biovision Research Products, Ca.

**Plasmid constructs, siRNA and transfection**

GFP vector and Par-4-GFP (C terminal) constructs were described previously (El-Guendy et al., 2003). The D123 to 123A, D200 to 200A, D209 to 209A, S124 to 124A, and S124 to 124D point mutants of Par-4 were made by site directed mutagenesis, thereby changing the sequence at D 123 and 200 from GAT to GCT (Alanine), S 124 from TCC to GCT (Alanine) or GAC (Aspartic acid). siRNA for caspase-8, caspase-9 and the non-targeting control were from Santa Cruz Biotechnology, Inc. Cells were transiently transfected with the indicated plasmids using lipofectamine reagent (Invitrogen Life Technologies, CA) as per manufacturer’s instructions, and appropriately processed 24 hours later.

**Antibodies and other reagents**

Cells were harvested and subjected to SDS-PAGE followed by Western blot analysis using appropriate antibodies as described (Vasudevan et al., 2006). Rabbit polyclonal antibody for Par-4 (sc-1807) was from Santa Cruz Biotechnology, Inc.
Antibodies for cleaved caspase-8 (9496) and caspase-9 (9502) were from Cell Signaling, Beverly, MA. The antibody for actin was from Sigma-Aldrich, St. Louis, MO. HRP conjugated secondary mouse (NA931) and rabbit (NA934) antibodies were from Amersham/GE Healthcare, Buckinghamshire, UK.

**In vitro caspase cleavage assay**

GST or GST-Par-4 purified from *E. coli* were incubated with the specified units of recombinant active caspase-8 for 1 hour at 37°C in reaction buffer containing 50mM Hepes pH 7.2, 50mM NaCl, 0.1% CHAPS, 10mM EDTA, 5% Glycerol and 10mM DTT. The mix was then subjected to Western blot analysis.

**Apoptosis assay and immunocytochemistry**

Cells were plated in chamber slides, transfected with the indicated plasmids (expressing GFP control or Par-4-GFP), treated with caspase inhibitors and TRAIL, and formalin fixed after the indicated times. The cells were subjected to immunocytochemistry for Par-4 followed by secondary antibody conjugated to Alexa Fluor 594 (red fluorescence) from Molecular Probes, Invitrogen. Nuclei were stained with DAPI (4, 6, diamidino-2-phenyl indole for cyan fluorescence) from Vector Laboratories, Inc., Burlingame, CA. The cells were visualized via confocal, fluorescent microscopy to examine localization and nuclear condensation as a measure of apoptosis. A minimum of 200 cells were counted in each experiment, which was performed at least three times (Gurumurthy et al., 2005).

**Results**

**Caspase-8 dependent nuclear translocation of Par-4 by TRAIL**

Par-4 is present primarily in the cytoplasm in cancer cells under non-apoptotic conditions. Ectopically over-expressed Par-4 localizes to the nucleus and the apoptotic
events that ensue are particularly dependent on this localization (El-Guendy et al., 2003). Since Par-4 plays an essential role in TRAIL inducible apoptosis, I asked whether endogenous Par-4 in PC-3 cells would translocate to the nucleus in response to TRAIL. Par-4 localization changed from that of cytoplasmic to nuclear in 24h post-TRAIL treatment (Figure 3.1 A), and the cells were shrunken in addition to reduction in cell numbers, indicating apoptosis. To further corroborate the change in Par-4 localization, I performed fractionation studies on untreated or TRAIL treated cells. Par-4 was present in both the cytoplasm and nucleus in untreated cells; however the nuclear levels of Par-4 increased within 12h of TRAIL exposure (Figure 3.1 B). Since apoptosis is not apparent at this time point, these data provide additional evidence that Par-4 translocation to the nucleus precedes execution of cell death, as previously established (El-Guendy et al., 2003).

In order to better study the dynamics of TRAIL mediated nuclear translocation of Par-4, I used a competitive, irreversible, peptide inhibitor of caspase-8. PC-3 cells were pre-treated with the inhibitor, and subsequently treated with TRAIL. A significant reduction in the number of apoptotic cells, as well as cells with nuclear Par-4 was observed when caspase-8 was inhibited following TRAIL treatment, as compared to the control with uninhibited activity of caspase-8 (Figure 3.2 A). Representative fluorescent microscopy images are shown in Figure 3.2 B.

Since Par-4 plays an important role in caspase-8 activation, it is possible that the nuclear translocation of Par-4 that is affiliated with caspase-8 activity is merely a facet of the TRAIL signaling pathway. To investigate whether, nuclear localization of Par-4 presents an appearance of being regulated by caspase-8 because of Par-4’s involvement in the TRAIL signaling pathway, or whether it is actively regulated by caspase-8, PC-3 cells were transfected with Par-4, treated with either caspase-8 or caspase-3 inhibitors, and 24h later, quantitatively analyzed for apoptosis. Apoptosis of the Par-4 transfectants strictly correlated with nuclear translocation of Par-4; conversely, upon
Figure 3.1: TRAIL induces nuclear translocation of Par-4

A

Vehicle

TRAIL

Par-4 – Green; Nucleus - Red

B

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<th>Nuclear fraction</th>
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</table>
Figure 3.1: TRAIL induces nuclear translocation of Par-4

A: PC-3 cells were treated with Vehicle or TRAIL for 24h, stained for Par-4 (green) and the nucleus (DAPI shown in Red) and visualized using a fluorescent microscope.

