CHARACTERIZATION OF THE ROLE OF THE PP2A-Aβ GENE, A PUTATIVE TUMOR SUPPRESSOR, IN CELL GROWTH AND TUMORIGENESIS

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To God who gives me the ability to pursue truth

To my wife Michal who inspires and supports me every day

To my mother for raising me to care for others

To my father who taught me to love science and learning.

CHARACTERIZATION OF THE ROLE OF THE PP2A-Aβ GENE, A PUTATIVE TUMOR SUPPRESSOR, IN CELL GROWTH AND TUMORIGENESIS

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CHARACTERIZATION OF THE ROLE OF THE PP2A-Aβ GENE, A PUTATIVE TUMOR SUPPRESSOR, IN CELL GROWTH AND TUMORIGENESIS

Publication No. _____

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The PP2A-Aβ gene (*PPP2R1B*) encodes the β isoform of the A subunit of serine/threonine protein phosphatase 2A. Mutations in PP2A-Aβ have been identified in a wide variety of human cancers. The important role of protein phosphatase 2A in down regulating cell growth suggests these mutations may contribute to cancer susceptibility and tumorigenesis by compromising the function of PP2A-Aβ and that PP2A-Aβ may act as a tumor suppressor. Screening of cancer patient DNAs revealed an association between a germline alteration of the PP2A-Aβ and breast carcinoma and identified alterations of PP2A-Aβ in lung carcinoma and ALL patient genomic DNAs.

The biochemical consequences of these PP2A-Aβ mutations on PP2A function were investigated by *in vitro* and *in vivo* coimmunoprecipitations between the PP2A-Aβ subunit and the B and C subunits of PP2A. These studies showed mutations in PP2A-Aβ confer a loss of function by reducing its ability to bind the B and C subunits, destabilizing the PP2A-Aβ containing PP2A complex.

The affect of the PP2A-Aβ gene on cell growth was analyzed by transfecting the PP2A-Aβ gene into cancer cell line deficient for wild type PP2A-Aβ and deriving stable cell lines. The PP2A-Aβ gene appeared to confer a relative disadvantage to transfected cells, resulting in a lower fraction of derived stable lines compared to controls. These cell lines were tested for proliferation and colony formation in soft agar. No significant difference was observed in the growth rate of PP2A-Aβ cell lines compared to controls. One of the PP2A-Aβ stable cell lines demonstrated dramatic suppression of colony formation in soft agar, but this was not confirmed in any additional PP2A-Aβ stable cell lines, leaving this finding inconclusive. The stable cell lines were also analyzed by Western blotting for changes in the Wnt signaling cascade. Cell lines expressing exogenous PP2A-Aβ are found to have lower levels of β-catenin compared to control cell lines. This suggests that the PP2A-Aβ gene is involved in regulating the Wnt signaling pathway, which is shown to be involved in cell growth control and is similarly affected by known tumor suppressor genes.

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LIST OF DEFINITIONS

ALL – acute lymphoblastic leukemia

APC – adenomatous polyposis coli

CAC – colon adenocarcinoma

cDNA – complementary deoxyribonucleic acid

DNA – deoxyribonucleic acid

FBS – fetal bovine serum

GSK – glycogen synthase kinase

IgG – immunoglobulin G

kD – kilodalton

LOH – loss of heterozygosity

mAB – monoclonal antibody

MAPK – mitogen-activated protein kinase

ml – milliliter

mM– millimolar

MTS – (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MTT – 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide

nm – nanometer

NSCLC – non-small cell lung carcinoma

PBS – phosphate buffered saline

PBS-T – phosphate buffered saline with tween-20

PCR – polymerase chain reaction

PP – protein phosphatase

RPM – rotations per minute

RT-PCR – reverse transcriptase polymerase chain reaction

SCLC– small cell lung carcinoma

SNP – single nucleotide polymorphism

SDS – sodium dodecyl sulfate

ul – microliter

uM– micromolar

CHAPTER ONE
Introduction

PROTEIN PHOSPHORYLATION

Protein Phosphatases

Functional regulation of proteins by reversible phosphorylation underlies almost every cellular process. The phosphorylation state of about one third of cellular proteins is regulated by the opposing actions of protein kinases and protein phosphatases (Groves et al., 1999). Protein phosphatases play an integral role in cellular signal transduction cascades which are responsible for regulating such important functions as cell growth, division, differentiation and apoptosis. A delicate balance between the activity of protein kinases and protein phosphatases allows very tight control over the activity of essential phosphoproteins. Protein phosphatases are separated generally into two classes: protein tyrosine phosphatases and protein serine/threonine phosphatases. The family of protein serine/threonine phosphatases can be further distinguished based on differing patterns of dephosphorylation of phosphorylase kinase and sensitivity to protein phosphatase inhibitor 1 and inhibitor 2. Type 1 protein phosphatase (PP1) preferentially dephosphorylates the β-subunit of phosphorylase kinase and is inhibited by inhibitor 1 and inhibitor 2. Type 2 protein phosphatases (PP2), conversely, dephosphorylate the α-subunit of phosphorylase kinase and are resistant to inhibitors 1 and 2. The type 2 protein phosphatases have been further characterized by their individual requirements for regulatory ions. Protein phosphatase type 2A (PP2A) shows no

apparent requirement for regulatory ions. PP2B requires regulation by Ca2+ and PP2C requires Mg2+ for activity (Mumby and Walter, 1993).

Protein Phosphatase 2A

Biochemical structure

PP2A is a heterotrimeric enzyme composed of a 36 kD catalytic C subunit, one of several regulatory B subunits of varying size, and a 65 kD structural/regulatory A subunit (Fig. 1-1). Human PP2A subunits include two C subunit isoforms (α, β), at least three families of B subunits with multiple isoforms, and two A subunit isoforms (α, β). The B subunit has been shown to regulate substrate specificity of PP2A. The A subunit consists of a scaffold of 15 non-identical protein repeats which is required for B and C subunit interaction (Walter and Mumby, 1993).

PP2A-C is ubiquitously expressed and its structure is highly conserved throughout evolution. There are two mammalian isoforms of PP2A-C, α and β, although PP2A-Cα is about 10 times more abundant than PP2A-Cβ probably since expression from the PP2A-Cα gene promoter is 7-10 times higher than expression from the PP2A-Cβ gene promoter. The enzymatic activity of the PP2A-C catalytic subunit is regulated and targeted by its association with the A and B subunits. PP2A-C subunit is found in cells as a heterotrimer with A and B subunits or complexed with A in an AC core dimer. Free PP2A-C subunit has not been detected in cells. The catalytic activity of C subunit alone appears to be higher in the absence of A and B subunits, suggesting the A and B subunits influence the level of activity of C in the heterotrimer. Efforts to better understand the function of PP2A-C have

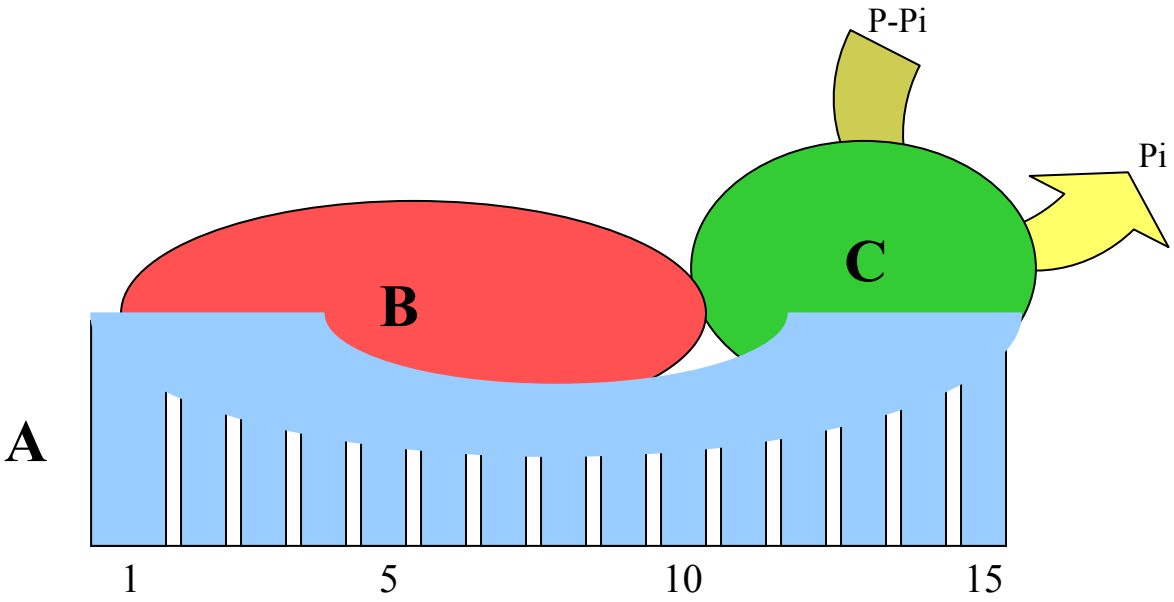

Figure 1-1. Protein phosphatase 2A holoenzyme. PP2A is a tripartite enzyme consisting of A, B and C subunits. The A subunit contains 15 HEAT repeats and serves as a scaffold to which the catalytic C and regulatory B subunit bind. HEAT repeats 1 through 10 are involved in B subunit binding and repeats 11 through 15 are important for C subunit binding.

been largely unsuccessful due to the difficulty in overexpressing C subunit in cells, possibly related to the tight cellular regulation of levels of PP2A-C subunit to maintain relatively constant levels. The importance of maintaining stable levels of PP2A-C in the cell is also illustrated by the embryonic lethal phenotype of mice lacking PP2A-Cα, the only PP2A subunit for which gene knock-out mice have been established (Janssens and Goris, 2001; Walter and Mumby, 1993).

