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MECHANISM OF CANCER SELECTIVE APOPTOSIS BY PAR-4

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ABSTRACT OF DISSERTATION

Sushma Gurumurthy

The Graduate School

University of Kentucky

2005

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ABSTRACT OF DISSERTATION
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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
at the University of Kentucky

By

Sushma Gurumurthy

Lexington, Kentucky

Director: Dr. Vivek Rangnekar, Professor, Radiation Medicine

Lexington, Kentucky

2005

ABSTRACT OF DISSERTATION

MECHANISM OF CANCER SELECTIVE APOPTOSIS BY PAR-4

Despite distinct dissimilarities, diverse cancers express several common pro-tumorigenic traits. We present here evidence that the pro-apoptotic protein Par-4 utilizes one such common tumorigenic trait to become selectively activated and induce apoptosis in cancer cells. Elevated PKA activity noted in cancer cells activated the apoptotic function of ectopic Par-4 or its SAC domain, which induces apoptosis selectively in cancer cells and not in normal or immortalized cells. PKA preferentially phosphorylated Par-4 at the T155 residue within the SAC domain in cancer cells. Moreover, pharmacological-, peptide- or siRNA-mediated inhibition of PKA activity in cancer cells resulted in abrogation of both T155 phosphorylation and apoptosis by Par-4. The mechanism of activation of endogenous Par-4 was similar to that of ectopic Par-4, and in response to exogenous stimuli, endogenous Par-4 induced apoptosis by a PKA and phospho-T155 dependent mechanism. Enforced elevation of PKA activity in normal cells resulted in apoptosis by the SAC domain of Par-4 in a T155-dependent manner. Together, these observations suggest that selective apoptosis of cancer cells by

the SAC domain of Par-4 involves phosphorylation of T155 by PKA. These findings uncover a novel mechanism engaging PKA, a pro-cancerous activity commonly elevated in most tumor cells, to activate the cancer selective apoptotic action of Par-4.

KEYWORDS: Par-4, apoptosis, cancer, PKA, T155, nuclear, cytoplasmic localization

Sushma Gurumurthy

January 2005

MECHANISM OF CANCER SELECTIVE APOPTOSIS BY PAR-4

By

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_____March 24, 2005_____

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DISSERTATION

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To My Parents, Lakshmi Gurumurthy and R.Gurumurthy

ACKNOWLEDGEMENTS

I'll always remember and cherish. I thank all my friends for being my family away from home and for all the fun times.

My family has been the cornerstone of all my endeavors. I thank my parents, my brother and my husband for their patience, prayers sacrifices and steadfast support. I am especially grateful to my parents, Mr. and Mrs Gurumurthy, for instilling in me the values that have helped me come this far and to them I dedicate this dissertation.

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INTRODUCTION

Apoptosis or Programmed cell death is a genetic and biochemical process essential to metazoans. It is an evolutionarily conserved pathway that is critical in developmental as well as adult physiological processes in multi-cellular organisms. During embryonic development, apoptosis is critical for organogenesis and ensuring the balance of cell lineage in multi-cellular tissues. In adult animals, apoptosis serves to maintain cellular homeostasis, protects against abnormal cell division, intracellular crisis such as DNA damage caused either by replicative error or external agents, protein mis-folding or ionic imbalances, and even regulating the response to infectious agents. At any given time in a cell, a balance between pro- and anti-apoptotic factors is maintained. Events that alter this ratio result either in excessive apoptosis or in lack of apoptosis, if not rectified, leads to disease conditions. Insufficient apoptosis can manifest as cancer or autoimmunity, while accelerated cell death is evident in acute and chronic degenerative diseases, immunodeficiency, and infertility. Here, we discuss the role of a pro-apoptotic protein prostate apoptosis response-4 (Par-4) in the pathways of apoptosis, and its role in disease processes such as neurodegenerative disorders and cancer.

Identification and Characterization of Par-4

Androgen-ablation causes apoptosis of androgen-dependent prostate cancer cells resulting in rapid involution of the tumor and is the mainstay of prostate cancer treatment. The effect of androgen-ablation is emulated in the rat ventral prostate gland, which serves as an excellent model to study apoptosis and prostate involution, by withdrawal of androgen. Several novel transcripts and proteins are induced post-castration in the regressing prostate and inhibitors of protein and RNA synthesis inhibit regression of the prostate, indicating that the gene products induced by androgen ablation are necessary for apoptosis. Importantly, the calcium channel inhibitor nifedipine causes inhibition of both apoptosis and prostate involution after castration, indicating a role for intracellular calcium elevation in apoptosis of the prostate. In a differential screen for genes induced during apoptosis of prostate cancer cells, we first identified the rat prostate apoptosis response-4 (Par-4) gene (Sells et al., 1994). Par-4 was up-regulated when androgen-independent prostate cancer cells were induced to undergo apoptosis upon treatment with a calcium ionophore ionomycin, mimicking the increase in intracellular calcium induced by androgen ablation. Par-4 was also induced in androgen-dependent luminal/secretory cell compartment of the rat ventral prostate after castration. Interestingly, Par-4 was not induced in other organs, such as the liver or kidneys, which express androgen receptors but do not undergo apoptosis and involution after castration. Moreover, the *par*-4 gene is not induced by growth stimulation, growth arrest or necrosis in cell culture paradigms. Subsequent studies identified the human Par-

4 gene, which shared a high degree of nucleotide and amino acid similarity with the rat Par-4 gene (Diaz-Meco et al., 1996; Johnstone et al., 1996).

The *par-4* gene encodes a 38 kilodalton protein and maps to human chromosome 12q21, a region that is unstable and often deleted in pancreatic and gastric cancer (Kimura et al., 1998; Schneider et al., 2003). The Par-4 sequence encodes a 332 amino acid protein, containing of two putative nuclear localization sequences in the N-terminal region and a leucine zipper domain and a nuclear export sequence in the C-terminal portion of the protein. These domains are 100% conserved in human, rat and mouse homologs of Par-4, suggesting that the function, localization and regulation of Par-4 is similar in all mammals. In addition, the Par-4 protein is conserved during evolution in vertebrates (Boghaert et al., 1997). Consistent with its pro-apoptotic function, Par-4 is highly expressed in involuting tadpole tail. The fact that the structure and function of Par-4 are evolutionarily conserved indicates the global significance of the apoptotic function of Par-4.

Par-4 is widely expressed in mammalian tissues. In the normal prostate gland, Par-4 is expressed in the mesenchyme surrounding the ventral part of the prostate and the basal glandular epithelial cells but is absent in adjacent differentiated ductal cells. A similar pattern is seen in epithelial cells of mammary gland and the retina where Par-4 is undetectable in terminally differentiated cells implying that Par-4 expression is down-regulated during differentiation.

Interestingly, withdrawal of androgen results in massive apoptosis of the ductal cells and this apoptotic process is mediated by Par-4 that is induced during androgen ablation. Upon testosterone ablation caused by castration, ductal cells undergo apoptosis, which peaks at day 3. Par-4 levels increase in ductal cells of the rat prostate on day 1 and day 3 post castration and diminish by day 5, suggesting that Par-4 induction is an early and transient event in apoptosis of prostate ductal cells (Sells et al., 1994). Interestingly, the anti-apoptotic protein Bcl-2 is normally well expressed in the ductal cells and androgen-withdrawal significantly decreases Bcl-2 expression. Consistently, an inverse correlation between Par-4 and Bcl-2 expression is noticed in human prostate tumors (Qiu et al., 1999a). Par-4 but not Bcl-2 is detected in primary and metastatic prostate tumors and in xenografts of human androgen-dependent CWR22 tumors, whereas the androgen-independent CWR22R tumors show pockets that stain for Bcl-2 but not Par-4. A similar inverse pattern of expression between Par-4 and Bcl-2 was also observed in lymphocytes from acute lymphoblastic leukemia patients (Boehrer et al., 2002).

Role of Par-4 in Inhibition of Cellular Transformation and Tumorigenesis

Evasion of apoptosis is one of the key requirements of oncogenic transformation (Hanahan and Weinberg, 2000) and one of the ways several cancers achieve this by down-regulating Par-4 expression. Par-4 levels are diminished in a majority of renal cell carcinoma (RCC) specimens or RCC cell lines relative to normal proximal renal tubular (PRT) cells within tumors or PRT

cell lines (Cook et al., 1999). Par-4 expression is decreased in a number of neuroblastoma cell lines (Kogel et al., 2001) and in cells of patients of acute lymphatic leukemia and chronic lymphocytic leukemia (Boehrer et al., 2001). Oncogenic *ras*, a potent oncogene involved in 40% of epithelial tumors, has been shown to down-regulate Par-4 expression at the mRNA and protein level via the Raf - MEK - ERK pathway (Barradas et al., 1999; Qiu et al., 1999b). Oncogenic Ras expression promotes a potent reduction of Par-4 protein and mRNA levels through a MEK-dependent pathway. Treatment of the cells with MEK-inhibitor PD98059 restored the levels of Par-4. In addition, the ectopic expression of constitutively active Raf-1 or MEK mutants but not phosphatidylinositol 3-kinase (PI3-kinase), was sufficient to decrease Par-4 levels, suggesting that other oncogenic pathways that utilize these molecules as intermediates can potentially repress Par-4 to promote cellular transformation. Restoration of Par-4 levels by ectopic expression results in reduced expression of ERK1 and ERK2 MAP kinases (Qiu et al., 1999b). Importantly, restoration of Par-4 levels in Ras-transformed cells by stable expression severely impairs colony formation in soft agar (Barradas et al., 1999; Qiu et al., 1999b). Interestingly, it appears that the mechanism by which Par-4 suppresses Ras-mediated cellular transformation is independent of the mechanism by which it induces apoptosis in these cells. Consistently, inhibition of NF-κB is essential for Par-4 to induce apoptosis (Barradas et al., 1999; Nalca et al., 1999), and Ras/ Par-4 stably expressing cells do not show any difference in NF-κB activity when compared to the Ras/ vector control cells.

Interestingly, the immunological profile of Par-4 -/- knockout mice shows an increased proliferative response of peripheral T cells together with increased cell cycle entry and inhibition of apoptosis, elevated NF-κB activity and decreased JNK activity (Garcia-Cao et al., 2003) These findings suggest that Par-4 loss or reduction contributes to the pathogenesis of lymphatic malignancies. It remains to be seen whether the reduction of Par-4 in the Par-4 -/- mice contributes to the development of other malignancies.   Further characterization of the tumor profile of these mice should shed more light on the role of Par-4 in tumor development, progression and metastasis.

Role of Par-4 in Neuronal Apoptosis and Neurodegenerative diseases

The role of Par-4 in neuronal function under normal and disease conditions is an intriguing and puzzling question that is being unraveled. Involvement of Par-4 in a variety of neuronal processes ranging from embryonic neuronal development, synaptic transport and neurotransmitter signaling to neurodegenerative diseases have been shown in the past few years. In the developing mouse brain, an asymmetric distribution pattern of Par-4 expression was observed in neuronal progenitor cells. This pattern was found to regulate ceramide-induced apoptosis during the proliferation and differentiation of stem cells. Neuronal stem cell apoptosis is necessary to prevent hyperproliferation of brain tissue during embryonic mouse brain development (Bieberich et al., 2003). This function of Par-4 is now being investigated for the use of pluripotent mouse and human stem cells in neural transplantion as selective apoptosis by Par-4

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prevents teratoma formation and enriches for neural precursors in ES cell-derived neural transplants (Bieberich et al., 2004).

Par-4 is expressed in neurons in the adult mouse brain. Interestingly, Par-4 was localized in the dentrites and the post-synaptic compartments of the synaptosome rather than the cell body of the neuron. Neurons of the cerebral cortex and the hippocampus, but not the cerebellum accumulate Par-4 in the synaptic region (Guo et al., 2001b). The post-synaptic compartment is enriched with cytoskeletal, transport and signaling machinery and is considered the central region involved in the modulation of neuronal signal transduction. The positioning of Par-4 in this region is intriguing, and it remains to be seen whether Par-4 has a functional role in the synaptosome under normal non-apoptotic conditions. In neurodegenerative diseases, on the other hand, there seems to be a connection between the synaptic localization of Par-4 and apoptosis. A recent study using an amylotrophic lateral sclerosis (ALS) mouse model identifies the synapse as a crucial cellular site for the cell death promoting actions of Par-4 in motor neurons. Accumulation of Par-4 in synaptosomes and post-synaptic density from the ventral horn of the spinal cord increased as the disease progressed and mediated production of pro-apoptotic cytosolic factors (Xie et al., 2005).

The involvement of Par-4 in neuronal apoptosis in neurodegenerative disease is well established. Elevated levels of Par-4 mRNA and protein have are responsible for apoptosis in dying neurons in patients, animal models and cell

culture models of a variety of neurodegenerative conditions such as Alzheimer's, Parkinsons, Huntington's diseases, amyotrophic lateral sclerosis (ALS) and ischemic brain injury or stroke (El-Guendy and Rangnekar, 2003; Mattson et al., 1999). For example in Alzheimer's disease patients, an 8 to 20 fold increase in the Par-4 protein levels was observed in hippocampal tissues and the inferior parietal cortex compared to normal samples (Guo et al., 1998; Xie et al., 2001). Exposure of primary neurons to apoptotic insults such as amyloid β-peptide, Neuronal growth factor (NGF) withdrawal or oxidative insults caused a rapid and significant increase in endogenous Par-4 levels leading to apoptosis of the cells {Guo, 1998 #85}. An interesting feature of Par-4 mediated neuronal apoptosis is that the mechanism involves loss of mitochondrial membrane potential leading to activation of downstream caspases. This mechanism of apoptosis by Par-4 is not commonly observed in the non-neuronal epithelial or fibroblast cell models tested in our laboratory indicating tissue-specific variation in mechanism of Par-4 action. Inhibition of Par-4 expression or function using antisense and RNA interference technology or dominant negative Par-4, respectively, abrogates apoptosis induced by the various agents in neuronal cell culture and *in vivo* animal models of diseases (Pedersen et al., 2000; Xie et al., 2005). Interestingly, in a feedback loop that could exacerbate the neuronal damage, Par-4 has been implicated in the abnormal processing of amyloid precursor protein (APP) and induced an aberrant production and secretion of Aβ-(1-142) (Guo et al., 2001a). This effect of Par-4 is inhibited by a recently identified antagonistic interaction partner in Apoptosis-Antagonizing Transcription Factor (AATF). AATF binds to the leucine

zipper of Par-4 and effectively inhibits aberrant Aβ production (Guo and Xie, 2004). This finding is fascinating because AATF is known to interact with DAP-like kinase (Dlk), a pro-apoptotic binding partner of Par-4 (Page et al., 1999). Both of these proteins interact with Par-4 through the leuzine zipper domain of Par-4. It is possible that under normal non-apoptotic conditions, antagonistic interaction with AATF restrict the functions of both Par-4 and Dlk. When apoptosis is induced, Dlk and Par-4 are modified or activated to promote interaction with each other and decreased affinity to AATF. The conditions that dictate binding and the implications of interaction of AATF as well as other proteins with the leucine zipper of Par-4 in the regulation of apoptosis as well as functions un-related to apoptosis are discussed in a later sections. Targeted inhibition of Par-4, by decreasing expression or using inhibitory partners such as AATF, is currently being investigated as a neuroprotective strategy for neuron degeneration.

Apoptosis Sensitizing Function of Par-4: Role of the Leucine Zipper and Partner Proteins.

Although Par-4 over-expression does not induce apoptosis in normal cells, Par-4 expression sensitizes cells to apoptosis by a wide variety of pro-apoptotic stimuli such as growth factor withdrawal, agents that elevate intracellular Ca2+, TNFα, UV, X-ray and gamma irradiation, or IFNγ. Inhibition of endogenous Par-4 protein levels by treatment with antisense or siRNA against Par-4 or functional interruption of Par-4 using a dominant negative Par-4 mutant abrogates

apoptosis by these apoptotic agents. Various reports have indicated that Par-4 plays a sensitizing function in various cell types including epithelial cells of the prostate, endothelial cells, neuronal cells, transformed fibroblasts and a variety of carcinomas. In its apoptosis-sensitizing role in these diverse cell backgrounds, Par-4 inhibits several pro-survival pathways, activates pro-apoptotic proteins and binds to several different partner proteins of varying function. The leucine zipper of Par-4 is the functional domain that is key to sensitization to apoptotic stimuli and is required for Par-4 to interact with a wide variety of proteins such as WT-1, atypical PKCs, p62, DLK/ZIP kinase, and THAP1; deletion of the leucine zipper domain abrogates this function of Par-4 (Fig 1.1). The leucine zipper domain functions as a kind of sensor to signaling pathways and regulates the apoptotic function of Par-4 by interacting with the appropriate partner protein.

Par-4 down-regulates the anti-apoptotic protein Bcl-2. The *bcl-2* gene was first identified in the t(14:18) translocation breakpoint from human follicular B cell lymphoma (Kimura et al., 1998; Reed et al., 1996). Bcl-2 has been found to be over-expressed in androgen independent prostate cancer. Over-expression of Bcl-2 enables prostate cancer cells to resist apoptosis induced by androgen-withdrawal, physiological death inducers such as TRAIL, or chemotherapeutic agents. (reviewed in (Bruckheimer et al., 1999). Qiu et al demonstrated that in immortalized mouse NIH 3T3 fibroblasts and prostate cancer PC-3 cells that over-expressed Par-4, there was a significant down-modulation of bcl-2 both at the promoter level and the protein level and that this down-regulation of *bcl-2*

was essential for Par-4 mediated apoptosis (Qiu et al., 1999a). Indeed, co-transfection of *bcl-2* rescued the cells from apoptosis induced by growth factor withdrawal. Par-4 exerts a pro-apoptotic role, augmenting chemosensitivity by down-regulating Bcl-2, promoting disruption of mitochondrial membrane potential and enforcing caspase-activation. It is interesting to note that in prostate cells, *bcl-2* is down-regulated by androgen withdrawal, the same signal that up-regulates Par-4, suggesting that Par-4 is an important molecular component involved in Bcl-2 down-modulation and induction of apoptosis (Qiu et al., 1999a). A better understanding of the mechanism of repression of *bcl-2* by Par-4 is now possible with recent findings by Cheema *et al.* that Par-4 utilizes WT1 to bind the *bcl-2* promoter and transcriptionally down-regulate Bcl-2 expression (Cheema et al., 2003). WT-1 binds to and upregulates bcl-2 promoter in the androgen independent cell line LNCaP/LN3. Co-expression of WT-1 and Par-4 leads to leucine zipper domain mediated interaction of Par-4 with WT-1 at the bcl-2 promoter followed by repression of the bcl-2 promoter (Fig. 1.1).

WT-1 is a tumor suppressor protein frequently mutated in Wilm's tumors, a rare form of kidney tumor. WT-1 mediates growth arrest mediated by several cytokines such as IL-1 and abrogates thapsigargin-induced apoptosis. Expression of Par-4 was able to override the WT-1 mediated abrogation of apoptosis in prostate cancer and melanoma cells (Johnstone et al., 1996; Sells et al., 1997). Although WT-1 is mainly expressed in the organs of the urinogenital tract, less is known about the status of WT-1 protein in prostate cancer. As WT-1

can differentially regulate bcl-2 depending on the availability of Par-4 and as bcl-2 is often over-expressed in advanced prostate tumors, it is pertinent to study the role of the interplay between WT-1 and Par-4 in the development of prostate cancer.

Par-4 inhibits the pro-survival pathway activated by atypical PKCs, ζPKC and λ/ιι PKC, by binding and inhibiting their kinase activities (Diaz-Meco et al., 1996). aPKCs are serine/threonine kinases that positively regulate cell proliferation and cell survival by activating transcription factors AP-1 and NF-κB, respectively. aPKCs also block apoptosis by phosphorylating pro-apoptotic protein FADD and preventing DISC formation. The interaction between Par-4 and ζPKC, mediated through the leucine zipper domain of Par-4 and the zinc finger in the regulatory domain of ζPKC, induces a conformational change leading to inhibition of ζPKC catalytic activity. Exposure to apoptotic stimuli such as UV irradiation, ceramide or TNFα triggers the interaction between endogenous Par-4 and endogenous ζPKC leading to a dramatic reduction of ζPKC enzymatic activity and an increase in apoptosis. Concordantly, replenishment of ζPKC levels by over-expression inhibits apoptosis by these agents (Barradas et al., 1999; Diaz-Meco et al., 1996). aPKCs activate NF-κB by functioning as IKKβ kinases. Par-4 potentiates TNFα-induced apoptosis by inhibiting NF-κB through the blockade of the aPKC-IKK signaling cascade. It was recently identified that aPKC and Par-4 are part of a ternary complex with an adapter protein, p62 (Chang et al., 2002). p62 has been suggested to play a

critical role in ζPKC-mediated NF-κB activation by recruiting ζPKC to the TNFα

signaling complex when the TNF receptor is activated by TNFα, IL-1β or other

ligands. p62 and Par-4 do not compete to bind to ζPKC but interact directly with

each other and form a ternary complex with ζPKC (Fig 1.1). Binding of p62 to the

ζPKC-Par-4 complex blocks Par-4 mediated inhibition of ζPKC kinase activity.