B: Cytoplasmic and nuclear fractions were isolated from TRAIL treated PC-3 cells and analyzed for Par-4 localization using western blot. GRP-78 and MnSOD were used as cytoplasmic markers and Lamin was used as the nuclear marker.
Figure 3.2: TRAIL mediated nuclear import of Par-4 is caspase-8 dependent

A

Apoptosis with TRAIL and caspase inhibitors

% Apoptosis and Nuclear Par-4

Vehicle Vehicle + Caspase8 inhibitor TRAIL TRAIL + Caspase8 Inhibitor

Apoptosis Nuclear Par-4

B

TRAIL Caspase-8 Inhibitor + TRAIL
Figure 3.2: TRAIL mediated nuclear import of Par-4 is caspase-8 dependent

A: PC-3 cells were treated with caspase-8 inhibitor for 6h, followed by treatment with vehicle or TRAIL for 12h and analyzed for apoptosis (measured by nuclear condensation using DAPI) and co-related with the number of cells with nuclear Par-4.

B: Representative pictures of TRAIL treated cells, with or without caspase-8 inhibitor. The nuclear DAPI staining is shown in red and immunostaining for Par-4 is shown in green.
inhibition of caspase-8 activity, both nuclear localization and apoptosis by Par-4 were attenuated. Inhibition of caspase-3 activity, however, allowed copious nuclear entry of Par-4 although apoptosis was blocked (Figure 3.3) indicating that nuclear import of Par-4 is an episode dynamically regulated by caspase-8 that precedes cell death.

Akt-1 is the major regulator of the nuclear – cytoplasmic shuttling of Par-4 in the cancer cells. Akt-1 mediated phosphorylation of Par-4 generates a binding site for the adaptor protein 14-3-3 θ, which interacts with and sequesters Par-4 in the cytoplasm by way of promoting cell survival (Goswami et al., 2005). Since caspase-8 regulates the nuclear translocation of Par-4, I decided to test the involvement of Akt-1 in this process. To examine whether Akt-1 is a direct substrate of caspase-8, PC-3 cells were treated with TRAIL for various time points (up to 24h), and analyzed for possible Akt-1 cleavage. TRAIL treatment did not alter Akt-1 levels significantly (Figure 3.4 A) although the minor reduction in protein amounts observed may be attributed to cell death and cleavage of Akt-1 by caspase-3 (Jahani-Asl et al., 2007).

I next examined the effect of TRAIL on 14-3-3 θ, as this particular protein has previously been reported to be an apoptotic target of many caspases, including caspase-8 (Nomura et al., 2003). A considerable decrease in protein amounts of 14-3-3 θ was noticed as early as 1h after TRAIL treatment, and at 12h, the protein was almost completely broken down signifying involvement in caspase-8 dependent release of Par-4 into the nucleus (Figure 3.4 B). The cells used in this study are cancer cells, which have high levels of PKA activity and therefore, an active form of Par-4; the only other requirement is for Par-4 to be translocated into the nucleus which is fulfilled by caspase-8 through 14-3-3 θ cleavage, thus promoting amplification of the TRAIL apoptotic signal.

Identification of Par-4 as a novel substrate of Caspases

In addition to the mutual regulation of Par-4 nuclear translocation by caspase-8 and transport of pro-caspase-8 to the plasma membrane by Par-4 (Chapter 2),
Figure 3.3: Nuclear Import of Par-4 depends on caspase-8 activity

PC-3 cells were transfected with GFP-Par-4 and treated with caspase-8 or caspase-3 inhibitors for 24h. The cells were then fixed and apoptosis was analyzed using nuclear condensation as measured by DAPI and co-related with nuclear Par-4.
**Figure 3.4**: Cleavage of 14-3-3 θ in response to TRAIL allows nuclear translocation of Par-4

**A**

<table>
<thead>
<tr>
<th>TRAIL:</th>
<th>0h</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
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**B**

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<td><img src="image20.png" alt="Actin Band" /></td>
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**Figure 3.4**: Cleavage of 14-3-3 in response to TRAIL allows nuclear transport of Par-4

A: Lysates of PC-3 cells treated with TRAIL for the indicated time points were analyzed for Akt expression levels by western blot. Actin was used as a loading control.

B: TRAIL treated PC-3 cell lysates were analyzed by western blot for expression levels of the adapter protein 14-3-3θ.
additional outcomes of the interaction of Par-4 with pro-caspase-8 (Chapter 2, Figure 2.5) are possible as well. As it is not clear whether Par-4 dissociates from caspase-8 following membrane translocation of the inactive zymogen, I examined the possibility that Par-4 could still be associated with the caspase following activation. Close association with an active protease implies an enzyme—substrate relationship and I inquired whether that was the case with caspase-8 and Par-4. Caspases choose their substrates based on consensus recognition and cleavage sequences; careful inspection of the Par-4 protein sequence revealed the presence of four putative caspase consensus sites (sequences specified in Table 3.1) at aspartic acid residues 123, 200, 209 and 287 (Figure 3.5). Cleavage of Par-4 at each of these sites would generate fragments of various sizes as described in Table 3.2. However, given that aspartic acids 200 and 209 are in close proximity, differences in fragment sizes will not be apparent if either or even both of the sites are utilized.