The four identified families of PP2A-B subunits (B, B', B'' and B'''), comprising at least 18 subunit isoforms, highlight the vast number of different PP2A holoenzymes that can be derived from combinations of one of each of the known A, B, and C subunits. The PP2A-B subunits associate with the AC core dimer to form a heterotrimer but do not appear able to bind either A or C subunits independently of the core dimer. The cellular localization, tissue specificity and structure of the PP2A-B subunits suggest they are involved in targeting the phosphatase activity of PP2A. For example, the B' family has three isoforms which localize to the cytoplasm, three isoforms which localize to the nucleus, and one isoform which is found in both nucleus and cytoplasm. Though several B' isoforms have a wide distribution of tissue expression, two isoforms are predominantly expressed in brain. While all of the B' family members have a highly conserved central region, they vary significantly at their N and C termini. This suggests the central region is involved in interaction with the AC core dimer and the more divergent ends may be involved in directing the subcellular localization and substrate specificity of the PP2A holoenzyme, for example guiding PP2A involvement in the ability of B' family subunits to down regulate cellular β-catenin levels in the Wnt signaling pathway (Janssens and Goris, 2001; Seeling et al., 1999).

The 65 kD PP2A-A subunit is present in two known isoforms, α and β, in mammalian cells. Though they are ubiquitously expressed and share 86% sequence identity, PP2A-Aβ appears generally to be expressed much less abundantly than PP2A-Aα, which is probably the reason relatively little is known concerning the specific role of PP2A-Aβ. The PP2A-A subunit is vital as a scaffold for the assembly of the PP2A heterotrimer, as the B and C subunits of PP2A are not capable of stable association in the absence of the A subunit (Walter and Mumby, 1993). The structure of this scaffolding subunit is unusual in being composed entirely of 15 tandem repeats. The N terminal 10 tandem repeats have been determined by site directed mutagenesis to be involved in binding of the B subunits, while the C terminal 5 tandem repeats are necessary for C subunit binding (Ruediger et al., 1994). These tandem repeats of a 39 amino acid sequence, are called HEAT (huntingtin/elongation/A subunit/TOR, where TOR is target of rapamycin) motifs. Such repeats are found in a variety of proteins including the huntingtin protein, an elongation factor necessary for protein synthesis, and the TOR kinase. The crystal structure of PP2A-Aα revealed each repeat to be composed of superimposed α-helices, which stack together to form a protein with a hook-like architecture. The evolutionarily conserved residues of PP2A-Aα localize to the intra-repeat turns, which connect the helices of the HEAT motifs, and are likely to be the sites of PP2A-A interaction with the B and C subunits (Janssens and Goris, 2001; Groves et al., 1999).

Roles in cell growth

PP2A provides the majority of intracellular serine/threonine specific phosphatase activity in cells. It functions in controlling cell growth by down-regulating the MAPK cascade, which is a common pathway of induction of cell proliferation and carcinogenic development by some oncogenes (Marshall, 1995). It also plays important roles in cell cycle checkpoint control and has been shown to inhibit nuclear telomerase activity (Lee et al., 1991; Li et al., 1997). PP2A is well known as a target for chemical tumor promoters as well as DNA tumor viruses. Okadaic acid, dinophysistoxin-1 and calyculin A, that inhibit PP2A activity, are potent tumor promoters (Fujiki and Suganuma, 1993). Polyoma T antigen and SV40 small T antigen bind to the PP2A-A subunit displacing the PP2A-B subunit leading to alteration of cellular PP2A activity, which is thought to mediate viral transformation (Sontag et al., 1993; Pallas et al., 1990). Additionally, some cellular oncogenes and other intracellular components of cell growth control, function through regulating PP2A activity. For example, the HOX11 oncogene interacts with PP2A inhibiting activity and disrupting a G2/M cell cycle checkpoint (Kawabe et al., 1997). Casein kinase 2α inhibits cellular transformation by Ras and suppresses cell growth by enhancing PP2A activity (Heriche et al., 1997). PP2A has also been implicated in regulation of Wnt signaling. PP2A-C subunit has been found to associate axin, a binding protein of glycogen synthase kinase-3β (GSK-3β) and β-catenin. PP2A-B' family subunits interact with the adenomatous polyposis coli (APC) protein which acts as a scaffolding protein for the assembly of the β-catenin, GSK-3β, axin complex and is central to Wnt signaling. Moreover, overexpression of B' family subunits in mammalian cells reduces the levels of β-catenin and β-catenin mediated transcription,

suggesting a suppressive role for the B' family subunits in Wnt mediated cell growth (Janssens and Goris, 2001). It has been suggested that protein phosphatases suppress cell growth, and act as tumor suppressors, by antagonizing protein kinases, many of which act as oncogenes such as *mos* and *raf* (Maxwell and Arlinghaus, 1985; Morrison et al., 1989).

Tumor Suppressor Genes

Definition

Tumor suppressor genes are classically defined as negative regulators of tumorigenesis which sustain loss-of-function mutations in the development of cancer (Haber and Harlow, 1997). Such genes have been discovered due to the interaction of their protein products with transforming proteins of DNA tumor viruses (p53 protein's interaction with large T antigen of SV40) as well as their role in familial cancers (*RB1* in familial retinoblastoma) (Marshall, 1991). The tumor suppressor *RB1* was initially identified, and eventually cloned, using polymorphic markers to evaluate development of homozygosity , or loss of heterozygosity (LOH), in specific alleles at 13q14 (Cavenee et al., 1983). These types of genes are involved in a wide variety of human cancers and are frequently detected through an apparent loss of alleles in polymorphic markers. The allelic loss signals the deletion of one of the two copies of the tumor suppressor gene with the remaining gene inactivated due to somatic or inherited mutations. Thus, regions of the human genome which demonstrate high frequency LOH in human cancers are likely to harbor as yet undiscovered tumor suppressor genes and are the subject of much investigation.

LOH at 11q23

Allelic loss at chromosome 11q22-24 has been associated with a variety of different cancers, including lung, colon, breast, cervical, head and neck, ovarian and melanoma (Rasio et al., 1995; Gustafson et al., 1994; Hampton et al., 1994; Hampton et al., 1994b; El-Naggar et al., 1996; Davis et al., 1996; Tomlinson et al., 1996). Chromosome transfer studies have shown that the introduction of a normal chromosome 11, or a derivative t(X;11) chromosome containing 11pter-q23, could reverse the tumorigenic potential of Wilms tumor, lung, breast and cervical carcinoma cells when introduced into nude mice (Weissman et al., 1987; Satoh et al., 1993; Negrini et al., 1994; Saxon et al., 1986). These studies suggest the presence of one or more functional tumor suppressor genes on chromosome 11q localized centromeric to the t(X;11) breakpoint at 11q23.

Identification of PPP2R1B

Based on this information, we evaluated and identified regions of LOH in lung cancer at 11q23 (Wang et al., 1999). We searched for candidate genes, within the region of LOH identified, whose biochemical properties could plausibly confer tumor suppressor activity. *PPP2R1B*, the gene encoding the β isoform of the A subunit of PP2A, fit these criteria. It was cloned and sequenced, and found to contain unique sequence coding 13 amino acids at its N-terminus, which differentiates it from its otherwise extensively homologous alternative isoform, PP2A-Aα (Wang et al., 1998).

We screened 135 cancer-derived and matching lymphoblastoid cell lines as well as 70 primary tumor tissues for mutations in the PP2A-Aβ gene-coding region. Mutations were

found in 15% (5/33) of primary lung tumors, 6% (4/70) of lung cancer cell lines and 13% (2/13) of primary colon tumors, including internal deletions, frameshifts and missense mutations (Table 1-1) (Wang et al., 1998).

