PROTEIN PHOSPHATASE 2A (PP2A) HOLOENZYMES REGULATE DEATH ASSOCIATED PROTEIN KINASE (DAPK) IN CERAMIDE-INDUCED ANOIKIS

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ABSTRACT
Ryan Cole Widau

PROTEIN PHOSPHATASE 2A (PP2A) HOLOENZYMES REGULATE DEATH ASSOCIATED PROTEIN KINASE (DAPK) IN CERAMIDE-INDUCED ANOIKIS

Modulation of sphingolipid-induced apoptosis is a potential mechanism to enhance the effectiveness of chemotherapeutic drugs. Ceramide is a pleiotropic, sphingolipid produced by cells in response to inflammatory cytokines, chemotherapeutic drugs and ionizing radiation. Ceramide is a potent activator of protein phosphatases, including protein phosphatase 2A (PP2A) leading to dephosphorylation of substrates important in regulating mitochondrial dysfunction and apoptosis. Previous studies demonstrated that death associated protein kinase (DAPK) plays a role in ceramide-induced apoptosis via an unknown mechanism. The tumor suppressor DAPK is a calcium/calmodulin regulated serine/threonine kinase with an important role in regulating cytoskeletal dynamics. Auto-phosphorylation within the calmodulin-binding domain at serine308 inhibits DAPK catalytic activity. Dephosphorylation of serine308 by a hitherto unknown phosphatase enhances kinase activity and proteasomal mediated degradation of DAPK.

In these studies, using a tandem affinity purification procedure coupled to LC-MS/MS, we have identified two holoenzyme forms of PP2A as DAPK interacting proteins. These phosphatase holoenzymes dephosphorylate DAPK at
Serine308 in vitro and in vivo resulting in enhanced kinase activity of DAPK. The enzymatic activity of PP2A also negatively regulates DAPK protein levels by enhancing proteasomal-mediated degradation of the kinase, as a means to attenuate prolonged kinase activation.

These studies also demonstrate that ceramide causes a caspase-independent cell detachment in HeLa cells, a human cervical carcinoma cell line. Subsequent to detachment, these cells underwent caspase-dependent apoptosis due to lack of adhesion, termed anoikis. Overexpression of wild type DAPK induced cell rounding and detachment similar to cells treated with ceramide; however, this effect was not observed following expression of a phosphorylation mutant, S308E DAPK. Finally, the endogenous interaction of DAPK and PP2A was determined to be required for ceramide-induced cell detachment and anoikis.

Together these studies have provided exciting and essential new data regarding the mechanisms of cell adhesion and anoikis. These results define a novel cellular pathway initiated by ceramide-mediated activation of PP2A and DAPK to regulate inside-out signaling and promote anoikis.

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LIST OF ABBREVIATIONS

A structural subunit of protein phosphatase 2A
APAF-1 apoptosis protease-activating factor 1
Bα regulatory subunit Bα of protein phosphatase 2A
Bδ regulatory subunit Bδ of protein phosphatase 2A
B'β regulatory subunit B'β of protein phosphatase 2A
C catalytic subunit of protein phosphatase 2A
CAPP ceramide-activated protein phosphatase
Caspase cysteine-aspartic protease
Ca²⁺/CaM calcium/calmodulin
CerK ceramide kinase
CHIP C-terminal HSC70-interacting protein E3 ubiquitin ligase
CIP2A cancerous inhibitor of PP2A
CLL chronic lymphocytic leukemia
Cyt c cytochrome c
C6 ceramide N-Hexanoyl-D-sphingosine
DAPK death associated protein kinase
DIC differential interference contrast
ECM extracellular matrix
ER endoplasmic reticulum
FADD fas-associating death domain-containing protein
FAK focal adhesion kinase
HEK human embryonic kidney
hTERT human telomerase
ILK integrin-linked kinase
INCAPS Indiana Center for Applied Proteomics
I2PP2A inhibitor 2 of protein phosphatase 2A
JNK c-Jun NH-terminal kinase
LC-MS/MS liquid chromatography separation, and tandem mass spectrometry
LT large T antigen
Mib1 mind bomb1
MMP mitochondrial membrane permeabilization
OA okadaic acid
PAK2 p21-activated kinase 2
PARP poly-ADP-ribose polymerase
PI3K phosphatidylinositol 3-kinase
PP2A protein phosphatase 2A
qRT-PCR quantitative reverse transcription PCR
RLC myosin regulatory light chain
siRNA small interfering RNA
SMase sphingomyelinase
SV40 simian virus 40
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ST</td>
<td>small T antigen</td>
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<tr>
<td>LT</td>
<td>large T antigen</td>
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<td>S308</td>
<td>serine 308</td>
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<td>TAP</td>
<td>tandem affinity purification</td>
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CHAPTER I:

Introduction

A. Mechanisms of Cell Adhesion

In multicellular organisms, cells do not exist in isolation. Instead, they interact with neighboring cells and the extracellular environment. The extracellular matrix (ECM) is part of this environment that serves as a scaffold onto which cells adhere throughout the body. It serves not only as a scaffold but also integrates environmental cues regarding its context within a tissue or organ that are required for proliferation, migration, differentiation and survival (reviewed in (127)). Cell adhesion to the ECM occurs mainly through integrin containing complexes, while cell-cell adhesion occurs through cadherin containing complexes. Integrins are a family of receptor adhesion molecules that play a role in cellular signaling to regulate many physiological processes including cytoskeletal organization, motility, transcription, proliferation, and survival (47). Integrins are obligate heterodimers containing two distinct chains called alpha and beta subunits. In mammals, 18 alpha and 8 beta subunits have been characterized that bind ECM proteins such as collagen, laminin, vitronectin and fibronectin to maintain adhesion and to regulate 'outside-in' signaling cues obtained from the ECM (47). Integrin signaling is mediated through focal adhesion kinase (FAK), integrin-linked kinase (ILK) and Shc (8). Together, these proteins regulate integrin-mediated survival signaling by activating phosphatidyl
inositol 3-kinase (PI3K)/Akt, Raf/extracellular signal-regulated kinase (ERK), and c-Jun NH-terminal kinase (JNK) pathways (8, 14, 57). In addition to ‘outside-in’ signaling, integrins play a crucial role in ‘inside-out’ signaling. Inside-out signaling is a rapid event on a timescale of <1 s, initiated by intracellular changes that change the ability of integrin extracellular domains to bind extracellular ligands (115). It is unclear if integrin-mediated adhesion occurs by conformational shape-shifting within a single receptor molecule (affinity) or by increased integrin clustering on the cell surface (avidity) (115). Regardless of increased affinity and/or avidity for integrins to their ligands, intracellular inside-out signaling plays a crucial role in controlling integrin-mediated adhesion (engagement) to the ECM, which in turn promotes survival signals. Disengagement of integrins from the ECM stops these survival signals and initiates cytoskeletal reorganization and cell death (91, 98). In cell culture, integrins are proteins involved in maintaining a cell’s ability to adhere to tissue culture dishes (80). The mechanisms by which cells maintain adherence through both outside-in and inside-out pathways are incompletely understood and of interest.

B. Apoptosis

A study on ischemic liver injury published in 1972 by Kerr et al. (56), described a novel form of cell death that differed from necrosis. Electron microscopy studies revealed that the structural changes that are seen during this type of cell death occurred in two stages. In the first stage, cells underwent
nuclear and cytoplasmic condensation and the breaking up of the cell into a number of membrane-bound bodies. In the second stage, these bodies were shed and taken up by other cells by phagocytosis (56). The authors termed this form of cell death as apoptosis, a Greek term to describe the “dropping off” or “falling off” of petals from flowers, or leaves from trees.

Apoptosis is a genetically programmed cellular process that results in the elimination of unwanted or damaged cells according to the rule “better death than wrong” (60). It is a well-conserved process that is essential for both embryonic and postembryonic development in both unicellular and multicellular organisms. In embryonic development, apoptosis plays an essential role in sculpting parts of the body such as the formation of the digits, regression of vestigial structures, and removing dangerous or injured cells (50). Many pathological conditions arise due to defects in apoptosis. Inhibition of apoptosis often leads to neoplasia and oncogenesis (114). Whereas inappropriate apoptosis in postmitotic cells (i.e. neurons) can lead to neurodegenerative disorders such as Alzheimer’s or Parkinson’s disease (22, 88). Thus, tight control of apoptosis during all stages of life is essential to the health of an organism.

Apoptosis is typically classified into two categories: the intrinsic and extrinsic pathways (Figure 1). The intrinsic apoptotic pathway is initiated by internal cellular stressors such as DNA damage, endoplasmic reticulum (ER) stress, defective cell cycle, loss of cell adhesion, hypoxia and loss of cell survival factors (reviewed in (60)). Depending on the intrinsic stressor, different pathways are initiated which converge at the mitochondria to induce mitochondrial
membrane permeabilization (MMP). Members of the Bcl-2 family of proteins play a key role in MMP and apoptosis. This family contains pro-apoptotic (i.e. Bax, Bak, Bid, Bad and Bim) and anti-apoptotic members (i.e. Bcl-2, Bcl-XL, Bcl-W and Mcl-1) proteins (reviewed in (60). The anti-apoptotic proteins, such as Bcl-2 (45), bind to pro-apoptotic proteins such as Bak and Bad (19, 137), to prevent the latter from oligomerizing and inserting into the outer membrane of the mitochondria. The insertion of these proteins into the mitochondria initiates MMP (30, 120). MMP causes the release of pro-apoptotic factors from the intermembrane space, including cytochrome c (cyt c). Cyt c then forms a death complex with apoptosis protease-activating factor 1 (APAF-1) and ATP/dATP, known as the apoptosome. The purpose of the apoptosome is to proteolytically cleave and active caspase-9. Caspase-9 is a member of a family of cysteine-aspartic proteases known as caspases. Active caspase-9 cleaves the effector caspases, Caspase-3, -6, and -7, resulting in widespread cleavage of a multitude of substrates. Substrates of active caspases include proteins with roles in cell survival and proliferation such as the DNA fragmentation factor (DFF), which induced DNA fragmentation after it is activated by caspase-3 (72). Other substrates include poly-ADP-ribose polymerase (PARP), nuclear lamins, and p21-activated kinase 2 (PAK2) (64, 89, 105). Analyzing the cleavage of these substrates is often used as an indirect measure of apoptosis.

The extrinsic apoptotic pathway (also known as “death receptor pathway”) occurs by ligand-induced activation of death receptors at the plasma membrane of a cell. These death receptors are a subset of the TNF receptor (TNFR) family,
including TNFR1, Fas/CD95, TRAIL-1 and-2, and TRAMP (reviewed in (60)). Activation of these receptors causes the recruitment of Fas-associating death domain-containing protein (FADD) within the death-inducing signaling complex (DISC). This results in the activation of Caspase-8 which in turn then cleaves the effector caspases, Caspase-3, -6, and -7, resulting in widespread cleavage of a multitude of substrates similar to the intrinsic pathway. Additionally, caspase-8 cleaves Bid, a BH3 domain protein, leading to MMP and represents the main link between the extrinsic and intrinsic apoptotic pathways (67).

Both the intrinsic and extrinsic pathways are further divided into three distinct phases: initiation, integration/decision, and execution/degradation (61). The initiation phase is complex and depends greatly on the death signal that occurs via the intrinsic or extrinsic pathway. The integration/decision phase is the activation of caspase and mitochondrial death effectors that push the cell to the “point of no return” leading to death (60). Finally, the execution/degradation phase is morphologically seen as cell shrinkage, chromatin condensation, nuclear fragmentation, blebbing, and phosphatidylserine exposure on the surface of the plasma membrane (Figure 1) (60, 77).