With this information in hand, I asked whether Par-4 was, in fact, a direct substrate of caspase-8 in vitro using recombinant caspase-8 and GST-Par-4. GST-Par-4 was incubated with the indicated amounts of recombinant caspase-8, and the integrity of Par-4 protein was analyzed by Western blot. Two major fragments of approximate sizes 25kd and 15kd, and a minor one of roughly 20kd were cleaved off from GST-Par-4 when incubated with caspase-8 (Figure 3.6 A). Based on the computed fragment sizes (Table 3.1), the three fragments might represent cleavage at the three residues 123, 200/209, and 287. In order to verify that Par-4 cleavage by caspase-8 is an authentic process that takes place in the cells, I activated endogenous caspase-8 in the PC-3 cells with TRAIL and examined the effect on Par-4 24h later. Two fragments of Par-4, similar to those produced by recombinant caspase-8 in vitro were generated with TRAIL treatment (Figure 3.6 B), pointing towards a role for TRAIL signaling in Par-4 break down.

So as to ascertain the role of caspase-8 in the cleavage of Par-4 in vivo along the TRAIL pathway, I pre-treated the PC-3 cells with caspase-8 and caspase-3 inhibitors and
Figure 3.5: Caspase-8 consensus sites in Par-4

- 123
- 200/209
- 287
- 332

- 40 Kd

- 123
- 200/209
- 287
- 332

- 23 Kd
- 20/209
- 201/210
- 15 Kd

- 123
- 124
- 200/209

- 14 Kd
- 9 Kd
Figure 3.5: Caspase consensus sites in Par-4:

Four different caspase-8 cleavage consensus sequences were found in Par-4, at aspartic acid residues 123, 200, 209 and 287. Cleavage of Par-4 at each of these sites will result in fragments of the indicated sizes. Among these, the 9kd fragment is of interest because it contains the functional core of Par-4.
Table 3.1: Putative caspase consensus sequences in Par-4

<table>
<thead>
<tr>
<th>Aspartate residue (Rat, Human)</th>
<th>Rat Sequence</th>
<th>Human Sequence</th>
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<tr>
<td>123, 134</td>
<td>EEP D</td>
<td>EEP D</td>
</tr>
<tr>
<td>200, 211</td>
<td>SLP D</td>
<td>NLL D</td>
</tr>
<tr>
<td>209, 220</td>
<td>LPQ D</td>
<td>LLQ D</td>
</tr>
<tr>
<td>287, 298</td>
<td>LMQ D</td>
<td>LMQ D</td>
</tr>
</tbody>
</table>

Table 3.1: Caspase consensus sequence in human and rat Par-4.

The caspase consensus sites in rat and human Par-4 and the corresponding consensus sequence.
Table 3.2: Possible Caspase cleavage fragments in Par-4

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Size</th>
<th>Domains</th>
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</thead>
<tbody>
<tr>
<td>Full length</td>
<td>38 KD</td>
<td>SAC, LZ</td>
</tr>
<tr>
<td>1-287</td>
<td>33 KD</td>
<td>SAC</td>
</tr>
<tr>
<td>124 – 332</td>
<td>24 KD</td>
<td>SAC, LZ</td>
</tr>
<tr>
<td>1 – 200</td>
<td>23 KD</td>
<td>SAC</td>
</tr>
<tr>
<td>124 - 287</td>
<td>19 KD</td>
<td>SAC</td>
</tr>
<tr>
<td>201 – 332</td>
<td>15 KD</td>
<td>LZ</td>
</tr>
<tr>
<td>1 – 123</td>
<td>14 KD</td>
<td>--</td>
</tr>
<tr>
<td>201 - 287</td>
<td>10 KD</td>
<td>--</td>
</tr>
<tr>
<td>124 – 200</td>
<td>9 KD</td>
<td>SAC</td>
</tr>
<tr>
<td>288 - 332</td>
<td>5 KD</td>
<td>LZ</td>
</tr>
</tbody>
</table>

Table 3.2: Caspase cleavage sites and fragments in Par-4.

A complete list of the various possible caspase cleavage products of Par-4 with their respective sizes and the major functional domains is given. The fragment length and sizes are indicated for rat Par-4; the molecular weight of human Par-4 fragments would be approximately 2kd higher.
Figure 3.6: Par-4 is a substrate of caspase-8 \textit{in vitro} and \textit{in vivo}

A

GST-Par-4: + + +

Caspase 8: - 2.5 U 5 U

B

TRAIL: - +

23kd

14kd
Figure 3.6: Par-4 is a substrate of caspase-8 \textit{in vitro} and \textit{in vivo}

A: \textit{In vitro} cleavage of Par-4 by caspase-8 - 1µg of GST-Par-4 was incubated with the indicated amounts of recombinant caspase-8 for 1h at 37°C and analyzed for Par-4 cleavage by western blot.

B: PC-3 cells were left untreated or treated with TRAIL for 24h and analyzed by western blot.
followed up with TRAIL. Subsequent to TRAIL treatment, an N terminal cleavage producing a 14kd fragment presumably containing the region from 1-123, susceptible to inhibition by caspase-8 but not caspase-3 inhibitor, was observed (Figure 3.7). The 23kd product was present constitutively in all the samples, suggesting this cleavage was not mediated by caspase-8, which is activated specifically in response to TRAIL. Although caspases are expected to maintain stringent criteria with regard to consensus sequence recognition, instances of promiscuous activity are not unheard of. Hence, I considered other caspases that might be responsible for generation of the 23kd fragment.