This shows that the functions of endogenous Par-4 and ζPKC are very tightly

controlled. However, little is known about the role of p62 in regulating ζPKC

activity that is not recruited to the ternary signaling complex or about the role of

p62 in the complex in the presence of diverse apoptotic signals.

Inhibition of pro-survival or anti-apoptotic pathways is not the mechanism

of action of Par-4, it also hetero-dimerizes with several pro-apoptotic molecules.

Recently the idea that Par-4 mediates apoptotic events from PML bodies or

PODs in the nucleus has emerged. In 1999, studies by Page *et al.* suggested

that DLK/ZIP kinase (ZIPK) bound and phosphorylated Par-4, and that this

function was essential for ZIPK mediated apoptosis (Page et al., 1999). These

findings were corroborated recently by John Reed's group when they found that

in response to several apoptotic stimuli such as IFNγ or As_2O_3, ZIPK associated

with the pro-apoptotic protein DAXX and that the interaction of ZIPK and Par-4

enhanced this association (Kawai et al., 2003). This work suggested that in

response to IFNγ or As_2O_3, the ZIPK- DAXX complex is recruited to the PODs

where they activate apoptosis. siRNA-mediated knockdown of Par-4 resulted in

suppression of apoptosis induced by IFNγ and As_2O_3 (Fig 1.1). Adding to the

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Fig. 1.1. Interaction Partners of Par-4

One of the most well studied aspects of Par-4 is the ability of the C-terminal leucine zipper domain to interact with a multitude of partner proteins that modulate the functions of Par-4. This figure is a schematic representation of the known interaction partners of Par-4

Fig. 1.1. Interaction Partners of Par-4
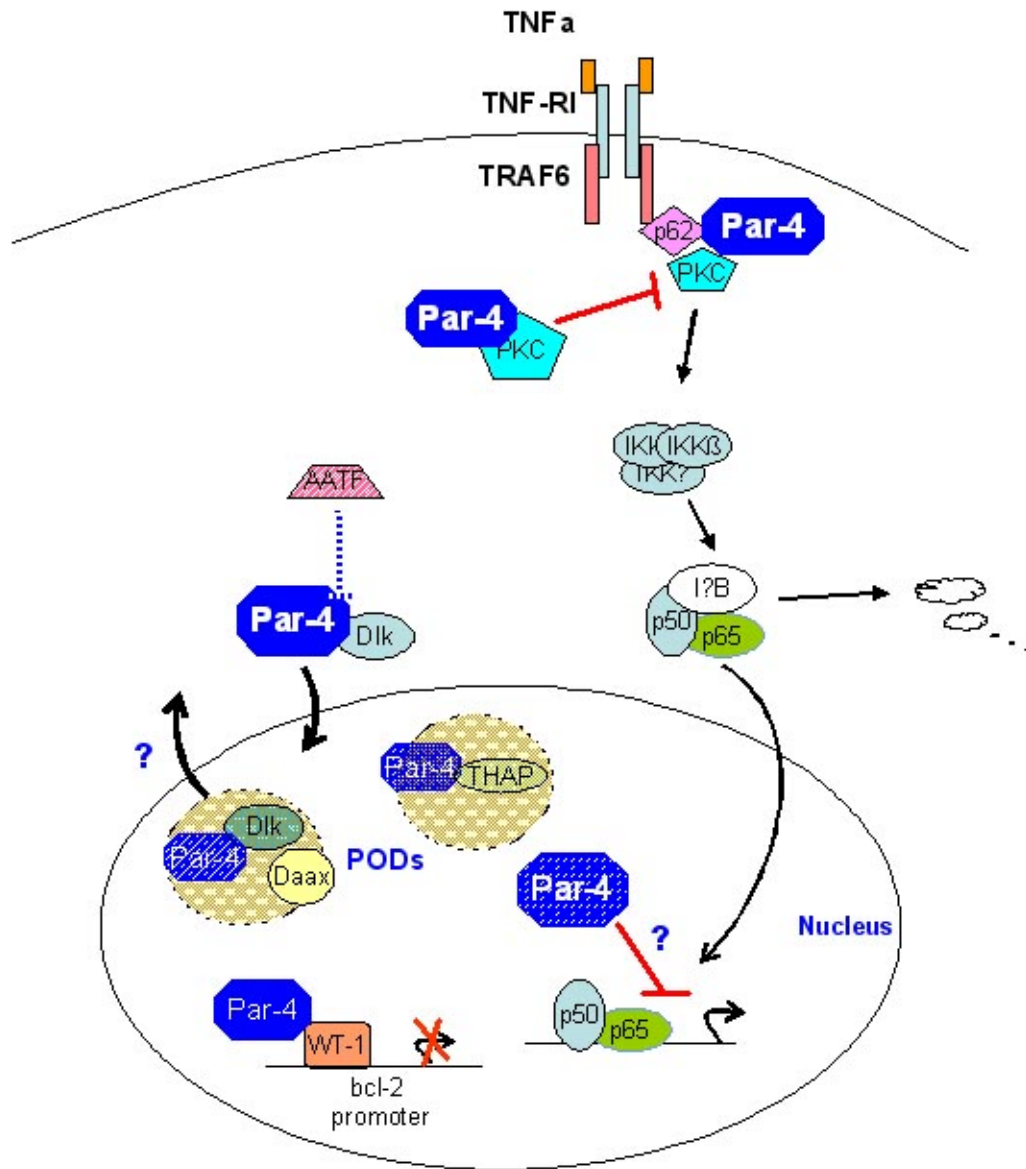
complexity of the situation, a recent finding in neuroblastomas identified AATF as an antagonistic binding protein that is capable of binding to both Par-4 as well as DLK (Fig. 1.1). AATF binds to Par-4 through the leucine zipper, the same domain that hetero-dimerizes with DLK (Guo and Xie, 2004). This finding provides a mechanism by which AATF could regulate the functions of two pro-apoptotic proteins under non-apoptotic conditions. How binding affinities between the three proteins is altered during apoptosis needs to be further investigated. In primary endothelial cells and human fibroblasts endogenous Par-4 colocalizes with THAP1 within PODs (Roussigne et al., 2003). In addition, Par-4 is a component of PML in blood vessels, a major site of PML expression *in vivo*. THAP1 is a novel nuclear pro-apoptotic factor associated with PML NBs, which interacts with the pro-apoptotic protein Par-4 and potentiates both serum withdrawal- and TNFα-induced apoptosis (Fig. 1.1). Par-4 is recruited to PODs in endothelial cells where PML plays a critical role. Thus, Par-4 is a global sensitizing agent, whose functions/partners are modified in a context-specific manner.

Apoptosis by Par-4 in Cancer Cells

Par-4 over-expression is sufficient to induce apoptosis in most cancer cells but normal primary or immortalized cells are resistant to its apoptotic action in the absence of a second apoptotic signal. Par-4 very efficiently induces apoptosis in all hormone independent cancer cell lines tested including lung, pancreatic, head and neck cancers and neuroblastomas as well as transformed cells such as Ras transformed NIH 3T3 fibroblasts (Chakraborty et al., 2001; El-

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Guendy et al., 2003; Nalca et al., 1999). Interestingly, Par-4 is able to override potential anti-apoptotic mechanisms such as high NF-κB activity, elevated expression of Bcl-xL or Bcl-2 present in these cell lines. The apoptotic function of Par-4 is also independent of the status of tumor suppressors such as p53 or PTEN. Interestingly, in cancers that arise in organs such as the prostate or the breast regulated by hormones such as estrogen or androgen, a unique dichotomy of Par-4 function is noted: Par-4 efficiently kills the more advanced hormone independent cancer cells whereas the hormone dependent cancer cells are resistant to the action of Par-4. Par-4 induces apoptosis in androgen independent prostate cancer cells such as PC-3 and DU-145 but not in androgen dependent prostate cancer cells such as LNCaP, LAPC-4, MDA PCa 2a and 2b (Chakraborty et al., 2001). Similarly, in breast cancer cells, Par-4 induces apoptosis in estrogen independent cells such as MDA MB-231, MDA MB-435 but is unable to do so in the estrogen dependent cells such as MCF-7 and T47D (El-Guendy et al., 2003).

The mechanism of apoptosis by Par-4 involves a unique co-parallel activation of a pro-death pathway together with the inhibition of a pro-survival pathway (Fig. 1.2). Par-4 activates the pro-death FasL-Fas-FADD-caspase-8 pathway by translocation of Fas and FasL to the cell membrane, and in parallel it inhibits the key pro-survival transcriptional activity of NF-κB (Chakraborty et al., 2001). Par-4 does not inhibit the ability of NF-κB to bind to DNA but abrogates the transcriptional activity of NF-κB, thereby preventing the expression of NF-κB-

Fig 1.2. Mechanism of Apoptosis by Par-4 in cancer cells.

In cancer cells, Par-4 induces apoptosis by a unique two pronged mechanism: First Par-4 activates a pro-apoptotic system by signaling the translocation of Fas and FasL to the cell membrane thereby activating the death receptor mediated apoptotic pathway. At the same time Par-4 shuts down a pro-survival pathway by inhibiting NF-κB transcriptional activity.

Fig. 1.2. Mechanism of Apoptosis by Par-4 in cancer cells
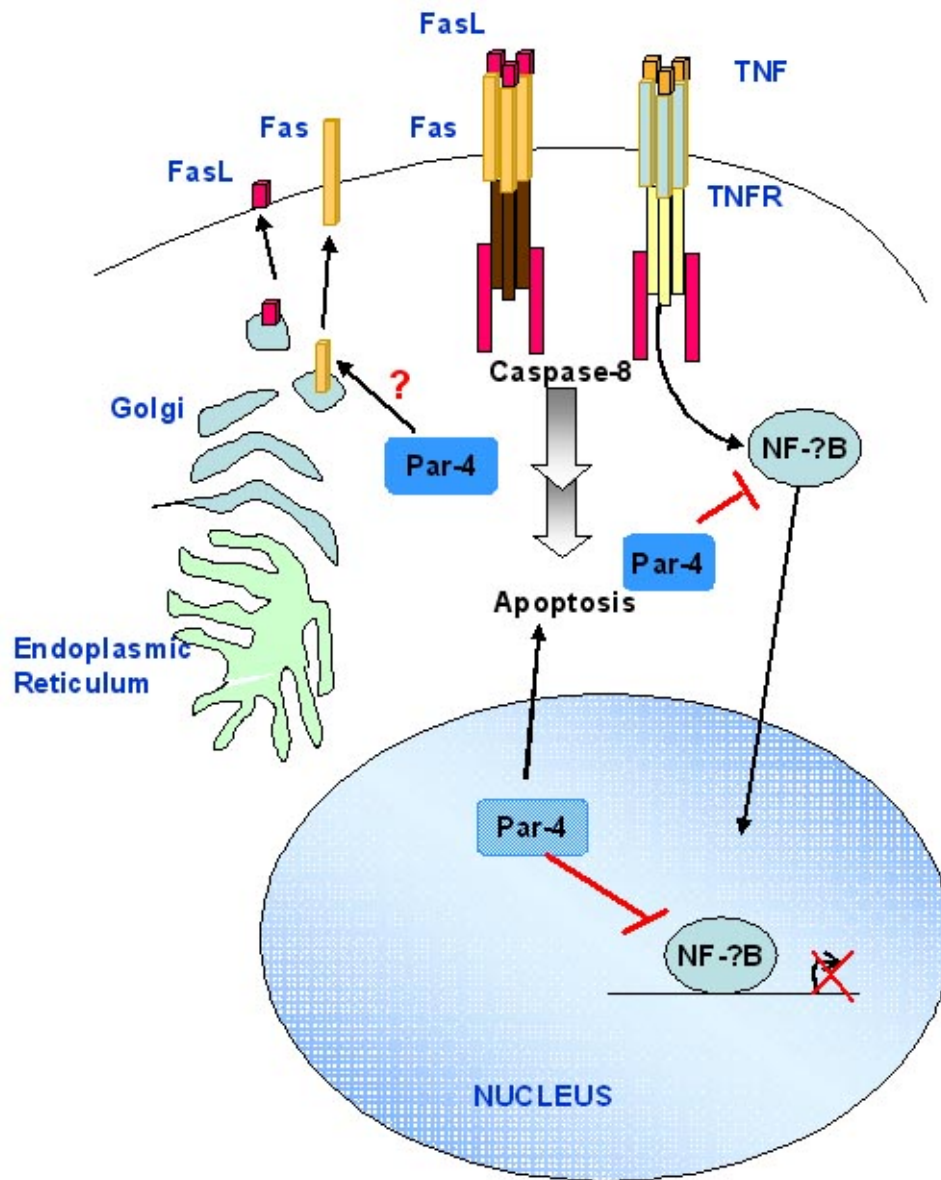
regulated anti-apoptotic genes. As a corollary, it is interesting to note that, MEFs derived from Par-4 -/- mice have elevated levels of NF-κB activity compared to matched Par-4 +/+ MEFs (Garcia-Cao et al., 2003). In addition to inhibition of NF-κB transcription Par-4, in parallel, activates the pro-death FasL-Fas-FADD-caspase-8 pathway by effecting the golgi dependent translocation of Fas and FasL to the cell membrane (Fig. 1.2). The trafficking of Fas and FasL by Par-4 triggers the interaction of death receptor Fas and FADD that is essential for the formation of a death-inducing signaling complex (DISC) (Chakraborty et al., 2001). Certain cancer cells, such as acute myeloid leukemia cells, acquire resistance to Fas induced apoptosis by phosphorylating FADD and thereby inhibiting DISC formation. It was recently found that Par-4 inhibits phosphorylation of FADD by inhibiting atypical PKC and promotes DISC formation (de Thonel et al., 2001). Activation of the Fas-FADD pathway is crucial for apoptosis by Par-4, as dominant negative or dominant interferring mutants of Fas, FADD, or caspase-8-blocked apoptosis by Par-4. In prostate cancer cells, the inhibition of NF-κB alone with IκB or the activation of the Fas pathway alone is not sufficient to induce apoptosis; but when used together IκB and Fas mimic Par-4 induced apoptosis. This implies that each pathway is necessary but not sufficient to induce apoptosis of prostate cancer cells and that Par-4 alone regulates both these pathways (Chakraborty et al., 2001).

Regression of Tumors by Par-4

Consistent with its pro-apoptotic and anti-transformation role in cell culture paradigms, Par-4 over-expression in mouse tumor models results in tumor regression. Subcutaneous tumors generated in nude mice with PC-3 cell implants and a single injection of adenovirus over-expressing Par-4 caused a dramatic reduction in tumor volume in <3 weeks compared to tumors injected with an adenovirus-producing GFP (Chakraborty et al., 2001). Stable over-expression of Par-4 decreased the development of tumors resulting from xenotransplanted A375-C6 melanoma cells in SCID mice and this diminished tumor growth correlated with increased tumor cell apoptosis (Lucas et al., 2001). In addition to nude mice, Par-4 also reduced orthotopic tumors in immuno-competent mice indicating that this function of Par-4 is independent of the immuno-deficient status of the nude mice. Intra-prostatic tumors were generated in C57BL/6 mice by injecting syngenic prostate cancer cells RM-1 into the prostate gland of the mice. Similar to the subcutaneous tumors, a single injection of adenovirus expressing Par-4 caused a significant reduction in tumor volume compared to the GFP expressing control virus demonstrating that tumor regression is a potent function of Par-4 (J.R. Herman, 2001). The mechanism by which Par-4 reduces in tumor size involves induction of apoptosis as assessed by TUNEL staining of the tumors. Similar to the mechanism of apoptosis in cancer cell cultures, regression of tumors by Par-4 is dependent on the activation of the Fas-FADD death receptor pathway and inhibition of NF-κB pathway. Tumors derived from cells stably expressing dn-FADD or RelA failed to show

regression (Chakraborty et al., 2001). Together, these findings suggest that Par-4 is an ideal candidate for therapy of tumors. However, not much is known about the role of Par-4 in two critical events of tumorigenisis including angiogenesis and local tissue invasion leading to metastasis of tumors. In a promising development, an *in vitro* study with melanoma cells suggested that Par-4 decreases migration in these cells. Over-expression of Par-4 in mouse melanoma B16 F1 cells decreases their migration ability in a ζPKC-dependent manner. The mechanism by which the migration was affected was not elucidated but in the presence of low levels of ζPKC, Par-4 did not affect cell migration (Sanz-Navares et al., 2001). It was hypothesized that in non-metastatic cells, the ζPKC-Par-4 complex provides a brake on migration and events that prevent Par-4 mediated down-regulation of ζPKC activity in melanoma cells may promote metastasis.

Structure, Function and Localization Analysis: Identification of <u>S</u>elective for <u>A</u>poptosis induction in <u>C</u>ancer cells (SAC) domain.

Par-4 is strongly localized in the nucleus in most cancer cell lines. On the other hand, in all the normal cells tested, over-expressed Par-4 in the absence of another apoptotic signal is predominantly localized in the cytoplasm and does not induce apoptosis. Concordantly, the ΔNLS2 mutant of Par-4 in which both NLS1 and NLS2 are deleted, is purely cytoplasmic and is unable to induce apoptosis (El-Guendy et al., 2003). Intriguingly, in hormone dependent breast cancer cells MCF-7 or prostate cancer cells LNCaP, Par-4 is purely cytoplasmic in localization

and does not induce apoptosis. However, derivatives of these cell lines known to be hormone independent such as MCF-7 stably expressing oncogenic Ras (MCF-7 Ras) or LNCAP stably expressing IL-6 (LNCaP-IL-6) show nuclear translocation of Par-4 and are susceptible to apoptosis by Par-4 (El-Guendy et al., 2003). These findings, taken together, suggest that nuclear entry is essential for direct apoptosis by Par-4. The mechanism by which Par-4 is excluded from the nucleus of normal cells, estrogen dependent breast cancer cell lines or androgen dependent prostate cancer cell lines is not known. Nuclear exclusion is possibly a highly efficient mechanism to protect healthy cells from the apoptotic action of Par-4, and one of the outcomes of tumor progression is probably diminished stringency in nuclear exclusion of Par-4 in advanced cancers relative to normal cells leading to increased nuclear presence of Par-4. Our ongoing studies will verify whether nuclear entry is a regulatory mechanism for apoptosis by Par-4 and identify the molecular basis for the differences underlying the nucleocytoplasmic trafficking of Par-4 in normal and cancer cells.

A deletion mutant of Par-4 that contains amino acids 137-195, including an intact NLS2, constitutively enters the nucleus in all normal and cancer cell lines. The 137-195 mutant has an expanded range in inducing apoptotic killing both hormone dependent and hormone independent cancer cell lines. In fact, amino acids 137-195, represents a unique core domain of Par-4 that is essential and sufficient for Fas/FasL translocation to the cell membrane, inhibition of NF-κB activity and induction of apoptosis in cancer cells. Interestingly, although this

core domain localizes to the nucleus, it does not induce apoptosis in normal cells and was therefore designated as the domain for Selective Apoptosis-induction in Cancer cells (SAC domain) (El-Guendy et al., 2003). This core domain is 100% conserved in rat, mouse, and human Par-4. These findings indicate that nuclear entry is essential but not sufficient for apoptosis by Par-4, and that Par-4 may require additional activation events; for example, posttranslational changes in the SAC domain; that occur selectively in cancer cells leading to induction of apoptosis. The SAC domain resembles neither the death domains nor the death effector domains of other pro-apoptotic proteins, nor does it show significant homology with other known proteins in GenBank; and unlike the previously characterized death domains and death effector domains, the SAC domain specifically induces apoptosis in cancer cells and not normal cells. This study will focus on identifying and characterizing the molecular differences between normal and cancer cells that selectively activate apoptosis by the SAC domain.