C. Anoikis

In adherent cells, such as epithelial cells, cell-matrix interactions mediated by integrins, and cell-cell interactions mediated by cadherins, together with actin organization and remodeling play a vital role in cell survival (49, 58, 133). ECM
adhesion is important for a cell to determine if it is in the correct location within the body. Apoptosis induced by loss of cell adhesion to the ECM or loss of cell-cell attachments, is a specialized type of cell death termed anoikis, a Greek word meaning ‘homelessness’ (31). Inhibition of anoikis is expected to confer a selective advantage upon pre-cancerous cells, giving them anchorage independence and affording them an increased survival time in the absence of matrix attachment. This anchorage independence eventually results in reattachment and colonization of secondary sites (metastasis) (32).

The biochemical events occurring during the execution phase of anoikis are similar to those in both intrinsic and extrinsic apoptosis. In contrast, initiation and integration phases of anoikis are mediated by different pathways, but converge to result in activation of caspases as in classical apoptosis. The initiation phase of anoikis can occur with disengagement of integrins from the ECM. This prevents the survival signaling mediated through integrin-mediated activation of FAK, ILK and Shc, previously discussed above (Figure 2). Loss of these survival signals initiates anoikis in multiple cell types (32). During the integration phase, as in classical apoptosis, members of the Bcl-2 family play an important role in promoting anoikis. Integrin mediated activation of ERK and PI3K/Akt results in the phosphorylation and subsequent proteasomal degradation of pro-apoptotic protein Bim. Loss of ECM contact inhibits ERK and PI3K/Akt signaling, thereby enhances Bim protein levels to promote MMP, caspase activation and anoikis (65, 99). The execution phase of anoikis is identical to classical apoptosis and morphologically seen as cell shrinkage, chromatin
condensation, nuclear fragmentation, blebbing, and phosphatidylserine exposure on the surface of the plasma membrane (60, 77).

Resistance to anoikis can be achieved by enhancing integrin-mediated survival signals (i.e. PI3K/Akt, MEK/ERK and NFkB), changing the pattern of integrin expression, and/or inhibiting apoptotic pathways (47). For example, overexpression of anti-apoptotic Bcl-2 proteins is a key step to achieving resistance (33). The constitutive activation of survival pathways can be achieved by a number of ways including overexpressing neurotrophic tyrosine kinase receptor (TrkB). This receptor is overexpressed in highly aggressive human tumors and confers resistance to anoikis by activating the PI3K/Akt pathway (26, 141). Studies have also shown integrin mediated activation of FAK can suppress anoikis (34). Conversely, reducing or silencing the expression of tumor suppressor proteins, such as death associated protein kinase (DAPK), is a key step to conferring resistance to anoikis and promoting metastasis in animal models (48). Additional studies demonstrated that DAPK can transduce an inside-out signal to convert integrins into an inactive conformation, thereby disrupting matrix survival signals, resulting in loss of cell adhesion and apoptosis (132).

In addition to cancer, anoikis plays a role in cardiovascular pathologies such as cardiac myocyte detachment in heart failure, plaque rupture in atherosclerosis and smooth muscle cell disappearance in aneurysms and varicose veins (18, 59, 129). The mechanism by which this may occur is through inflammatory cell secretion of proteases (e.g. elastase and cathepsin G) that are
able to degrade adhesive glycoproteins such as fibronectin, and induce anoikis (81). In these examples, anoikis is seen as detrimental and probably occurs due to overcompensating for a dysfunctional healing process (18). Overall, the events that initiate anoikis by the inside-out signaling are unclear. Identifying the molecular pathways and proteins involved in this important process may lead to new therapeutics.

D. Ceramide-Induced Apoptosis

Sphingolipids are components of the lipid membrane that control various aspects of cell growth and proliferation (111). Ceramides are a product of sphingolipid metabolism and are generated in response to cellular stress and cytokine production. Ceramides are derived by formation of a peptide bond between sphingosine and a fatty acid during de novo synthesis. In addition, ceramide is generated by the activation of sphingomyelinases (SMases) that hydrolyze sphingomyelin to produce ceramide. The generation of ceramide is mainly associated with anti-proliferative responses and apoptosis and can be initiated by death receptors such as TNFR1, chemotherapeutics agents (i.e. daunorubicin, camptothecin, fludarabine, etoposide and gemcitabine) or ionizing radiation (35, 90, 111, 113). Ceramide directly binds to the inhibitor 2 of protein phosphatase 2A (I2PP2A), enhancing the activity of PP2A (84), leading to dephosphorylation of Bcl-2 and Bax to result in MMP and apoptosis (96, 108, 135). Another well characterized ceramide target protein is ceramide kinase
(CerK) and its activation enhances ceramide-induced apoptosis (40). In addition to activating PP2A and CerK, ceramides may directly form large protein-permeable channels that allow for the release of cyto c from the mitochondria (118). More recent laboratory studies have yielded new ceramide target proteins which include, DAPK, which promotes ceramide-induced apoptosis through an unknown mechanism.

Although ceramide-induced apoptosis has been studied extensively in cultured neurons and other type of cells treated with cell-permeable analogs of ceramide, such as C6 ceramide, the ceramide signaling pathway that leads to the activation of effector caspases and apoptosis is not clear (13, 44, 94, 125). Ceramide has also been implicated in anoikis and is accompanied by fragmentation of the Golgi apparatus via an unknown mechanism (46). It has been proposed that modulation of sphingolipid-induced apoptosis by chemotherapeutic agents, may enhance the effectiveness of cancer therapy and thus a better understanding of these pathway(s) activated by sphingolipids is important (5, 83, 104).

E. Death Associated Protein Kinase (DAPK)-Structure and Function

Death-associated protein kinase (DAPK) is a calcium/calmodulin (Ca^{2+}/CaM)-dependent serine/threonine kinase that regulates multiple signaling pathways including cell apoptosis, autophagy, survival, motility, and adhesion (12, 54, 62, 132, 143) (Figure 3). DAPK functions as a positive mediator of
apoptosis induced by a variety of stimuli including INFγ, TGFβ, ceramide, and the oncogenes c-myc and p53 (for a review see (12)), and as negative mediator of apoptosis induced by TNFα (54, 71). Forced expression of DAPK results in morphological changes including cell rounding, shrinking, detachment, and anoikis in multiple cell types (62, 132). In animal studies, the expression level of DAPK was inversely correlated with the metastatic potential of tumors and reintroduction of DAPK into the metastatic tumors initiated anoikis (48). DAPK has also been suggested to be a tumor suppressor and in human cancers, DNA methylation within the promoter of DAPK is a frequent event and strongly correlates with the rates of recurrence and metastasis (12). A point mutation within the promoter of DAPK reduces it’s expression and results in hereditary predisposition to chronic lymphocytic leukemia (CLL) (101).

DAPK is a large multi-domain protein that forms many intracellular signaling complexes. These protein-protein interactions give DAPK distinct biological roles including autophagy, apoptosis and survival (121). DAPK has five functional domains, including the kinase, calmodulin, ankyrin repeats, cytoskeletal and death domains (Figure 4). The amino terminal kinase domain interacts with a member of the microtubule family, microtubule associated protein 1B (MAP1B) to regulate autophagy (43). The calmodulin binding domain interacts with Ca²⁺/CaM to regulate the kinase activity (54, 117). The ankryrin repeat domain interacts with Src, LAR protein phosphatase, E3-ligase DIP-1/Mib1, and actin stress fibers (23, 53, 131) to regulate the activities and localization of DAPK. The cytoskeletal domain forms interactions with cathepsin
B (71) to negatively regulate DAPK and promote TNFα-mediated apoptosis. The carboxyl terminal death domain interacts with a number of proteins including the netrin-1 receptor UNC5H2, ERK, TNFR1, FADD, and TSC2, and these associated proteins regulate its apoptotic functions.

Recent studies focusing on the posttranscriptional control of DAPK have identified a complex network regulating the protein levels of DAPK. Translational repression of DAPK occurs by the interferon-γ-activated inhibitor of translation (GAI)T complex (85). Posttranslational control of DAPK protein levels is regulated by at least two distinct E3 ubiquitin ligases, C-terminal HSC70-interacting protein E3 ubiquitin ligase (CHIP) (145) and Mind bomb1 (Mib1) (53), which polyubiquitinate DAPK resulting in proteasomal degradation. In addition, the lysosomal protease Cathepsin B (71) negatively regulates protein levels of DAPK. Finally, a small alternatively spliced form of DAPK, sDAPK, was shown to cause decreased stability of full-length DAPK independent of the proteasome or lysosome (70).

The catalytic activity of DAPK is regulated by Ca²⁺/CaM and by auto-phosphorylation of serine308 (S308), which resides within the CaM-binding domain (54, 117). Auto-phosphorylation of S308 prevents calmodulin binding, which is necessary for the kinase activity of DAPK; thus, S308 phosphorylation negatively regulates DAPK activity (54, 117). Despite the obvious importance associated with dephosphorylation of S308, the phosphatase that dephosphorylates this site has not been extensively characterized. In addition, other recent studies have suggested that the catalytic activity of DAPK is
regulated by phosphorylation of additional sites. The kinase has been identified as a substrate for Src, Erk, and p90RSK. Phosphorylation of DAPK at Y491/Y492 by Src reduces the catalytic activity and these phosphorylation sites are reciprocally regulated by leukocyte common antigen-related tyrosine phosphatase (LAR) (131). Phosphorylation of S289 by p90 ribosomal S6 kinase (RSK) 1 and 2 also suppress the catalytic activity of DAPK (4). However, phosphorylation of S735 by ERK leads to enhanced the catalytic activity (17). The mechanisms by which these newly identified phosphorylation sites regulate the kinase activity are unclear.

The unphosphorylated, active form of DAPK is rapidly ubiquitinated and degraded by the proteasome (54). With this in mind, we propose that an unknown phosphatase controls the activation of DAPK in a two-step mechanism. First, the kinase is dephosphorylated by the activated phosphatase to enhance Ca$^{2+}$/CaM binding, relax autoinhibition, and promote activation of DAPK. Second, dephosphorylation induces a conformational change, potentially exposing a ubiquitination site which attenuates the expression level of the activated pool of DAPK through targeting for proteasomal degradation, thereby providing an additional mechanism to limit DAPK activity. Thus, the S308 phosphatase not only controls DAPK activation, but also the cellular levels of DAPK. Recent evidence suggests that a “PP2A-like” phosphatase may control S308 phosphorylation; however, the specific holoenzyme form(s) of PP2A involved in this event are unknown (39).
Ceramide is a potent activator of DAPK and this kinase is necessary for ceramide-induced cell death in multiple cell types, but the cellular mechanism leading to death is unclear (54, 93, 136). The activation of DAPK by ceramide is not thought to be through a direct association, but rather through a ceramide-activated phosphatase; however, the identity of this phosphatase remains uncertain. Once active, DAPK phosphorylates substrates including myosin regulatory light chain at S19 to regulate cytoskeletal dynamics, cell adhesion, and migration (11, 54, 63, 117, 132) (Figure 3). In addition, activation of DAPK by an unknown mechanism was recently shown to regulate inside-out signaling to suppress β-integrin mediated cell adhesion likely through disrupting the association of talin and CDC42 (62, 132). These and other recent studies highlight the role for DAPK in multiple signaling pathways, all of which are dependent on its kinase activity. Clearly, identification of the S308 phosphatase(s) and its mode of activation will greatly enhance our understanding of how this kinase is regulated in vivo.

F. Protein Phosphatase 2A (PP2A)-Structure and Function

As mentioned in the previous section, a PP2A-like phosphatase is proposed to dephosphorylate DAPK at S308. PP2A is a serine/threonine protein phosphatase and tumor suppressor that regulates numerous cellular processes including proliferation, differentiation and apoptosis (28). The native forms of PP2A consist as a core dimer and a heterotrimeric holoenzyme. The core dimer
(AC) consists of a scaffolding/structural subunit (A) and a catalytic subunit (C) which associate with a regulatory B subunit (B) to form a heterotrimeric holoenzyme (Figure 5). The heterotrimeric holoenzyme is the most predominant form of PP2A in the cell (51).