The mitochondrial apoptotic pathway is involved in spontaneous apoptosis of cells due to ‘culture shock’ and induces a basal level of caspase-9 activity in certain cells (Bailly-Maitre et al., 2002; Pennati et al., 2004). Since about 10% of PC-3 cells undergo spontaneous apoptosis in culture, these cells may tolerate low levels of caspase-9 activity. Moreover, these cells exhibit a Type I response to TRAIL, and as a result, caspase-9 activity is not enhanced beyond the basal level even upon treatment; this may account for the constitutive, unaltered presence of the 23kd fragment in these cells. In order to test this possibility, I used a siRNA approach on the H460 cells, which suffer a Type II response to TRAIL. I used siRNA to knock-down caspases -8 and -9 from these cells which was verified by western blot. The cells were then treated with TRAIL for the indicated time frames and analyzed for Par-4 cleavage by western blot. TRAIL treatment of the control cells resulted in generation of the two distinct 23kd and 14kd fragments of Par-4. As expected, knock-down of caspase-8 led to inhibition of the N-terminal cleavage but, did not affect the 23kd cleavage product. Caspase-9 knock-down, on the other hand, inhibited generation of the 23kd fragment, indicating a specific role for the two different caspases in Par-4 break down (Figure 3.8).
Figure 3. 7: Par-4 cleavage by caspase-8 in response to TRAIL

<table>
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<tr>
<th></th>
<th>IETD</th>
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<tr>
<td>TRAIL</td>
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</tr>
</tbody>
</table>

23KD

14KD
Figure 3. 7 : Par-4 cleavage by caspase-8 in response to TRAIL

PC-3 cells were treated with vehicle, caspase-8 inhibitor (IETD) or caspase-3 inhibitor (DEVD) for 6h followed by TRAIL for 24h. The cell lysates were then analyzed by western blot for Par-4 cleavage.
Figure 3.8: Collaboration of Caspases -8 and -9 to generate Par-4 functional fragment
Figure 3.8: Collaboration of Caspases -8 and -9 to generate Par-4 functional fragment

H460 cells were transfected with siRNA for Caspase-8, Caspase-9 or non-specific controls for 48h. The cells were then treated with TRAIL for 3h or 6h or left untreated and the lysates were examined on a western blot for cleavage of Par-4. Knockdown of Caspases -8 and -9 were checked by western and Actin was used as the loading control.
Delineation of the sites of caspase cleavage in Par-4

Having clarified that Par-4 is unmistakably cleaved into at least two fragments by caspases -8 and -9 in response to TRAIL (and possibly other apoptotic stimuli), our next aim was to define the actual sites in Par-4 responsible for the generation of these fragments. According to the putative caspase site estimate (Table 3.1), I determined that residues 123 and 200/209 were the ones most likely to generate fragments of sizes 24/23 kd and 15/14 kd. Point mutations in GFP-Par-4 cDNA were made so as to change each of these residues from aspartic acid to alanine and following transfection of PC-3 and H460 cells with the mutants, GFP-Par-4/D123A, GFP-Par-4/D200A and GFP-Par-4/D209A, I analyzed them for cleavage. The D123A mutant failed to generate the 14 kd fragment in both PC-3 and H460 cells, whereas the 24/23 kd product was intact (Figure 3.9 A, B). All the fragments observed in wild type Par-4 were also obtained from the D209A mutant, indicating this was not a genuine site (Figure 3.9 A). On the contrary, when the Type II H460 cells were transfected with the mutants, then treated with TRAIL, the 23kd fragment was absent from the D200A transfectants, in addition to a decreased amount of the 14kd product (Figure 3.9 B). Interestingly, the D200A mutant despite an intact aspartic acid at 123, yielded only to an inefficient cleavage at this site, giving rise to a weak 14kd fragment. It is evident from these data that caspase-8 cleaves Par-4 at amino acid 123 in the N terminus, generating a 14kd fragment containing the region from 1-123, while caspase-9, responsible for the 23kd fragment cleaves at residue 200 to produce the fragment comprised of amino acids 1-200.
Figure 3.9: Caspases cleave Par-4 at Aspartate residues 123 and 200

A

Par-4  D123A  Par-4  D209A

23kd

14kd

B

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>TRAIL</th>
</tr>
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<tbody>
<tr>
<td>GFP</td>
<td>Par-4</td>
<td>Par-4</td>
</tr>
<tr>
<td>123A</td>
<td>200A</td>
<td>123A</td>
</tr>
<tr>
<td>23Kd</td>
<td>14Kd</td>
<td>23Kd</td>
</tr>
</tbody>
</table>

23Kd

14Kd
Figure 3.9: Caspases cleaves Par-4 at aspartate 123 and 200

A: PC-3 cells were transfected with GFP-Par-4 or GFP-Par-4/D123A for 24h and the cell lysates were analyzed by western blot using Par-4 antibody. In a similar experiment, H460 cells were transfected with GFP-Par-4 and GFP-Par-4/D209A and analyzed by western blot.

B: H460 cells were transfected with GFP-Par-4, GFP-Par4/D123A or GFP-Par-4/D200A mutants for 24h and the cell lysates were analyzed by western blot using Par-4 antibody.
Regulation of cleavage by phosphorylation

A casual glance at the Par-4 protein sequence reveals the presence of a Casein Kinase-2 (CK-2) phosphorylation site immediately after the caspase-8 consensus at residue 124 (Figure 3.10A). Since CK-2 is known to play a role in regulating TRAIL inducible apoptosis (Ravi and Bedi, 2002), I was interested in finding out whether this site would regulate cleavage of Par-4 as well. To this end, an unphosphorylatable mutant, GFP-Par-4/S124A and a phospho-mimetic mutant GFP-Par-4/S124D were tested for caspase-8 based cleavage in the PC-3 cells. The S124A mutant was subject to cleavage even by the basal activity of caspase-8 present in the untreated PC-3 cells, whereas the phospho-mimetic mutant S124D was not cleaved in spite of higher caspase-8 activity upon TRAIL signaling (Figure 3.10B). This suggests CK-2 may regulate access of caspase-8 to the site at 123 as CK-2 site phospho-mimetic form of Par-4 is not cleaved while unphosphorylated molecules of Par-4 are a subject of cleavage.