Tumor	Diagnosis	Allele	Mutation (cDNA)	Predicted effect
H1450	SCLC	1	Δ867 bp (717-1583	Δ289 codons (230-518)
		2	1540 A to G	504 Asp to Gly
H838	NSCLC	1	51 G to C	8 Gly to Arg
		2	wild-type not detected	inactive
H220	SCLC	1	Δ143 bp (1584-1726)	frameshift, (519-601)
		2	normal	none
H2009	NSCLC	1	germline, 298 G to A	90 Gly to Asp
		2	normal	none
T11	NSCLC	1	1056 A to G	343 Lys to Glu
		2	normal	none
T9	NSCLC	1	Δ135 bp (1057-1191)	Δ45 codons (344-388)
		2	wild-type not detected	inactive
T12	NSCLC	1	Δ135 bp (1057-1191)	Δ45 codons (344-388)
		2	222 C to T	65 Pro to Ser
T64	NSCLC	1	298 G to A	90 Gly to Asp
		2	normal	none
T68	NSCLC	1	298 G to A	90 Gly to Asp
		2	normal	none
T24	CAC*	1	Δ191 bp (1315-1505)	frameshift, (422-601)
		2	1663 T to C	545 Val to Ala
T25	CAC	1	331 T to C & 1372 T to C	101 Leu to Pro & 448 Val to Ala
		2	wild-type not detected	inactive

Table 1-1. Summary of PP2A-Aβ mutations in lung and colon cancer.
*Colon adenocarcinoma. Mutations are indicated in the sense orientation. Δ: deletion. The mutations in the primary tumors were not found in adjacent normal tissue DNA.

RT-PCR screening of the tumors for somatic or inherited mutations in the PP2A-Aβ gene showed that all but two produced a single 1.8 kb PCR product. Lung cancer cell line

H1450 produced a weak 1.8 kb product as well as a strong 1 kb product. DNA sequencing of the 1 kb product from cell line H1450 revealed that the gene contains an internal deletion of 867 bp which would result in a truncated PP2A-Aβ protein with an in frame deletion of 289 codons (amino acid residues 230 to 518). The DNA sequence of the 1.8 kb PCR product from cell line H1450 uncovered a point mutation at position 1540 where an A to G transition results in a substitution of a neutral glycine for an acidic aspartate. DNA sequencing of PCR amplification products from non small-cell lung carcinoma (NSCLC) cell line H2009 revealed it carries one allele with a G298→A transition. The matched lymphoblastoid cell line BL2009 also carries one allele with a G298→A transition, which suggests that the lung cancer patient from whom these cells are derived harbors a germline mutation in the *PPP2R1B* gene (Wang et al., 1998).

Objectives of the dissertation research

The goal of the studies presented here was to examine the role of the PP2A-Aβ gene in cell growth control and cancer development. Since mutations in the PP2A-Aβ had already been identified as I began my work, the primary objective was to investigate the ability of PP2A-Aβ to act as a tumor suppressor gene.

Large scale screening of genomic DNA for alterations in the PP2A-Aβ gene in cancer patients indicated the PP2A-Aβ gene is altered in additional cancer types and in families with cancer susceptibility. The functional impact of these mutations on the PP2A enzyme was investigated using *in vitro* and *in vivo* biochemical analysis of the PP2A-Aβ. The mutations

were found to disrupt assembly of the PP2A holoenzyme. In order to demonstrate tumor suppression by the PP2A-Aβ gene and in an effort to identify a mechanism by which it occurs, it was necessary to develop an *in vivo* cell model. Stable, PP2A-Aβ gene expressing cancer cell lines were derived and were tested for reduced tumorigenicity using cell proliferation assay and soft agar colony forming analysis. Lysates from these cells were Western blotted to identify changes in the levels of phosphorylation regulated intracellular proteins.

CHAPTER TWO
PP2A-Aβ Gene Polymorphisms and Cancer Susceptibility

INTRODUCTION

The most common cancers of adults include lung and breast cancer. Acute lymphoblastic leukemia (ALL) is the most common cancer in childhood. 1,200,000 new cases of cancer occur each year in the United States (Greenlee et al., 2001). Many types of cancer carry mutations in tumor suppressor genes, which often map to genomic regions that demonstrate loss of heterozygosity (LOH) in tumors. LOH at 11q22-24 has been demonstrated in carcinoma of the breast, lung and ALL (Arai et al., 1996; Takeuchi et al., 1999). The putative tumor suppressor *PPP2R1B* was identified at 11q22-24, which showed loss of function mutations in lung and colon cancer (Wang et al., 1998). Mutations of *PPP2R1B* have also been described in breast carcinoma (Calin et al., 2000) but have not been evaluated in ALL.

The G298A missense mutation in *PPP2R1B* changes codon 90 of the PP2A-Aβ subunit from GGC(Gly)→GAC(Asp). The G90D alteration was found in two primary non-small cell lung carcinomas (NSCLC), in a patient-derived NSCLC cell line (H2009) and in the germline DNA of the same patient (Wang et al., 1998). G90D occurs in a region of the PP2A-Aβ subunit important for binding to the regulatory subunits. In the crystal structure of the highly homologous Aα protein this glycine residue is adjacent to a conserved hydrophobic amino acid (Groves et al., 1999). Since hydrophobic regions are predicted to be

sites of subunit interactions, the change to a charged aspartate residue could disrupt subunit function.

The germline occurrence of the G90D polymorphism raised the possibility that this alteration is a heritable polymorphism which confers susceptibility to cancer. To establish this correlation, we screened genomic DNA collected from patients with lung cancer, breast cancer and ALL for the presence of the G90D polymorphism.

MATERIALS AND METHODS

Screening of genomic DNAs

We screened genomic DNA collected from patients with lung cancer, breast cancer, and ALL for the G90D polymorphism. Analysis was done using a novel technology developed by Nanogen, Inc. (San Diego, CA). This method utilizes a microelectronic DNA chip which allows binding of an oligonucleotide probe and the conduct of a specific hybridization reaction in the presence of a controlled electric field. Oligonucleotide probes (5'-CCT AGT GGG AGG TCC TGA CTT TGC-3') were prepared using a 5' biotinylated nucleotide and covalently bound to a streptavidin treated DNA chip. Indicator probes (5'-GAA ATT TCA CTG G-3' and 5'GAA AGG TCA CTG A-3') were prepared as 5' fluorescently labeled oligonucleotides. Exon 3 of *PPP2R1B* was PCR amplified using primers 5'-GTC CTG TTT GTT GTC ACT TCC-3' and 5'-GCA GCT TAT AAA TAC TCA CC-3'. Purified PCR amplicons were applied to the NanoChip system according to

manufacturer's protocol. 540 DNAs were screened using this detection system. These results were confirmed, and the remaining controls were assessed, by sequence analysis.

RESULTS

G90D frequency in cancer patients

The G90D polymorphism was observed in 7/387 cancer patient genomic DNAs, whereas non-cancer control genomic DNAs showed 1/329 occurrences of the G90D polymorphism (Table 2-1). These controls included 75 Caucasian adults derived from population based sampling as well as 254 newborns with ethnic distribution similar to that of the population of all samples.

Table 2-1 ♦ Frequency of *PPP2R1B* G90D SNP in cancer patient and control groups

PPP2R1B	Breast carcinoma (n=99)	Lung carcinoma (n=138)	Acute lymphoblastic leukemia (n=150)	Non-cancer controls (n=329)
G90D heterozygous	3 (3%)[a]	1 (0.7%)[b]	3 (2%)[c]	1 (0.3%)
wild type	96 (97%)	137 (99.3%)	147 (98%)	328 (99.7%)

[a]P=0.0399, [b]P=0.5, [c]P=0.089 (Fisher exact 2-sided test)

G90D in families susceptible to cancer

The breast cancer patients were from families with a history of breast and other cancers. Four of these families had a member that was positive for the G90D polymorphism. To examine the association of the G90D alteration with cancer development, we screened 3 of these families for whom DNA was available. Each of the family members screened (9/9) carries the G90D polymorphism, and 7/9 (78%) of those individuals are diagnosed cancer patients (Fig. 2-1). The G90D carriers without cancer are not of sufficient age to rule out increased cancer risk. We found a total of 11/391 (2.8%) cancer patients who carry the G90D alteration in their germline DNA.

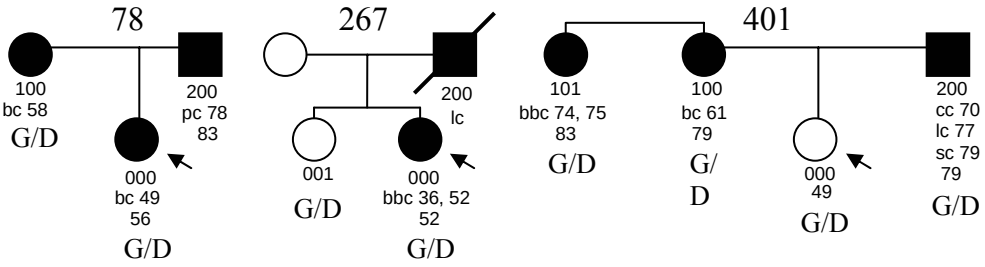

Figure 2-1. Association of G90D SNP with multiple cancers in families with a history of cancer. Abridged pedigrees of families BCA78, BCA267 and BCA401 (DNA from family BCA133 was unavailable for further screening). Filled symbols indicate individuals with cancer. The diagnosis (bc, breast cancer; bbc, bilateral breast cancer; cc, colon cancer; lc, lung cancer; pc, prostate cancer; sc, skin cancer) for each patient is listed below the symbols followed by the age at diagnosis (including multiple diagnoses is some cases), and the age at pedigree assessment. Arrows signify individuals identified in the initial screen. G/D indicates individuals that were heterozygous for the G90D SNP of *PPP2R1B*.