The structural A subunit regulates holoenzyme composition and binds to both the catalytic and regulatory subunits. It exists in two non-redundant isoforms (α and β) and mutations in several types of human cancers interrupt binding to the catalytic subunit, thus resulting in an overall decrease in phosphatase activity (20, 106, 107, 134). Cell transformation by the simian virus 40 (SV40) small t antigen occurs by binding to the A subunit and prevents holoenzyme formation (92, 138).

The catalytic C subunit also exists in two non-redundant isoforms (α and β) that share 97% identity (6, 37, 38). Endogenous catalytic inhibitors have been described for PP2A; cancerous inhibitor of PP2A (CIP2A), inhibitor 2 of PP2A (I2PP2A, also known as SET), and type 2A-interacting protein (TIP) (55, 68, 78). Pharmacological inhibitors of PP2A include fostriecin, okadaic acid and calyculin A. Fostriecin is in phase I clinical trials and is showing promise as a potential anti-cancer therapy (66). Activation of the catalytic activity of PP2A can be stimulated with ceramide and the novel compound, FTY720 (also known as fingolimod). Ceramide is well known to activate PP2A and likely acts through ceramide directly binding to I2PP2A, thereby displacing the endogenous inhibitor of PP2A, enhancing its catalytic activity (15, 25, 84). FTY720, a novel PP2A activator, leads to cell cycle arrest and apoptosis in human B and T-cell
leukemias, including BCR/ABL-transformed myeloid and lymphoid cells and chronic myelogenous leukemia in blast crisis (86). FTY720 is in phase III clinical trials as a small molecule immunosuppressant.

The regulatory B subunit is the substrate targeting subunit for PP2A and is categorized into four distinct families with several different nomenclatures. The B (B55 or PR55), B’ (B56 or PR61), B” (PR72) and B’’’ (1). Multiple isoforms exist within each family and they share significant amino acid homology as well as some of the same substrates, whereas regulatory B subunits in different families lack amino acid homology and have distinct functions (reviewed in (27)). Overall, 20 regulatory subunits have been identified giving diversity to the holoenzymes, enabling PP2A to have selectivity and a wide range of functions within the cell. The regulatory B subunit directs the subcellular localization and enzymatic kinetics of the catalytic subunit of PP2A (95, 123). Studying the regulatory B subunits has led to the identity of new PP2A substrates involved in various cell-signaling pathways, including ceramide-induced apoptosis (108). Members of the B’ family dephosphorylate proto-oncogenes c-Myc and Pim-1 and negatively regulate their activity, resulting in the enhancement of ubiquitination and proteasomal degradation of these proteins (7, 76). Members of the B family (Bα and Bδ), have opposing roles in the TGFβ/Activin/Nodal pathway. The Bα subunit prevents lysosomal degradation of the ALK4 and ALK5 receptor whereas the Bδ subunit inhibits ALK4 activity (10). However, in another study Bα and Bδ played a redundant role in removing the inhibitory phosphorylation site S259 on Raf-1, leading to positive regulation of Raf1-MEK1/2-ERK1/2 signaling. These studies
highlight the complexity of specific PP2A holoenzymes and the complex roles they play in pathway activation, inactivation and in regulating protein turnover.

Because PP2A plays roles in both proliferation and apoptosis, identification of holoenzyme specific targets is necessary to determine if a small-molecule phosphatase inhibitor or small-molecule phosphatase activator should be utilized in anti-cancer therapies (79).

G. Rationale

It is widely accepted that one mechanism by which DAPK can induce many physiological changes in cells, including cell death and cell adhesion, is through pathway-specific protein interactions. In order to determine additional DAPK interactions, our laboratory conducted a tandem affinity purification coupled to mass spectrometry using DAPK as bait. In this screen, we identified the regulatory Bα subunit of protein phosphatase 2A (PP2A) as a candidate DAPK binding partner. Additional experiments indicated that the highly homologous Bδ regulatory subunit also associates with DAPK. As described above, PP2A is a multi-subunit complex and the regulatory subunits give it its substrate specificity. This dissertation, thus, focuses on these newly identified DAPK interacting proteins, PP2A-ABαC and ABδC, and determines their role in DAPK-induced cell death and cell adhesion.
Figure 1: Extrinsc versus intrinsic caspase activation cascades in apoptosis. *Left:* extrinsic pathway. *Right:* intrinsic pathways. (Adapted from Kroemer et al., 2007 Physiol Rev (60))
Figure 2: Mechanisms of Anoikis. *Top:* Attachment to the extracellular matrix and stimulation of growth factor signaling cascades suppresses the activity of apoptotic factors. *Bottom:* Detachment from the matrix or growth factor deprivation shuts down these signaling cascades and promotes MMP. (Adapted from Reddig and Juliano, 2005 Cancer Metastasis Rev (102))
Figure 3: Regulation of DAPK. A) DAPK is regulated by multiple signals at the level of transcription and at the protein level. B) The DAPK death signaling network. (Adapted from Bialik and Kimchi 2006 Annu Rev Biochem (12))
**Figure 4: Protein domains of DAPK.** (Adapted from Bialik and Kimchi 2006 Annu Rev Biochem (12))
Figure 5: PP2A holoenzyme composition. PP2A isoforms of the structural A, regulatory B and catalytic C subunits. (Adapted from Sablina and Hahn 2008 Cancer Metastasis Rev (110)).
CHAPTER II:

PP2A Holoenzymes Regulate DAPK Activity and Stability

A. Summary

The tumor suppressor, Death-associated protein kinase (DAPK) is a Ca^{2+}/CaM regulated Ser/Thr kinase with an important role in regulating cytoskeletal dynamics, apoptosis and cellular homeostasis. Auto-phosphorylation within the calmodulin-binding domain at S308 prevents Ca^{2+}/CaM binding and inhibits DAPK catalytic activity. Dephosphorylation of S308 by a hitherto unknown phosphatase enhances the kinase activity and proteasomal-mediated degradation of DAPK. In this chapter, we utilized a protein affinity purification technique coupled to tandem mass spectrometry in an effort to identify novel DAPK interacting complexes. Subsequently, we identified two holoenzymes of protein phosphatase 2A (PP2A), ABαC and ABδC, as DAPK interacting proteins. These holoenzymes interact via the cytoskeletal binding domain of DAPK and dephosphorylate S308 in vitro and in vivo. Dephosphorylation of S308 enhances Ca^{2+}/CaM binding to DAPK, resulting in enhanced kinase activity in vitro. In addition to activating DAPK, we determined PP2A negatively regulates DAPK protein levels by enhancing its proteasomal-mediated degradation. Together, our results provide a mechanism by which PP2A holoenzymes control the kinase activity and protein stability of DAPK.
B. Introduction

Complex signal transduction cascades control multiple physiological processes such as cellular growth, proliferation and apoptosis. Within these cascades exist protein-protein networks that are sensitive to biological stimuli and regulate a cell’s response to its environment. A common mechanism used by cells to respond to environmental cues is modification of these signal transduction cascades through reversible protein phosphorylation. The addition of a negatively charged phosphate group to a serine, threonine or tyrosine residue by a protein kinase, or removal of a phosphate by a protein phosphatase can alter the activity of targeted proteins. Protein phosphorylation and dephosphorylation reactions can affect the not only target protein’s activity, and function but also half-life, or subcellular localization of the substrate; therefore, the underlying molecular mechanisms controlling this reversible post-translational modification are of great physiological importance (79).

Death-associated protein kinase (DAPK) is a Ca\(^{2+}\)/CaM-dependent Ser/Thr kinase that regulates many cellular signaling cascades including cell apoptosis, autophagy, survival, motility, and adhesion (1-5). The mechanisms governing the activation of this important kinase are unclear. However, it is known that the catalytic activity of DAPK is regulated by Ca\(^{2+}\)/CaM and by auto-phosphorylation of S308, which resides within the calmodulin-binding domain (3,13). This auto-phosphorylation of S308 prevents calmodulin binding, which is necessary for the kinase activity of DAPK; thus, S308 phosphorylation negatively
regulates DAPK activity (3,13). The unphosphorylated, active form of DAPK is rapidly ubiquitinated and degraded by the proteasome (54), thereby providing an additional mechanism to limit DAPK activity. Thus, the S308 phosphatase is proposed to not only control DAPK activation, but also cellular DAPK levels. Despite the obvious importance associated with dephosphorylation of S308, the phosphatase that dephosphorylates this site has not been extensively characterized. Identification of novel DAPK-protein complexes, including a DAPK-phosphatase(s) complex, is needed to elucidate the mechanisms by which this kinase regulates many physiological processes.

To identify novel DAPK-protein complexes we utilized tandem affinity purification (TAP), using DAPK as bait. Using this approach in conjunction with other biochemical techniques, we identified two specific holoenzymes of PP2A. PP2A is a Ser/Thr protein phosphatase that regulates numerous cellular processes including proliferation, differentiation and apoptosis (27). The predominant form of PP2A is a heterotrimeric holoenzyme consisting of a scaffolding/structural subunit (A), a regulatory subunit (B), and a catalytic subunit (C). The regulatory B subunit is the substrate targeting subunit for PP2A and is categorized into four distinct families B, B', B'' and B''' (19). Multiple isoforms exist within each family and they share significant amino acid homology as well as some of the same substrates, whereas regulatory B subunits in different families lack amino acid homology and have distinct functions (for a review see (27)). The diversity of holoenzymes enables PP2A to have selectivity and a wide range of functions within the cell.
In the current study we demonstrated that PP2A interacts with DAPK and determined the effects of PP2A on DAPK activity in HEK293 and HeLa cells. Results from these studies suggest that PP2A affects the activities and cellular levels of DAPK through protein dephosphorylation.
C. Experimental Methods and Procedures

i. Materials and Reagents.

MG132, chloroquine, doxycycline, protease inhibitor cocktail, phosphatase inhibitor cocktail-1, N-Hexanoyl-D-sphingosine (ceramide-C6), FLAG peptide (DYKDDDDK), anti-FLAG M2-agarose and Proteosilver Silver Stain kit were from Sigma (St. Louis, MO). Okadaic Acid (OA) was from EMD (Gibbstown, NJ). FTY720 was from ALEXIS Biochemical (San Diego, CA). Absolute QPCR Mixes were from ABgene (Rockford, IL). Fugene 6 transfection reagent was purchased from Roche Diagnostics (Indianapolis, IN). DharmaFect-1 siRNA transfection reagent was from Dharmacon (Lafayette, CO). DAPK substrate peptide was from TOCRIS (Ellisville, MO). PP2A immunoprecipitation Phosphatase Assay Kit was from Millipore (Temecula, CA). \[^{32}\text{P}\]-ATP was from MP Biomedicals, Inc (Irvine, CA). zVAD-FMK was from BD Biosciences (San Jose, CA). Recombinant adenoviruses were produced at ViraQuest Inc. (North Liberty, IA).

ii. Antibodies.

Antibodies to DAPK (DAPK55), p-S308 DAPK, FLAG M2 and vinculin were purchased from Sigma (St Louis, MO). Anti-DAPK (DAP-3) was from BD Biosciences (San Jose, CA). PP2A catalytic C, structural A, and regulatory B subunit antibodies were from Cell Signaling (Beverly, MA). The generation and
characterization of affinity-purified B\(\alpha\)/B\(\delta\) antibody was as reported previously (122). Anti-Omni probe (D-8) and anti-PARP were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). TrueBlotTM anti-mouse secondary antibody was from eBioscience (San Diego, CA).

iii. Tissue Culture and Transient Transfection.

Human embryonic kidney (HEK) T-Rex cell lines harboring pcDNA5/TO (EV), pcDNA5/TO-B\(\alpha\)-FLAG or pcDNA5/TO-B\(\delta\)-FLAG were generated previously (1). Expression of B\(\alpha\)-FLAG and B\(\delta\)-FLAG was accomplished by treating with 2 \(\mu\)g/mL doxycycline for 48 h at 37\(^\circ\)C as previously described (1). HeLa and HEK293 cells were obtained from ATCC (Bethesda, MD). HEK T-Rex, HeLa, and HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transient transfection of HeLa and HEK293 cells was carried out using equal amounts of total plasmid DNA (adjusted with the corresponding empty vectors) together with Fugene 6 transfection reagent according to the manufacturer's guidelines. Short-interfering RNAs (siRNAs) for B\(\alpha\) were obtained from Dharmacon (target sequence 5’UGUAGUAGGAGUCUCUAUAC-3’) as well as a SMARTpool for B\(\delta\). Nontargeting siRNAs were purchased from Dharmacon and used as negative control. DharmFect 1 was used for the siRNA transfection.
iv. Tandem Affinity Purification.