Generation of the functional core of Par-4 by cleavage

Given that every cellular activity serves a purpose, I examined the functional significance of Par-4 cleavage at the two sites 123 and 200. It is apparent from the Par-4 sequence that cleavage at both these sites will generate the fragment 124-199 that effectively contains the functional core of Par-4 (Figure 3.11A), the SAC domain and I examined whether such cleavage indeed occurs. The H460 cells were chosen because they are Type II and would stimulate profuse activation of caspase-9, as well as caspase-8. I observed the appearance of the 14kd fragment as early as 3 hours following TRAIL treatment and increase in the 23kd cleavage product starting at 6 hours. A 9kd fragment of Par-4 was observed at 24 hours, suggesting sufficient cleavage at both 123 and 200 ultimately results in accumulation of the possible SAC fragment (Figure 3.11B).
Figure 3.10: Cleavage of Par-4 by caspase 8 is determined by phosphorylation.
Figure 3.10: Cleavage of Par-4 by caspase-8 is determined by phosphorylation

A: The N-terminal caspase-8 consensus is followed by a Casein kinase-2 phosphorylation site as shown in the schematic; this phosphorylation likely regulates substrate specificity for caspase-8.

B: PC-3 cells were transfected with GFP-Par-4, GFP-Par-4/124A or GFP-Par-4/124D for 24h and analyzed by western blot using Par-4 antibody for cleaved fragments of Par-4.
Figure 3.11: Possible generation of the functional core fragment, SAC, of Par-4 by caspases.

A

1 123 200 287 332

14KD SAC 9KD LZ 15KD

23KD

B

TRAIL: 0h 1h 3h 6h 12h 24h

Par-4

23kd 14kd 9kd
Figure 3.11: Possible generation of the functional core of Par-4 by caspases

A: Schematic of Par-4 primary structure with representations of caspase cleavage sites and resulting fragment sizes.

B: H460 cells were treated with TRAIL for the indicated time points and analyzed for Par-4 cleavage by western blot.
Discussion

TRAIL is a widely recognized cancer selective killer agent and extensive research is underway to examine the pathways and mechanisms operating downstream of this cytokine. I have demonstrated here that Par-4, another protein that causes cancer selective apoptosis upon over expression, is actively regulated along the TRAIL pathway. TRAIL signaling increases nuclear import of Par-4 and results in cleavage of Par-4 into its active form, both regulated chiefly by caspase-8.

Par-4 translocation to the nucleus is increased with TRAIL signaling in approximately 12 hours following treatment. The structural organization of the cell and nucleus is destroyed as a result of the breakdown of cytoskeletal proteins such as actin, fodrin and gelsolin, and the nuclear matrix protein lamin (Philchenkov et al., 2004). As a result of this breakdown, the nuclear/cytoplasmic barrier may be disrupted in a caspase dependent manner leading to passive diffusion of proteins into the nucleus. However, in the case of Par-4, increase in nuclear amounts is seen earlier than lamin B break down (Figure 3.1 B). Additionally, inhibition of caspase-3 activity, which is responsible for lamin B cleavage, does not affect Par-4 translocation suggesting Par-4 is being actively transported to the nucleus in response to TRAIL. The kinase Akt is known to be an important arbitrator of TRAIL sensitivity (Chen et al., 2001) and here, cleavage of 14-3-3 θ, an Akt signaling intermediate, by caspase-8 was found to be highly co-relative to the increase in Par-4 nuclear import suggesting Par-4 might be a key component of Akt mediated TRAIL resistance in various cell types.

Par-4 is identified as a novel substrate of caspases that undergoes cleavage during apoptosis. In addition I identified four putative consensus caspase sequences in Par-4 and verified two to be genuine sites responding to caspases -8 and -9. Although in vivo the N terminal site alone is subject to cleavage by caspase-8, in vitro, more than one site was recognized and cleaved by caspase-8, forming 3 fragments of sizes 23, 19,
and 14 KD. This may be due to the fact that caspases display promiscuous, overlapping activity in the presence of non limiting concentrations of substrate. The residue 287 is a genuine caspase-8 consensus (LXXD) and is most likely cleaved by caspase-8 under conditions that are similar to those that support cleavage at the N terminus. The amino acids from 287 until 332 (the end of the protein) include the leucine zipper, which mediates most of the protein-protein interactions of Par-4, including that with caspase-8. Cleavage at this site would confer enormous advantage to an apoptotic cell because Par-4 can be relieved from certain inactivating and non-apoptotic interactions such as with Akt-1 and Topoisomerase-1. I did not, however, study this site because our primary interest was in the doubly efficient, functional core of Par-4, the SAC domain. Moreover, cells that are resistant to the over expression of full length Par-4 are sensitive to mutants that are deleted for most of the C terminal amino acids i.e. SAC and 1-204 (El-Guendy et al., 2003). Since cleavage at 287 would effectively eliminate only the leucine zipper, I did not pursue this further.