CONCLUSIONS

These results suggest that the G90D polymorphism in the *PPP2R1B* gene is associated with a low frequency of inherited cancer susceptibility. This is the first report of genomic alterations of *PPP2R1B* in patients with ALL. LOH at 11q22-24 has been observed in ALL and *PPP2R1B* may be a gene affected by this LOH.

To our knowledge, this is the first report of a polymorphism in a protein phosphatase subunit that may be associated with cancer predisposition. The G90D polymorphism of *PPP2R1B* has also been observed in breast (Calin et al., 2000), ovarian (Campbell and Manolitsas, 1999), cervical carcinoma (Pulido et al., 2000), and parathyroid adenoma (Hemmer et al., 2002) suggesting that this polymorphism may contribute to the etiology of a wide variety of cancer types.

It has been hypothesized that the G90D polymorphism is a non-pathological variant (Campbell and Manolitsas, 1999). The potential for the G90D alteration to alter the function of the Aβ subunit is consistent with data showing that a single amino acid substitution in the A subunit significantly alters the interaction with regulatory subunits (Ruediger et al., 1999). Confirmation of the significance of the G90D polymorphism to cancer pathology requires demonstration of a functional alteration in PP2A due to the G90D polymorphism.

CHAPTER THREE
Functional Analysis of PP2A-Aβ Gene Mutations

INTRODUCTION

Frameshifts, deletions and point mutations of *PPP2R1B* were identified in primary lung cancers, colon cancers and lung cancer cell lines at the DNA level by RT-PCR and sequence analysis. These findings established the possibility that such mutations contribute to lung cancer development and progression. The mechanism by which these mutations influenced cancer development was unclear. Of particular interest were the mutations occurring in the H1450 cell line and in the H2009 cell line. The H1450 cell line contains an Asp504→Gly point mutation in one allele of *PPP2R1B* and a 351 amino acid deletion in the other *PPP2R1B* allele. The H2009 cell line carries a Gly90→Asp polymorphism in one allele of *PPP2R1B*. The mutations in cell line H1450 alter the C-terminus of the PP2A-Aβ protein, which is important for the binding the catalytic C-subunit. The polymorphism in cell line H2009 affects the N-terminus of the PP2A-Aβ subunit which is involved in binding the regulatory B-subunits (Wang et al., 1998). Determining whether this N-terminal alteration affects the binding of regulatory B-subunits, and, if so, identifying the B subunits affected, could give important insights into the pathways by which alterations in the PP2A-Aβ protein lead to cancer. These alterations in the PP2A-Aβ subunit could affect PP2A enzyme function by decreasing the affinity of PP2A subunits for each other, thereby destabilizing the holoenzyme. We utilized both in vivo and in vitro approaches in order to study the biochemical effect of such mutations on PP2A-Aβ protein translation and function.

MATERIALS AND METHODS

Western Blotting

H1450, CCD-8 and COS7 cells were lysed in cold RIPA lysis buffer with protease inhibitors, and centrifuged to remove cell membrane proteins. Proteins were separated by polyacrylamide gel electrophoresis, and gels were transferred using a Trans-Blot SD (Bio Rad) to a Millipore Immobilon-P membrane. Immunodetection was carried out with goat anti-human PP2A-Aβ immunoglobulin and secondary horseradish peroxidase-conjugated donkey anti-goat immunoglobulin (Santa Cruz Biotechnology). Immunodetection was carried out with enhanced chemiluminescence (Amersham Life Sciences). Goat anti-human actin immunoglobulin was added as a control.

In vivo co-immunoprecipitation

H1450 and CCD-8 cell lysates were prepared as described and immunoprecipitated with rabbit anti-human PP2A-C immunoglobulin (Promega) and protein G-agarose (Roche). The precipitates were collected by centrifugation, and the pellet was washed with cold phosphate-buffered saline. The precipitate was then denatured at 95^{o} C in SDS loading buffer and analyzed on an 8% SDS-polyacrylamide gel. Protein immunoblot detection was carried out with purified goat anti-human immunoglobulin against PP2A-Aβ and PP2A-C and secondary horseradish peroxidase-conjugated donkey anti-goat immunoglobulin (Santa Cruz Biotechnology).

Construction of wild type and mutant *PPP2R1B* expression vectors

The *PPP2R1B* cDNA was used to make tagged and untagged expression constructs for mutational analysis. The following constructs were generated: P2Aβ - the coding region of the *PPP2R1B* cDNA was digested out of a TOPO TA (Invitrogen) cloning vector using BstXI and NotI, and gel purified. This fragment was then ligated into a pcDNA3.1B-His-V5 vector (Invitrogen) with an intact stop codon, leaving this construct without the His and V5 epitope tags. P2AβT - The coding region of the *PPP2R1B* cDNA was also amplified using a forward primer containing a BstXI site (5'-CTG CAG AAC CAA TGC ATT GGA GGA GGA GAA AGA ACA TG-3') and a reverse primer containing a NotI site (5'-TTT TCC TTT TGC GGC CGC TCT TGT ATG CCA ATG CAA GAA CAC T-3'). These primers were designed complementary to the stop codon sequence in order to eliminate the stop codon, allowing translation of the His and V5 epitope tags. The amplification product was gel purified and ligated into a TOPO TA vector. The *PPP2R1B* cDNA insert was digested with BstXI and NotI, gel purified and ligated into pcDNA3.1B-His-V5 in frame with the epitope tags.

The P2Aβ and P2AβT expression vectors were then subjected to mutagenesis in order to construct the G298A and Δ519-601 mutations of *PPP2R1B*. G298A primer pairs 5'-GCA GCT GGG AAA TTT CAC TGA CCT AGT GGG AGG TCC-3' and 5'-GGA CCT CCC ACT AGG TCA GTG AAA TTT CCC AGCTGC-3' and Δ519-601 primer pairs 5'-CCA CTT TAT TCT GCA TTA ATT GCT TTA CAG GGA GAA GTG AAG CC-3' and 5'-GGC

TTC ACT TCT CCC TGT AAA GCA ATT AAT GCA GAA TAA AGT GG-3' were designed for use with the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

In vitro co-immunoprecipitation

The *PPP2R1B* wild-type and mutant expression constructs, described above, as well as expression constructs of the PP2A B subunits were transcribed and translated *in vitro* using the T7 TnT Quick Coupled Transcription/Translation System and the T3 Coupled Transcription/Translation System (Promega) both with either ^{35}S-labeled methionine or unlabeled methionine. The *in vitro* translations reactions were incubated at 30^{o} C for 2 hours. Nine microliters of labeled translation mix from a single subunit was combined with 5ul of unlabeled translation mix from another single subunit and incubated together for 4 hours at 30^{o} C to allow the independently translated subunits to interact and form holoenzyme complexes. Subsequently, 1ug of monoclonal V5 epitope antibody (Invitrogen) or 5 ul of anti-B56γ polyclonal rabbit serum were added to the translation mixtures as well as 200 ul of X-100 buffer (0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-pH 8.0, 3 mM $MgCl_2$, 1 mM Dithiothreitol, 50 uM Leupeptin) and agitated by rotation for 1 hour at 4^{o} C. Twenty ul of a slurry of protein A sepharose beads in phosphate-buffered saline (~10 ul of beads), were added to the mixtures and the immunoprecipitation was agitated by rotation for 1 hour at 4^{o} C. The immunoprecipitations were then washed with 1 ml of cold X-100 buffer (Kremmer et al., 1997), vortexed for 5 seconds, and centrifuged for 5 minutes at 2500 rpm. The immunoprecipitations were washed in this manner 3 times. The resulting bead pellets were suspended in 20 ul of SDS loading buffer, denatured at 95^{o} C, and 10 ul were loaded

onto SDS polyacrylamide gels for electrophoresis. The gels were dried and exposed to film for 7-14 days.

Transient transfection and coimmunoprecipitation

COS7 cells were simultaneously cotransfected with 5 ug each of an untagged B56γ expression construct and either the P2AβT or the G90D-P2AβT construct using Lipofectamine 2000 (Invitrogen) and OptiMem transfection media (Invitrogen). After 48 hours the cells were lysed, lysates from replicate transfections were pooled and incubated with either 1 ug of V5 antibody (Invitrogen) or purified mouse IgG, and 10 ul of protein A sepharose beads. Immunoprecipitations were incubated for 2 hours at 4^{o} C then washed 3 times with cold X-100 buffer, each sample being vortexed for 5 seconds with each wash. The pellets were resuspended in SDS loading buffer, loaded on an SDS polyacrylamide gel and electrophoresed. Western blots were prepared as described, blotted with polyclonal B56γ antibody and in some cases stripped and blotted with V5 antibody.