Full length human DAPK was cloned into TAP vector (Stratagene) using a standard PCR-based cloning strategy. HEK293 cells were seeded in 15-cm plates and then transiently transfected with DAPK-TAP (15 µg of plasmid/plate). TAP protocol was essential as described by the manufacturer’s protocol. The final eluted bound samples were concentrated and submitted to the Indiana Center for Applied Proteomics (INCAPS) for analysis, including tryptic digestion, high performance liquid chromatography separation, and tandem mass spectrometry (MS/MS) to determine peptide sequences.

v. Western Blotting and Immunoprecipitation.

Western blotting and immunoprecipitation were performed as described previously (52). Cell extracts were prepared in a lysis buffer containing 0.1% Nonidet P-40 (NP40), 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 10 mM sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, protease inhibitor mixture, and phosphatase inhibitor mixtures including microcystin LR, cantharidin, (−)-p-bromotetramisole, and OA (Sigma phosphatase inhibitor cocktail-1), where appropriate. For immunoprecipitation, lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.1% NP40, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1 mM EDTA, 0.15 M NaCl, 10 mM sodium fluoride, 2 mM sodium vanadate and protease inhibitors). Cell lysates were clarified by centrifugation,
and the supernatant was pre-cleared by incubation with Trueblot IgG (eBioscience) beads. For each immunoprecipitation, 1 mL aliquots of lysates (1 mg protein) were incubated with 4–8 µg of DAP-3 antibody at 4°C for 3 h. The immune complexes were then isolated by the addition of 40 µL of protein G beads and incubation for 2 h. Flag-tagged proteins were isolated by incubating 1 mL aliquots of lysates (1 mg protein) with 40 µL of a 50% slurry of anti-FLAG agarose at 4°C for 3 h. Immune complexes were washed three times with lysis buffer to reduce nonspecific binding. The immune complexes were resolved by electrophoresis and analyzed by western blotting.

vi. Reverse Transcription-PCR.

RNA was extracted with TRIzol reagent (Invitrogen) and 0.5 µg of RNA was used as template for reverse transcription (RT) using Superscript first strand cDNA synthesis kit (Invitrogen). The resulting cDNAs were resuspended in 20 µL H₂O. The cDNA levels of specific genes were measured by quantitative real time PCR using Absolute QPCR Mixes (ABgene) and an ABI 7500 Real Time PCR system (Applied Biosystems). The gene-specific primers used for QPCR were sense hHPRT1 5’-CCT TGG TCA GGC AGT ATA ATC CA-3’ and antisense hHPRT1 5’-GGT CCT TTT CAC CAG CAA GCT-3’, hDAPK1 sense 5’-CCC GGA AAA AAA TGG AAA CA-3’ and antisense hDAPK1 5’-TGG ACA GGA ATG ACC TGG ATA AT-3’. All samples were amplified in duplicate and every experiment was repeated independently at least 2 times. Relative gene
expression was converted using the $2^{-\Delta \Delta Ct}$ method against the internal control HPRT1 housekeeping gene as previously described (146).

vii. Purification of PP2A Holoenzymes

PP2A ABαC and ABδC holoenzymes were prepared as previously described (2). Briefly, HEK T-Rex cell lines harboring pcDNA5-TO (EV), Bα-FLAG-pcDNA5-TO, or Bδ-FLAG-pcDNA5-TO were treated with the tetracycline analog, doxycycline (2 µg/mL), for 48 h to induce protein expression. Cells were lysed in buffer containing 20 mM Tris-HCl, pH 7.6, 0.1% Igepal CA-630, 150 mM NaCl, 3 mM EDTA, 3 mM EGTA, and protease inhibitor cocktail. The clarified cell lysates were incubated with 20 µL of a 50% slurry of anti-FLAG agarose for 4 h. Bound proteins were washed twice with PAN buffer (10 mM PIPES, pH 7.0, 17 µg/mL aprotinin, and 100 mM NaCl) containing 0.5% Igepal CA-630, once with PAN buffer, and once in phosphatase assay buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.25 mg/ml bovine serum albumin). Bound proteins were eluted by incubation for 1 h at 4°C in 100 µl of phosphatase assay buffer containing 100 µg/ml FLAG peptide. The amount of PP2A catalytic subunit in the purified PP2A holoenzymes was determined by SDS-PAGE and silver staining using serial dilutions of bovine serum albumin as standards as previously described (2). These values were used to calculate the protein concentration of purified PP2A holoenzymes based upon stoichiometric levels of the A, B, and C subunits in each preparation. Aliquots of the purified
holoenzymes were either assayed for phosphatase activity or subjected to SDS-PAGE followed by silver stain or immunoblot analysis.

viii. Phosphatase and Kinase Assays

Protein phosphatase assays were performed essentially as previously described (1). HEK293 cells were transfected with FLAG-DAPK for 48 h, DAPK was immunoprecipitated from clarified cell lysates (1 mg of protein) using 40 µL anti-FLAG agarose for 4 h in lysis buffer (0.1% NP40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 10 mM sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, protease inhibitor cocktail and phosphatase inhibitor cocktails). The FLAG-DAPK immune complexes were washed once in lysis buffer containing both protease and phosphatase inhibitors and twice in phosphatase assay buffer (25 mM Tris-HCL, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.25 mg/ml bovine serum albumin) and then resuspended in 40 µL of the same buffer. The washed immune complexes were incubated with purified ABαC and ABδC holoenzymes (~100 ng); FLAG peptide eluates for EV-expressing cells were used as a control. Following a 30 min incubation (with agitation) at 37°C, the phosphatase reactions were terminated by washing twice in phosphatase assay buffer containing 100 nM okadaic acid and subjected to immunoblot analysis using antibodies recognizing p-S308-DAPK. For the experiments coupled to an in vitro kinase assay, twenty percent of the reaction products from the phosphatase assay were analyzed by immunoblotting and the
remaining 80% was used in a kinase assay. For the kinase assay, the beads were washed extensively in kinase assay buffer (50 mM MOPS, pH 7, 10 mM magnesium acetate, 1 mM dithiothreitol, 10 mM CaCl₂, 10 µM calmodulin and 100 nM okadaic acid). The kinase reactions were carried out as previously described (76) with minor modifications. In each kinase reaction (40 µL), 100 µM of DAPK peptide substrate (TOCRIS), and [γ-³²P]-ATP (200 cpm/pmol) diluted in 1 mM ATP. Incubation was carried out for 30°C for up to 22.5 min with agitation, collecting 10 µL of the reaction at each time point. The amount of [γ³²P]-ATP incorporation into the synthetic DAPK peptide substrate was analyzed as previously described using a scintillation counter (85).

ix. Calmodulin Overlay Assay

The calmodulin overlay assay was performed as previously described (54). Briefly, immunoprecipitated DAPK was treated with PP2A holoenzymes in an in vitro phosphatase assay. Following SDS-PAGE, DAPK was Western-blotted and incubated with biotinylated CaM (Sigma) in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM Ca²⁺ and 5% nonfat dry milk. Calmodulin was detected with streptavidin-conjugated horseradish peroxidase (The Jackson Laboratory).
x. Statistical Analysis and Quantification.

All experiments were carried out using independent cell transfections, in triplicate. Statistical analysis was performed using Students t-test and graphs were created using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Western blotting images are representative of the repeated experiments. All densitometry analysis of western blotting data was normalized to vinculin levels.
D. Results

i. Identification of regulatory Bα subunit of PP2A as a DAPK binding protein.

To identify novel DAPK interacting proteins in HEK293 cells, we exploited a tandem affinity purification (TAP) approach that consists of two specific binding and elution steps that are designed to minimize non-specific interactions (Figure 6). Shelley Dixon, a technician in the lab, generated a fusion protein consisting of full-length human DAPK and the TAP tag (DAPK-TAP) that was expressed in HEK293 cells to form endogenous protein interactions and complexes. The TAP tag contains protein A, a tobacco etch virus (TEV) cleavage site and a calmodulin binding peptide (CBP) derived from skeletal muscle myosin light chain kinase. In the first step, the DAPK-TAP protein complexes are purified on IgG sepharose, which binds to protein A within the TAP tag. To eliminate non-specific proteins bound to protein A, the protein A tag is cleaved with tobacco etch virus (TEV) enzyme. In the second step, DAPK-TAP protein complexes are purified with the calmodulin binding peptide in the presence of calcium on calmodulin sepharose. DAPK-TAP protein complexes are eluted by calcium chelation with EGTA. Finally, the affinity-purified proteins were sent to the Indiana Center for Applied Proteomics (INCAPS) for identification using LC-MS/MS. Several proteins were identified by LC-MS/MS using this TAP screen (data not shown). One of these
identified proteins, the regulatory B$\alpha$ of PP2A, and was selected for further examination.

ii. PP2A-B$\alpha$ and PP2A-B$\delta$ holoenzymes associate with DAPK.

To validate the TAP results, we examined the ability of DAPK to co-immunoprecipitate with B$\alpha$ and the highly homologous (90% identity) B$\delta$ isoform subunit of PP2A. Although B$\delta$ is a non-redundant protein, it has been determined to share some of the same substrates as B$\alpha$ (1). Thus, we examined the ability of B$\delta$ to bind to DAPK. Co-immunoprecipitation (coIP) experiments were performed using HEK T-REX stable cell lines expressing either empty vector (EV), FLAG-B$\alpha$ (B$\alpha$), or FLAG-B$\delta$ (B$\delta$) described in Methods. Western analysis of the FLAG immune complexes confirmed endogenous DAPK immunoprecipitates with B$\alpha$ and the closely related isoform B$\delta$; no DAPK was detected in the control immune complexes (Figure 7A). Additionally, both the structural (A) and catalytic (C) subunits of PP2A were detected in the FLAG-B subunit eluates, whereas no PP2A subunits were observed in FLAG immune complexes isolated from lysates of control cells. Similarly, western analysis of FLAG-DAPK immune complexes revealed that endogenous B$\alpha$ subunit co-immunoprecipitated with FLAG-DAPK (Figure 7B). Unfortunately, no commercial antibody specifically to B$\delta$ exists, thus we were unable to determine if endogenous B$\delta$ immunopurified with FLAG-DAPK. To determine if DAPK binds other PP2A holoenzymes, we tested its ability to interact with B'$\beta$', a member of the B’ family that shares little homology to
members of the B family. HEK293 cells were transiently transfected with FLAG-Bα, Bδ, or B′β. Western analysis of the FLAG immune complexes confirmed endogenous DAPK immunoprecipitates with Bα and Bδ but not B′β, indicating in vivo specificity for the targeting subunits of PP2A (Figure 7C). Together, these data indicate that the two closely related PP2A holoenzymes, ABαC and ABδC, interact with DAPK.

iii. PP2A-Bα and PP2A-Bδ holoenzymes associate with DAPK at the cytoskeletal domain.

To determine the region of DAPK that mediates its interaction with PP2A, fragments of DAPK corresponding to its known domains (Figure 8A) were transfected into HEK T-Rex cell lines that express either FLAG-Bα, FLAG-Bδ or an empty vector (EV). Immunoprecipitation of these DAPK domains revealed that only the DAPK cytoskeletal domain (Cyto), encompassing residues 628-1215, was sufficient for binding to ABαC and ABδC holoenzymes (Figure 8B). Collectively, these results indicate the PP2A holoenzyme associates with the cytoskeletal domain of DAPK through the regulatory Bα and Bδ subunits.

iv. PP2A negatively regulates the cellular levels of DAPK.