Specific Aims of this study

Par-4 is a pro-apoptotic protein implicated in various physiological and pathological apoptotic pathways. In most cancer cells, over-expressed Par-4 localizes strongly in the nucleus and spontaneously induces apoptosis in these cells. By contrast, in normal/immortalized cells, Par-4 is predominantly cytoplasmic and does not induce apoptosis, indicating that the nuclear presence of Par-4 is essential for apoptosis. The minimal region of Par-4 required for apoptosis, the SAC domain, constitutively enters the nucleus and has an

Fig.1.3. Model for Mechanism of Spontaneous Apoptosis by Par-4

Par-4 specifically induces apoptosis in cancer cells but not normal cells. This model suggests that mechanism for activation of apoptosis by Par-4 requires two specific events, namely, post-translational, possibly phosphorylation event/s in the SAC domain and nuclear translocation of Par-4.

Fig.1.3. Model for Mechanism of Spontaneous Apoptosis by Par-4
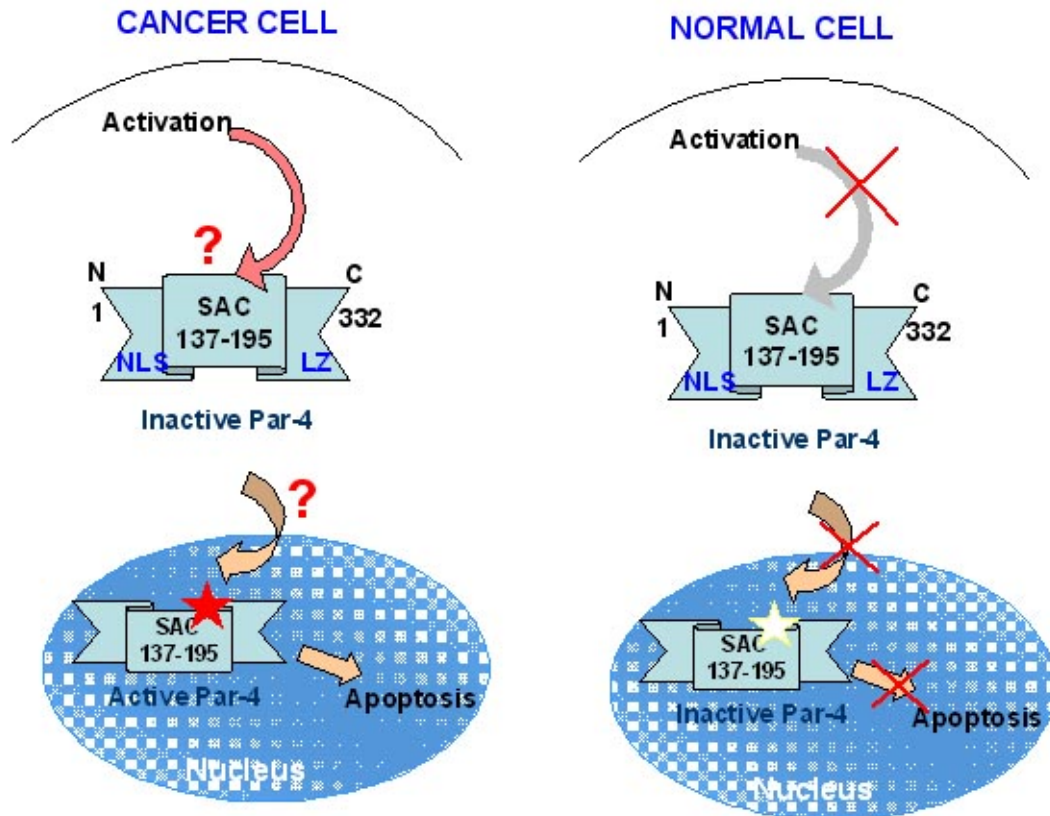
expanded range in inducing apoptosis of all cancer cell lines tested. Interestingly, although this mutant localizes to the nucleus, it does not induce apoptosis in non-transformed normal/immortalized cells (El-Guendy et al., 2003). These findings indicated that besides nuclear entry, Par-4 requires additional activation event(s) in order to induce apoptosis and that cancer cells possess the ability to activate the SAC domain of Par-4 (Fig 1.3):

Hypothesis: Activation of apoptosis by Par-4 requires two specific events, namely, post-translational, possibly phosphorylation event/s in the SAC domain and nuclear translocation of Par-4 (Fig. 1.3). The specific objectives of this study are to:

1. Identify the candidate amino acid residues in the SAC domain of Par-4 that could be phosphorylated and establish their importance in apoptosis by site directed mutagenesis.

2. Verify whether phosphorylation of residues S154 or T155 present in the SAC domain are involved in activation of Par-4 function

3. Identify the kinase that phosphorylates either S154 or T155

4. Identify the mechanism behind the cancer selective activation of apoptosis by the SAC domain; verify if the same kinase that phosphorylates either S154 or T155 is elevated in cancers

5. Show that apoptosis by endogenous Par-4 in response to stimuli is regulated by the same mechanism as ectopic Par-4 in cancer cells

CHAPTER 2

MECHANISM OF CANCER SELECTIVE APOPTOSIS BY PAR-4

Introduction

Tumors often show heterogeneity at many levels including morphology, expression of pro-survival traits and down-modulation of apoptotic or tumor suppressor traits. Despite tumor heterogeneity, several predominant traits exist within similar types of cancers. Such traits are prospective targets for therapeutic intervention strategies. However, because most cancer therapy protocols have an accompanying toxicity for normal cells, there are ongoing efforts directed toward the identification of apoptotic molecules that are highly selective for cancer cells. Our recent studies have identified Par-4 protein as a unique pro-apoptotic molecule that induces apoptosis in hormone-independent but not hormone-dependent cancer cells or in primary or immortalized normal cells (El-Guendy et al., 2003). We describe here a novel mechanism underlying the selective activation of the apoptotic function of Par-4 in cancer cells.

The *par-4* gene was identified in a search for genes induced during apoptosis in prostate cancer cells (Sells et al., 1994). The gene maps to human chromosome 12q21, a region that is unstable and often deleted in pancreatic and gastric cancers (Schneider et al., 2003). Expression of Par-4 is diminished in renal cell carcinoma (Cook et al., 1999), neuroblastoma (Kogel et al., 2001), acute lymphatic leukemia and chronic lymphocytic leukemia (Boehrer et al.,

2001). In addition, the *par-4* gene is down-regulated by oncogenes such as Ras, Raf or Src, (Barradas et al., 1999; Qiu et al., 1999b), and restoration of Par-4 levels results in the inhibition of oncogene-induced cellular transformation (Nalca et al., 1999). Interestingly, ectopic Par-4 over-expression in cancer cells is sufficient to induce apoptosis in these cells and cause regression of tumors. Direct apoptosis by Par-4 occurs by a unique mechanism that involves co-parallel activation of the pro-death FasL-Fas-FADD-caspase-8 pathway and inhibition of NF-κB pro-survival pathway (Chakraborty et al., 2001). Par-4 activates the Fas pathway by inducing the translocation of Fas and FasL to the cell membrane, and parallel inhibition of the transcription activity of NF-κB, to allow the apoptosis pathway to progress unhindered (Chakraborty et al., 2001). Apoptosis in cancer cells by Par-4 is independent of potential anti-apoptotic roadblocks, such as high NF-κB activity, Bcl-xL or Bcl-2 levels, and the status of tumor suppressors such as p53 or PTEN (Chakraborty et al., 2001).

Par-4 is a 332 amino acid protein that is composed of a leucine zipper domain in the carboxy-terminal region and two putative nuclear localization signal (NLS) domains (El-Guendy et al., 2003). These domains are fully conserved in human, rat and mouse Par-4 (El-Guendy and Rangnekar, 2003). We recently reported that nuclear localization of Par-4 by NLS2 is essential for apoptosis by Par-4 (El-Guendy et al., 2003). In certain cancer cells and normal or immortalized cells, Par-4 does not enter the nucleus and is unable to induce apoptosis (El-Guendy et al., 2003). Consistently, a deletion mutant of Par-4,

containing amino acids 137-195 with intact NLS2 domain, constitutively enters the nucleus and has an expanded range in inducing apoptosis of all cancer cell lines tested (El-Guendy et al., 2003). In fact, the region containing amino acids 137-195 represents a unique core domain of Par-4 that is essential and sufficient for Fas/FasL translocation to the cell membrane, inhibition of NF-κB activity and induction of apoptosis in cancer cells. Interestingly, although this core domain localizes to the nucleus, it does not induce apoptosis in normal/immortalized cells, and was therefore designated as the domain Selective for Apoptosis induction in Cancer cells (SAC domain) (El-Guendy et al., 2003). These findings indicate that nuclear entry is required but not sufficient for apoptosis by Par-4, and that Par-4 may require additional activation events; such as, posttranslational changes in the SAC domain, that occur selectively in cancer cells leading to induction of apoptosis.

One of the most common posttranslational modifications by which the function of cellular proteins may be rapidly switched on or off is phosphorylation. The phosphorylation events are affected by tyrosine or serine (S)/threonine (T) kinases. cAMP dependent protein kinase or PKA is one such S/T kinase that is involved in the regulation of gene expression, metabolism, cell growth and differentiation. PKA is a broad-spectrum kinase that phosphorylates a wide variety of substrates and is therefore very tightly regulated by differential expression of subunits and sub-cellular localization. The PKA holoenzyme is a tetramer composed of two catalytic (C) subunits that are held in an inactive state

by association with regulatory (R) subunits. Binding of cAMP to the R subunits causes the C subunits to dissociate allowing phosphorylation of substrates (Corbin and Keely, 1977; Corbin et al., 1973; Gibbs et al., 1992; Wang et al., 1991). PKA occurs in mammalian cells in two distinct isoforms, PKA-I and PKA-II, which differ only in their regulatory subunits (that are termed RI for PKA-I and RII for PKA-II). PKA-I is generally associated with proliferation, and is often over-expressed in human cancer cell lines and in primary tumors. On the other hand, preferential expression of PKA-II is found in normal non-proliferating tissues and in growth-arrested cells (reviewed in (Tortora and Ciardiello, 2002)). In addition to the current dogma for PKA regulation, recent discoveries implicate the existence of cAMP-independent activation of PKA (Cvijic et al., 2000).

The role of PKA in cancers is not fully understood. On the one hand, germline mutations in PKA-I lead to the Carney complex, a multiple neoplasia syndrome associated with a greater PKA response to cAMP due to compensatory increases in the other PKA subunits (reviewed in (Stergiopoulos and Stratakis, 2003)). On the other hand, PKA-I is found to be over-expressed in several cancer cells, and is often associated with a poor prognosis in cancer patients (Cho-Chung et al., 2002). In addition, increased expression of PKA-I is seen following cellular transformation by certain growth factors, such as TGF-α, or oncogenes, such as *ras, myc* and *erb*B-2 (Tortora and Ciardiello, 2002). Although PKA is primarily an intracellular enzyme, there is growing evidence that there is a form of PKA that is secreted into the extracellular space. Extracellular

PKA functions in a cAMP-independent manner and the levels of this form of PKA are especially high in the growth media of cultured cancer cells, as well as, in plasma samples from cancer patients (Cho et al., 2000; Cvijic et al., 2000). Furthermore, over-expression of the catalytic subunit of PKA (PKAc) in fibroblast cells leads to transformation of the fibroblasts; this finding directly implicates PKA in the process of oncogenesis (Wu et al., 2002). In a recent study of breast cancer patients, elevation of PKA activity was found to be the cause of tamoxifen resistance in breast cancer (Michalides et al., 2004). Thus, ample evidence in literature suggests that PKA plays a critical role in the development of the cancer phenotype. PKA exerts its cellular functions by phosphorylation of various substrate proteins that include transcription factors such as CREB and NF-κB, pro-apoptotic proteins such as Bad, or tumor suppressor proteins such as LKB1 (Collins et al., 2000; Harada et al., 1999; Zhong et al., 1998).

In the present study, we investigated the mechanism by which Par-4 or the SAC domain selectively induces apoptosis in cancer cells but not in primary normal or immortalized cells. We report that PKA-mediated phosphorylation of T155 residue in Par-4 is essential for the apoptotic function of Par-4. Moreover, we found that the PKA enzymatic activity is elevated in transformed and cancer cell lines, relative to normal/immortalized cells, leading to selective activation of the apoptotic function of Par-4 in cancer cells but not in non-transformed cells. Thus, the pro-apoptotic molecule Par-4 utilizes elevated PKA, a pro-survival

alteration commonly occurring in several types of cancer cells, for activation, in order to induce selective apoptosis of the cancer cells.

MATERIALS AND METHODS

Cell lines

Androgen-dependent prostate cancer cells LNCaP; androgen-independent prostate cancer cells PC-3, DU145, or LNCaP/IGFBP5 (an isogenic derivative of LNCaP); immortalized human prostate epithelial cells PZ-HPV-7; normal primary prostatic cells PrE or PrS; mouse embryo fibroblast (MEF) cells, NIH 3T3 fibroblast cells and NIH 3T3/Ras transformed fibroblast cells; human lung cancer cells A549, H157, H838 and H460; and human breast cancer cells MCF-7, MDA MB 231 and MDA MB 435 have been described by us (El-Guendy et al., 2003). Normal bronchial epithelial cells NHBE were purchased from Clonetics Corp. (San Diego, CA). Human embryonic lung fibroblast cells HEL were from Tim Kowalik (University of Massachusetts, Worcester, MA).

Plasmid constructs chemical reagents and antibodies.

pCB6$^+$ vector, pCB6$^+$-Par-4, GFP-SAC domain and GFP-137-187 constructs, and NF-κB-luciferase reporter construct were described previously (El-Guendy et al., 2003). The S154 to 154A or T155 to 155A point mutant of Par-4 was made by site directed mutagenesis, so as to change the sequence at S154 from TCC to GCC or T155 from AGC to GCC. GFP-SAC domain/155A and GFP-137-187/155A constructs were prepared by PCR amplification using Par-4/155A as template, followed by ligation in pcDNA3.1/CT-GFP-TOPO vector (Invitrogen Corp., CA). The expression construct for the catalytic subunit of PKA

(PKAc) was kindly provided by Sankar Ghosh (Yale Medical School, CT). PKA inhibitory peptide PKI and 8-Cl-cAMP were from Calbiochem. Vincristine, doxorubicin and DAPI were from Sigma Chemicals Co. (St. Louis, MO). Antibodies for Par-4, PKA, pCREB, and total CREB, as well as the Annexin V kit for apoptosis assays were from Santa Cruz Biotechnology, Inc. (Palo Alto, CA). The actin antibody was from Sigma Chemical Co. (St. Louis, MO). The phospho-T155-Par-4 antibody was raised in rabbits against the peptide KRKLREKRRS**(PO4T)**GVVNIP (representing amino acids 148-161 of Par-4 with phosphorylated T155 residue). Small interfering (si) RNA oligonucleotide duplexes directed against human Par-4 or human PKA α or β catalytic subunits were from Dharmacon, Inc. (Lafayette, Co).

Transfection and RelA/NF-κB reporter assays.

Cells were transiently transfected with the indicated plasmid constructs by using lipofectamine-plus (from Invitrogen Life Technologies, CA) according to the manufacturer's instructions. Cells were harvested after 48 h, and whole-cell lysates were resolved by SDS-PAGE, transferred to PDVF membranes and subjected to Western blot analysis to ascertain expression of the constructs; or to luciferase and β-galactosidase assays, to quantify and normalize NF-κB activity, as previously described (Chakraborty et al., 2001).

PKA activity assay.

The *in vitro* PKA enzymatic assay was performed using the cAMP-dependent PKA Signatect Assay kit from Promega Corp. (Madison, WI). Briefly, cells were suspended in extraction buffer (25 mM Tris-pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, leupeptin, aprotinin and PMSF) and homogenized using a cold homogenizer. Aliquots of cell lysate (5 µl) were incubated with PKA assay buffer (200 mM Tris-pH7.4, 100 mM MgCl2, 0.5 mg/ml BSA), 25 mM cAMP, PKA biotinylated substrate peptide and (γ-^{32}P)-labeled ATP at 30 C. After 15 minutes, the reaction was terminated with guanidinium hydrochloride and spotted on streptavidin-coated phosphocellulose paper. Incorporation of γ-^{32}P into PKA biotinylated substrate peptide was measured in a scintillation counter, and the PKA activity was normalized to protein concentration of the cell lysates and expressed as pmoles of ATP/min/μg protein.

Apoptosis assay and indirect immunofluorescence.

Cells, plated in chamber slides, were transiently transfected with Par-4, mutant Par-4, or PKAc constructs or subjected to the indicated treatments. The cells were subjected to immunocytochemistry (ICC) by using Par-4 or PKA antibody followed by secondary antibody conjugated to Alexa Fluor 488 (green fluorescence) or Texas red (red fluorescence), and nuclei were stained with DAPI (cyan fluorescence) and visualized by confocal microscopy. In each experiment, a total of at least 300 transfectants expressing the constructs were scored for apoptosis based on condensed nuclei, or by staining with FITC conjugated-

Annexin V, (Chakraborty et al., 2001). The data presented are mean values of three independent experiments $\pm$ standard deviation.

Metabolic phospholabeling and immunoprecipitation.

Cells were transfected with expression constructs for Par-4 or the indicated point mutants, and 36h post-transfection, the cells were washed with phosphate-free DMEM, incubated overnight with (^{32}P) orthophosphate (200 µCi/ml). Cells were lysed with lysis buffer (50 mM Tris-HCl-pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% w/w Triton X-100, 1mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 µM microcystin-LR, 0.1% v/v 2-mercaptoethanol, and complete proteinase inhibitor mixture) and Par-4 was immunoprecipitated from the cleared lysate with anti-Par-4 antibody (5 µg of antibody conjugated to 5 µl of protein G-Sepharose beads). The immunoprecipitates were washed ten times with lysis buffer and subjected to SDS-PAGE, transferred to a nylon membrane followed by autoradiography. The same blots were subjected to Western blot analysis with anti-Par-4 antibody as a loading control.

***In vitro* phosphorylation assays on immunoprecipitated Par-4.**

NIH 3T3 cells were transiently transfected with Par-4 or the indicated point mutants, the cells were lysed after 48h and Par-4 was immunoprecipitated as described above. Kinase assays were performed *in vitro* by adding purified PKA

enzyme and (γ-^{32}P) ATP in kinase buffer (10 mM MgCl2, HEPES-pH 7.2). Immunoprecipitated proteins were incubated in a total volume of 40µl at 30°C with 1 units/ml PKA, in kinase (10 mM MgCl2, HEPES pH7.2), 100 µM (γ-^{32}P) ATP (1000 cpm/pmol). After incubation for 15 min, incorporation of phosphate into Par-4 was determined by resolving the samples by SDS-PAGE, followed by transfer to PVDF nylon membrane and autoradiography. The same blots were subjected to Western blot analysis with anti-Par-4 antibody as a loading control.

Transfections with siRNA.

Cells, in six-well plates, were transiently transfected with siRNAs using Oligofectamine (Invitrogen Corp.). Briefly, 4 µg of siRNA against Par-4 or PKA α and β catalytic subunits or control siRNA was mixed with 175 µl of Opti-MEM (fresh RPMI medium without antibiotics) and complexed with a mixture of 3 µl of Oligofectamine and 15 µl of Opti-MEM for 20 min at room temperature. The complex was diluted to obtain a final volume of 1 ml and added to the cells. After 4 h, the cells were replenished with 500 µl of Opti-MEM containing 30% fetal calf serum and incubated for 24 h. siRNA transfections were repeated after 24 h, and after a total of 48 h, the cells were processed for either apoptosis, PKA activity assays or Western blot analysis. The siRNA for Par-4 (5'-GAUGCAAUUACACAACAGAdTdT-3'); siRNA for PKA catalytic subunit α (catalog number M-004649-00) and PKA catalytic subunit β (catalog number M-004650-00) were purchased from Dharmacon, Inc., Lafayette, CO.

RESULTS

T155 residue is essential for apoptosis by Par-4.

We recently reported that nuclear localization is essential for apoptosis by Par-4 (El-Guendy et al., 2003). Par-4 does not enter the nucleus in normal cells or in hormone-dependent cancer cells, such as the prostate cancer cells LNCaP or breast cancer cells MCF-7, and is unable to induce apoptosis. Moreover when NLS2 is deleted, Par-4 loses its ability to induce apoptosis. The SAC domain mutant of Par-4 constitutively enters the nucleus and has an expanded range in inducing apoptosis of all cancer cell lines tested. Interestingly, although this mutant localizes to the nucleus, it does not induce apoptosis in non-transformed normal/immortalized cells (El-Guendy et al., 2003). These findings indicated that besides nuclear entry, Par-4 or the SAC domain require additional activation event(s) in order to induce apoptosis.