RESULTS

Truncation of PP2A-Aβ protein in H1450 cell line

To determine if the sequence alterations in *PPP2R1B* might produce biochemical changes in translation of PP2A-Aβ , we performed immunoblot analysis with anti-PP2A-Aβ immunoglobulin. Lung cancer cell line H1450 has two *PPP2R1B* alterations, a deletion in one *PPP2R1B* allele, and a missense mutation in the other allele of *PPP2R1B*. We were

particularly interested in whether the deletion mutation would prevent translation of the PP2A-Aβ protein. Whole cell lysates of H1450 cells were found to contain an immunoreactive, truncated PP2A-Aβ protein of 45 kD in addition to the wild-type 65 kD PP2A-A protein (Fig. 3-1). The deletion in this truncated PP2A-Aβ protein might be expected to compromise a highly conserved region of the protein necessary for PP2A-Aβ to form functional heterotrimers with PP2A-B and PP2A-C (Mumby, M.C. and Walter, G., 1993).

Truncated PP2A-Aβ subunit has decreased affinity for PP2A-C subunit

PP2A-A, PP2A-B and PP2A-C subunits were coimmunoprecipitated with a polyclonal antibody against the PP2A-C subunit, to determine whether binding of the truncated PP2A-Aβ to PP2A-C was altered. Coimmunoprecipitation using H1450 whole cell lysates showed no 45-kD truncated PP2A-Aβ coprecipitating in the immune pellet, compared with similar coimmunoprecipitations performed using whole cell lysates from normal human lung cells. There was also a decrease in the amount of coimmunoprecipitating 62 kD PP2A-A protein. Because the antibody used to detect the PP2A-A subunit does not distinguish PP2A-Aβ from PP2A-Aα, this 62 kD band represents the amount of wild-type PP2A-Aα subunit and Asp504→Gly mutated PP2A-Aβ subunit in the precipitate. As the wild-type PP2A-Aα has unchanged affinity for the C-subunit, it is possible that the decrease in 62 kD coimmunoprecipitating protein represents a decrease in the Asp504→Gly mutant PP2A-Aβ.

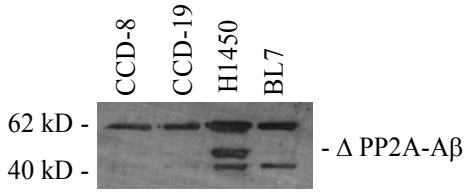

Figure 3-1. Identification of PP2A-Aβ proteins by immunoblotting. PP2A-Aβ was detected in lysates of normal lung tissue samples CCD-8 and CCD-19, cell line H1450, and BL7 lymphoblastoid control cells, with anti-PP2A-Aβ immunoglobulin. The 65-kD PP2A-Aβ protein was detected in all cells tested, and H1450 lysates contain an additional 45-kD band representing the mutant subunit. The 40-kD band is an actin control.

This suggests that both the truncated and the missense A subunits have decreased affinity for the C subunit (Fig.3-2).

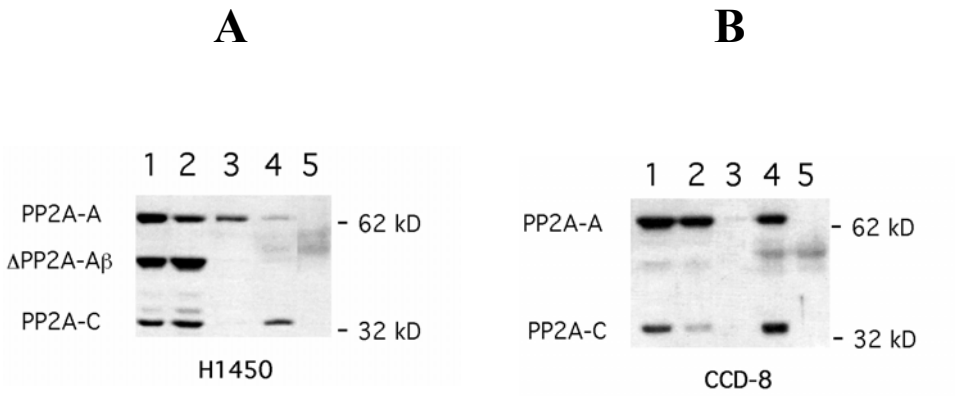

Figure 3-2. Coimmunoprecipitation of PP2A-A and PP2A-C subunits.
(**A**) Coimmunoprecipitation of PP2A-A and PP2A-C subunits from H1450 cells with anti-PP2A-C immunoglobulin. Immunoblots of whole cell lysates with both anti-PP2A-A and anti-PP2A-C show (lane 1) full-length PP2A-A (65 kD), truncated ΔPP2A-Aβ (45 kD), and PP2A-C (36 kD). The supernatant after immunoprecipitation with anti-PP2A-C (lane 2) contains PP2A-A, ΔPP2A-Aβ, and PP2A-C. The supernatant after washing of the immunoprecipitation (lane 3) contains PP2A-A, indicating that it is dislodged from the complex during the wash. The immunoprecipitate (lane 4) contains decreased amounts of full-length PP2A-A and PP2A-C but lacks ΔPP2A-Aβ, which suggests that ΔPP2A-Aβ cannot bind PP2A-C. The control (lane 5) represents the reactivity of anti-PP2A-C immunoglobulin and horseradish peroxidase-conjugated anti-immunoglobulin in the absence of cell lysates. (**B**) Immunoprecipitation of PP2A-A and PP2A-C subunits from normal lung tissue with anti-PP2A-C immunoglobulin. The whole-cell lysate (lane 1) contains PP2A-A (65 kD) and PP2A-C (36 kD). The supernatant after immunoprecipitation with anti-PP2A-C contains PP2A-A and PP2A-C (lane 2). The supernatant after washing (lane 3) lacks PP2A-A, indicating that normal PP2A-A remains bound to PP2A-C during the wash procedure. The immunoprecipitate contains both PP2A-A and PP2A-C (lane 4). Lane 5 is a control lane with antibodies without cell lysate. The goat anti-PP2A-Aβ immunoglobulin was cross-reactive with the PP2A-Aα isoform as well as with PP2A-Aβ.

PP2A-Aβ binds B56γ *in vitro*

Three families of B subunits have been shown to interact with the PP2A-Aα subunit, but because of differences between the PP2A-Aα subunit and the PP2A-Aβ subunit, it was not known which families of B subunits would be able to interact with the PP2A-Aβ subunit. We tested the ability of PP2A-Aβ to bind several different members of the B' and B'' families of PP2A-B subunits by coimmunoprecipitation of *in vitro* translated protein subunits using an epitope tagged PP2A-Aβ to precipitate wild-type PP2A-B subunits. We found that PP2A-Aβ does not bind the B55, B56α, or B56β subunits of PP2A. We also found that PP2A-Aβ does bind two members of the B'' family, PR48 and PR72, with higher affinity for PR72 than for PR48, and binds to B56γ of the B' family (Fig.3-3 A).

G90D altered PP2A-Aβ has reduced affinity for B56γ *in vitro*

We next tested the ability of and epitope tagged, G90D altered PP2A-Aβ protein to bind those subunits that bind to wild-type PP2A-Aβ. We discovered that, though the G90D altered PP2A-Aβ protein had little change of affinity for PR48 and PR72, the G90D alteration decreased the affinity of PP2A-Aβ for the B56γ subunit (Fig.3-3 A).

In order to confirm this observation, and rule out interference of the epitope tag, the reciprocal coimmunoprecipitation was carried out using untagged constructs. We *in vitro* translated wild-type PP2A-Aβ, G90D mutant PP2A-Aβ and B56γsubunits. These were coimmunoprecipitated using a polyclonal antibody against the B56γ subunit. We found