Caspases are primarily involved in dismantling the cell during apoptosis, although certain caspase targets are known to be activated by cleavage. Here, the cleavage of Par-4 by caspases -8 and -9 should be considered as activating since a Par-4 molecule that has undergone cleavage by both caspases would essentially become SAC (124-199). The SAC domain has been demonstrated in various cell culture (El-Guendy et al., 2003) and animal (Zhao et al., 2007) models to efficiently cause apoptosis and cancer resistance. The SAC domain, once produced, provides the cell with an additional thrust into the apoptotic pathway, as it is capable of inhibiting pro-survival NF-kB activity (El-Guendy et al., 2003). Generation of SAC can be considered as the point of no return for a cell en route apoptosis; therefore, it is not surprising that two different caspases from distinct apoptotic pathways collaborate to produce this fragment. Isolated activation of either caspase will not generate SAC and this probably acts as a failsafe mechanism to guard the cells against unwarranted apoptosis. Although caspase -8 and -9 share
substrates such as effector caspase-3, there are no known instances of co-operation between the two as that reported here with Par-4.

Another interesting issue regarding the generation of the 124-199 fragment by the two caspases is the sequence of events. In the Type II H460 cells, caspase-9 is not active to begin with; activation of caspase-8 leads to caspase-9 activation via Bid cleavage and mitochondrial cytochrome c release. Therefore in these cells, Par-4 may not be cleaved at residue 200 prior to being cleaved at 123. In the Type I PC-3 cells, in contrast, caspase-9 is not activated in response to TRAIL, yet Par-4 is cleaved at residue 200 by the basal activity of caspase-9 and low amounts of the 23kd fragment, which does not increase with TRAIL, are observed. This 23kd fragment, however, cannot become a substrate of caspase-8, as it lacks the leucine zipper domain that is responsible for Par-4 – caspase-8 interaction. This again, could be a safeguard to protect the cells against apoptosis in response to minor stress that might activate caspase-9. Despite the stipulation that 123 and 200 be cleaved in that sequence in order to generate 124-199, the 23kd fragment occasionally appears to be accumulating when cleavage at 123 is inhibited. This could simply be a function of the stoichiometric availability of Par-4 protein under conditions of caspase-8 deficiency rather than the product of caspase-9 becoming a substrate of caspase-8.

In essence, Par-4 is regulated along the TRAIL pathway, both by being transported into the nucleus and by caspase mediated cleavage to endow the cells with uninhibited apoptotic activity.

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Chapter Four

Summary and Future Directions

Par-4 is a unique pro-apoptotic, tumor suppressor known for causing selective apoptosis of cancer cells and, in this study, I report a novel role for endogenous Par-4 in the extrinsic apoptotic pathway induced by TRAIL. Par-4 is present both in the cytoplasm and nucleus in cancer cells and its function is tightly regulated by localization. Moreover, Par-4 has a modular structure – function relationship, with discrete domains contributing to distinct functions. Although many key discoveries have been made concerning Par-4, the majority of the data was obtained from ectopic over-expression systems. Hence, this work sought to establish the role of endogenous Par-4 in apoptosis and identified that Par-4 is an essential component of the TRAIL induced extrinsic pathway besides being cleaved by caspase-8 and translocated to the nucleus.

Par-4 is intimately involved in trafficking caspase-8 to the cell membrane upon death receptor signaling, facilitating caspase-8 activation, as a result. I also discovered that a greater part of the succession of molecular events in the aftermath of TRAIL – receptor engagement, such as receptor aggregation, formation of the DISC in the lipid rafts, activation of caspase-8 and consequent activation of downstream initiator and effector caspases, are contingent upon Par-4 activity. These discoveries reveal a critical function for Par-4 in death receptor signaling. Likewise, in a parallel installment, Par-4 is also regulated by caspases creating a functional regulatory loop. Accordingly, Par-4 is transported into the nucleus by caspase-8 activity in addition to being cleaved, at more than one site by the concerted action of caspases -8 and -9. Cleavage of Par-4 at sites 123 and 200 would result in the fragment 124-199, which includes the SAC domain. These observations support the hypothesis that the extrinsic and intrinsic cell death pathways collaborate in an effort to generate the apoptotic functional core of Par-4 (Figure 4.1).
Figure 4.1: A model for mutual regulation of Par-4 and caspase-8 in the TRAIL pathway

TRAIL

DR4/5

FADD

Pro-Casp-8

Par-4

Casp-8

14-3-3

1-123

SAC

Pro-Casp-8

Casp-9

Nucleus

Par-4

SAC

NF-κB
Figure 4.1: Mutual regulation of Par-4 and caspase-8:

Par-4 mediates membrane transport and activation of pro-caspase-8 following TRAIL signaling. The activated caspase-8 then mediates nuclear translocation of Par-4 by breaking down 14-3-3. Caspase -8 and -9 in collaboration also generate a SAC containing fragment of Par-4; full length and cleaved Par-4 both inhibit NF-κB. All these events consequently lead to apoptosis of the cell.
Biological systems abound with instances of feedback regulation, where the effects of a pathway are tempered by regulation of the regulator. Par-4, similarly, is required for activation of caspase-8, following which, Par-4 is itself cleaved by the protease. Although both these events are interconnected, whether they are mutually relevant functionally is an issue for deliberation. Par-4 plays an important role in conveying caspase-8 to the actively forming DISC in the membrane. This event must occur with full length Par-4 (i.e. prior to being cleaved by caspase-8) because, first of all, Par-4 binds to caspase-8 with its leucine zipper (Figure 2.5) and evidently cleavage at either residue 200 or 287 would render Par-4 incompetent in this regard. Moreover, only the pro form of a caspase would be recruited to the DISC, thus a Par-4 molecule that interacts with the protease zymogen at this stage cannot be subjected to any proteolytic activity. In addition, even the basal caspase-8 activity present in the cell is diminished with Par-4 siRNA (Figure 2.3 A) indicating caspase-8 cannot act on Par-4 or any other substrate unless its activation is first aided by Par-4. Although these observations suggest activation of caspase-8 by Par-4 precedes Par-4 cleavage by caspase-8, it does not entirely preclude the possibility that two functionally distinct pools of Par-4 regulate, and are being regulated by caspase-8 concurrently in a cell.