To identify a potential activation switch in Par-4, we focused on the amino acid sequence of the SAC domain, and noted the presence of two PKA consensus phosphorylation sites at residues S154 and T155 for PKA (Fig. 2.1A). To test the functionality of these sites, we mutated the S154 or T155 residues to 154A or 155A, respectively, in full length Par-4. The ability of these mutants to induce apoptosis was tested in PC-3 cells, which are known to undergo apoptosis with Par-4. The cells were transiently transfected with vector, wild type Par-4, 154A or 155A mutant constructs and assayed for apoptosis by scoring

Fig. 2.1. T155 is essential for the apoptotic function of Par-4.

A)   Schematic representation of NLS1, NLS2, S154, T155 and the SAC domain of full length Par-4.

B)   PC-3 cells were transiently transfected with vector, Par-4, ΔNLS2 or the point mutant 154A or 155A, and expression of Par-4 and mutants was examined by Western blot analysis using the Par-4 antibody or actin antibody

Fig. 2.1. T155 is essential for the apoptotic function of Par-4.
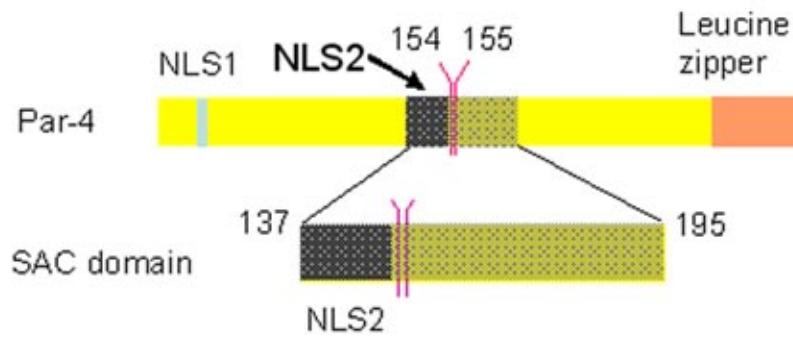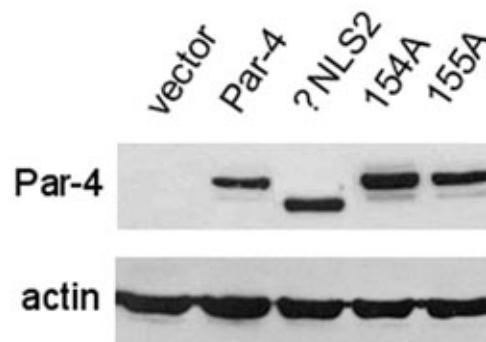
Fig. 2.1. T155 is essential for the apoptotic function of Par-4.

C and D) The ability of Par-4 or the mutants to induce apoptosis at 48 h post-transfection was examined in PC-3 cells by either ICC with the Par-4 antibody and secondary antibody conjugated to Alexa Fluor 488 (green fluorescence) followed by nuclear staining with DAPI (D, left panel) or by staining with FITC conjugated-Annexin V antibody (D, right panel). Cells expressing Par-4 or mutant proteins were scored for chromatin condensation or Annexin V positive cells indicative of apoptosis by confocal microscopy as described in Materials and Methods, and quantitative data are shown in panel C. Nomarski images show total number of cells in each field (D, right panel).

Fig. 2.1. T155 is essential for the apoptotic function of Par-4.
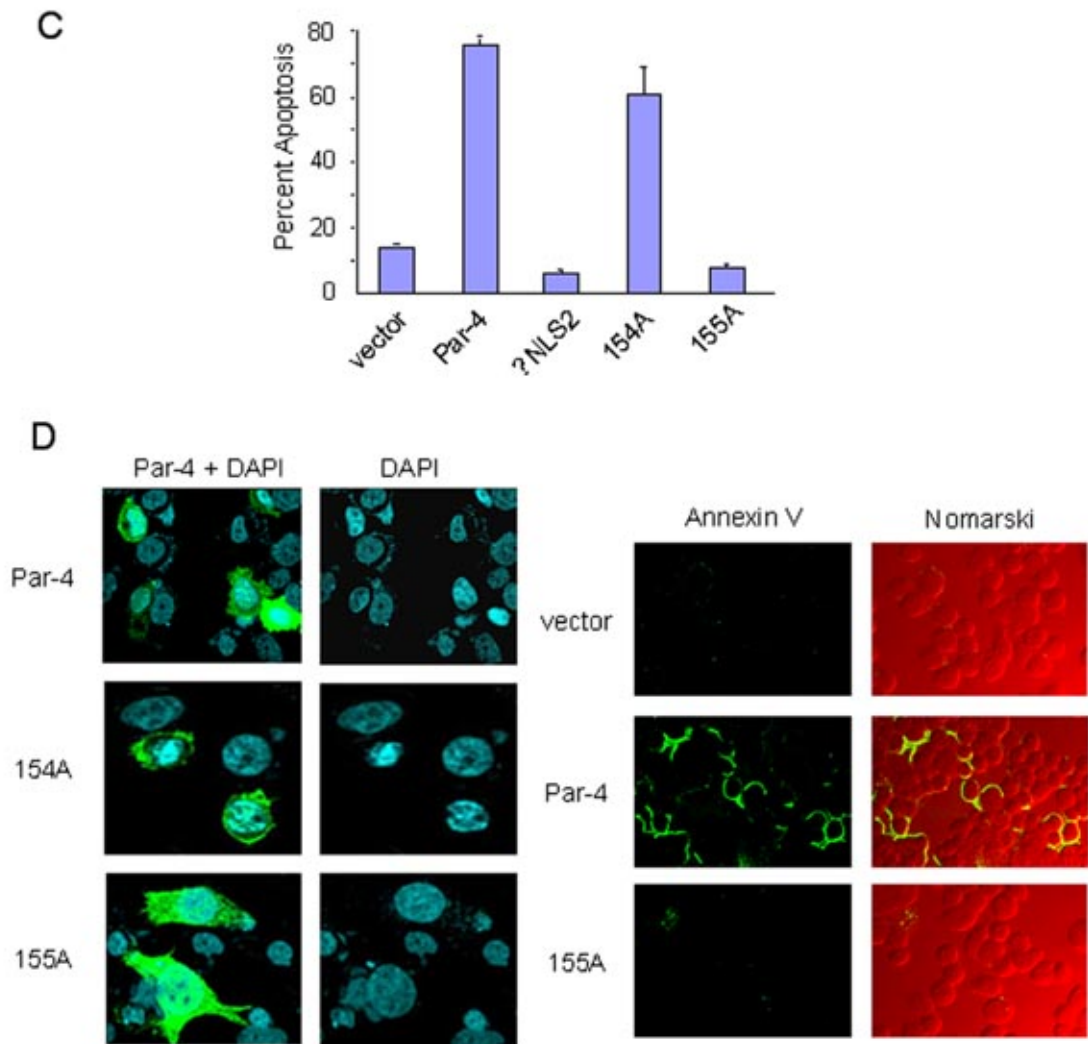
Fig. 2.1. T155 is essential for the apoptotic function of Par-4.

E)   Inhibition of NF-κB transcriptional activity by Par-4 or its mutants (E), PC-3

cells were transfected for 48 h with NF-κB-luc reporter construct and β-

galactosidase plasmid together with vector, Par-4 or mutant constructs. Whole-

cell lysates were subjected to luciferase assays, and luciferase activity was

normalized to the corresponding β-galactosidase activity.
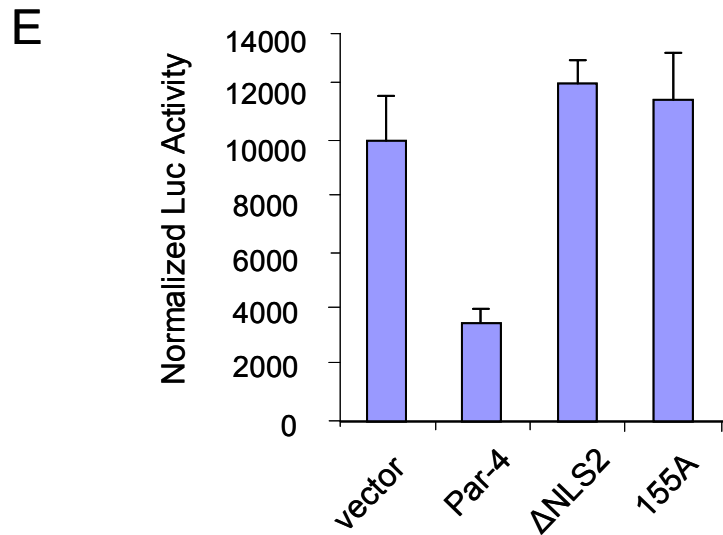
Fig. 2.1. T155 is essential for the apoptotic function of Par-4.

F)   PC-3 cells were transiently transfected with expression constructs for GFP-SAC or GFP-SAC/155A mutant and expression of the mutants was confirmed by Western blot analysis with the Par-4 or actin antibody

G and H)   The ability of the mutants to induce apoptosis was quantified by confocal microscopy). In panel H, DAPI has been psuedo-colored red, and the yellow fluorescence resulting from the overlay with the green fluorescent GFP-fusion proteins, indicates nuclear localization.

Fig. 2.1. T155 is essential for the apoptotic function of Par-4.
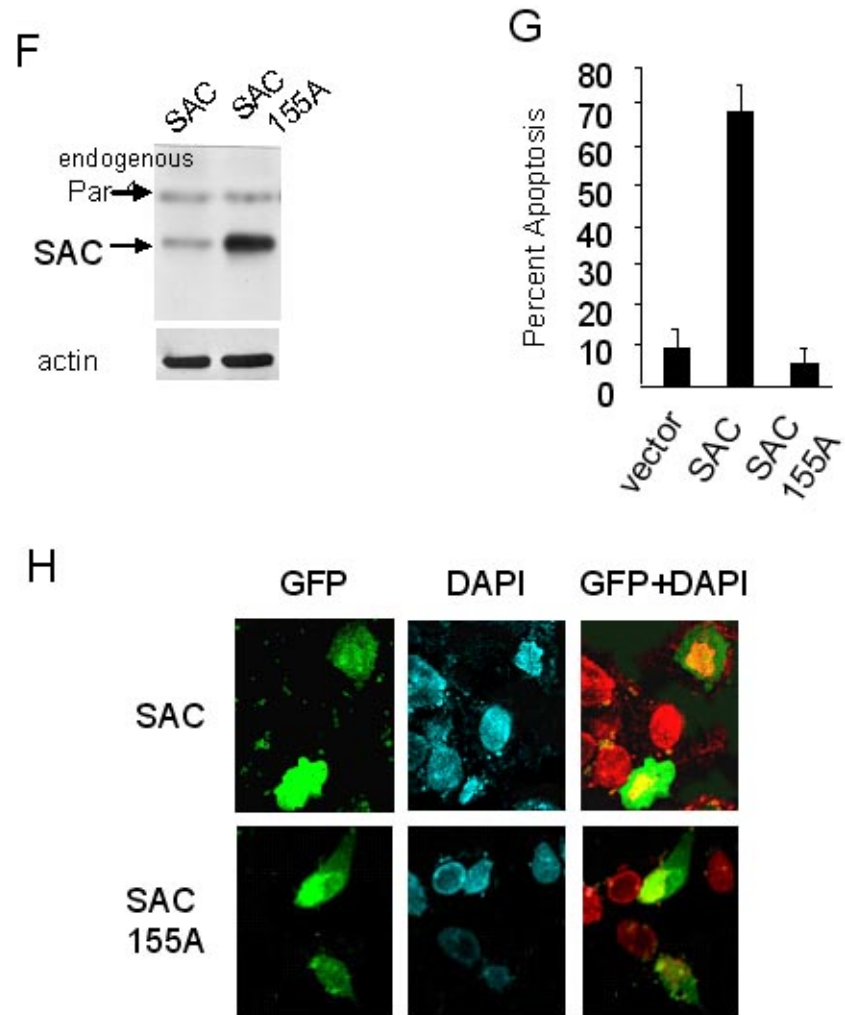
either condensed DAPI stained nuclei (Fig. 2.1D left panel) or Annexin V positive cells (Fig. 2.1D right panel). The deletion mutant ΔNLS2, lacking NLS1 and NLS2 that does not translocate to the nucleus (El-Guendy et al., 2003), was used as a control. We found that the 154A mutant induced apoptosis similar to wild type Par-4, but the 155A mutant and ΔNLS2 were unable to induce apoptosis (Fig. 2.1, C and D; data for cytoplasmic retention of ΔNLS2 are similar to those described previously in reference 12, and therefore not shown). All the constructs were expressed equally well, as assessed by Western blot analysis (Fig. 2.1B) and immuno-cytochemistry (Fig. 2.1D); suggesting that lack of apoptosis by 155A is not due to reduced expression. These findings suggested that T155 is essential for the apoptotic function of Par-4 and that the T155A mutation abolishes its apoptotic activity in PC-3 cells.

We then tested whether the functions of Par-4 essential for its apoptotic activity, such as inhibition of NF-κB transcription were altered by the T155A mutation. PC-3 cells were transiently transfected with Par-4, 155A mutant, or ΔNLS2 and vector for control and NF-κB-luciferase reporter construct, and whole-cell extracts were tested for the ability of Par-4 or the mutants to inhibit NF-κB activity. As seen in Fig. 2.1E, relative to vector, Par-4 inhibited NF-κB-dependent luciferase activity but the 155A mutant or ΔNLS2 was unable to inhibit this activity. A study of the kinetics of inhibition of NF-κB activity and apoptosis over a 48 h period indicated that, similar to our previous findings (El-Guendy et al., 2003), inhibition of NF-κB activity by Par-4 preceded apoptosis (data not

shown as the findings are similar to those reported in reference (El-Guendy et al., 2003). Moreover, the 155A mutant neither inhibited NF-κB activity nor induced apoptosis over this time period (data not shown). These findings suggested that the T155A mutation in Par-4 led to loss of its apoptotic potential.

Next, because the SAC domain of Par-4 was necessary and sufficient for cancer cell apoptosis by Par-4, we tested whether T155 was crucial for apoptosis by the SAC domain. The T155 residue in the SAC domain was mutated to 155A, and PC-3 cells were transiently transfected with the SAC domain construct or SAC/155A mutant construct and tested for apoptosis induction. Expression of the constructs was ascertained by Western blot analysis (Fig. 2.1F). As seen in Fig. 2.1 (G and H), the 155A mutant of the SAC domain completely lost its ability to induce apoptosis in PC-3 cells. The proximity of T155 to the NLS raised the possibility that phosphorylation of T155 might play a role in nuclear entry of Par-4. However, confocal microscopy indicated that the T155A mutant of Par-4 or of the SAC domain translocated to the nucleus yet failed to induce apoptosis (Fig. 2.1, C & H).

To ascertain that the loss of the apoptotic potential of Par-4 or the SAC domain resulting from the T155A mutation was not limited to the PC-3 cell background, we tested the 155A mutant of Par-4 or of the SAC domain in NIH 3T3/Ras cells, as well as in various lung cancer, breast cancer, and prostate cancer cell lines (see Fig. 2.2, G & H; or Fig. 2.3, B-D). Similar to our findings in

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PC-3 cells, Par-4 or the SAC domain induced apoptosis in all the cells tested but the Par-4/155A or SAC/155A mutant failed to induce apoptosis in these cells (also see Figs. 2 and 3). Together, these findings indicated that the T155 residue is essential for apoptosis by Par-4 or the SAC domain.

PKA phosphorylates Par-4 at T155.

Par-4 protein contains several S and T residues that are potential phosphorylation sites for PKA, PKC and CKII (El-Guendy et al., 2003). In order to determine whether regulation of the apoptotic function of Par-4 is linked to phosphorylation, we determined whether Par-4 is phosphorylated *in vivo*. NIH 3T3/Ras cells were transfected with the Par-4 expression construct or vector for control and then metabolically labeled with (^{32}P) orthophosphate. The lysates were subjected to immuno-precipitation with the rabbit polyclonal Par-4 antibody or with pre-immune serum as control. The immuno-precipitates were resolved by SDS-PAGE, transferred onto a nylon membrane, and detected by autoradiography. The ^{32}P-labeled protein (approximately 40 kDa) that immunoprecipitated with the Par-4 antibody (Fig. 2.2A, upper panel) was further confirmed as Par-4 by subjecting the same blot to Western blot analysis with Par-4 antibody (Fig. 2.2A, lower panel). The amino acid context of T155 (KRRST) suggests that it is a consensus phosphorylation site (RXXS/T) for PKA. To ascertain that Par-4 is indeed phosphorylated by PKA and that Par-4

Fig. 2.2. PKA phosphorylates Par-4 at T155.

A)   Par-4 is a phosphorylated protein, NIH 3T3/Ras cells were transiently trasfected with vector or Par-4 for 12 h, incubated in phosphate-free medium overnight, and then metabolically labeled with 200 µCi ^{32}P-orthophosphate for 5 h. Whole-cell extracts were subjected to immuno-precipitation with Par-4 or control IgG antibody, resolved by SDS-PAGE, and transferred to PVDF membrane. The blot was autoradiographed (upper panel), and finally probed with the Par-4 antibody (lower panel).

B)   PKA phosphorylates Par-4 at T155, NIH 3T3 cells were transiently transfected with vector, Par-4, 154A or 155A expression construct for 24 h and whole-cell extracts were immuno-precipitated with the Par-4 antibody or IgG control antibody. The immuno-precipitated proteins were subjected to an *in vitro* phosphorylation reaction with purifed PKA enzyme and (γ-^{32}P)-labeled ATP, and then the radiolabeled proteins were resolved by SDS-PAGE, transferred to PVDF membrane and autoradiographed. The blot was finally probed with Par-4 antibody to determine Par-4 or mutant protein level. Following densitometric scanning of the blots, the amount of ^{32}P-label incorporated into ectopic Par-4 or mutant protein by PKA was normalized to the corresponding protein level detected on the Western blots, and relative amount of ^{32}P-label incorporated (i.e., PKA phosphorylation) is shown at the bottom of the panel (B).

Fig. 2.2. PKA phosphorylates Par-4 at T155.
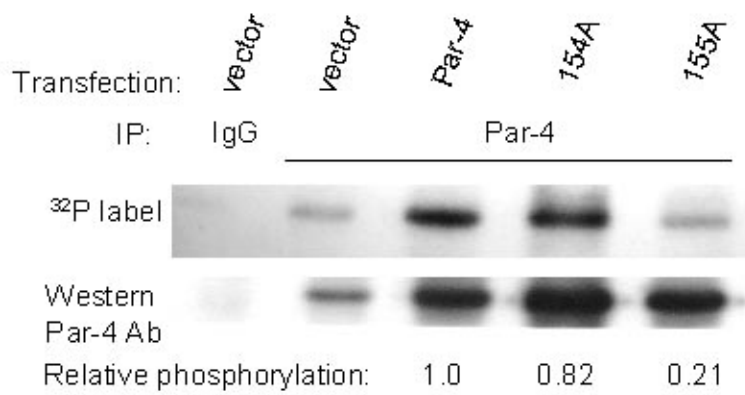
Fig. 2.2. PKA phosphorylates Par-4 at T155.

C) PKA phosphorylates Par-4 *in vivo* , we used 137-187 and 137-187/155A expression constructs, which were first tested for expression by transient transfection followed by Western blot analysis with Par-4 or actin antibody (C, right panel). To examine whether PKA phosphorylates Par-4 *in vivo* (C, left panel), NIH 3T3/Ras cells were transiently transfected with vector, 137-187 or 137-187/155A mutant expression construct in the presence or absence of PKA-inhibitory peptide PKI for 24 h, then metabolically labeled and immuno-precipitated with the Par-4 antibody or IgG control antibody. The immuno-precipitates were subjected to SDS-PAGE, transferred to nylon membranes and autoradiographed (C, left upper panel). Finally, the blots were probed with the Par-4 antibody (C, left lower panel), as described above in panels A and B.
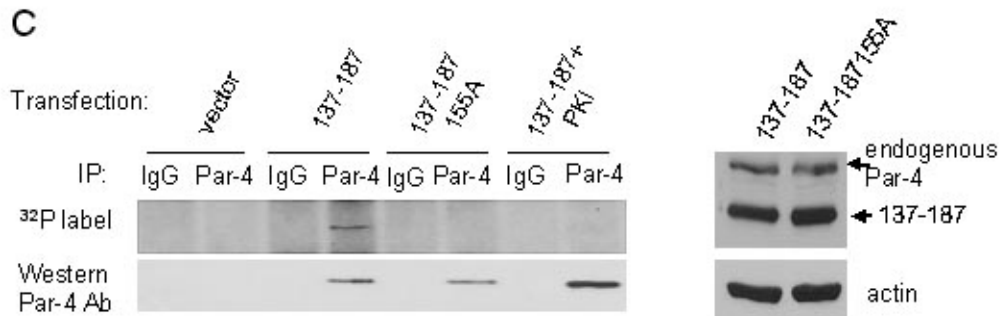
Fig. 2.2. PKA phosphorylates Par-4 at T155.