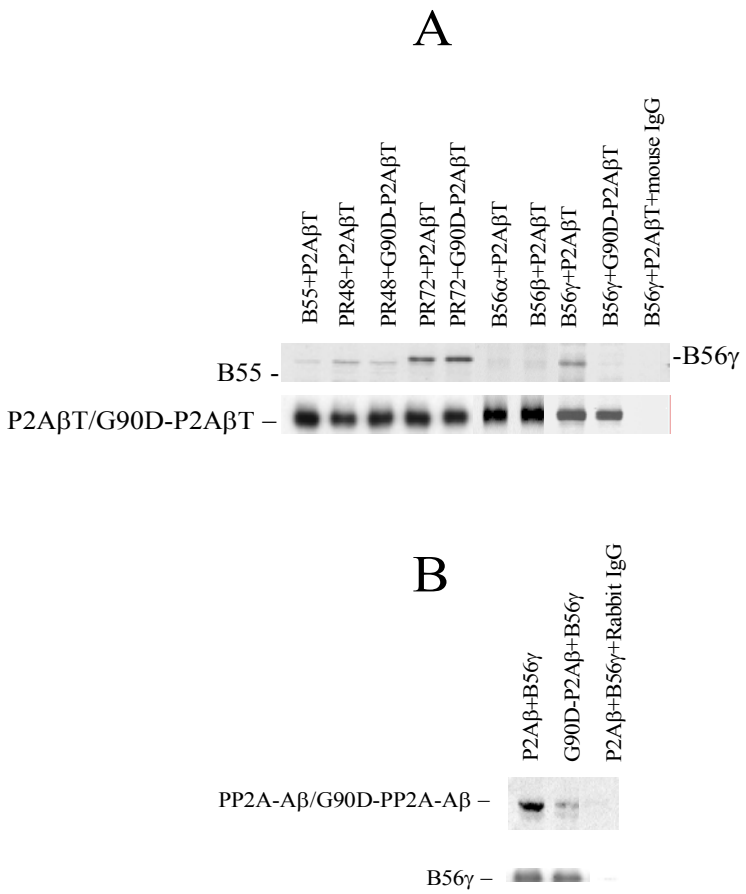

Figure 3-3. *In vitro* **coimmunoprecipitation of PP2A-B subunits with the PP2A-Aβ subunit.** (A) *In vitro* translated and ^{35}S labeled PP2A-B subunits and V5 epitope tagged wildtype (P2AβT) and mutant (G90D-P2AβT) PP2A-Aβ subunits were coimmunoprecipitated using V5 antibody. The top gel shows the ability ofboth P2AβT and G90D-P2AβT to bind PR48 and PR72, whereas P2AβT does not bind B55, B56α or B56β. However, P2AβT does bind to B56γ and G90D-P2AβT has significantly reduced affinity for B56γ. The bottom gel shows the relative amounts of V5 epitope tagged PP2A-Aβ subunits in each of the immunoprecipitates for a loading control. (B) The top gel shows coimmunoprecipitation of ^{35}S labeled wildtype (PP2A-Aβ) and mutant (G90D-PP2A-Aβ) with B56γ using B56γ anti-serum. PP2A-Aβ (left lane) binds B56γ with higher affinity than G90D-PP2A-Aβ (middle lane). Rabbit IgG was used (right lane) as a negative control. The bottom gel is a western blot of the coimmunoprecipitate for B56γ, as a loading control.

untagged, wild-type PP2A-Aβ is able to bind B56γ, and untagged G90D mutant PP2A-Aβ has reduced affinity for B56γ compared to wild-type (Fig. 3-3 B).

In vivo coimmunoprecipitation of B56γ and PP2A-Aβ

As further evidence that our *in vitro* observations were relevant in living cells, we performed *in vivo* coimmunoprecipitations of PP2A-Aβ and B56γ. COS7 cells were transiently cotransfected with expression constructs for both PP2A-Aβ and B56γ. The cells were then lysed and PP2A subunits were coimmunoprecipitated using V5 antibody against the epitope tag of the P2AβT expression construct. We observed that PP2A-Aβ and B56γ subunits bind each other in vivo (Fig. 3-4).

CONCLUSIONS

These data demonstrate that mutant PP2A-Aβ is translated in the cell and both deletions and point mutations of *PPP2R1B* found in human cancers functionally alter the ability of the PP2A-Aβ subunit to associate with the B and C subunits of the PP2A holoenzyme. Of the mutations in the H1450 cell line, both show a decreased affinity for the catalytic C subunit, as would be predicted because of the location of the mutations in the C terminus of the PP2A-Aβ subunit. Loss of the C subunit from complexes containing mutant PP2A-Aβ would cause these complexes to be catalytically inactive and functionally null,

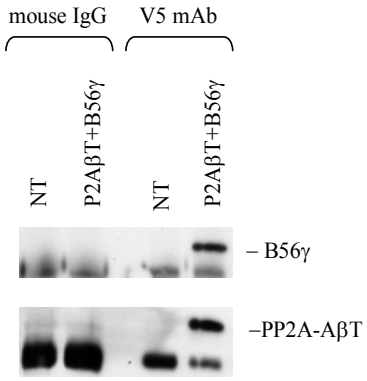

Figure 3-4. *In vivo* coimmunoprecipitations of PP2A-Aβ and B56γ.
In vivo coimmunoprecipitation of PP2A-AβT and B56γ from transfected COS7 cell lysates using V5 monoclonal antibody. In the upper panel precipitates were blotted for B56γ, showing it only in the V5 antibody immunoprecipitate. In the lower frame the same blot was stripped and blotted for the PP2A-AβT using V5 antibody, confirming its coprecipitation with B56γ *in vivo*. Mouse IgG was used as a negative control. (NT indicates non-transfected cell lysates.)

resulting in hyperphosphorylation of substrates for which the PP2A-Aβ holoenzyme is specific.

It was recently shown that the PP2A-Aβ subunit could bind PR72 of the B'' family of subunits, but did not bind B55 of the B family or B56α of the B' family (Ruediger et al., 2001). The ability of the PP2A-Aβ subunit to bind the B56γ subunit is particularly interesting in light of the fact that PP2A-Aβ appears unable *in vitro* to bind the B56α and B56β subunits of the same family. This suggests a high level of binding specificity even among B subunits within the same family, and may provide insight into specific functions of PP2A-Aβ related to B56γ alone. The ability of the isolated point mutation resulting in the G90D alteration of PP2A-Aβ to significantly decrease the affinity of B56γ for PP2A-Aβ *in vitro* is further evidence for the specificity of binding between PP2A subunits and for the significant effect even small mutations in *PPP2R1B* can have on the function of the PP2A holoenzyme. It also suggests the G90D alteration of PP2A-Aβ changes the function of PP2A in cells, and may disrupt the delicate phosphorylation dependent circuitry regulated by PP2A activity. The observation that overexpression of the B56γ subunit down regulates the levels of cellular β-catenin (Seeling et al., 1999), suggests that the G90D alteration of PP2A-Aβ may allow levels of cellular β-catenin to increase, and overexpression of PP2A-Aβ may down regulate levels of β-catenin in cancer cells deficient for functional PP2A-Aβ.

CHAPTER FOUR
The Effects of the PP2A-Aβ Gene on Cell Growth and Wnt Signaling

INTRODUCTION

The identification of loss of function mutations of *PPP2R1B* in lung, breast and cervical carcinomas (Wang et al., 1998; Calin et al., 2000; Pulido et al., 2000) suggests a role for *PPP2R1B* in tumor suppression. An important proof of this would be exogenous expression of the *PPP2R1B* gene in a cancer cell line deficient for the gene, subsequent identification of a growth suppression phenotype and, ideally, discovery of a mechanism or pathway by which such growth suppression occurs.

Earlier studies for tumor suppressor genes on chromosome 11 indicated introduction of certain portions of chromosome 11 into cancer cell lines suppressed their growth and tumorigenicity (Satoh et al., 1993; Weissman et al., 1987). This identified chromosome 11 as the location for one or more tumor suppressor genes. Testing of *PPP2R1B* in a similar manner would confirm it as one of the tumor suppressor genes responsible for the earlier observations.

Using a cell line which carries a loss of function mutation in one of the alleles of *PPP2R1B* (H2009), the effect of exogenous *PPP2R1B* on cell growth and signaling cascades was examined.

MATERIALS AND METHODS

Development of stable cell lines

H2009 cell lines, growing in RPMI 1640 (Invitrogen) with 10% FBS (Invitrogen) and 2% Antibiotic/Antimycotic (Invitrogen), were transfected using FuGene 6 (Roche) and Optim-Mem transfection media (Invitrogen) with the empty pcDNA3.1B mammalian expression vector, the P2AβT vector expressing wild type *PPP2R1B* or the G90D-P2AβT vector expressing *PPP2R1B* with a loss of function G90D mutation. 48 hours after transfection 350ug/ml of G418 antibiotic (Roche) was added and the cells were selected for 2-4 weeks. Individual G418 resistant colonies of cells were picked using autoclaved toothpicks and plated into individual wells of a 48 well plate and expanded in culture until they reached a number sufficient for screening by Western blot. Clonal populations of G418 cells demonstrating expression of either P2AβT or G90D-P2AβT were maintained in culture with 350ug/ml G418 for further characterization.

Growth rate and soft agar analysis

Stable cell lines were plated in duplicate at 10^4 cells per well in 6 well plates. G418 cell growth media was replaced daily. Cells from individual wells (2 wells/cell line/day) were trypsinized and counted with a hemocytometer daily for 4 days.

Stable cell lines were also analyzed using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Cells were plated in triplicate at 5000 cells per well in 96 well plates for 4 days of growth analysis. Each day, 20 ul of MTS reagent was added to one

set of triplicate wells of each cell line and plates were incubated at 37^{o} C. The absorbance of the wells at 490 nm was read with a 96 well plate reader after 1 and 2 hours of incubation.

The underlayer of the soft agar assay plates was made using 0.5% agar in RPMI 1640 containing 10% FBS, 2% Antibiotic/Antimycotic and 350 ug/ml G418. This solution was heated to 45^{o} C and 1 ml was used to cover the bottom of each well of a 6 well plate. 0.3% agar in RPMI 1640 with 10% FBS, 2% Antibiotic/Antimycotic and 350 ug/ml G418 made up the overlayer of soft agar, which was heated to 37^{o} C. 10^4 cells were resuspended in 500 ul of overlayer agar and plated on top of the underlayer in 6 well plates. After the soft agar solidified, 1 ml of cell growth media with 350 ug/ml of G418 was added to each well. This media was changed every 2 days. 2 weeks after plating, colonies were stained with 1 ml of a 1 mg/ml solution of MTT in cell growth media and incubated at 37^{o} C overnight. Colonies were visualized and counted for triplicate wells of each cell line analyzed.