The cycle of Par-4 first activating caspase-8 before being cleaved itself points towards a feedback regulatory loop. The caspase-8 cleavage site in Par-4 is the N terminal Aspartic acid at 123. In the absence of further cleavage, the resulting fragment of Par-4 would be 124-332. A mutant of Par-4 that is deleted for the N terminal 25 amino acids displays stronger apoptotic ability than full length Par-4, suggesting the presence of an N terminal inhibitory domain (El-Guendy, 2002).

Par-4 interacts with itself to form oligomers of varying molecular masses. Based on Par-4 interaction with caspase-8, and the fact that over expression of Par-4 triggers death via a caspase-8 mediated pathway, it is possible that the oligomers of Par-4 form a platform onto which caspase-8 molecules are brought into close proximity for auto catalytic activation; a similar, FADD independent, phenomenon has been noted during
ER stress mediated activation of caspase-8 (Breckenridge et al., 2002; Ng et al., 1997). The homo-oligomerization of Par-4 may amplify caspase-8 activation in the absence of DISC components and this process would be more efficient in the absence of the N terminal amino acids (Gao et al., 2006), providing one more rationale for cleavage. As this pathway reaches a threshold in the Type II cells where caspase-9 is activated, the cells are presumed to have reached a point of no return. The dying cell can now afford to have Par-4 cleaved in the middle of the caspase-8 activating fragment (124-332) to generate 124-199, as amplification of the death signal along the extrinsic axis may no longer be required. Incidentally, the N terminal 36 amino acids of Par-4 have an inhibitory effect on the synthesis of c-FLIP, an inhibitor of caspase-8 activation (Gao et al., 2006). This could be another positive feedback mechanism to ensure prompt and profuse activation of caspase-8 upon reception of apoptotic signal. Additionally, Par-4 is translocated to the nucleus during TRAIL signaling, presumably to inhibit the cell survival NF-κB pathway, and offer reinforcement to the apoptotic pathway in progress.

TRAIL, similar to Par-4, is a cytokine implicated in the phenomenon of cancer selective apoptosis and therefore, it is not wholly unexpected that Par-4 is a chief downstream component in the pathway. However, Par-4 is not implicated in all of the physiological functions of TRAIL. For instance, TRAIL is involved in activation induced proliferation of T and B cells (Chou et al., 2001), whereas the Par-4 knockout mice display accelerated development of these lymphocytes upon activation (Lafuente et al., 2003), indicating opposing functions under the circumstance. On the other hand, most apoptotic functions of TRAIL are perhaps mediated by Par-4. Par-4 is intimately involved in neuronal cell death in certain neurodegenerative diseases and not surprisingly, TRAIL expression in the neurons is scanty under normal conditions. Moreover, TRAIL induces apoptosis in the granulosa cells of the atretic follicles in the ovary (Inoue et al., 2003), where evidently, Par-4 expression is also elevated (Boghaert et al., 1997). Par-4 might, therefore, prove to be a generic regulator of caspase-8 activation downstream of TRAIL.
The reciprocal regulation of Par-4 and caspase-8 in response to TRAIL reveals a complex relationship between two apoptotic proteins and opens up numerous questions. First of all, although I know that Par-4 causes membrane translocation of caspase-8, the specifics of this trafficking are not clearly understood. Further studies should be undertaken to examine the regulation of membrane trafficking of caspase-8 by Par-4. For instance, Par-4 has an N-myristoylation consensus at the N terminus; whether Par-4 is in fact myristoylated and whether such modification has implications on caspase-8 trafficking and propagation of the TRAIL apoptotic signal are valid questions that can be addressed. Numerous proteins, such as Src, Yes and Akt are myristoylated to enable association with the membrane and cytoskeleton (Cross et al., 1984). Interestingly, activity of N-myristoyltransferase, the enzyme responsible of myristoylating proteins, is elevated in several human cancers (Magnuson et al., 1995; Rajala et al., 2000; Selvakumar et al., 2007). This may be a specific mechanism contributing to the cancer selective utilization of Par-4 by TRAIL for caspase-8 recruitment. Secondly, as phosphorylation of Par-4 at threonine 155 appears to be a necessary event for TRAIL induced apoptosis, the role of this phosphorylation with regard to caspase-8 interaction and traffic, as well as DISC formation can be investigated further. Par-4 is an obligatory component of caspase-8 activation in response to TRAIL in our experimental system. However, I do not know whether activation of caspase-8 under all conditions entails Par-4 mediated membrane translocation. In order to answer this question, the requirement of Par-4 for this process has to be tested in response to various other death receptor ligands such as TNF-α and FasL and in other experimental models such as TCR/BCR activation. The Par-4 knockout does not phenocopy the caspase-8 knockout indicating that Par-4 may be expendable in the extrinsic apoptotic pathway during embryogenesis and perhaps, across cell types.