D)   To characterize the phospho-T155 (pT155) antibody, MEFs were transfected with GFP, GFP-Par-4 or GFP-155A plasmids, with or without the PKAc expression construct for 24 h, and Western blot analysis for phospho-T155, Par-4, PKA catalytic subunit and actin was performed.
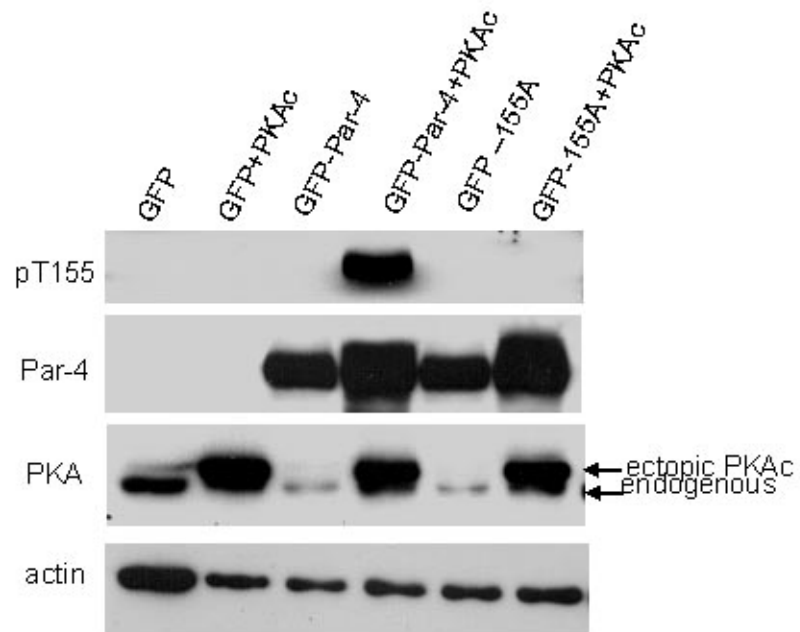
Fig. 2.2. PKA phosphorylates Par-4 at T155.

E and F)   pT155 antibody does not recognize 155A mutant of Par-4. NIH

3T3/Ras cells (E) or PC-3 cells (F), cells were transiently transfected with vector,

Par-4, 154A or 155A plasmids (E & F, left panels), or with GFP, GFP-Par-4 or

GFP-Par-4/155A plasmid and treated for 24 h with vehicle or 20 µM or 40 µM

PKI (E & F, right panels), and then subjected to Western blot analysis with

pT155, Par-4 or actin antibodies.
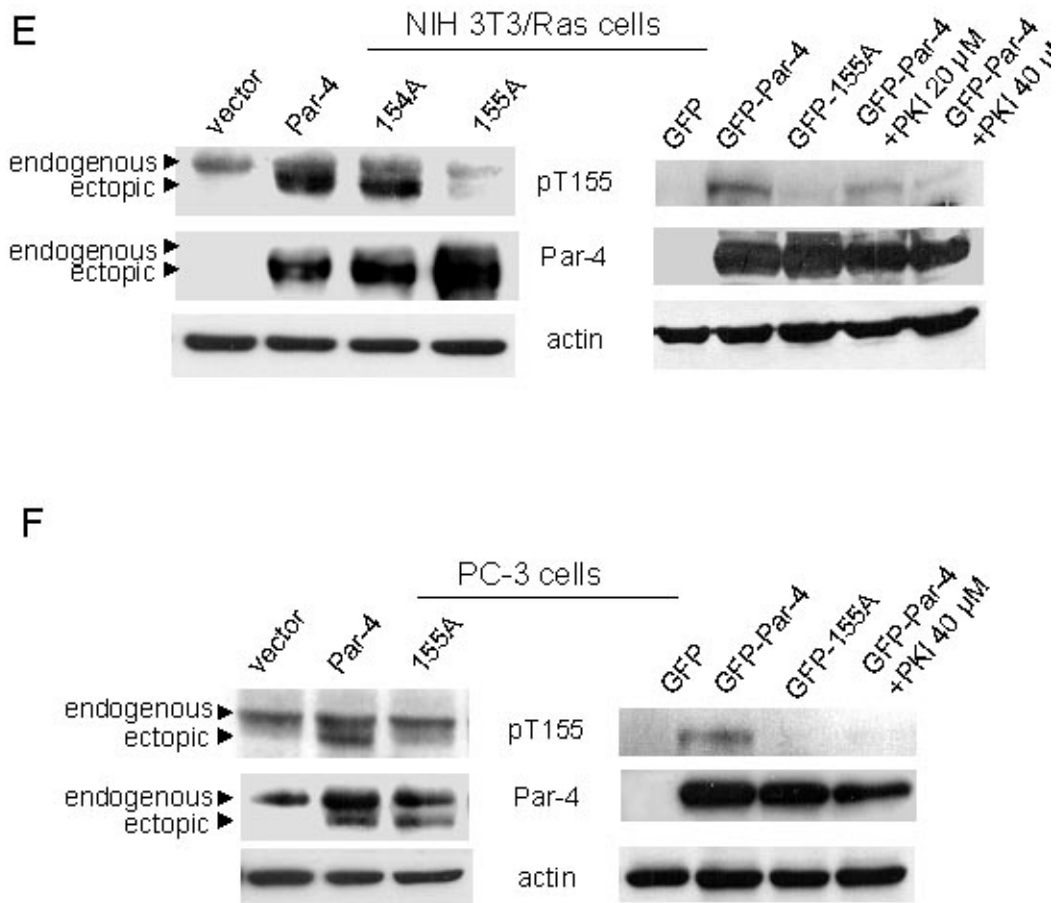
Fig. 2.2. PKA phosphorylates Par-4 at T155.

G)   To determine whether inhibition of PKA activity inhibits apoptosis by Par-4, PC-3, NIH 3T3/Ras or MDA MB 231 cells were transiently transfected with GFP or GFP-Par-4. The PC-3 transfectants were treated with vehicle, PKI 20 μM or PKI 40 μM; and NIH 3T3/Ras or MDA MB 231 cells were treated with PKI 20 μM; and assayed for apoptosis, as described in Fig. 1.

H)   To ascertain that inhibition of PKA activity by PKI inhibits apoptosis by the SAC domain in diverse cancer cell lines, the cells were transfected with SAC expression construct and then treated with 20 μM PKI peptide or control and scored for apoptosis.

I)   To confirm that PKI inhibits PKA activity, PC-3 cells, NIH 3T3/Ras or MDA MB 231 cells were treated with control or 20 μM PKI peptide for 48 h, and whole-cell lysates were tested for PKA activity by using the cAMP-dependent PKA Signatect Assay kit.

Fig. 2.2. PKA phosphorylates Par-4 at T155.
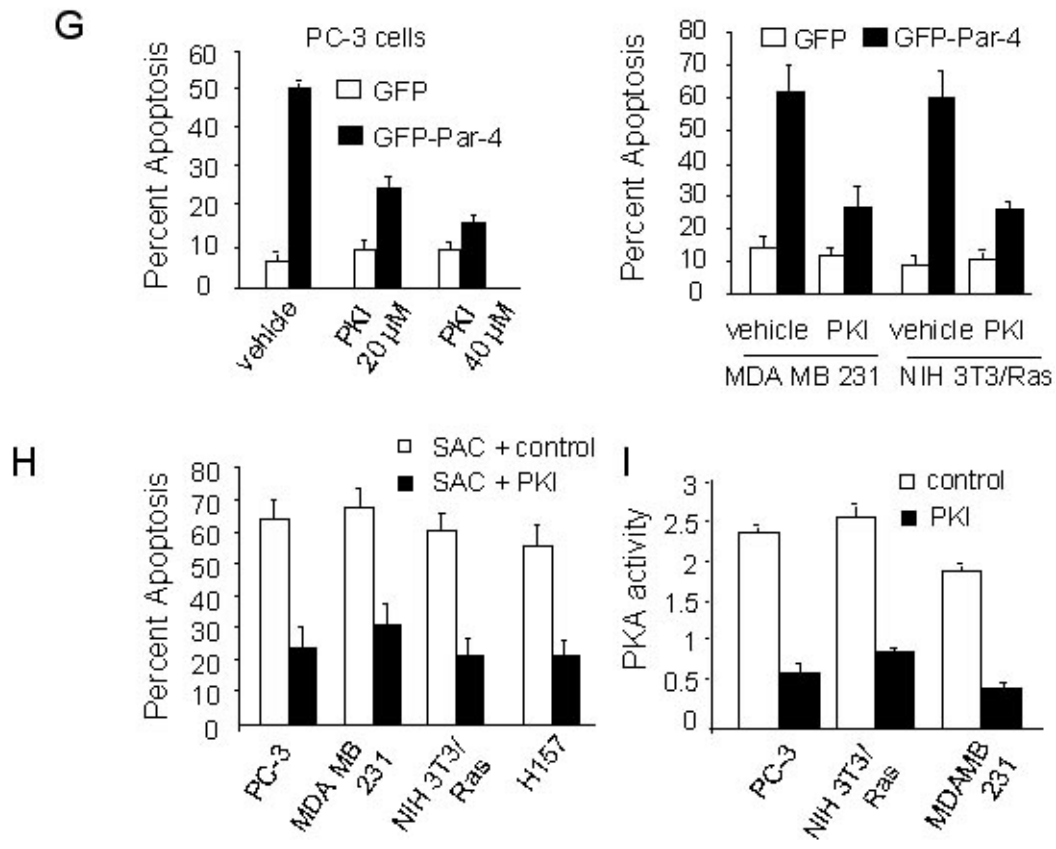
phosphorylation occurs at T155, we performed an *in vitro* phosphorylation assay

with PKA on Par-4 or mutants immuno-precipitated from NIH 3T3 cells, which are

resistant to apoptosis by Par-4. Relative to endogenous Par-4 (which showed

basal levels of phosphorylation in cells transfected with vector), ectopic wild type

Par-4 and the 154A mutant but not 155A mutant showed elevated levels of PKA

phosphorylation (Fig. 2.2B), suggesting that T155 is a primary site of

phosphorylation by PKA.

We next determined whether Par-4 was phosphorylated at T155 residue

by PKA in NIH 3T3/Ras cells. Par-4 has putative phosphorylation sites for

several protein kinases throughout its sequence that could potentially

overshadow the phosphorylation of T155. In order to focus on the effect of PKA

on T155 in metabolic labeling experiments, we used a deletion mutant of Par-4

that contained aa 137-187, as an *in vivo* substrate, in which S154 and T155 are

the only two potential phosphorylation sites. The cells were transiently

transfected with 137-187 or 137-187/155A mutant and subjected to Western blot

analysis to ascertain expression of the constructs (Fig. 2.2C right panel); or to

metabolic labeling, followed by immuno-precipitation with the Par-4 antibody or

control IgG (Fig. 2.2C, left panel). As seen in Fig. 2.2C (upper panel), 137-187

showed phosphorylation that was abolished when the T155 residue was altered

to 155A, suggesting that T155 is the primary phosphorylation site in the SAC

domain. Moreover, when the cells were treated with PKA inhibitory peptide PKI

and then subjected to metabolic labeling followed by immuno-precipitation of

labeled proteins, we noted that inhibition of endogenous PKA activity with PKI abolished phosphorylation of 137-187 (Fig. 2.2C, left-upper panel). Western blot analysis confirmed immuno-precipitation of comparable amounts of Par-4 mutant proteins with the Par-4 antibody (Fig. 2.2C, left-lower panel).

To ascertain phospho-labeling of the T155 residue of Par-4, we raised an antibody toward the phospho-T155 residue of Par-4, as described in Materials and Methods. To characterize the specificity of this antibody toward the phospho-T155 residue of Par-4, MEF cells were transfected with GFP, GFP-Par-4, or GFP-Par-4/155A mutant plasmid in the presence or absence of a plasmid expressing the PKA catalytic subunit (PKAc). As seen in Fig. 2.2D, the phospho-T155 antibody recognized a Par-4 band only in transfectants that expressed both GFP-Par-4 and PKAc, but not in those that expressed GFP-Par-4 in the absence of PKAc or expressed GFP or GFP-Par-4/155A mutant, indicating that the phospho-T155 antibody is specific for the phospho-T155 residue of Par-4.

We next used the phospho-T155 antibody to test whole-cell lysates of NIH 3T3/Ras or PC-3 cells transfected with either Par-4 or the 155A mutant of Par-4. Consistent with the phospho-labeling data, the phospho-T155 antibody recognized a potentially phosphorylated form of Par-4 only when NIH 3T3/Ras or PC-3 cells were transfected with Par-4 but not when transfected with the 155A mutant (Figs. 2 E and F, left panels). Moreover, the phospho-T155 band of Par-4 was abolished when the cells were treated with PKA specific inhibitor PKI; further

confirming that PKA phosphorylates Par-4 at T155 (Figs. 2 E and F, right panels). Together, these findings indicated that in cells, such as NIH 3T3/Ras and PC-3, that are susceptible to apoptosis by Par-4, PKA activity phosphorylates Par-4 at T155.

Inhibition of PKA activity inhibits apoptosis by Par-4 or SAC domain.

To ascertain that PKA activity is necessary for the apoptotic function of Par-4, PC-3, NIH 3T3/Ras, or MDA MB 231 cells were transiently transfected with GFP or GFP-Par-4 construct and treated with either control or PKA inhibitory peptide PKI. The cells were then scored for apoptosis. As seen in Fig. 2.2G, PKI significantly ($P<0.001$) inhibited the induction of apoptosis by Par-4 in all three cell lines. We also tested whether apoptosis induced by the SAC domain was regulated by PKI by transfecting PC-3, NIH 3T3/Ras, MDA MB 231, or H157 cells with the SAC domain construct in the presence of control or PKI peptide. As seen in Fig. 2.2H, relative to control, PKI inhibited apoptosis by the SAC domain.

To ascertain that PKI indeed inhibited PKA activity, PC-3, NIH 3T3/Ras or MDA MB 231 cells were treated with PKI or control peptide and intracellular PKA activity was determined. As seen in Fig. 2.2I, PKI strongly inhibited the endogenous PKA activity relative to the control peptide. These findings suggest that the apoptotic ability of Par-4 is dependent on the presence of active PKA

enzyme and inhibition of PKA enzymatic activity abolishes the apoptotic ability of Par-4.

Cancer cells have elevated PKA activity, which correlates with the apoptotic ability of the SAC domain.

The findings at this stage of the study suggested that apoptosis by Par-4 depends on the presence of an intact PKA consensus site T155 and active PKA enzyme. Moreover, the differential apoptotic ability of ectopic Par-4 in cancer versus normal cells raised the possibility that cancer cells may have elevated basal PKA activity levels compared to normal counterparts. A literature search indicated that PKA, especially PKA type I, is highly expressed in various transformed and cancer cells compared to corresponding non-transformed normal/immortalized cells (Cho et al., 2000; Cho-Chung et al., 2002; Tortora and Ciardiello, 2002). However, as PKA is a tightly regulated enzyme, it was not clear whether elevated protein levels of the PKA subunits correlate with elevated catalytic activity. Therefore, we sought to determine whether PKA activity was indeed elevated in cancer cells relative to normal/immortalized cells. We assayed the enzymatic activity of PKA in several panels of transformed or cancer cell lines, as well as, their normal/immortalized counterparts. As seen in Fig. 2.3A (left panel), NIH 3T3/Ras cells showed over 10 fold higher PKA activity than the immortalized NIH 3T3 parental cells. A panel of lung cancer cells including A549, H838, H157, and H460, showed 5-12 fold higher PKA activity relative to normal primary bronchial epithelial cells NHBE (Fig. 2.3B, left panel). The breast

60

cancer cells MCF-7, MDA MB 231 and MDA MB 435 showed 47-82 fold higher PKA activity compared to normal immortalized mammary epithelial cells MCF10A (Fig. 2.3C, left panel). A panel of prostate cancer cells including LNCaP, LNCaP derivative LNCaP/IGFBP5 and PC3 cells showed 4-13 fold higher PKA activity than normal primary prostate epithelial PrE cells or immortalized prostate epithelial PZ cells (Fig. 2.3D, left panel). These findings indicated that transformed or tumor cells had elevated PKA activity relative to the corresponding normal or immortalized cells.

The apoptotic ability of Par-4 is mediated by the SAC domain with an intact T155 residue. To test whether elevated PKA activity correlates with susceptibility to apoptosis by the SAC domain, we transfected the normal/immortalized and cancer cells with vector, SAC domain, or SAC/155A expression construct and assayed for apoptosis 48 h post-transfection. Interestingly, the SAC domain of Par-4 induced apoptosis only in the cells with elevated PKA, i.e., in the transformed or tumor cells and not in the normal/immortalized cells (Fig. 2.3A-D, right panels). By contrast, the SAC/155A mutant failed to induce apoptosis in the cell lines tested (Fig. 2.3A-D, right panel). These findings indicated that PKA activity is elevated in transformed/cancer cells compared to normal/immortalized cells and that elevated PKA activity correlates with the ability of the SAC domain to induce apoptosis in a T155-dependent manner. Thus, elevation of PKA activity is a key factor that activates cancer cell-specific apoptosis by Par-4. To test if elevated PKA activity in cancer cells

Fig. 2.3. Cancer cells have elevated PKA activity.

A-D, left panel)   Whole-cell lysates were prepared from various normal/immortalized or cancer cell lines and *in vitro* PKA enzymatic assay was performed using the cAMP-dependent PKA Signatect Assay kit (A-D, left panel).

A-D, right panel)   The cell lines were transiently transfected with vector, GFP-SAC or GFP-SAC/155A constructs and the transfectants were visualized using confocal microscopy for GFP fluorescence of Par-4 or mutant constructs and DAPI staining for apoptosis.

E)   Par-4 was preferentially phosphorylated in cancer cells compared to normal cells, whole cells lysates from prostate cancer cell lines LNCaP, LNCaP/IGFBP5 and PC-3, and immortalized prostate epithelial cell line PZ were subjected SDS-PAGE, transferred to PVDF membranes and probed with phospho-T155 antibody, Par-4 antibody and finally actin antibody as loading control.

Fig. 2.3. Cancer cells have elevated PKA activity.