Western blotting

10^6 cells were harvested with trypsin (Roche), pelleted by centrifugation, washed with cold PBS and pelleted again by centrifugation. Cell pellets were stored at –20^{o} C until lysis. Cell pellets were resuspended in 50 ul of lysis buffer (7 ml RIPA buffer with 1 tablet of Complete Protease Inhibitor w/EDTA (Roche), 70 ul of 1M sodium orthovanadate and 70 ul of sodium fluoride) and lysed on ice for 45 minutes. Cell lysates were centrifuged at 14,000 rpm for 10 minutes and the supernatant transferred to a new tube. Cell lysate protein concentrations were measured by Bradford assay. 10 ug of total cell lysate protein was suspended in SDS loading buffer, denatured at 95^{o} C for 5 minutes, loaded on an SDS

polyacrylamide gel and separated by electrophoresis. The SDS gel was transferred to a Millipore Immobilon-P membrane using a Trans-Blot SD (Bio Rad). Blots were blocked in PBS-T with 5% milk for 1 hour at room temperature or overnight at 4^{o} C. They were then washed twice with PBS-T for 10 minutes, incubated in PBS-T and 5% milk with primary antibody for 1 hour, washed twice with PBS-T for 10 minutes, incubated in PBS-T and 5% milk with secondary antibody, and washed 4 times with PBS-T for 10 minutes each before application of ECL and exposure to film.

RESULTS

Stable cell line growth analysis

Multiple independent transfections and selections were performed in order to isolate and screen for stable cell lines containing each vector (pcDNA3.1B, G90D-P2AβT, P2AβT). Though sufficient stable clones containing pcDNA3.1B and G90D-P2AβT were readily obtained, stable P2AβT clones were significantly more difficult to derive (Table 4-1). Two of these stable P2AβT clones were selected which expressed relatively high levels of the V5 tagged PP2A-Aβ construct. One of the G90D-P2AβT stable clones was selected that expressed V5 tagged PP2A-Aβ at a similar level to the P2AβT clones. Three of the pcDNA3.1B clones were also selected for control comparison. Growth rates as analyzed by cellular metabolism of an MTS tetrazolium compound were measured over 3 days. Stable cell lines containing pcDNA3.1B vector only showed slightly faster growth overall compared to G90D-P2AβT and P2AβT stable lines. There was no significant difference between the

Vector	G418 resistant clones screened	Stable clones	% positive
pcDNA3.1B	11	9	82%
G90D-P2AβT	11	6	55%
P2AβT	51	11	22%

Table 4-1. Percentage of stable clones obtained per G418 resistant clones screened

growth rate of G90D-P2AβT and P2AβT stable lines that correlated with expression level of *PPP2R1B* (Figure 4-1).

Soft agar colony formation

Cellular ability to grow colonies in soft agar is a demonstration of anchorage independent cell growth, a hallmark of malignant cells, and has been shown to correlate well with in vivo cellular tumorigenicity. To test the effect of *PPP2R1B* on the tumorigenicity of the H2009, we plated stable cell lines containing pcDNA3.1B vector only, G90D-P2AβT or P2AβT in soft agar. We found that the pcDNA3.1B and G90D-P2AβT stable cell lines successfully form colonies in soft agar at variable rates. The P2AβT-1 cell line also formed

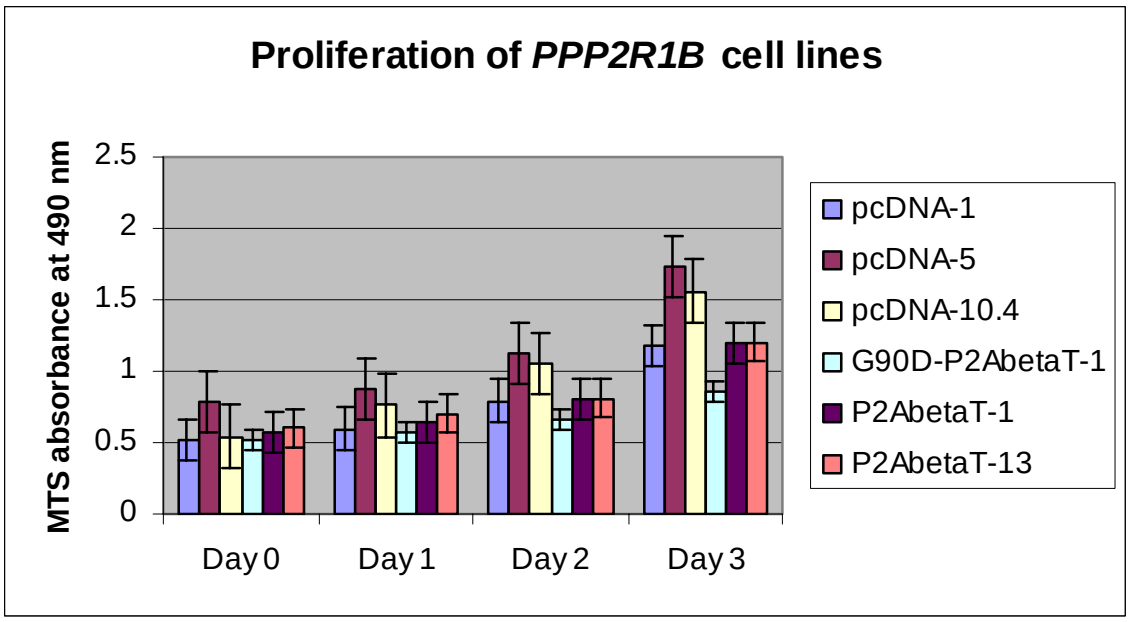

Figure 4-1. Proliferation comparison of *PPP2R1B* transfected stable cell lines. Cell lines were plated at equal density on Day 0 and monitored through 3 days of proliferation measured by the cells' ability to metabolize MTS. Though some variations in proliferation are observed, *PPP2R1B* does not appear to significantly alter cell proliferation compared to control cell lines.

colonies at rates comparable to control cell lines. The P2AβT-13 stable cell line formed 98% fewer soft agar colonies than the G90D-P2AβT-1 stable cell line (Table 4-2).

PPP2R1B regulates β-catenin levels

Overexpression of B56 family subunits of PP2A was shown to down regulate intracellular levels of β-catenin (Seeling et al., 1999). We found B56γ, one of the subunits shown to down regulate β-catenin levels, to have decreased affinity for the G90D mutation of PP2A-Aβ. These observations suggested the overexpression of *PPP2R1B* in the H2009

cancer cell line, which is haploinsufficient for *PPP2R1B*, might decrease the level of cellular β-catenin. We prepared lysates of our stable cell lines and did Western blots to measure the levels of β-catenin. We found the levels of β-catenin decreased in the P2AβT-1 and P2AβT-13 cell lines compared to lysates from the G90D-P2AβT-1 and pcDNA3.1B cell lines (Fig. 4-2). It is possible that higher levels of *PPP2R1B* overexpression would cause greater suppression of intracellular β-catenin. However, though the levels of V5 tagged PP2A-Aβ

	Number of colonies at 14 days, ave. (s.d.)		
Clone	Experiment 1	Experiment 2	Experiment 3
pcDNA-1	158 (3)	221 (31)	215 (28)
pcDNA-2	328 (7)		
pcDNA-5	176 (20)	506 (66)	562 (17)
pcDNA-10	203 (2)		
pcDNA-10.4	275 (36)	437 (19)	562 (27)
G90D-P2AβT-1	258 (41)	95 (23)	130 (7)
P2AβT-1	184 (35)	231 (14)	296 (26)
P2AβT-13	5 (4)	2 (3)	2 (1)

Table 4-2. Soft agar colony formation in *PPP2R1B* transfected H2009 NSCLC cell lines. Each experiment represents the average of 3 plates (standard deviation). Though all cell lines were capable of producing colonies in soft agar, the P2AβT-13 was the only cell line that showed a consistently and significantly suppressed ability to form colonies in soft agar compared to vector only and G90D-P2AβT control cell lines.