As our previous studies suggested that the dephosphorylated form of DAPK is ubiquitinated and degraded (53, 54, 145), we examined the effects of a well-
characterized PP2A inhibitor, okadaic acid, (OA) and a PP2A activator (FTY720) on DAPK protein levels. We treated HeLa cells with low concentrations (1-5 nM) of OA to inhibit PP2A activity and examined the cellular levels of DAPK using western blotting. This concentration of OA has been shown to be relatively specific for inhibiting PP2A while not affecting the other OA sensitive phosphatases PP1 and PP2B (124). Inhibition of PP2A by OA slightly enhanced the cellular levels of endogenous DAPK (Figure 9A). A recent study by Neviani et al. (86) identified a novel activator of PP2A, FTY720. To determine if enhanced activation of PP2A altered endogenous DAPK protein levels, we treated HeLa cells with increasing amounts of FTY720 in the presence or absence of OA. FTY720 reduced the cellular levels of endogenous DAPK in the absence of OA but this effect could be reversed by co-treating cells with 1 nM OA (Figure 9B). The maximum concentration of FTY720 that we were able to use in these experiments was 5 µM as rapid cell detachment and cell death occurred at higher dosages. These data indicate a “PP2A-like” enzyme negatively regulates the protein levels of DAPK.

To determine if directly targeting PP2A to DAPK would alter its cellular levels, HEK293 cells were transiently transfected with the targeting subunits Bα, Bδ or B'β. With forced expression of the Bα or Bδ regulatory subunits, we observed a decrease in the endogenous protein levels of DAPK (Figure 9C). No reduction in endogenous DAPK protein levels was observed in control cells (EV) or cells overexpressing B'β, a result consistent with our co-immunoprecipitation studies (Figure 9C). These findings further validate the in vivo specificity of the
Bα and Bδ subunits in targeting PP2A to DAPK. Finally, siRNA mediated knockdown of Bα or Bδ separately, or in combination (Bα/δ), resulted in an increase in endogenous protein levels of DAPK (Figure 9D). Since the effects of siRNA-mediated knockdown of Bα and Bδ on DAPK levels were indistinguishable, only Bα was used in subsequent experiments. Collectively, these data indicate the enzymatic activity of specific holoenzymes of PP2A negatively regulate DAPK protein levels.

v. PP2A targets DAPK for proteasomal degradation.

Previous studies have shown activation (dephosphorylation of p-S308) of DAPK leads to enhanced degradation by the proteasome (53, 54, 145) and lysosome (71). To distinguish between proteasomal and lysosomal degradation, we induced the expression of Bα or Bδ in HEK-TRex cells using the tetracycline analog, doxycycline (2 µg/mL) and then treated the cells with either the proteasome inhibitor, MG132 [10 µM] or the lysosomal inhibitor, chloroquine [100 µM] for 6 h. Cells treated with proteasomal inhibitor MG132 could rescue the Bα- or Bδ-induced decrease in DAPK protein levels, whereas the lysosomal inhibitor could not (Figure 10A). No change in the steady state level of DAPK1 mRNA was observed when overexpressing Bα or Bδ either in absence or presence of MG132 or chloroquine, indicating that the observed PP2A-induced decrease in DAPK occurs at the posttranslational level and not at the transcriptional level (Figure 10B). Together, these results indicate that the association of PP2A with
DAPK negatively regulates the cellular levels of DAPK via a proteasome-dependent process.

vi. Regulation of DAPK phosphorylation by PP2A holoenzymes.

A recent study by Gozuacik et al. (39) implicated a “PP2A-like” enzyme in the control of p-S308 dephosphorylation. While these investigators demonstrated that the AC core dimer of PP2A could dephosphorylate p-S308, no evidence was presented as to if PP2A could be targeted to DAPK to facilitate this process. To determine if PP2A targeted to DAPK by either the Bα or Bδ subunits could dephosphorylate DAPK at S308, ABαC or ABδC holoenzymes were immunopurified using a previously described method (Figure 11A) (1, 2). The relative purity of the isolated PP2A holoenzymes was examined with SDS-PAGE followed by silver stain and western blotting (Figures 11B and 11C). Our analyses confirmed that the purified complexes contain the three PP2A subunits (A, B, and C) with only minor amounts of contaminating proteins. To determine if the purified holoenzymes were active, they were tested for their phosphatase activity using a generic phospho-peptide in an in vitro phosphatase assay in the presence or absence of OA. The results of this assay confirmed that both purified PP2A complexes could dephosphorylate the phospho-peptide in an OA-sensitive manner (Figure 11D). To determine if DAPK could be dephosphorylated by the purified PP2A holoenzymes, FLAG-DAPK immune complexes were isolated from lysates of HEK293 cells prepared in the presence of the phosphatase inhibitors
as described in Methods. After washing to remove the phosphatase inhibitors, the immunopurified DAPK was incubated with purified PP2A-Bα or PP2A-Bδ that had been pre-incubated with or without the PP2A inhibitor OA. Immunoblot analysis of the reaction mixtures revealed that PP2A-Bα and PP2A-Bδ holoenzymes exhibited appreciable phosphatase activity towards p-S308; no dephosphorylation was observed in the control reactions (i.e. FLAG peptide eluates from FLAG immune complexes of cells expressing empty vector) and in the reactions containing OA (Figure 11E). To determine if phosphorylation of S308 in DAPK could be altered by siRNA-mediated knockdown of endogenous Bα subunit, HeLa cells were first transfected with siRNA for Bα (siBα) or a control siRNA and then DAPK was immunoprecipitated. These results revealed a significant increase in the ratio of p-S308 to total DAPK protein levels compared to cells treated with a control siRNA (siScr) (Figure 11F). Together, these data confirm that PP2A ABαC and PP2A ABδC are cellular phosphatases that dephosphorylate p-S308 on DAPK.

vii. PP2A activates DAPK.

Given that previous studies have shown that dephosphorylation of S308 results in increased kinase activity of DAPK (54, 117, 145), we next sought to determine if dephosphorylation of DAPK by PP2A-Bα and PP2A-Bδ holoenzymes would increase the activity of DAPK. DAPK immune complexes were dephosphorylated using the purified ABαC and ABδC holoenzymes and
kinase activity was subsequently measured using a synthetic DAPK substrate peptide as illustrated (Figure 12A). The results of this assay show that DAPK treated with ABαC and ABδC prior to the kinase assay exhibited increased activity towards the DAPK substrate (Figure 12B). Consistent with this enhanced kinase activity the PP2A-treated (dephosphorylated) DAPK bound Ca\(^{2+}/\text{CaM}\) with higher affinity (Figure 12C). These findings demonstrate that DAPK is activated by both PP2A holoenzymes (ABαC and ABδC).

E. Discussion

Previous studies established that the activities of DAPK can be regulated by Ca\(^{2+}/\text{CaM}\), autophosphorylation of S308 within its calmodulin-binding domain, and ubiquitin-mediated degradation (54, 117, 145). Activation of the catalytic activities of DAPK is initiated by dephosphorylation of S308 by a PP2A-like phosphatase (39), and subsequent to its dephosphorylation and activation, DAPK protein levels are modulated by ubiquitin-mediated proteasomal degradation (53, 54, 145). These studies suggest a network of protein modifications and protein-protein interactions act to regulate the activities of this Ser/Thr protein kinase. In this chapter, we extend these studies by identifying and characterizing two novel S308 phosphatases that control the activation and stability of DAPK.

Protein-protein interactions can be identified using affinity purification techniques such as TAP coupled to tandem mass spectrometry. Previous studies
utilizing this approach were successful in identifying novel phosphatase-substrate interactions such as the association of transcription factor TRIP-Br1 with PP2A-ABαC holoenzyme (144). Using this methodology, we identified a potential DAPK interacting protein, PP2A regulatory subunit Bα. We extended these findings and confirmed a physical interaction between DAPK and PP2A ABαC holoenzyme as well as a closely related (>90% shared amino acid homology) PP2A holoenzyme, ABδC. This interaction occurs via the Bα and Bδ targeting subunits of PP2A and the cytoskeletal domain of DAPK. PP2A regulatory subunits share little homology between families; however isoforms within a family are highly homologous. We determined the in vivo specificity for PP2A ABαC and ABδC holoenzymes by showing that PP2A AB’βC, of the B’ family, did not interact with endogenous DAPK. Consistent with this, Bα and Bδ containing PP2A holoenzymes were shown to dephosphorylate Raf1, whereas B’β could not (1).

Manipulation of the cellular levels of regulatory B subunits is helpful in identifying signaling pathways and substrates of PP2A due to the inability to overexpress or knockdown the catalytic subunit of PP2A in mammalian cells (9, 128). Pharmacological inhibition of PP2A, as well as siRNA-mediated depletion of the Bα or Bδ regulatory subunits of PP2A results in increased cellular levels of DAPK. Consistent with these observations, pharmacological activation or overexpression of Bα or Bδ regulatory subunits led to decreased cellular levels of DAPK while not affecting the steady state mRNA transcript. Moreover, treating cells with MG132 to inhibit proteasomal degradation rescued PP2A induced
degradation of DAPK, whereas treatment with chloroquine to inhibit lysosomal proteases had no effect on the cellular levels of DAPK.

It was proposed that the autophosphorylation at S308 imposes charge-dependent interaction of the CaM regulatory segment of DAPK with the catalytic cleft and the ATP binding site, and lowers the susceptibility of the enzyme to activation by CaM by reducing the binding capacity of the enzyme to its activator, CaM (117). In the basal state DAPK is phosphorylated at S308 and is rapidly dephosphorylated in response to proapoptotic stimuli such as ceramide and TNFα (54, 117). Dephosphorylation at S308 by an unknown phosphatase(s) attenuates autoinhibition by releasing the calmodulin-binding region from the catalytic cleft and allows binding of Ca^{2+}/CaM to DAPK. We were able to show that purified and active PP2A holoenzymes PP2A-Bα and -Bδ could dephosphorylate S308, enhance Ca^{2+}/CaM binding as well as the kinase activity of DAPK in vitro.

Consistent with our results, studies have linked the phosphorylation status of proteins to protein turnover via the lysosome or ubiquitin-proteasome system (10, 69). Distinct holoenzymes of PP2A have been shown to affect the protein stability and turnover of multiple substrates. PP2A-AB’αC and AB’βC dephosphorylate oncogenes c-Myc and Pim-1, respectively. This dephosphorylation enhances their proteasomal-mediated turnover (7, 76). PP2A does not exclusively enhance degradation but can also stabilize proteins. For example, dephosphorylation of TRIP-Br1, ALK4 and ALK5 by PP2A ABαC is protective against degradation (10, 144). The unphosphorylated form of DAPK is
targeted for polyubiquitination by at least two recently discovered E3-ligases, CHIP and Mib1 (53, 145). One possible mechanism by which dephosphorylation of DAPK may enhance proteasomal degradation is that dephosphorylation may cause a conformational change in DAPK to expose a once hidden ubiquitination site. Proteasomal-mediated degradation of DAPK occurs rather than inactivation of DAPK by re-autophosphorylation (54). This paradigm is analogous to those that have been identified for temporally regulating other apoptotic regulatory proteins such as p53 and retinoblastoma and may be a common mechanism used to rapidly modulate the activities of these important factors (116, 139).