Immortalized and transformed fibroblasts

A

Primary bronchial epithelial and lung cancer cells

B

Immortalized mammary epithelial and breast cancer cells

C
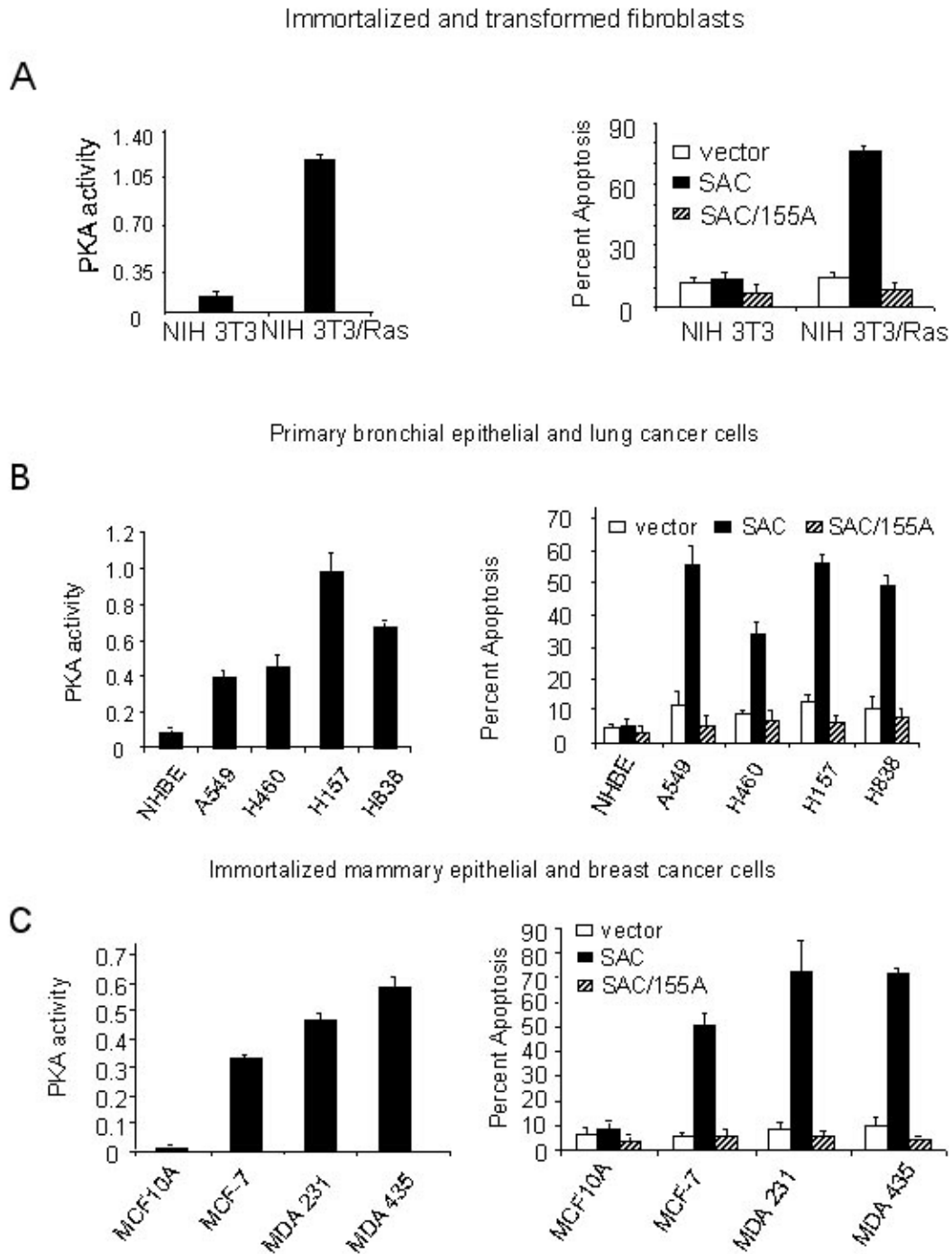
Fig. 2.3. Cancer cells have elevated PKA activity.
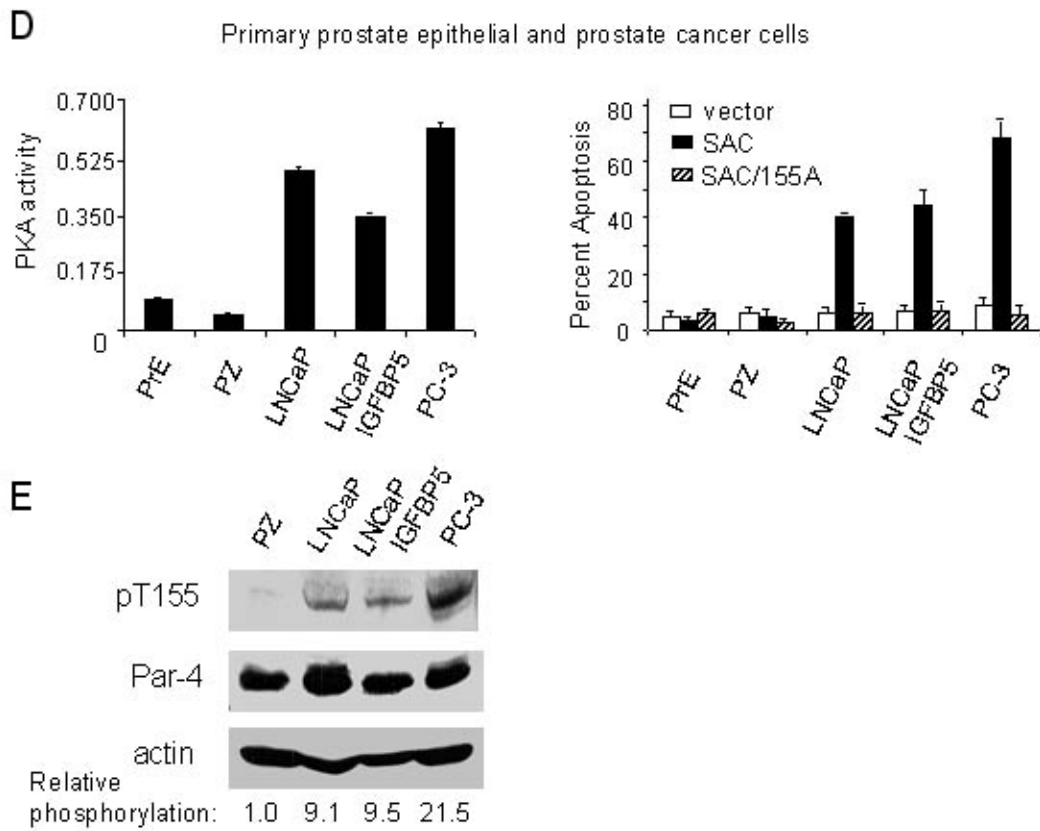
correlated with increased phosphorylation of Par-4 in cancer compared to normal cells, whole-cell extracts of prostate cancer cells LNCaP, LNCaP/IGFBP5 and PC3 cells along with immortalized prostate epithelial PZ cells were analyzed for phosphorylation of Par-4 at T155 by Western blot analysis using a phospho-Par-4 T155 antibody. As seen in Fig. 2.3E (left panel), when the phospho-T155 Par-4 levels were normalized to the total Par-4 levels, the cancer cells showed much higher levels of phospho-T155 Par-4 compared to the PZ cells. The pattern of occurrence of phospho-Par-4 correlated with the PKA activities of the cells and with the ability of the SAC domain to induce apoptosis in the respective cells. The findings from these experiments indicate that Par-4 is preferentially phosphorylated in cancer cells at T155 and that the relative extent of phosphorylation correlates with PKA activity and the apoptotic ability of Par-4 in these cells.

Enforced elevation of PKA activity in normal cells activates the apoptotic potential of the SAC domain of Par-4 in a T155-dependent manner.

The above experiments indicated that in addition to nuclear entry, Par-4 must be phosphorylated at T155 by PKA in order to induce apoptosis. Normal/immortalized cells do not allow Par-4 to translocate to the nucleus and may not contain adequate levels of active PKA enzyme for T155 phosphorylation; and are therefore resistant to apoptosis by Par-4. This observation of inadequate levels of PKA in normal/immortalized cells is further supported by the fact that the SAC domain construct is unable to induce

apoptosis in normal/immortalized cells, despite its predominantly nuclear localization (Fig. 2.4A). We, therefore, tested whether enforced elevation of PKA activity in normal cells renders them sensitive to apoptosis by Par-4, SAC domain or SAC domain/T155A mutant. To activate endogenous PKA, normal MEFs were transfected with the Par-4 or SAC domain expression construct and treated with cAMP analog 8-Cl-cAMP or vehicle as control, and the cells were examined by confocal microscopy for nuclear translocation of ectopically expressed proteins and apoptosis. Treatment with cAMP significantly increased apoptosis by the SAC domain compared to the vehicle-treated cells (Fig. 2.4B). By contrast, cAMP treatment did not increase apoptosis by Par-4 or SAC domain/T155A mutant (Fig. 2.4B). Consistently, cAMP did not alter the cytoplasmic localization of Par-4, or the nuclear localization of the SAC domain or SAC domain/T155A mutant (Fig. 2.4A).

Similarly, when MEFs were co-transfected with the expression construct for Par-4, SAC domain or SAC domain/155A mutant and PKAc, we noted that the co-transfectants expressing the SAC domain and PKAc underwent apoptosis (Fig. 2.4, C and D). By contrast, PKAc did not induce apoptosis in conjunction with vector, Par-4 or the SAC domain/155A mutant (Fig. 2.4D, left panel). Expression of the constructs was ascertained by Western blot analysis (Fig. 2.4E). Transfection with PKAc construct resulted in an increase in PKA catalytic activity as seen in Fig. 2.4D, right panel. These findings suggest that neither Par-4, which fails to enter the nucleus, nor the SAC domain/T155A mutant, which

Fig. 2.4. Elevation of PKA activity in normal cells activates the apoptotic potential of the SAC domain of Par-4 in a T155-dependent manner.

A and B)   MEF cells were transiently transfected with vector, GFP-Par-4, GFP-SAC or GFP-SAC/155A expression constructs, and treated with either vehicle or 8-Cl-cAMP (10 μM) for 48 h and examined for intracellular localization of Par-4 or mutants (A) or apoptosis (B).

C)   MEF cells were co-transfected with expression constructs for GFP-Par-4, GFP-SAC or vector, and PKAc for 48 h and the transfected cells were visualized under a confocal microscope by GFP fluorescence for Par-4 or mutants or by immuno-staining with PKAc antibody followed by Texas Red-conjugated secondary antibody, and for apoptosis by DAPI staining .

D, left panel)   Apoptotic cells were scored and the data presented as percent apoptosis.

D, right panel)   PKA activity was determined using cAMP-dependent PKA Signatect Assay kit).

E)   Protein expression was examined by Western blot analysis with antibodies for GFP, PKA catalytic subunit or actin **.**

Fig. 2.4. Elevation of PKA activity in normal cells activates the apoptotic potential of the SAC domain of Par-4 in a T155-dependent manner.

Fig. 2.4. Elevation of PKA activity in normal cells activates the apoptotic potential of the SAC domain of Par-4 in a T155-dependent manner.
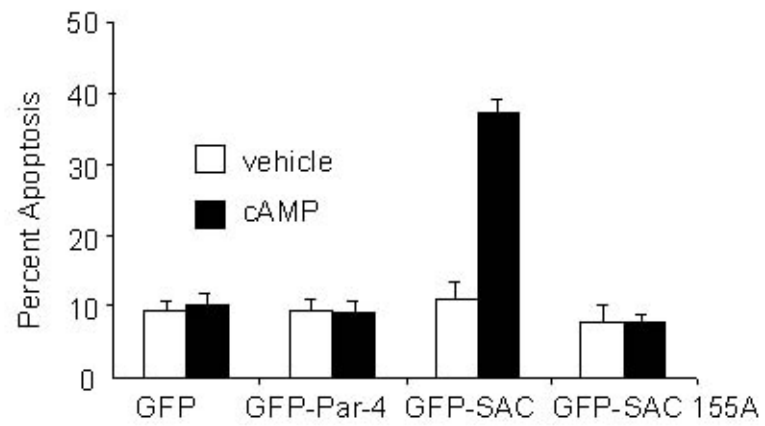
Fig. 2.4. Elevation of PKA activity in normal cells activates the apoptotic potential of the SAC domain of Par-4 in a T155-dependent manner.

Fig. 2.4. Elevation of PKA activity in normal cells activates the apoptotic potential of the SAC domain of Par-4 in a T155-dependent manner.
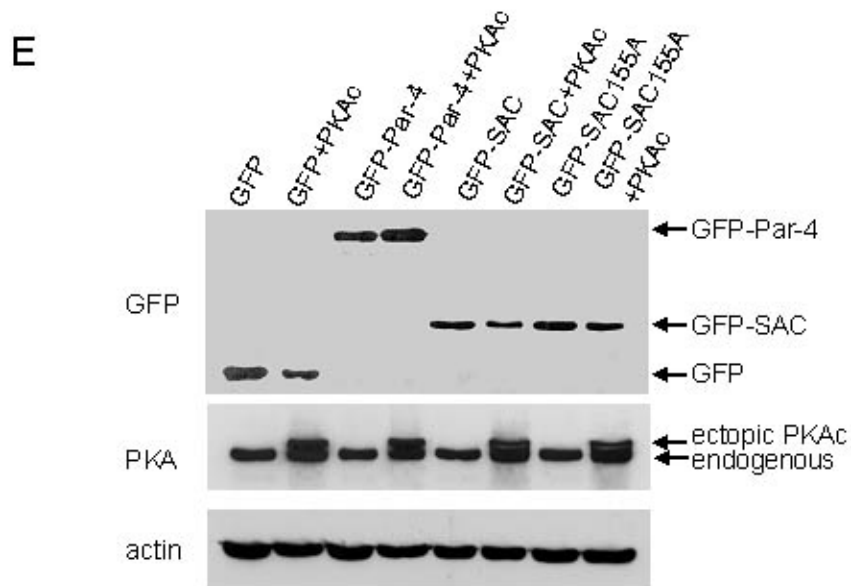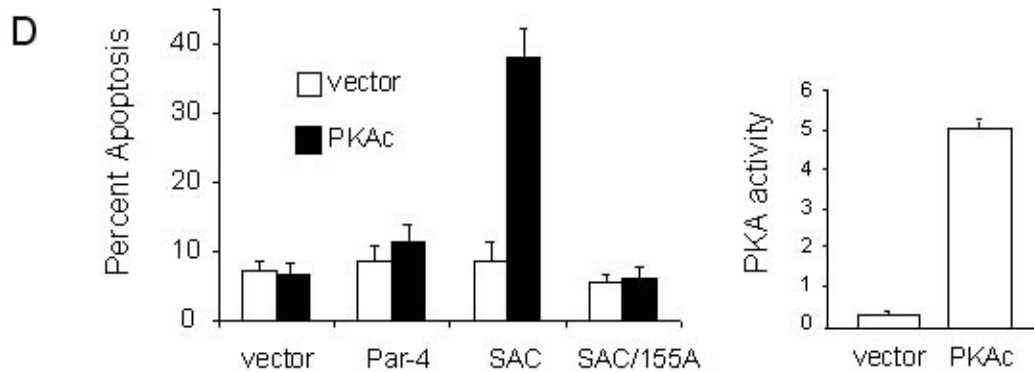
enters the nucleus but lacks the PKA phosphorylation site, causes apoptosis of the normal cells in the presence of elevated PKA activity. Thus, the localization of Par-4 does not depend upon its phosphorylation status, and nuclear localization of Par-4 is required but not sufficient for ectopic Par-4 to induce apoptosis.

Exogenous apoptotic stimuli activate endogenous Par-4 to induce apoptosis in a PKA- and T155-dependent manner.

Our findings thus far suggested that apoptosis by ectopic Par-4 is critically dependent on the phosphorylation of T155 by PKA. To determine whether endogenous Par-4 also induces apoptosis in a T155- and PKA-dependent manner, we used primary MEFs to study the role of the cAMP/PKA pathway in apoptosis by endogenous Par-4. Consistent with the findings with ectopic Par-4, endogenous Par-4 in MEFs was predominantly cytoplasmic in localization (Fig. 2.5A). Treatment with cAMP, which induced phosphorylation of the T155 residue of Par-4 (Fig. 2.5B), did not alter the localization of Par-4 (Fig. 2.5A), and did not cause apoptosis (Fig. 2.5C). Treatment with low doses of doxorubicin (which do not elevate PKA levels {Srivastava, 1998}, caused translocation of Par-4 to the nucleus but did not induce apoptosis (Fig. 2.5, A and C). Interestingly, co-treatment with cAMP (to cause phosphorylation of Par-4 at T155 through the cAMP/PKA pathway) and doxorubicin resulted in translocation of Par-4 to the nucleus with a significant increase in apoptosis (Fig. 2.5, A & C). Importantly, apoptosis inducible by this combination of cAMP and doxorubicin was blocked by

Fig. 2.5. Endogenous Par-4 induces T155- and PKA-dependent apoptosis.

A)   Localization of Par-4 and apoptosis in response to cAMP and doxorubicin treatment, MEFs were treated with vehicle, 10 µM 8-Cl-cAMP, 100 nM doxorubicin, 8-Cl-cAMP plus doxorubicin, or 8-Cl-cAMP, doxorubicin, plus 20 µM PKI and subjected to immuno-cytochemical analysis with Par-4 antibody followed by FITC conjugated secondary antibody (green). The nuclei were stained with DAPI (pseudo-colored red) and visualized under a confocal microscope (A) and scored for apoptosis.

B)   Phosphorylation of T155 by cAMP, MEFs were treated with 10 μM cAMP, cAMP plus PKI 20 μM, or cAMP plus PKI 40 μM, and whole-cell lysates were subjected to Western blot analysis with pT155, Par-4, pCREB, total CREB or actin antibodies (B, upper panel). PKA activity was determined in the lysates using cAMP-dependent PKA Signatect Assay kit (B, lower panel).

C)   Apoptosis in response to cAMP and doxorubicin, Cells from (A) were scored for apoptosis as described in Fig. 2.1.

Fig. 2.5. Endogenous Par-4 induces T155- and PKA-dependent apoptosis.
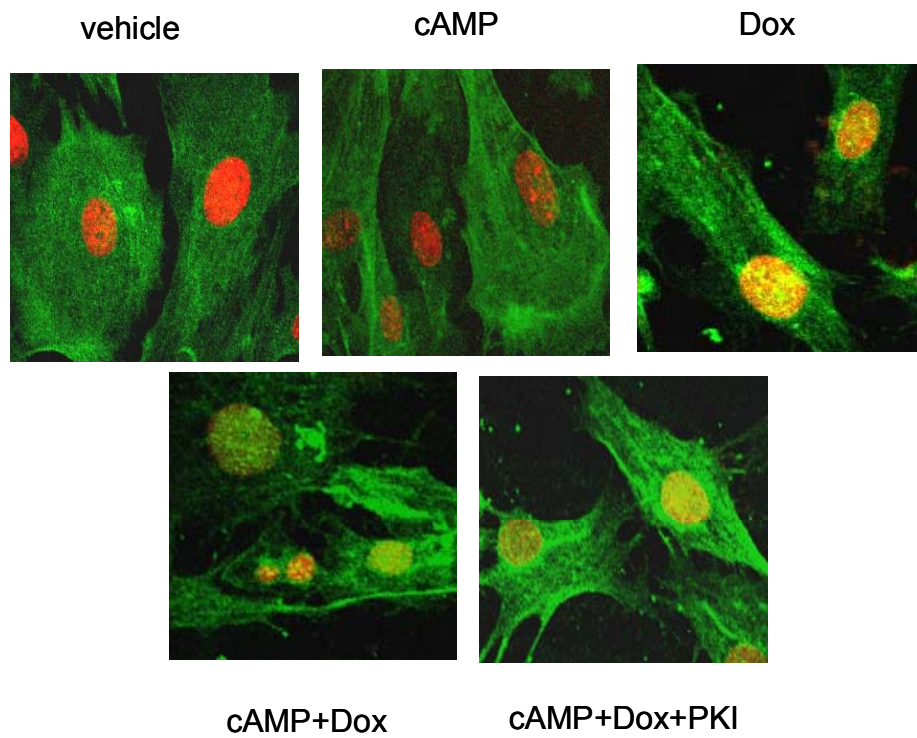
A

vehicle   cAMP   Dox

cAMP+Dox   cAMP+Dox+PKI

Fig. 2.5. Endogenous Par-4 induces T155- and PKA-dependent apoptosis.
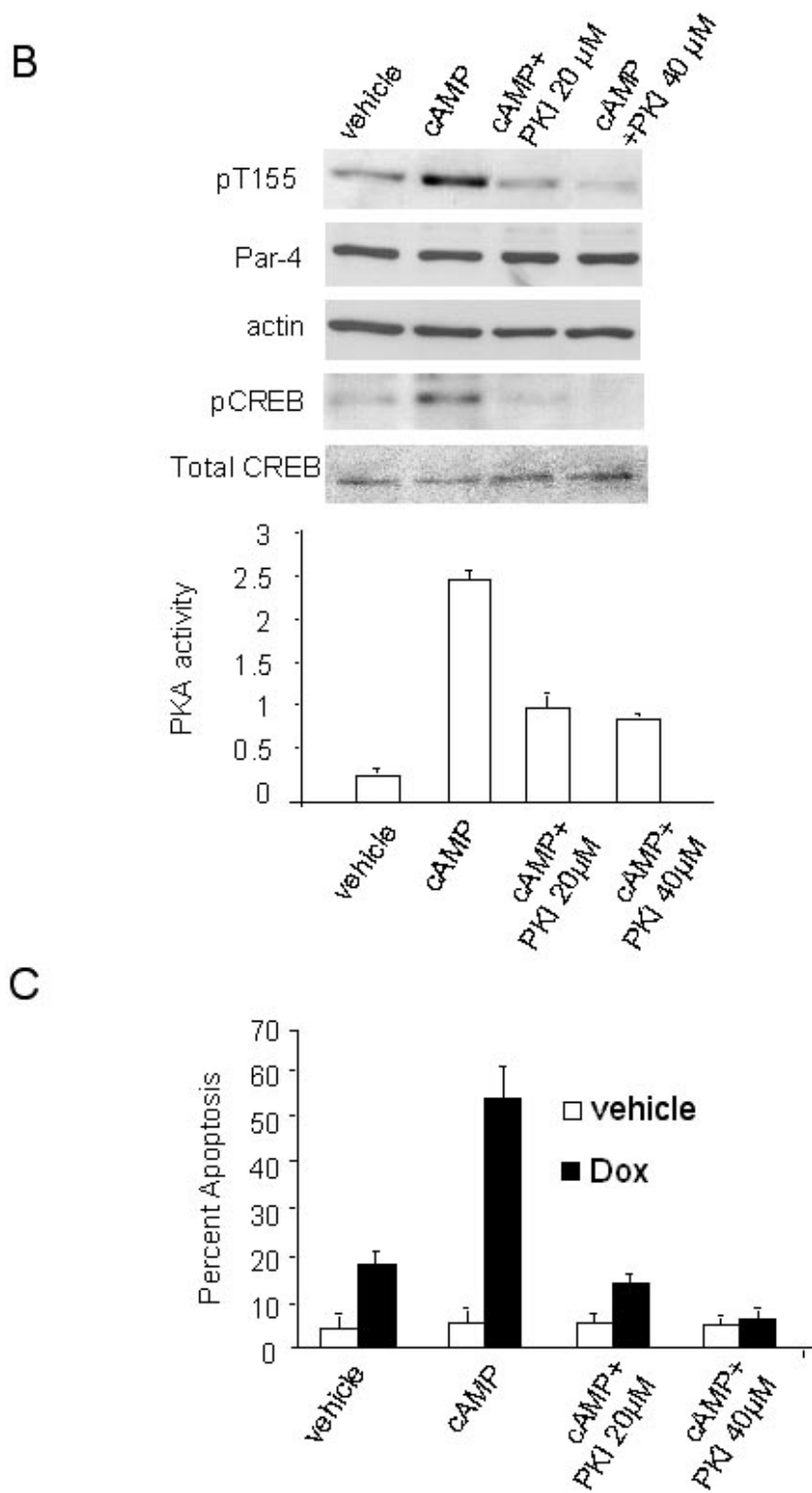
Fig. 2.5. Endogenous Par-4 induces T155- and PKA-dependent apoptosis.