Caspase-8 interacts with the leucine zipper of Par-4. The domain of caspase-8 that is responsible for this interaction, however, is not known. Caspase-8 binds to FADD with its DED (Death Effector Domain), which mediates protein – protein interactions,
precluding the involvement of this region in its interaction with Par-4. Yet, it is possible that Par-4 interacts with the DED motif during trafficking, prior to caspase-8 being tethered to FADD. Hence, the domain of caspase-8 responsible for its interaction with Par-4 would be an interesting line of inquiry. Subsequently, if the interactions of Par-4 and FADD with caspase-8 turn out to be mutually exclusive, it would be interesting to study how Par-4 is brought in to the vicinity of the DISC and activated caspase-8 for subsequent cleavage.

Par-4 interacts with itself to form dimers, tetramers, and octamers (Gao et al., 2006); such oligomerization can have an impact on the interaction of Par-4 with caspase-8. I do not know whether caspase-8 can bind to all forms of Par-4, or whether the multimeric state of Par-4 determines the strength of caspase-8 interaction. In addition, cleavage of Par-4 at aspartate 287 should diminish Par-4 self association and this might have an effect on Par-4 feeding back onto caspase-8 amplification. Future studies are necessary to investigate whether oligomerization of Par-4 can influence interaction with and cleavage by caspase-8.

Comprehensive studies are required in order to determine the relevance of Par-4 cleavage to the overall cell death program. First of all, the impact of any post-translational modification, such as phosphorylation, that can limit caspase access to the site at 287 should be examined. Subsequent studies could examine the role of prevailing conditions, such as cell type and apoptotic vs. anti-apoptotic signal ratio in determining, via such post-translational modification, whether or not cleavage feeds back onto the caspase cascade to complete the apoptotic program.

Cleavage of Par-4 generates at least 3 functionally distinct fragments, the N terminal fragment, central fragment and C terminal region. Among these, the first to be cleaved and the most prominent is the N–terminal 14 KD fragment. Although some observations suggest this region has an inhibitory function and would act as a dominant
negative in isolation, further studies are required to clarify the function of this cleavage product. In general, although existing data point towards amplification of Par-4 apoptotic potential by the cleavage events, further studies with a mutant deficient in cleavable residues at both positions 123 and 200 (123-200 AA) are necessary to verify the contribution of cleavage to cell death by Par-4. Along the same lines, the smallest fragment of 9KD has to be verified as SAC containing before concluding that cleavage of Par-4, indeed, contributes to apoptosis.

In essence, this study identified the role of cancer selective apoptotic protein Par-4, in the TRAIL – caspase-8 apoptotic axis and uncovered an essential function for Par-4 in the activation of caspase-8 via regulating translocation of pro-caspase-8 to the membrane. In turn, Par-4 is conveyed to the nucleus in addition to being cleaved by the activated caspases, completing an entire regulatory loop.

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Appendix

LIST OF ABBREVIATIONS

AATF: Apoptosis-Antagonizing Transcription Factor
AD: Alzheimer's disease.
ALS: Amyotrophic lateral sclerosis.
APAF-1: Apoptotic Protease Activating Factor-1
ARC: Apoptosis Repressor with CARD
βAPP: Beta amyloid precursor protein.
BAR: Bifunctional Apoptosis Regulator
cAMP: cyclic adenosine monophosphate
CD95: Cluster of Differentiation 95
cFLIP: FLICE inhibitory protein.
cFLIPs: FLICE inhibitory protein, short form
cFLIPL: FLICE inhibitory protein, long form
CKII: Casein kinase II.
DAP kinase: death associated protein kinase.
DAPI: 4',6'-diamidino-2-phenylindole hydrochloride.
DISC: Death Inducing Signaling Complex.
Dlk: DAP-like kinase
DMEM: Dulbecco’s modified Eagle medium.
DTT: Dithiothreitol.
ERK: Extracellular Signal-Regulated kinase.
FADD: Fas associated death domain protein.
FasL: Fas ligand.
FBS: Fetal bovine serum.
FLICE: FADD like ICE (caspase 8).
GFP: Green fluorescence protein.
HEL: Human embryonic lung fibroblast
HEPES: N-2-hydroxyethylpiperazine N’-2-ethanesulfonic acid.
IAPs: Inhibitor of Apoptosis Proteins.
ICC: immunocytochemistry
IGF: Insulin growth factor.
IFNy: Interferon γ
IkB: inhibitor of κB.
IL-2: Interleukin-2
Jnk: c-Jun N-terminal kinase
kD: kilodalton
MEF: Mouse Embryonic Fibroblasts
NES: Nuclear exclusion sequence.
NGF: Neuronal growth factor
NF-κB: Nuclear factor-κB
NHBE: Normal bronchial epithelial cells
NLS: Nuclear localization sequence.
NSCLC: Non Small Cell Lung Carcinoma
PAK: p21 Activated Kinase
Par-4: Prostate apoptosis response 4.
PBS: phosphate buffered saline
PKA: Protein kinase A.
PKC: Protein kinase C.
PML: Promyelocytic leukemia.
Rb: Retinoblastoma
RCC: Renal cell carcinomas.
RIPA: Radio Immuno Precipitation Assay
SAC: Selective Apoptosis-induction in Cancer cells
SCLC: Small cell lung carcinoma
SDS: Sodium dodecylsulfate.
SDS-PAGE: Sodium dodecylsulfate polyacrylamide gel electrophoresis.
siRNA: small interfering RNA
**THAP**: Thanatos-associated protein.

**TNF-α**: Tumor necrosis factor alpha.

**TNF-R**: TNF receptor.

**TRAIL**: Tumor necrosis factor-related apoptosis-inducing ligand

**WT1**: Wilms’ tumor 1.
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MEETINGS AND CONFERENCES