Overall, these studies are the first to identify distinct holoenzymes of PP2A that regulate the cellular activities and protein levels of DAPK. The activation of these protein phosphatases and the physiological response to activation of DAPK are further explored in Chapter III.
Figure 6: Identification of PP2A regulatory subunit Bα as a potential DAPK binding protein by TAP. A) Schematic of DAPK-TAP tagged fusion protein containing full-length human wild type DAPK fused to the TAP tag consisting of a calmodulin binding peptide, TEV cleavage site and Protein A. B) Schematic of TAP method in five sequential steps (i) purification of protein A tag on IgG sepharose, (ii) cleavage of Protein A tag with TEV, (iii) purification of calmodulin binding peptide in the presence of calcium on calmodulin sepharose, (iv) elution by calcium chelation, (v) identification by LC MS/MS. (Modified from Gingras et al. 2005 J. Physiol. (36))
Figure 7: DAPK interacts with PP2A holoenzymes ABαC and ABδC

A) HEK T-Rex doxycycline inducible stable cell lines: empty vector (EV), FLAG-Bα or FLAG-Bδ were lysed 48 h post-doxycycline induction, and FLAG-tagged subunit complexes were isolated from the lysates using anti-FLAG agarose. Bound proteins were analyzed by western blotting. B) HeLa cells expressing FLAG-DAPK were lysed after 48 h transduction, and DAPK complexes were isolated from lysates using anti-DAPK or control IgG. C) HEK293 cells expressing FLAG-EV, -Bα, -Bδ, or B’β were lysed after 48 h post transfection, and FLAG complexes were isolated from lysates using anti-FLAG agarose.
Figure 8: The cytoskeletal domain of DAPK is required to interact with Bα and Bδ. A) Schematic of Omni-tagged DAPK domain plasmids and corresponding amino acid numbers. B) Omni-DAPK domain plasmids shown in (A) were transiently transfected into HEK T-rex cells. Cell lysates prepared 48 h post doxycycline induction and immunoprecipitated with anti-FLAG agarose and analyzed by western blotting.
Figure 9: PP2A negatively regulates DAPK protein levels.

A) HeLa cells were treated with increasing amounts of PP2A inhibitor, okadaic acid (OA) for 24 h. Cells were lysed and analyzed for total endogenous DAPK levels. B) HeLa cells treated with increasing amounts of PP2A activator, FTY720 for 48 h in the presence or absence of OA (1 nM). C) HEK293 cells transiently transfected with pcDNA5/TO (EV), Bα-FLAG/pcDNA5/TO (Bα), Bδ-FLAG/pcDNA5/TO (Bδ), or Bβ'-FLAG/pcDNA5/TO (Bβ'). Cells were lysed 48 h post-transfection and analyzed by western for endogenous DAPK levels. Quantification of DAPK to vinculin is shown (*p<0.05). D) HeLa cells transfected with siRNA for scrambled control (siScr), siBα, siBδ, or siBα/δ for 72 h. Cells
were lysed and analyzed by western. Quantification of DAPK to vinculin is shown (*p<0.05).
Figure 10: PP2A enhances proteasomal-mediated degradation of DAPK.

**A)** HEK T-Rex cells stably transfected with pcDNA5/TO (EV), Bα-FLAG/pcDNA5/TO (Bα), or Bδ-FLAG/pcDNA5/TO (Bδ) were treated with doxycycline to induce PP2A regulatory subunit expression. After 48h doxycycline, cells were treated with vehicle, chloroquine [100 µM] or MG132 [10 µM] for 6 h and subjected to analysis by immunoblot. **B)** qRT-PCR of steady state mRNA level of DAPK and housekeeping gene HPRT in HEK T-Rex cells in parallel with (A).
Figure 11: Dephosphorylation of DAPK by PP2A holoenzymes.

**A)** Schematic of experimental procedure used to isolate ABαC and ABδC holoenzymes and then perform *in vitro* phosphatase assays. HEK T-Rex cells stably transfected with pcDNA5/TO (EV), Bα-FLAG/pcDNA5/TO (Bα), or Bd-FLAG/pcDNA5/TO (Bδ) were treated with doxycycline to induce PP2A regulatory subunit expression for 48 h and lysed. FLAG-tagged B subunit complexes were isolated from the lysates using anti-FLAG-agarose. Bound proteins were washed, eluted with FLAG peptide resolved by SDS-PAGE, and subjected to silver stain...
analysis or immunoblot. **B)** Silver stain analysis of FLAG eluates; the positions of PP2A-A (A), FLAG-B, and PP2A-C (C) subunits are denoted. **C)** Western blot analysis of FLAG eluates. **D)** The isolated PP2A holoenzymes were assessed for activity in the absence or presence of okadaic acid (OA) using a generic phospho-peptide as the substrate. **E)** HEK293 cells were transfected with FLAG-DAPK. After 48 h transfection, the FLAG-DAPK proteins were isolated using anti-FLAG-agarose. The immunoprecipitates were incubated with purified active PP2A ABαC (Bα), ABδC (Bδ), or mock (EV) holoenzyme in an *in vitro* phosphatase reaction in the presence or absence of okadaic acid. Quantification of S308-DAPK to total DAPK is shown (*p<0.05). **F)** HeLa cells transfected with siRNA for Scrambled control (siScr) or siBα for 72 h. Cells were lysed and DAPK was isolated by anti-DAPK antibody and analyzed by western. The ratio of S308-DAPK to total DAPK is shown and representative of repeat experiments.
Figure 12: PP2A enhances the kinase activity of DAPK.

A) Schematic of experimental procedure used to perform in vitro phosphatase/kinase assays. B) HEK293 cells were transfected with FLAG-DAPK. After 48 h transfection, the FLAG-DAPK proteins were isolated with anti-FLAG-agarose. The immunoprecipitates were incubated with purified active PP2A ABαC (Bα), ABδC (Bδ), or mock (EV) holoenzyme in an in vitro phosphatase reaction. For western blotting, 20% of the immunoprecipitate was utilized for total p-S308 and total DAPK levels. The remaining 80% of the immunoprecipitate was washed extensively in the presence of okadaic acid [100 nM] in kinase assay buffer. The washed immunoprecipitate was incubated in the
presence of a synthetic DAPK substrate peptide in an in vitro kinase reaction using [$\gamma^{32}$P]-ATP. Samples collected at time points and [$\gamma^{32}$P]-ATP incorporation into the synthetic peptide was quantitated using a scintillation counter. Western of p-S308 and total DAPK levels is shown. C) Western blotting and CaM overlay of immunoprecipitated endogenous human DAPK treated with PP2A AB$\alpha$C (B$\alpha$), AB$\delta$C (B$\delta$), or mock (EV) holoenzyme in an in vitro phosphatase reaction. The CaM overlay was carried out using biotinylated CaM (100 nM) in Tris buffer containing 10 mM Ca$^{2+}$.
A. Abstract

Previous studies have demonstrated that the tumor suppressor death associated protein kinase (DAPK) plays a role in ceramide-induced apoptosis via an unknown mechanism. DAPK is a calcium/calmodulin regulated serine/threonine kinase with an important role in regulating cytoskeletal dynamics and cell adhesion. Auto-phosphorylation within the calmodulin-binding domain at serine308 inhibits DAPK catalytic activity. Dephosphorylation of serine308 by PP2A ABαC and ABδC holoenzymes enhance kinase activity and proteasomal mediated degradation of DAPK (Chapter II). We determined that ceramide causes a capsase-independent cell detachment in HeLa cells, a human cervical carcinoma cell line. Subsequent to detachment, these cells undergo caspase-dependent apoptosis due to lack of adhesion, termed anoikis. Overexpression of wild type DAPK induced cell rounding and detachment similar to cells treated with ceramide; however, this effect was not observed following expression of phosphorylation mutant, S308E DAPK. Finally, the endogenous interaction of DAPK and PP2A was determined to be required for ceramide-induced cell detachment and anoikis. Together these studies provide interesting and new insight into the mechanisms by which ceramide regulates cell adhesion and anoikis. We have defined a cellular pathway initiated by ceramide-mediated
activation of PP2A and DAPK to regulate inside-out signaling and promote anoikis.
B. Introduction

Apoptosis is a highly coordinated cellular process that is important in the development and maintenance of tissue homeostasis in multicellular organisms (126). Apoptosis can be triggered by a variety of stimuli including a loss of cell adhesion to the extracellular matrix, a process called anoikis (31). In adherent cells, such as epithelial cells, cell-matrix interactions through integrin adhesions play a vital role in cell survival (49, 133). Integrin adhesion regulates the organization and remodeling of actin cytoskeleton and actin cytoskeleton dynamics also plays an important role in maintaining the cell-matrix interaction through integrin adhesion (58). Loss of integrin-mediated adhesion through inside-out mechanisms initiates cellular cascades leading to mitochondrial membrane permeabilization and anoikis (18, 32, 60, 61).

Ceramide is a pleiotropic, sphingolipid produced by cells in response to radiation, inflammatory cytokines and chemotherapeutic drugs (35, 90, 111, 113). Modulation of sphingolipid-induced apoptosis is a potential mechanism to enhance the effectiveness of chemotherapeutic drugs. Ceramide is a potent activator of protein phosphatases including PP2A leading to dephosphorylation of substrates important in regulating mitochondrial dysfunction, apoptosis and anoikis (5, 15, 25, 46, 108). Ceramide is a potent activator of DAPK and this kinase is necessary for ceramide-induced cell death in multiple cell types, but the cellular mechanism leading to death is unclear (3, 15, 16). The activation of DAPK by ceramide is thought to be mediated by a ceramide-activated
phosphatase; however, the identity of this phosphatase remains uncertain (39).

Once active, DAPK phosphorylates substrates including myosin regulatory light chain at Ser19 to regulate cytoskeletal dynamics, cell adhesion, and migration (3-5,13,17). In addition, activation of DAPK has been shown to suppress β-integrin mediated cell adhesion through disrupting the association of talin and CDC42 (4).

In these studies the mechanisms of ceramide-induced apoptosis were examined. It was determined that ceramide treated cells lose cell adhesion in a caspase-independent manner. Prolonged loss of cell adhesion leads to caspase activation and anoikis. Finally, it was determined that ceramide activates PP2A leading to dephosphorylation of serine308 and activation of DAPK that is required for ceramide induced cell detachment and anoikis.
C. Experimental Procedures

i. Adenoviral Transduction

HeLa cells were transduced with recombinant adenovirus directing expression of either DAPK (Ad-DAPK), green fluorescent protein (Ad-GFP), a control scrambled shRNA (Ad-shScr), or an shRNA for depletion of endogenous DAPK (AD-shDAPK) for 48 h. Cells were washed with PBS, trypsinized and collected in 1% paraformaldehyde for analysis using a Beckman Coulter cell counter or in RIPA lysis buffer for SDS-PAGE.

ii. Cell Detachment Assays

Cell detachment was quantified by counting only adherent cells. Briefly, cells were washed twice in PBS to remove detached cells. Adherent cells were then trypsinized and counted with a Beckman Coulter cell counter. The extent of cell detachment is defined as (the number of control cells – the number of treated cells)/(the number of control cells). For the HEK293 detachment assay, control cells were transiently transfected with EV. Treated cells were transfected with Wt-DAPK, S308A or S308E. In the ceramide-induced anoikis experiments, HeLa cells were transduced with adenovirus for 48 h and then incubated in the presence of DMSO (control) or C6-Ceramide (treated) for 16 h.
iii. Statistical Analysis

All experiments were carried out using independent cell transfections, in triplicate. Statistical analysis was performed using Students t-test and graphs were created using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Western blotting and DIC images are representative of the repeated experiments. All densitometry analysis of western blotting data was normalized to vinculin levels.
D. Results

i. DAPK Autophosphorylation Prevents DAPK-Induced Cell Detachment

DAPK was reported to suppress β-integrin signaling and cell adhesion through disruption of talin and CDC42 interactions (62, 132). To determine if dephosphorylation of p-S308 and activation of DAPK resulted in altered levels of cell detachment, HEK293 cells were transiently transfected with wild type DAPK (WT) or two phospho-site mutant forms of DAPK (S308A and S308E). The S308A DAPK mutant represents an active, dephosphorylated form of DAPK, whereas the S308E mutant is inactive (117). Cell rounding and detachment was visualized by DIC microscopy (Figure 13A). The transfected HEK293 cells were analyzed to determine the relative levels of cell detachment, which was quantitated by counting the number of attached cells as described in the Methods. The relative levels of DAPK expression in the transfected cells were examined using immunoblotting (Figure 13B). Expression of WT-DAPK resulted in 40% cell detachment, while expression of the active form of DAPK (S308A) increased this to 60% overall cell detachment, a result consistent with previous studies showing increased kinase activity of this mutant (117) (Figure 13C). In contrast, expression of the inactive form of DAPK (S308E) completely attenuated the ability of DAPK to induce cell detachment; no significant cell detachment was observed when compared to empty vector transfected cells. These data are
consistent with previous studies highlighting a role for DAPK in inhibiting cell adhesion (62, 132).

ii. Ceramide induces caspase-independent cell detachment leading to anoikis.