D)   Vincristine induces T155 phosphorylation of Par-4 , HEL cells were treated with vehicle or 100 nM vincristine for 24 h and 48 h, and Western blot analysis was performed on the whole-cell lysates with pT155, Par-4 or actin antibodies.

E)   Vincristine-inducible apoptosis, HEL cells were treated with vehicle or vincristine, in the presence or absence of 20 µM or 40 µM PKI peptide, or transiently transfected with vector or GFP-Par-4 155A plasmid and assayed for apoptosis as described in Fig. 2.1.

F)   Effect of the various treatments on PKA activity, whole-cell lysates prepared from the cells after treatments, as described in panel E, were tested for PKA enzymatic assay by using the cAMP-dependent PKA Signatect Assay kit.

G)   Inhibition of Par-4 or PKA expression inhibits apoptosis by vincristine, HEL cells were transiently transfected with 10 µM nonspecific control siRNA, siRNA for Par-4 or siRNA for PKA α+β duplexes, and then treated with either vehicle or 100 nM vincristine, and assayed for apoptosis as described in Fig. 2.1.

H)   Whole cell lysates from the siRNA transfected cells were subjected to Western blot analysis with antibodies against PKA catalytic subunits α, β, Par-4 and actin.

I)   Effect of the various treatments on PKA activity, whole-cell lysates prepared from the cells after treatments, as described in panel F, were tested for PKA enzymatic assay by using the cAMP-dependent PKA Signatect Assay kit.

Fig. 2.5. Endogenous Par-4 induces T155- and PKA-dependent apoptosis.
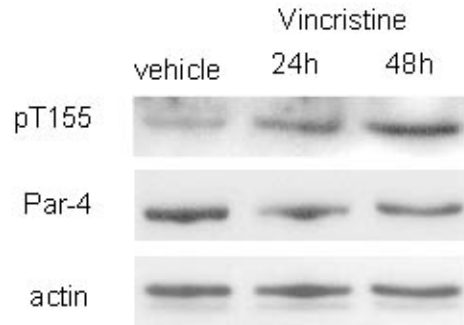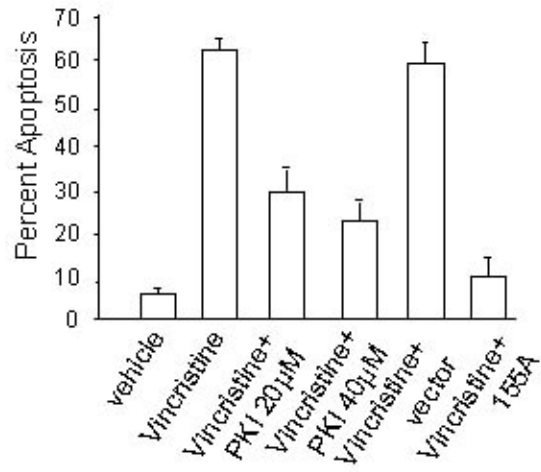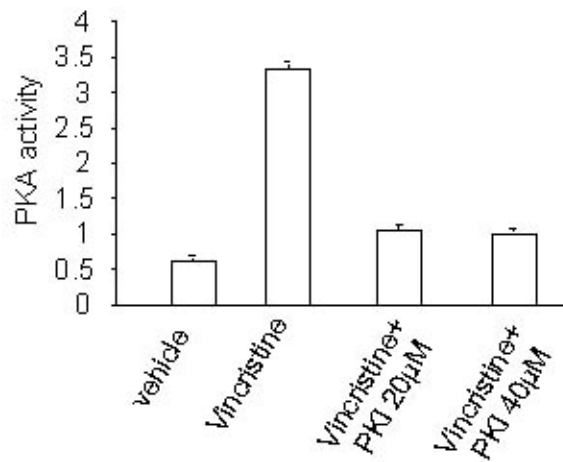
Fig. 2.5. Endogenous Par-4 induces T155- and PKA-dependent apoptosis.
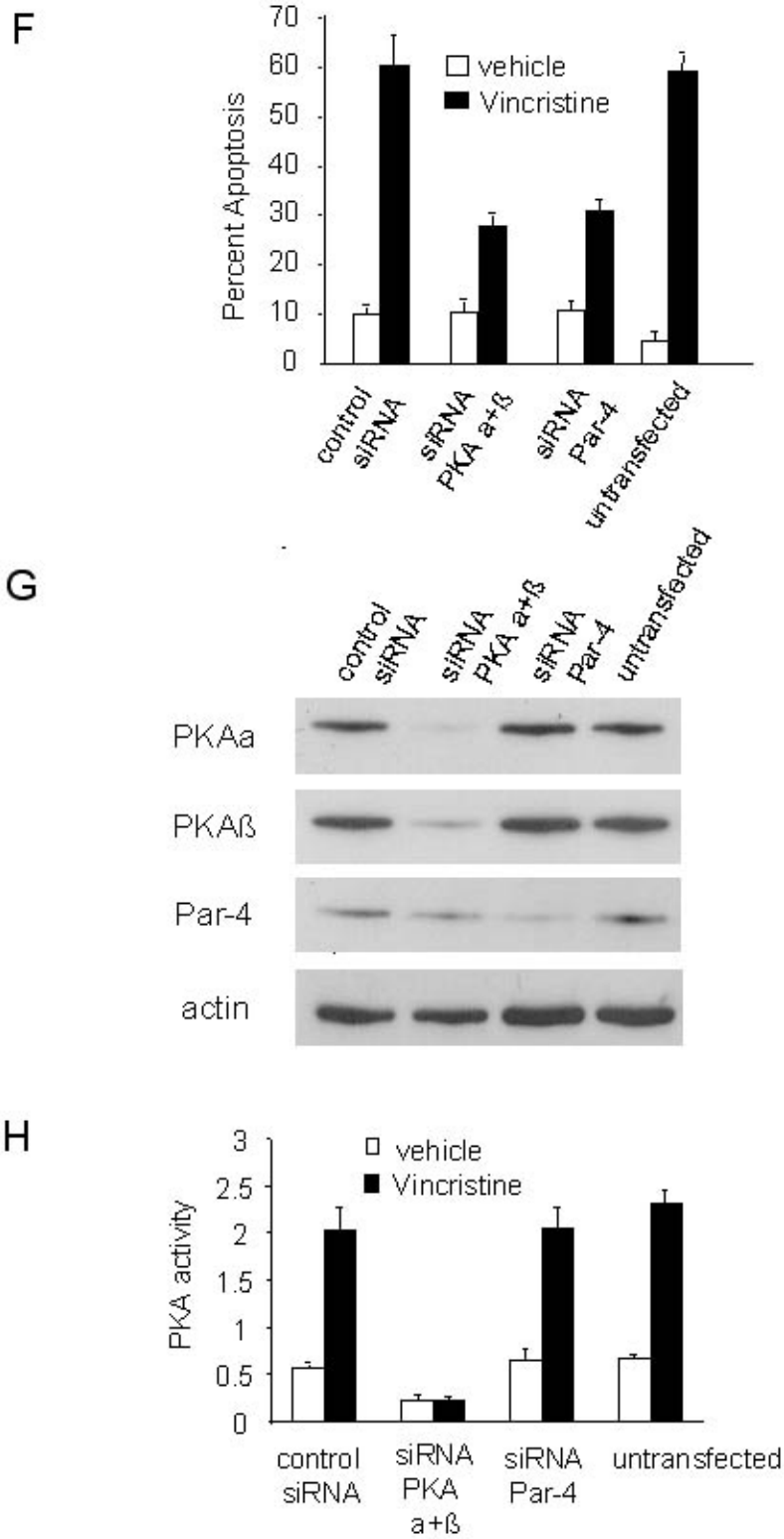
inhibiting PKA with PKI treatment (Fig. 2.5, A & C). The efficacy of cAMP or PKI was confirmed by determining phospho-CREB protein levels by Western blot analysis (Fig. 2.5B, upper panel) and by assaying the PKA enzymatic activity of the cells (Fig. 2.5B, lower panel).

Moreover, we studied vincristine for PKA- and phospho-T155/ Par-4-dependent apoptosis of primary human fibroblast cell line HEL. Vincristine, a microtubule inhibitor and widely used chemotherapeutic agent, is a potent inducer of apoptosis and is known to induce PKA to cause apoptosis (Srivastava et al., 1998b). As the first step in determining whether vincristine activates T155-dependent apoptosis by Par-4, we tested whether vincristine can induce phosphorylation of Par-4 at T155. As seen in Fig. 2.5D, vincristine treatment led to an increase in phospho-T155 levels in HEL cells, but total Par-4 levels remain unaltered. Vincristine treatment induced apoptosis and co-treatment with PKI resulted in a significant decrease in vincristine-induced apoptosis (Fig. 2.5E), indicating that vincristine induces PKA-dependent apoptosis. Moreover, ectopic expression of the 155A mutant of Par-4, but not control vector, completely abrogated vincristine-inducible apoptosis in these cells. The precise mechanism by which the 155A mutant serves as a dominant negative to inhibit endogenous Par-4 function is currently being investigated in our laboratory.

To further ascertain that vincristine-inducible apoptosis was Par-4- and PKA-dependent, siRNAs targeted against either Par-4 or the α and β catalytic

subunits of PKA were used to knockdown endogenous levels of the respective proteins in HEL cells (Fig. 2.5H), and the apoptotic effect of vincristine on these cells was studied. As seen in Fig. 2.5G, apoptosis inducible by vincristine was significantly diminished in cells with either siRNA for Par-4 or siRNA for PKA α+β compared to the nonspecific control siRNA or the untransfected control cells. Transfection of the siRNA for PKA α+β in the HEL cells led to a decrease in the basal PKA activity and abolished vincristine-inducible PKA activity (Fig. 2.5I). PKA activity in cells transfected with control siRNA or siRNA for Par-4 were similar to the untransfected control indicating that the siRNAs used did not exert nonspecific effects on PKA activity. Taken together, these findings imply that in normal cells subjected to an exogenous apoptotic stimulus, endogenous Par-4 induces apoptosis in a T155- and PKA-dependent manner (i.e., similar to ectopic Par-4 in cancer cells), and that inhibition of either PKA or Par-4 can abrogate apoptosis.

We also examined the role of PKA- and phospho-T155 Par-4 in the action of vincristine in lung cancer cells H157. Similar to our observations in HEL cells, vincristine induced apoptosis in H157 cells and this process was inhibited by PKI, siRNA Par-4, siRNA PKA α+β or 155A (data not shown). Thus, vincristine induces apoptosis by a mechanism that is dependent on PKA and T155 residue of Par-4 in both normal and cancer cells.

DISCUSSION

The findings of this study uncover a novel mechanism wherein a pro-cancerous trait, such as elevated PKA activity, is effectively utilized by Par-4 for selective apoptosis of cancer cells. We noted that cancer cells show a marked elevation in the enzymatic activity of PKA compared to their normal counterparts; PKA phosphorylates Par-4 at the T155 residue within the SAC domain and PKA-mediated phosphorylation of T155 is critical for induction of apoptosis by Par-4 (Fig. 2.6). Apoptosis by Par-4 requires trafficking of Fas to the cell membrane, to activate the Fas death receptor pathway, and co-parallel inhibition of cell survival NF-κB activity; and phosphorylation of Par-4 at T155 is essential for both Fas trafficking and NF-κB inhibition (this study, and data not shown). The enzymatic activity of PKA in cancer and normal cells correlates with the phosphorylation status of Par-4 in these cells and with the susceptibility of the cells to apoptosis by Par-4. Normal/immortalized cells contain low basal PKA activity and are unable to phosphorylate Par-4 at T155; moreover, they do not translocate Par-4 to the nucleus, and consequently are resistant to apoptosis by Par-4 (Fig. 2.6). By contrast, cancer cells that show nuclear entry of Par-4 and have elevated PKA activity are sensitive to apoptosis by Par-4 (Fig. 2.6). Consistent with this dual requirement of PKA phosphorylation and nuclear entry for apoptosis, the SAC domain, which translocates to the nucleus in all cell types, induces apoptosis only in cancer cells because they show elevated PKA activity. As PKA promotes survival in various cellular contexts and shows elevated expression and activity in a broad range of cancer cells, PKA-dependent phosphorylation

Fig. 2.6. Schematic representation of the PKA- and T155-dependent apoptosis by Par-4 in normal and cancer cells.

Based on our findings, we propose the following model for differential apoptosis by Par-4 in cancer and normal cells. PKA activity, which is constitutively elevated in cancer cells, causes phosphorylation of Par-4 at the T155 residue. The translocation of phospho-T155 to the nucleus results in inhibition of NF-κB activity and apoptosis. By contrast, PKA activity and T155 phosphorylation are relatively low in normal cells and, in addition, Par-4 translocation to the nucleus is blocked by an unknown mechanism. Consequently, normal cells fail to undergo apoptosis with Par-4. However, pro-apoptotic stimuli, such as cAMP or vincristine, cause PKA-dependent phosphorylation of T155; and doxorubicin or vincristine can cause translocation of Par-4 to the nucleus. Therefore, vincristine alone, or a combination of cAMP and doxorubicin, can induce apoptosis of the normal cells in a PKA- and T155 Par-4-dependent mechanism. The mechanism of nuclear translocation of Par-4 is currently under investigation.

Fig. 2.6. Schematic representation of the PKA- and T155-dependent apoptosis by Par-4 in normal and cancer cells.
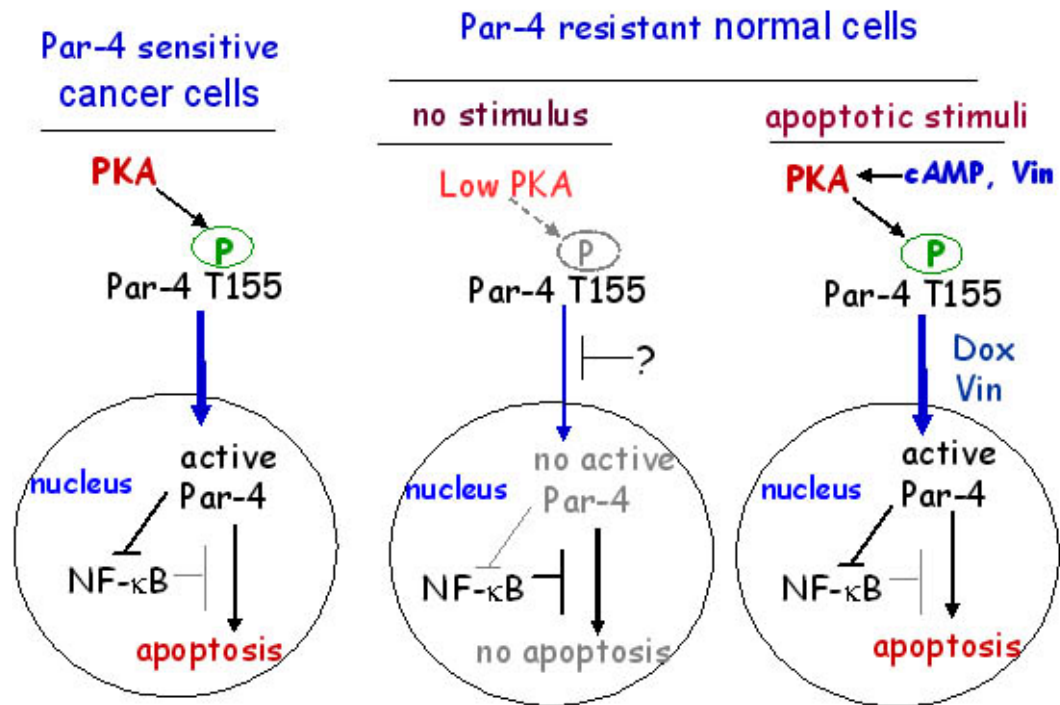
represents a common mechanism for activation of the apoptotic function of over-expressed Par-4 in cancer cells.

The findings of this study implicate the T155 phosphorylation site in the activation of Par-4-mediated cell death by PKA. T155 flanks the nuclear localization sequence NLS2 and is conserved in rat, mouse and human Par-4. An intact T155 site is required for Par-4-mediated cell death and mutation of T155 to 155A results in loss of apoptosis by ectopic Par-4. By contrast, mutation of S154, a putative PKA phosphorylation site in Par-4, to 154A, had no effect on the ability of ectopic Par-4 to induce apoptosis, indicating that T155 was the primary site of action of PKA in Par-4. Furthermore, inhibition of PKA function with PKI or PKA expression with siRNAs toward the PKA catalytic subunits, abrogated apoptosis by ectopic Par-4 or by the SAC domain, suggesting that the presence of an active form of PKA is essential for apoptosis. Both *in vivo* and *in vitro* assays indicated that the T to A mutation at residue-155 resulted in significantly diminished PKA phosphorylation of Par-4. On the other hand, the S to A mutation at residue-154 did not substantially affect the phosphorylation status of the T155 residue. Only exogenous Par-4 induces apoptosis on its own in cancer cells, whereas endogenous Par-4 is necessary for apoptosis when cells are treated with vincristine or cAMP plus doxorubicin (Fig. 2.6). Similar to exogenous Par-4, endogenous Par-4 is phosphorylated at T155 in response to vincristine or cAMP treatment, and induces apoptosis in response to the exogenous stimuli in a phospho-T155/PKA dependent manner. Together, these

observations suggest that PKA triggers Par-4 phosphorylation primarily at the T155 residue.