Ceramide is a potent activator of some phosphatases including PP2A and PP1 (15, 25). Ceramide also activates DAPK via dephosphorylation at S308 and DAPK is required for ceramide-induced apoptosis in multiple cell types (54, 93, 136). As we have shown that PP2A dephosphorylates DAPK at serine308 to activate it, we sought to determine if this is the mechanism whereby ceramide might induce apoptosis. Firstly we determined if ceramide induces cell detachment prior to apoptosis. A control experiment in which HeLa cells were treated with C6 ceramide (50 μM) for up to 24 h showed that ceramide induces a caspase-dependent apoptosis, as evidenced by the progressive cleavage of the caspase substrate, poly-ADP-ribose polymerase (PARP), within 8 hrs of ceramide treatment (Figure 14A). As expected, addition of z-VAD-FMK (100 μM), a cell permeable, pan-caspase inhibitor, blocked ceramide-induced PARP cleavage (Figure 15A). In parallel experiments, both floating and adherent cells were counted after ceramide treatment to quantify cell detachment. After 8 hrs of ceramide treatment, HeLa cells began to detach from the tissue culture plates with nearly 60% of the cells floating after 16 hrs (Figure 14B). Addition of z-VAD-FMK did not reduce the number of floating cells, suggesting that cell detachment
induced by ceramide treatment is independent of caspase activation. To
determine whether the cells treated with ceramide were able to reattach,
ceramide-detached HeLa cells treated either in the presence or absence of z-
VAD-FMK were collected and washed to remove ceramide and then reseeded
onto dishes in medium containing the inactive ceramide analog,
dihydroceramide. Following 8 h incubation, both re-attached and floating cells
were counted. As shown in Figure 14C, more than 75% of the floating cells were
viable and could reattach when collected after 8 hrs of ceramide treatment.
However, after more than 8 hrs of ceramide treatment, the floating HeLa cells
progressively lost their ability to reattach and by 24 hrs of ceramide treatment,
the percentage of cells capable of reattaching had decreased to 2%. Although
inclusion of z-VAD-FMK did not prevent ceramide-treated HeLa cells from
detaching (Figure 14B), it significantly increased the proportion of detached cells
that were capable of reattachment after 24 hrs of ceramide treatment from 2% to
35% (Figure 14C). These results suggest that prolonged cell detachment in the
presence of ceramide induces an irreversible loss of cellular adherence, probably
due to induction of anoikis.

Ceramide treated HeLa cells do not exhibit typical morphological
characteristics of classical apoptosis, such as membrane blebbing, cytoplasmic
condensation or nuclear fragmentation prior to their detachment from tissue
culture plates. To determine whether ceramide induces caspase activation prior
to cell detachment, detached or adherent cells were collected separately at the
indicated times after ceramide treatment and then analyzed by western blotting
to detect caspase activity by monitoring the cleavage of PARP (Figure 14D). Surprisingly, the adherent population of HeLa cells, even in the presence of prolonged ceramide treatment (24 hrs), had nearly undetectable levels of PARP cleavage. In contrast, within the population of detached cells there was a progressive increase in the levels of PARP cleavage, which begins within 8 hours of ceramide treatment (Figure 14D). Detached cells collected after 16 hrs of ceramide treatment were found to be mostly apoptotic with less than 25% of the cells capable of reattachment (data not shown). The finding that caspase-dependent apoptosis occurs only in HeLa cells that have lost cellular adhesion contacts due to ceramide treatment together with the viability of cells soon after detachment (Figure 14C) suggests that ceramide-treated cells are not committed to apoptosis prior to detachment from the tissue culture plate and the observed apoptosis is induced by the loss of cell adhesion (anoikis).

iii. DAPK is required for ceramide-induced anoikis in human cervical carcinoma cells.

Ceramide was previously shown to inhibit β-integrin-mediated cell adhesion to extracellular matrices in HeLa cells to promote cell detachment and anoikis by an unknown mechanism (46). Interestingly, forced expression of DAPK in HEK293T cells also caused inactivation of β-integrins and resulted in cell detachment and anoikis (132). This finding, together with our knowledge that ceramide activates both PP2A and DAPK (54, 117, 136), prompted us to
determine if PP2A mediated activation of DAPK is required for ceramide-induced anoikis.

We first confirmed that treatment of cells with ceramide caused dephosphorylation of p-S308 in DAPK. As shown in Figure 15A, treatment of HeLa cells with 50 µM C6-ceramide induced a time dependent dephosphorylation of DAPK as well as a decrease in its cellular levels. Next, HeLa cells were transduced with recombinant adenovirus directing expression of either DAPK (Ad-DAPK), green fluorescent protein (Ad-GFP), a control scrambled shRNA (Ad-shScr), or an shRNA for depletion of endogenous DAPK (Ad-shDAPK). At 48 h post-infection, the relative expression levels of DAPK in the infected cells was analyzed by western blotting, which clearly indicated overexpression and effective depletion of DAPK using the appropriate viruses (Figure 15B). In parallel, at 48 h post-transduction ceramide was added and the infected cells were incubated for an additional 16 h before analysis to determine the levels of cell detachment in the infected cells. As shown in Figure 15C, ceramide induced nearly 65-68% cell detachment in the control cell populations expressing either Ad-GFP or Ad-shScr, respectively. In contrast, HeLa cells infected with Ad-DAPK significantly increased cell detachment to 78%, while cells with reduced expression of DAPK (Ad-shDAPK) had significantly reduced cell detachment to 51%. These data suggest ceramide-mediated activation of DAPK promotes cell detachment and anoikis.
iv. PP2A is required for activation of DAPK and anoikis.

Ceramide is a potent activator of PP2A, however no studies have highlighted a role for PP2A in regulating anoikis. Based on our findings, PP2A ABαC and ABδC are S308 DAPK phosphatases. We hypothesized that regulatory Bα subunit of PP2A is required for ceramide-mediated activation of DAPK and anoikis. To test our hypothesis, HeLa cells were transiently transfected with Bα-targeted siRNA (siBα) for depletion of endogenous Bα or a control, scrambled siRNA (siScr). After 48 h post-transfection, cells were treated with ceramide for 8 to 24 h as indicated (Figure 16A). Cells were then analyzed by western blotting to examine the levels of DAPK and DAPK phosphorylated at S308 (pS308-DAPK) as well as the relative levels of cell detachment. Depletion of PP2A-Bα significantly reduced dephosphorylation of DAPK at S308 and prevented ceramide-induced degradation of DAPK (Figure 16A). Consistent with reduced levels of active, dephosphorylated DAPK, cell detachment was also reduced by 20% in cells treated with siBα (Figure 16B). These data suggest ceramide-mediated activation of the PP2A ABαC holoenzyme regulates the endogenous activities and cellular levels of DAPK to promote cell detachment and anoikis.
Studies examining the mechanism by which ceramide induces apoptosis have focused on the ability of this compound to activate ceramide-activated protein phosphatases (CAPPs). The CAPPs, principally PP2A but also PP1, are thought to induce apoptosis by dephosphorylating specific target proteins that include the mitochondrial apoptosis regulators Bcl-2 and Bax (15, 16, 109). However, the potential significance of the dephosphorylation of protein kinase DAPK in the regulation of ceramide-induced apoptosis signaling has not been considered.

Enhanced enzymatic activity of DAPK negatively regulates cell adhesion to substratum including tissue culture dishes, collagen and fibronectin (132). Wang et al. demonstrated that DAPK-induced loss of adherence was attributed to inhibition of β-integrin signaling and either forcibly activating β-integrins or plating cells on poly-L lysine could block the anti-adhesion effects. Forced expression of DAPK does not induce apoptosis or alter surface expression of integrins responsible for attachment ((132), and data not shown). This finding led to the proposal that for cells dependent on adhesion to the extracellular matrix for viability, DAPK’s ability to interfere with integrin function could directly contribute to cell death (anoikis). A possible mechanism by which DAPK regulates cell adhesion is through phosphorylation of an unidentified substrate and/or modulating cytoskeletal dynamics by phosphorylating myosin regulatory light
chain (RLC) at Ser19. Altogether, this previous study has provided interesting results that largely coincide with our overall hypothesis.

In our current study, we show that the phosphorylation status of DAPK-S308 is crucial for adhesion of cells to tissue culture plates. Expression of wild type or an active DAPK mutant DAPK-S308A (117), rapidly caused apoptotic cell morphological changes, whereas expression of an inactive, phosphomimetic form of DAPK (S308E) had no effect. Although the exact mechanism by which DAPK may modulate cell attachment is unknown, its ability to phosphorylate myosin II RLC to activate myosin motor activity (11, 52), its association with the cytoskeleton (23, 62), and its ability to regulate integrin function (62, 132) are all consistent with a role for DAPK in regulation of cell attachment to substrates.

Finally, these studies demonstrate that the dephosphorylation of endogenous DAPK by PP2A to activate its catalytic activity in cells treated with ceramide is intimately associated with regulating the balance between cell adhesion and detachment induced anoikis. Importantly, we have discovered that caspase activation by ceramide does not occur until after the ceramide treated cells detach from the substratum. In adherent cells such as HeLa cells, loss of cell adhesion to the substratum leads to anoikis (31). These results suggest a novel pathway by which ceramide can lead to anoikis in adherent cells by inducing cell detachment from the substratum. These results are complementary and an extension to a study linking ceramide-induced disruption of the Golgi complex to anoikis (46). These findings reveal that there is a relationship between DAPK activity and cell detachment, suggesting that in response to
ceramide treatment the rapid dephosphorylation of S308 by PP2A leading to activation of DAPK correlates with loss of cell adhesion. Downregulation of either endogenous DAPK or PP2A regulatory subunit Bα blocked ceramide-induced cell detachment and anoikis, thus providing physiological evidence for a crucial role for PP2A to activate DAPK.

In summary, our data not only provide new evidence to extend previous studies (62, 132) showing DAPK plays a role in anoikis, but also reveal a physiological stimulus and signaling pathway through which DAPK is activated. Based on the results of this study, the following model describing how DAPK regulates ceramide-induced apoptosis is proposed (Figure 17). Activation of PP2A ABαC and ABδC holoenzymes by ceramide results in dephosphorylation of the autophosphorylation site, S308, within the calmodulin-binding region of the ser/thr protein kinase DAPK and increased cytoplasmic calcium (73, 74). Dephosphorylation of DAPK-S308 serves to enhance calcium/calmodulin binding and activation of DAPK (52, 54, 117). Active DAPK can phosphorylate myosin II RLC to activate myosin motor activities as well as inactivate β-integrin signaling (54, 62). These events promote cell detachment from the substratum and subsequent apoptosis (anoikis). Active DAPK is also targeted by two distinct E3 ubiquitin ligases (CHIP and Mib1) for proteasomal degradation, which serves to reduce cellular levels of DAPK and terminate phosphorylation of substrates (53, 54, 145). Future studies aimed at identifying the DAPK substrate(s) involved in mediating anoikis will aid in our understanding of the complex mechanisms
governing cell adhesion and the ability of DAPK to induce indirect apoptosis through the anoikis pathway.
**Figure 13: Dephosphorylation at S308 promotes loss of adhesion.**

**A)** HEK293 cells transfected with FLAG-DAPK Wt, S308A, or S308E for 48h. Cell rounding and detachment are visible by DIC microscopy. **B)** Western of relative expression of DAPK Wt and mutant plasmids. **C)** Quantification of detached HEK293 cells following forced expression of DAPK Wt and phosphorylation mutants. The percentage of cell detached was determined as described in the Methods.
Figure 14: Ceramide induces caspase-independent cell detachment.

A) Western blotting to detect cleavage of poly-ADP-ribose-polymerase (PARP) by caspases in lysates of HeLa cells treated with ceramide (50 µM) for indicated times in the absence or presence of the cell-permeable pan-caspase inhibitor, z-VAD-FMK (100 µM). B) Quantification of detached HeLa cells following treatment with ceramide. The percentage of cell detached was determined as described in the Methods. C) Quantification of ceramide detached HeLa cells that become reattached following washing and culture in culture medium containing the inactive ceramide analog, dihydroceramide. D) Western blotting to detect full length (116 kDa) and cleaved (85 kDa) PARP in total cell lysates from attached or detached (floating) HeLa cells collected after ceramide treatment for indicated times.
Figure 15: DAPK is required for ceramide-induced anoikis.

A) Levels of total DAPK and DAPK phosphorylated at S308 were measured by western blotting after immunoprecipitation in Hela cells treated with 50 µM ceramide for 0-24 h. B) HeLa cells transduced with adenovirus (Ad) for Ad-GFP, Ad-DAPK, Ad-ShScr or Ad-shDAPK were treated with or without ceramide for 16 h and analyzed by western. C) HeLa cells transduced with adenovirus (Ad) in (B) were assayed for cell detachment.
Figure 16: PP2A is required for activation of DAPK and Anoikis.

A) HeLa cells transfected with control Scr siRNA or siRNA directed against Bα. At 48hrs post-transfection cells were treated with ceramide for 0, 8, 16, 24 h. Lysates were prepared and DAPK immunoprecipitated and analyzed by western blotting. B) HeLa cells transiently transfected with siScr or siBα were assayed for cell detachment in response to 16 h ceramide.
Figure 17: Schematic representation of the activities of DAPK and PP2A in regulation of ceramide-induced anoikis. See text for details. Ca^{2+}/CaM, calcium-calmodulin; PP2A-Bα, PP2A-Bδ, protein phosphatase 2A associated with the regulatory subunits Bα or Bδ; Mib1, Mind bomb1 E3 ubiquitin ligase; CHIP, C-terminal HSC70-interacting protein E3 ubiquitin ligase.
Chapter IV:

Conclusions and Future Studies

A. Conclusions

These studies demonstrate that two distinct holoenzymes of PP2A (AB\textsubscript{αC} and AB\textsubscript{δC}) are novel regulators of DAPK. These phosphatases not only control the activation but also the steady state protein levels of DAPK as a means to limit prolonged activation (Chapter II). The proteins involved and mechanisms regulating inside-out signaling during anoikis remain elusive to this day. Using an in vitro model of ceramide-induced anoikis in human cervical carcinoma cells, the functional relevance of the endogenous interaction of PP2A and DAPK in cell adhesion was examined. We determined that both PP2A and its downstream substrate DAPK were required for cells to lose cell adhesion and die by anoikis (Chapter III). Together these studies provide exciting and essential new data regarding the mechanisms regulating cell adhesion. These studies are significant in their expansion of our understanding of the proteins involved in both ceramide-induced cell death and the process of anoikis. The studies in this thesis also provide fundamental information to understand the mechanisms of the tumor suppressor proteins PP2A and DAPK.
B. Future Studies

i. Determine the detailed mechanism of PP2A-induced ubiquitination of DAPK.

The steady state levels of DAPK are regulated posttranslationally by the ubiquitin-proteasome and the lysosomal degradation pathways (53, 54, 71, 145). My studies have highlighted that two distinct holoenzymes of PP2A negatively regulate the protein levels of DAPK, posttranslationally. I further determined the ubiquitin-proteasome but not the lysosomal degradation pathway is likely induced by these PP2A holoenzymes. I hypothesize that dephosphorylation of DAPK by PP2A induces a conformational change exposing an unidentified ubiquitination site(s), leading to polyubiquitylation and degradation of the kinase. Two DAPK E3-ubiquitin ligases were discovered by our lab, CHIP and Mib1 (53, 145). It is of interest to determine if either of these known ubiquitin ligases are responsible for PP2A-induced degradation of DAPK. Experiments using siRNA-mediated knockdown of these E3-ligases in cells expressing recombinant EV, Bα and Bδ (i.e. Figure 9C) should elucidate if either plays a role. It will also be of interest to identify the lysine residue(s) in DAPK that are ubiquitinated. To narrow down the region on DAPK that is targeted by ubiquitin, domain deletion plasmids of DAPK could be transfected in cells expressing recombinant EV, Bα and Bδ. The proteasomal inhibitor MG132 would then be used to determine if any of the DAPK domain deletion plasmids are targeted for PP2A-induced degradation. The
deletion plasmids containing the cytoskeletal binding domain would be required for this experiment, as my studies have shown the cytoskeletal region of DAPK is required for binding the Bα and Bδ (Figure 8B). Loss of the cytoskeletal domain would most likely prevent the ubiquitination of DAPK even if the ubiquitination site does not reside in the cytoskeletal-binding domain of DAPK. Studies highlighting the E3-ligase and ubiquitination site for PP2A-induced degradation of DAPK will give helpful insights into the mechanisms that limit prolonged activation of the kinase. Small molecules or peptides designed to mimic the region of DAPK that is ubiquitinated or at the E3-ligase responsible for targeting DAPK may be a strategy to prolong the activities of this tumor suppressor protein.

ii. Determine if myosin light chain phosphorylation plays a role in DAPK-induced cell detachment.

Studies in Chapter II and III demonstrate that PP2A targets DAPK for dephosphorylation at S308 resulting in enhanced kinase activity and cell detachment. The role of endogenous PP2A and DAPK was further highlighted in a novel model of ceramide-induced anoikis in which we determine both proteins were necessary for cells to lose adhesion by an unidentified inside-out signaling mechanism, possibly involving the actin cytoskeleton.

Actin cytoskeletal dynamics includes actin polymerization, depolymerization, and filament reorganization and these dynamic processes are
controlled by a number of actin binding proteins including myosin II (29). Myosin II motor activity is determined by the phosphorylation state of the 20-kDa myosin regulatory light chain (RLC) on serine 19. Kinase phosphorylation of serine 19 (i.e. MLCK and DAPK) stimulates actin-activated myosin II ATPase activity to cause movement of actin filaments and generation of force (3). Activation of myosin II motors is reversed by the activity of myosin RLC phosphatase. Alterations in RLC phosphorylation and myosin II motor activity have been linked to depolymerization of actin cytoskeleton and cell detachment, resulting in apoptosis (anoikis) (82, 112). Consistent with this, I hypothesize that DAPK enhances the phosphorylation of myosin regulatory light chain, causing a loss of cell adhesion in ceramide treated cells. To determine if alterations in RLC phosphorylation induced by ceramide treatment is responsible for anoikis, RLC could be manipulated in experiments with adenoviral transduction and a pharmacological inhibitor. Adenoviral myosin RLC constructs have already been generated for constitutively active (S19E), inactive (S19A) or wild type proteins. An inhibitor for myosin II motor activities, blebbistatin, will be used in separate experiments and given prior to ceramide treatment. Myosin RLC adenoviral infected or blebbistatin treated HeLa cells will be quantitated to determine the percent (%) cell detachment following ceramide treatment. If myosin RLC phosphorylation is important to maintain cell adhesion in ceramide treatment, then over expressing a constitutively active or dominant negative RLC is predicted to result in reduced or enhanced cell adhesion, respectively when compared to wt RLC. Blebbistatin inhibits myosin II motor activities and could be
used to link myosin RLC phosphorylation to actomyosin motor function in cell adhesion. Blebbistatin will be used to confirm that myosin RLC phosphorylation, which results in activation of myosin II motor activity, plays a role in ceramide-induced apoptosis. These studies will provide helpful insight into a potential mechanism by which PP2A induced activation of DAPK promotes cell detachment and anoikis.

iii. Determine if DAPK plays a role in cell transformation

DAPK is a known tumor suppressor protein with roles in suppressing metastasis in vivo (48). Expression is lost in numerous types of cancer due to methylation of its promoter (21, 87, 97, 142). Our studies have highlighted a role DAPK plays in initiating anoikis, a key step in preventing cells from acquiring anchorage independence and transformation. We hypothesize that DAPK may be an important tumor suppressor regulating anoikis and thus we propose inactivation of DAPK may be required for anchorage independence and cellular transformation.

SV40 is a DNA tumor virus that encodes three proteins through alternative splicing, SV40 large T (LT), small T (ST), and 17kT antigens (reviewed in (119). The LT and 17kT antigens bind to retinoblastoma family members as well as p53 resulting in their inhibition (75, 103). The ST antigen binds to structural A subunit of PP2A, preventing the formation holoenzymes, thereby suppressing its activity (92). Together these viral oncoproteins promote anchorage independence and
cellular transformation (reviewed in (110)). Indeed, PP2A is an important tumor suppressor and reduced levels of PP2A activity has been detected in a number of different types of human cancer (106, 107, 130). This can occur when cells upregulate an endogenous PP2A inhibitor (i.e. SET, CIP2A and I2PP2A), highlighting the important role of its tumor suppressive activity (55, 68, 78).

Pioneering studies on cellular transformation by William Hahn and Robert Weinberg determined most all types of human cells could be immortalized by expressing LT, the catalytic subunit of human telomerase (hTERT), and an oncogenic allele of Ras (41). However, to fully transform human cells, the enzymatic activity of PP2A had to be suppressed by ST. These fully transformed human cells (expressing: LT, hTERT, Ras, and ST) were capable of anchorage independence and tumor formation in immunodeficient mice (41, 42, 100, 140). This model provides an experimental system for the study of PP2A and its novel downstream substrate DAPK in human cell transformation. This system (cell line) is proposed to test the hypothesis that inactivation of DAPK is required for anchorage independence and cellular transformation.

To test this hypothesis, we have obtained human fibroblast cell lines from William Hahn’s laboratory (Harvard University) that stably express LT, hTERT, and oncogenic RAS, but not ST. We have also obtained an expression plasmid for ST antigen from the laboratory of Kathleen Rundell (Northwestern University). A preliminary experiment will determine if expression of the ST antigen (inhibitor of PP2A) can prevent activation of DAPK in ceramide-treated HeLa cells. To determine if DAPK activity is required to suppress anchorage independence and
transformation, fibroblasts obtained from the Hahn lab will be infected with a lentiviral shRNA to DAPK. These fibroblasts are unable to fully transform without blocking the activity of PP2A with the ST antigen. I predict that stable knockdown of DAPK (shRNA) will result in enhanced cellular transformation, bypassing the requirement for ST antigen in colony formation assays. Additional experiments should include expressing the constitutively active mutant DAPK (S308A) in fully transformed cells (LT, hTERT, Ras and ST) to determine if DAPK could then suppress anchorage independence by initiating anoikis. Promising results from these proposed in vitro studies could be validated in vivo by injecting these cells in a tumor xenograft mouse model. Tumor size and the metastatic potential are predicted to be diminished in cells expressing S308A. These experiments would provide helpful insights into the role for DAPK in anoikis and cellular transformation and may provide new rationale targets for cancer therapy.

C. Overall Summary and Clinical Implications

In summary, our current studies identify a novel function for distinct holoenzymes of PP2A in regulating the cellular activities of the tumor suppressor DAPK. We have studied this endogenous protein interaction in a novel model of ceramide-induced anoikis and determined that both PP2A and DAPK negatively regulate cell adhesion. Steps at clarifying the mechanisms of cell detachment have been initiated in the laboratory but many unanswered questions still exist.
DAPK expression is currently used as a diagnostic biomarker for disease and loss of expression inversely correlates with severity, metastasis, recurrence and survival rates of cancer patients (12). This correlation exists in multiple types of cancer including cervical, acute and chronic lymphoblastic leukemia, gastric carcinoma and breast cancer (12). Ceramide is currently in preclinical trials as a novel and promising cancer therapeutic (24). Based on our studies, we predict tumors containing low cellular levels of DAPK will be less responsive to ceramide therapy. As such, determining the expression level of DAPK from tumor biopsy prior to ceramide regimen may predict the ceramide sensitivity and clinical outcome for these patients.
REFERENCES


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