Several studies have noted that PKA is over-expressed in transformed cells and in various cancers (Tortora and Ciardiello, 2002). However, because the activity of PKA is tightly controlled by its regulatory subunit and cAMP, it was unclear whether this elevation in expression results in concomitant increase in the enzyme activity. The findings of this study indicated that PKA activity is significantly elevated in various types of cancer cell lines such as lung, breast and prostate cancer cells, compared to their normal/immortalized counterparts. Moreover, there was a direct correlation between the PKA activity of the cells and apoptotic ability of Par-4 via the SAC domain. Cells with low PKA activity, such as the normal/immortalized cells, were resistant to Par-4 or the SAC domain; and cancer cells with elevated PKA activity were susceptible to Par-4 (this study, and reference (El-Guendy et al., 2003). This observation is further supported by the recent findings of Dalla-Favera and coworkers that c-myc inducible up-regulation of the catalytic subunit of PKA (PKAc) is essential for transformation of normal fibroblasts (Wu et al., 2002). Moreover, stable transfection of normal fibroblast cells with the PKAc or PKA type I lead to transformation and anchorage-independent colony formation in soft agar (Wu et al., 2002). Several other reports have shown that free PKAc (also known as extracellular PKA) is elevated and secreted in several cancers leading to increased cell survival, migration and metastasis of the cancers (Cho-Chung et al., 2002; Cvijic et al., 2000). By

directly activating PKAc, oncogenes can induce the cAMP/PKA signal transduction pathway in the absence of other cAMP-inducing stimuli. Therefore, elevation of PKA expression and activity is an integral part of the process of transformation. This distinct feature of cancer cells is effectively utilized by ectopic Par-4 for its own activation and apoptosis induction. Interestingly, although endogenous Par-4 is phosphorylated by elevated levels of PKA activity in cancer cells, it fails to induce apoptosis, suggesting that the apoptotic action of Par-4 is hindered by an active intracellular mechanism. This constitutive anti-apoptotic mechanism directed against endogenous Par-4 is currently under investigation in our laboratory.

The SAC domain construct was able to induce apoptosis in all cancer cells tested, but was unable to induce apoptosis in primary normal or immortalized cells. Because PKA elevation is one of the important features contributing to the selective apoptotic action of Par-4 in cancer cells, it was conceivable that enforced elevation of PKA enzymatic activity in normal cells may enable Par-4 to induce apoptosis in normal cells. Indeed, elevation of PKA activity in normal cells induced the ability of the SAC domain, which localized predominantly to the nucleus, to induce apoptosis in MEFs. By contrast, full length Par-4 is predominantly localized in the cytoplasm and is unable to induce apoptosis despite enforced elevation in PKA activity in normal cells. The mechanism for this selective action of the SAC domain was hitherto unknown. Our findings suggest that Par-4 phosphorylation at T155 by PKA, as well as, nuclear translocation is

essential for Par-4 and the SAC domain to induce apoptosis. Cancer cells susceptible to full length Par-4 have elevated PKA activity for phosphorylation of T155 and are also able to translocate Par-4 to the nucleus. On the other hand, hormone-dependent cancer cells, such as LNCaP or MCF-7, are unable to localize full length Par-4 to the nucleus despite elevated PKA activity; and normal cells are unable to phosphorylate T155 owing to low PKA levels or to cause nuclear localization of Par-4. Apoptosis by Par-4, therefore, requires two critical steps: phosphorylation of T155 by PKA and entry into the nucleus (Fig. 2.6). Both these steps are feasible for the SAC domain in all cancer cells or for Par-4 in hormone-independent cancer cells, but not in normal cells; thus, accounting for the tumor selective activation and apoptotic action of Par-4 via its SAC domain.

In summary, ectopic Par-4 is sufficient to induce apoptosis in cancer cells, whereas endogenous Par-4 is critical for apoptosis induced by vincristine or cAMP plus doxorubicin. PKA activity is constitutively elevated in cancer cells and causes phoshorylation of the T155 residue of Par-4. This phosphorylation event is critical for apoptosis by Par-4. In normal cells, basal PKA activity is relatively low and phosphorylation of T155 fails to occur; therefore, normal cells are resistant to apoptosis by ectopic Par-4. Elevation of PKA in normal cells by cAMP/doxorubicin or vincristine induces apoptosis by a mechanism that is dependent on PKA-mediated phosphorylation of the T155 residue of endogenous Par-4. Thus, phosphorylation of the T155 residue of Par-4 by PKA is critical for

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apoptosis. This work provides a novel insight into the mechanism underlying differential sensitivity of normal and cancer cells.

CHAPTER 3

DISCUSSION AND FUTURE DIRECTIONS

Par-4 is an interesting pro-apoptotic protein that plays a role in apoptosis in a wide range of cellular contexts involving both physiological and disease processes. The different localization of Par-4 protein in normal and cancer cells, as well as the presence of the NLS, NES and LZ domains, and several putative phosphorylation sites clearly indicate that the apoptotic function of Par-4 is tightly regulated at multiple levels. It is well documented that the leucine zipper domain of Par-4 is capable of binding to a wide variety of partner proteins and this regulates the apoptotic functions of Par-4. However deletion of this region does not abrogate the apoptotic functions of Par-4 in cancer cells and interestingly, leads to constitutive nuclear localization of the Par-4 mutant. These observations indicate that the C-terminal region plays a predominantly regulatory role in the functioning of Par-4 exercising control over both cellular localization and apoptosis function through the NES and hetero-dimerization by the leucine zipper respectively. The amino acid sequence of Par-4 protein can therefore be divided, based on function, into the regulatory domain composed of the NES and LZ domain in the C-terminus and a minimal apoptotic effecter domain composed of the centrally located NLS2 and the region between aa residues 137-195 called the SAC domain.

What regulates localization and activation of Par-4 apoptotic function?

In trying to understand the mechanism by which apoptosis by Par-4 was activated we built upon two important observations: i) varied localization of ectopic and endogenous Par-4 in normal vs cancer cells and ii) apoptosis by SAC mutant preferentially in cancer cells despite strong nuclear presence in the normal cells. In this study we identified that both endogenous and ectopic Par-4 are regulated by the same mechanism. For Par-4 apoptotic function to be activated, the minimal apoptotic SAC domain needs to be phosphorylated at T155 and Par-4 protein should be localized and expressed in the nucleus of the cell. In an untransformed normal cell, Par-4, both endogenous as well as ectopic, is excluded from the nucleus, predominantly localized to the cytoplasm and very poorly phosphorylated at T155 and the cell is maintained in non-apoptotic state. Under apoptotic conditions, such as with treatment with vincristine, there is induction of PKA activity, which phosphorylates T155 and translocaties Par-4 to the nucleus, resulting in apoptosis. Although there are some clues, the mechanism that controls the localization of Par-4 at the moment is not very clear. The presence of the NLS and NES in the Par-4 sequence suggests that there is a dynamic process that shifts between the opposing functions of the NES and NLS domains. In normal cells, both over-expressed full length Par-4 as well as endogenous Par-4 is retained in the cytoplasm indicating that the C-terminal region strongly regulates nuclear exclusion. Deletion of the C-terminal region relieves the exclusion from the nucleus indicating that this region is essential for retention of Par-4 in the cytoplasm under normal circumstances. This function is

probably determined by the nuclear exclusion sequence or the partner protein bound to the leucine zipper or both. Further studies are required to identify what relieves the nuclear exclusion of Par-4 in cancer cells and in normal cells undergoing apoptosis. In contrast to the findings in this study it was noticed that, in neuronal apoptosis, Par-4 increasingly localized in the post-synaptic density (PSD) of the synaptosome, away from the cell body and nucleus, during induction of apoptosis. Although this indicates the criteria for apoptosis by Par-4 may vary with the cellular context, it needs to be verified by our laboratory by including cell culture models representative of a wider variety of tissue types.

Significance of PKA mediated regulation of Par-4 function

In this study, we identify for the first time that the apoptotic function of Par-4 is regulated at the level of the central apoptotic SAC domain. This regulation is mediated by the phosphorylation of the T155 residue by cAMP-dependent kinase or PKA, a prolific kinase, a molecule that is active in cells in any given tissue at any given time. Par-4 regulates several aspects of functioning of a mutli-cellular organism ranging from embryonic development to tissue differentiation to maintaining cellular homeostasis. It is possible that this step in activation of Par-4 apoptosis may have evolved as a mechanism to protect tissues in case of erroneous or excessive PKA signaling. With a discovery that connects PKA and Par-4, it is now possible to envision a role for Par-4 in physiological apoptotic settings that may have been overlooked.

With PKA playing such a variety of apoptotic as well as non-apoptotic roles, how is apoptosis by Par-4 regulated under specific conditions? All these functions of PKA are kept in check and appropriately regulated by very tightly controlling the activation and functional localization of the enzyme. These are made possible by the development of well differentiated sub-types of the regulatory subunits and organellar tethering by the different A-kinase anchor proteins or AKAPs. For example, in an anti-apoptotic function, PKA phosphorylates and inhibits apoptotic action of mitochondrial protein Bad. This however, occurs only in response to survival factors and anchoring of type II PKA to the mitochondria by mitochondrial AKAPs creating a focused subcellular kinase/substrate interaction (Harada et al., 1999). In contrast, in response to treatment with microtubule inhibitors such as vincristine, PKA promotes apoptosis by hyper-phosphorylating and inhibiting its function of Bcl-2, an anti-apoptotic mitochondrial protein (Srivastava et al., 1998b). Further studies are required to understand the complexities of the interaction between PKA and Par-4 and the induction of apoptosis. Knowledge of sub-cellular localization of Par-4 and PKA sub-types under varied signaling circumstances is essential. These studies should include in vitro models of a vast array cells types including developmental models as well as in vivo models of the same.

Elevation of T155 Phosphorylation and Apoptosis in Cancers and Other Diseases

Molecular alterations that promote increased phosphorylation of T155 or relieve the nuclear exclusion of Par-4 will pre-dispose cells to spontaneous apoptosis by Par-4. In one of the most exciting findings of this study, we found that cancer cells show a marked elevation in the enzymatic activity of PKA compared to their normal counterparts; this is responsible for induction of apoptosis by Par-4 or SAC mutant in cancer cells. cAMP mediated activation of PKA is used in the therapy of certain cancers such as certain leukemias and in combination with retinoic acid in lung and ovarian cancers (Smith et al., 2005; Srivastava et al., 1998a; Srivastava et al., 1999a; Srivastava et al., 1999b; Srivastava et al., 2000). If cancer cells already have elevated PKA activity and phosphorylated Par-4, what is the need to further activate this pathway to induce apoptosis? It is critical to adequately answer these questions to better understand the modifications that happen to endogenous Par-4 in these cancers.

While these findings have therapeutic implications for the use of Par-4 or SAC in the treatment of cancer there may be other disease conditions where elevated PKA activity can result in deleterious consequences due to activation apoptosis by Par-4. For example in Alzheimer's disease (AD), where Par-4 has been implicated, 30-50% of the cells positive for hyperphosphorylated tau showed elevation of Par-4 protein levels (Guo et al., 1998). Abnormally hyperphosphorylated tau protein is associated with poor prognosis in patients

with AD. Recent studies identified PKA as the kinase that initiates phoshorylation of the multiple sites of Tau protein (Liu et al., 2004). Together, these findings suggest that elevated PKA activity may be responsible for hyperphopshorylated tau as well as activated Par-4 in the neurons of AD. In other diseases like chronic heart failure, sustained beta adrenergic receptor (AR) stimulation has been shown to promote cardiac myocyte apoptosis by activation of Ca(2+)/calmodulin kinase II and PKA (Zhu et al., 2003). In addition to PKA, the T155 site also bears a consensus site for phosphorylation by calmodulin kinase II. The role of Par-4 apoptosis in cardiac myocytes as well as other disease conditions where PKA or other kinases that may phosphorylate Par-4 are elevated needs to be investigated.

Interestingly, endogenous Par-4, despite being nuclear and strongly phosphorylated at T155, does not induce apoptosis in the cancer cell lines tested indicating that these cancers have evolved an inhibitory mechanism that possibly protects the cells from apoptosis by Par-4. The possibility of an interaction partner that is elevated in cancers, that sequesters and blocks apoptosis by Par-4 is currently being investigated in our lab.

This study has made significant advances in understanding the regulation of Par-4 function at the posttranslational level in normal and cancer cells. As many questions the findings in this study may have helped answering, there are as many if not more new unanswered questions that need to be investigated.

Significance of phosphorylation of T155

The results of this study indicate that phosphorylation of Par-4 at residue T155 is critical for apoptosis by Par-4. How this phosphorylation event alters Par-4 to activate its apoptotic function is not known. Does adding a phosphate moiety to T155 alter the 3-dimensional structure of Par-4 such that it can interact with apoptotic effecter proteins? Does this event affect just the core apoptotic domain from 137-195 or does it have a more global effect on the entire sequence of Par-4. This question can be studied by comparing the properties of full length Par-4 with those of the SAC mutant as well as with their respective 155A mutants. A good starting point would be to study why the 155A mutant of Par-4 inhibits apoptosis induced by exogenous insults. It has been observed in this study in vincristine induced apoptosis as well a other studies in our lab, that the 155A mutant of Par-4 functions as a dominant negative and blocks apoptosis induced by a wide variety of apoptotic insults. It is not known whether the SAC 155A mutant behaves similar to the Par-4 155A mutant in abrogating apoptosis induced by exogenous insults. If it does, this implies that phosphorylation of T155 affects only the region between aa 137-195 and does not have implications on the entire sequence of Par-4. This question can also be addressed by testing whether the mutation of T155 to A interferes with the ability of Par-4 to bind to the known partners of Par-4 such as ζPKC, Dlk or THAP through the leucine zipper domain. This manner of regulation is seen in the case of p65, a component of the NF-κB heterodimer. Binding of the C-terminus of p65 to CBP is dependent on PKA mediated phosphorylation of p65 at a centrally located residue S276.

Phosphorylation of p65 at S276 triggers a conformational change in the C-terminal domain that allows interaction between p65 and CBP (Zhong et al., 1998).

The mechanism by which the 155A mutant protects the cells from exogenous apoptotic stimuli is another fascinating yet challenging question that needs to be investigated. One potential mechanism may be that the 155A mutant binds to endogenous Par-4 via the leucine zipper and prevents its activation by the signaling pathway initiated by the stimulus. This raises the question of whether ratios of phosphorylated to un-phosphorylated form of Par-4 are critical in determining the decision to undergo apoptosis. It is possible that under non-stimulated conditions, the ratio of phospho- vs non-phospho- form of Par-4 is maintained in favor of the un-phosphorylated Par-4. During apoptosis induced by external stimuli, the threshold is probably breached when the amounts of pro-apoptotic T155-phosphorylated Par-4 far outweigh the non-apoptotic non-phosphorylated Par-4 thereby leading to apoptosis. As interesting as these possibilities may seem, these are difficult to test with a simple experimental model system. It is, however, important to keep these possibilities in mind and test them when the opportunity arises in the future.

Regulation of T155 phosphorylation under physiologic apoptotic

conditions

The results from this study have shown that phosphorylation of the Par-4 T155 residue by PKA activates the Par-4 apoptotic action. Elevated PKA enzymatic activity in cancer cells therefore activates over-expressed Par-4 leading to apoptosis in these cells. Normal cells under non-apoptotic circumstances have low PKA activity and this serves to keep Par-4 in an inactive form, thereby preventing apoptosis. Par-4 has been shown to sensitize cells to apoptosis regardless of initiating agent and not all of them are known to activate the PKA signaling pathway. It is quite possible that T155 may be phosphorylated by kinases other than PKA under different apoptotic conditions. Examination of the sequence around T155 (K-R-R-S-**T**-G-V-V) revealed that T155 could be a putative phosphorylation site for kinases such as calcium/calmodulin-dependent kinase II (CaM kinase II) (R-X-X-**S/T**-V), Ribosomal S6 kinase (RSK) (K/R-X-X-**S/T**). Further studies are needed to identify known or novel kinases that regulate T155 phosphorylation of Par-4 upon apoptotic stimulation, whether these kinases differ according to the nature of the stimulus or whether they are common to apoptosis in general. Another appealing prospect is that Par-4 associates with phosphatases, maintaining T155 in a de-phosphorylated state of under normal or anti-apoptotic conditions. Recent studies by the Weinberg group suggest that loss of phosphatase PP2A is one of the requirements for the cellular transformation of normal human cells (Rangarajan et al., 2004). It is possible that, in addition to elevation of PKA enzymatic activity in cancer cells, loss of

PP2A or a similar phosphatase during tumorigenesis is also responsible for the increased phosphorylation of T155 and therefore apoptosis by Par-4 in cancer cells. Another interesting question is whether the leucine zipper domain of Par-4 negatively impacts phosphorylation of T155 if it is bound to an antagonistic protein. In other words, does the phosphorylation status of T155 of Par-4 depend on the protein bound to the leuzine zipper domain?

Mechanism of SAC induced apoptosis

It was initially thought that the LZ was critical for apoptosis by Par-4. However deletion of the LZ had no effect on spontaneous apoptosis by Par-4 in sensitive cells. Further, the region from 137-195 comprising the SAC domain proved to be the minimal region of Par-4 that was necessary and sufficient for apoptosis by Par-4. Although this region of Par-4 is undoubtedly the apoptotic effecter of Par-4, less is known regarding how this domain causes apoptosis. Further studies are required to identify the mechanism of apoptosis by this domain and the SAC 155A mutant can be used as an excellent control in these experiments. A good place to start would be to identify protein interaction partners that are unique to this domain of Par-4. The study may be designed to differentiate between partners that are apoptotic effecter proteins and partners that bind to a non-apoptotic or dormant form of SAC. Another focus of this study could be delineating the location of the ultimate site/sites of action of SAC within the cell. Though the knowledge that the SAC mutant is enriched in the nucleus has been with us for some time, nothing is known regarding what or how this

molecule causes apoptosis from within the nucleus. The search for apoptosis specific SAC binding partners should be designed to identify nuclear specific candidates and their significance evaluated using knockout or silencing strategies. Apoptosis is a series of events that engages a number of organelles. Although the nuclear localization of Par-4 is essential for apoptosis by Par-4 and the SAC domain is predominantly expressed in the nucleus, there is some SAC expressed in the cytoplasm. Also one of the better-known mechanisms of actions of Par-4 is translocation of Fas/FasL to the membrane through the golgi and this is essentially a cytoplasmic event. Why, then, does Par-4 or SAC have to be in the nucleus to induce apoptosis? Since the SAC domain lacks the leucine zipper, the chances of it affecting transcription through protein interaction, such as interaction with WT1 in the case of bcl-2 down-regulation, seem to be low. One sub-organelle in the nucleus known to play a role in apoptosis is the PML body. Par-4 is known interact with two different PML specific pro-apoptotic proteins, Dlk along with Daxx and THAP, under apoptotic conditions. However, their interaction is again dependent on the presence of the leucine zipper of Par-4, a domain that is lacking in the SAC mutant. Given all these details, it seems that identification of candidate partner or target proteins by which the SAC domain induces apoptosis will be novel and definitely exciting.

In summary, in this study we identified that activation of apoptotic function of Par-4 requires two separate events; phosphorylation of T155 residue by PKA and translocation of Par-4 to the nucleus. Cancer cells are susceptible to

apoptosis by ectopic Par-4 as cancer cells have elevated levels of PKA enzymatic activity that effectively phosphorylates T155 in overexpressed Par-4 and can translocate Par-4 to the nucleus, by an unknown mechanism, Normal cells lack either of these criteria and hence are resistant to apoptosis by Par-4 in the absence of an exogenous apoptotic stimuli. In normal cells ectopic and endogenous Par-4 are regulated by a similar mechanism in response to apoptotic stimuli.

APPENDIX

LIST OF ABBREVIATIONS

AATF: Apoptosis-Antagonizing Transcription Factor
AD: Alzheimer.s disease.
ALS: Amyotrophic lateral sclerosis.
αMEM: Minimum essential medium.
ATRA: all trans-retinoic acid.
βAPP: Beta amyloid precursor protein.
BP5: Binding protein 5.
cAMP: cyclic adenosine monophosphate
cFLIP: FLICE inhibitory protein.
CKII: Casein kinase II.
CREB: cAMP response element Binding protein
DAP kinase: death associated protein kinase.
DAPI: 4',6'-diamidino-2-phenylindole hydrochloride.
DISC: death inducing signaling complex.
Dlk: DAP-like kinase
DMEM: Dulbeco's modified Eagle medium.
DTT: dithiothreitol.
ERK: Extracellular Signal-Regulated kinase.
ES: Embryonic stem cells
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.
IFNγ: Interferon γ
IκB: inhibitor of κB.
IL-1: Interleukin-1
Jnk: c-Jun N-terminal kinase
kDa: 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.
Par-4: Prostate apoptosis response 4.

pCREB: phosphor-CREB
PBS: phosphate buffered saline
PKA: Protein kinase A.
PKC: Protein kinase C.
PKI: Protein kinase A Inhibitor.
PML: Promyelocytic leukemia.
PrE: Prostate epithelial cells
PrS: Prostate stromal cells
PSD: postsynaptic density.
RCC: Renal cell carcinomas.
SAC: Selective Apoptosis-induction in Cancer cells
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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