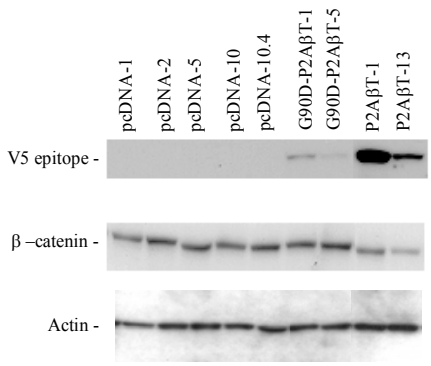

Figure 4-2. β–catenin expression in P2AβT stable cell lines. Stable cell lines with vector only (pcDNA), mutant *PPP2R1B* (G90D- P2AβT) or wildtype *PPP2R1B* (P2AβT) were lysed and Western blotted. The top blot with V5 epitope antibody indicates the levels of expression of the G90D-P2AβT and P2AβT constructs in the stable cell lines. β-catenin antibody in the middle blot shows the levels of cellular β-catenin in 10ug of total cell lysate from each cell line. Levels of β-catenin in P2AβT-1 and P2AβT-13 cell are decreased compared with control cell lines. The bottom blot shows the levels of actin as a loading control.

protein expression are higher in the P2AβT-1 cell line than in the P2AβT-13 cell line, the level of β-catenin suppression appears greater in the P2AβT-13 cell line, suggesting this is not a dosage dependent suppression.

CONCLUSIONS

The technical difficulty encountered in deriving stable clones expressing the P2AβT construct at high levels is notable, especially compared to the increased fraction of stable lines produced when using the G90D-P2AβT construct and even higher fraction with the pcDNA3.1B construct. This observation suggests the possibility that the PP2A-Aβ gene imbued a mild disadvantage on the transfected cells, allowing a smaller fraction to express the construct at a high level and continue to proliferate to cell line stability.

There appears to be no consistent correlation between the rate of cell growth, as measured by MTS reagent metabolism, and expression level of V5 epitope tagged PP2A-Aβ. Though pcDNA3.1B transfected cells had somewhat higher growth rates than G90D-P2AβT or P2AβT transfected cells, the fact that P2AβT-1 and P2AβT-13 cell lines actually grew faster than G90D-P2AβT-1 in culture suggests the levels of expression of *PPP2R1B* in P2AβT-1 and P2AβT-13 are insufficient to affect doubling time, or that *PPP2R1B* overexpression does not impact doubling time.

The suppression of soft agar colony formation observed in P2AβT-13 is statistically significant, and consistent with the degree of suppression observed in such assays done with

known tumor suppressor genes. However, when P2AβT-1 and P2AβT-13 are compared, the lack of suppression observed in P2AβT-1 combined with its higher level of V5 tagged PP2A-Aβ compared to P2AβT-13, makes it impossible to conclude the overexpression of *PPP2R1B* in P2AβT-13 is responsible for its suppressed ability to form colonies in soft agar, until this can be replicated by additional independent cell lines. It is likely that either P2AβT –1 or P2AβT –13 represents the true colony forming phenotype conferred by *PPP2R1B* overexpression, and that events separate from the transfection of *PPP2R1B* account for the confounding phenotype observed.

Elevated levels of β-catenin have been observed in multiple cancer types and correlated with an up regulation of cell growth. The ability of transfection with P2AβT to decrease β-catenin levels in H2009 cells when G90D- P2AβT transfection has no effect, suggests that the G90D mutant disrupts the ability of *PPP2R1B* expression to regulate β-catenin levels and that expression of *PPP2R1B* is important in regulating Wnt signaling. Though the affects of this down regulation of β-catenin were not observed in growth or soft agar colony assays, they may have been observed in the low percentage of stable P2AβT clones obtained by G418 selection, which suggests there is strong selection against overexpression of *PPP2R1B*, consistent with its proposed function as a tumor suppressor.

CHAPTER FIVE
Discussion

GENERAL DISCUSSION

The initial discovery of mutations in the PP2A-Aβ gene (*PPP2R1B*) in lung and colon cancer has been joined by reports of additional mutations of this PP2A subunit in breast, ovarian, cervical and colon carcinoma, and parathyroid adenoma (Calin et al., 2000; Campbell and Manolitsas, 1999; Pulido et al., 2000; Takagi et al., 2000; Hemmer et al., 2002). The PP2A-Aα gene (*PPP2R1A*) has been shown to carry a low frequency of alterations in breast and lung carcinomas and melanoma, and to have decreased protein expression in gliomas in the absence of mutations (Calin et al., 2000; Colella et al., 2001). Cancer associated mutations are not limited to PP2A subunit genes, as the *PPP1R3*, which encodes the regulatory subunit 3 of PP1, is found to be mutated in colorectal and ovarian carcinoma (Takakura et al., 2000). These findings suggest the PP2A-Aβ gene is the first of a new family of tumor suppressor genes comprised of genes encoding the subunits of the PP2A holoenzyme and possibly other phosphatase encoding gene products. An example is *PTEN*, a protein tyrosine phosphatase that is mutated in sporadic cancers and familial cancer syndromes, and acts as a tumor suppressor by negative regulation of the PI3K/Akt pathway by dephosphorylation of phosphatidylinositol (3, 4, 5)-triphosphate (PtdIns-3,4,5-P3) (Simpson and Parsons, 2001). It is likely that further cancer causing mutations will be identified in the regulatory and structural subunit genes of PP2A.

Evidence for alterations of the PP2A-Aβ contributing to inherited cancer susceptibility is observed in the presence of the G90D polymorphism in breast cancer patients' germline DNA and in the germline DNA of their high cancer risk family members. All of the individuals carrying the G90D alteration are heterozygotes and so far only one tumor has been shown to be a G90D homozygote. This suggests that some mutations of PP2A-Aβ may not fit the classical two-hit tumor suppressor gene model, but rather that they disrupt the delicate balance of interacting PP2A subunits, even in a heterozygote state, creating an intracellular environment which is susceptible to cancer development. The low frequency of PP2A-Aβ polymorphisms observed is likely due in part to the fact that though we screened several hundred patients, we only examined a single locus of the 1.8 kb PP2A-Aβ, leaving the possibility of having missed other alterations in the coding sequence that may contribute to cancer risk. Nevertheless, even a low frequency occurrence of the G90D polymorphism can be of tremendous significance in those cancer susceptible families where it is identified as a tool for early diagnosis and therapeutic management of cancer risk.

Functional analysis of PP2A-Aβ gene mutations occurring in human cancer has shown varying effects of individual mutations on the PP2A-Aβ protein and its ability to form heterotrimers. We have shown that a C-terminal point mutation (Asp504Gly) and C-terminal deletion (Δ230-518) in cell line H1450 both affect the ability of PP2A-Aβ to bind the catalytic PP2A-C subunit. We have also observed an N-terminal point mutation (Gly90Asp) in cell line H2009 that decreases PP2A-Aβ affinity for the B56γ subunit. Further analyses of cancer associated mutations have shown an N-terminal point mutation (Pro65Ser) and an N-terminal internal deletion (Δ344-388) which lower PP2A-Aβ affinity for the B'' family

subunit PR72, and two C-terminal point mutations (Val448Ala, Val545Ala) which abrogate PP2A-Aβ binding of PP2A-C subunit and B'' family subunit PR72 (Ruediger et al., 2001). These findings suggest mutations of PP2A-Aβ promote alterations in cell growth either by decreasing the total enzymatic activity of the PP2A-Aβ containing holoenzyme through changes in C subunit binding, or by lowering the activity of the holoenzyme toward specific substrates targeted by individual B subunits. The PR72 subunit, for example, may be important in targeting the PP2A holoenzyme to a specific substrate in the nucleus consistent with the observation that PR72 has a nuclear localization signal sequence (Ruediger et al., 2001). The only PP2A-Aβ gene mutation tested and not yet found to have a functional impact on PP2A is a Gly8Arg point mutation. This point mutation occurs in a 13 amino acid sequence at the extreme N-terminus of PP2A-Aβ. The Gly8Arg is of particular interest because it is located in a region which shares no homology with PP2A-Aα and may interfere with biological activity which is specific for the PP2A-Aβ subunit. That the Gly8Arg mutation has yet to be associated with the disruption of a specific PP2A subunit may be because it has not be analyzed against all known PP2A subunits, or because it regulates binding of an as yet undiscovered PP2A-Aβ interacting protein.

One of the challenges encountered in studying the protein effects of PP2A-Aβ gene mutations was the high protein sequence homology it shares with PP2A-Aα. These two isoforms are indistinguishable on Western blot due to their similar molecular weights (~1 kD difference) and the lack of a specific antibody which can differentiate the two isoforms. When we began this work antibodies were available to PP2A-Aα which cross reacted with

PP2A-Aβ, but there was no antibody against PP2A-Aβ. We were hopeful the previously mentioned 13 amino acid PP2A-Aβ unique N-terminal sequence would provide a basis for the development of a polyclonal peptide antibody. However, three attempts, with multiple test animals each time, yielded no specific PP2A-Aβ antiserum. These findings suggest it will be difficult to derive a useful PP2A-Aβ specific antibody. For this reason we constructed both wild type and epitope tagged PP2A-Aβ expression constructs for use in immunoprecipitation assays. *In vitro* and *in vivo* immunoprecipitation experiments showed the epitope tags of our PP2A-Aβ gene constructs did not alter their binding ability compared to wild type, confirming their suitability for use in stable transfections.

Selection pressure against the introduction of tumor suppressor genes into cancer cell lines would be expected and has been observed in the difficulty of obtaining stable, high expressing clones in cell model systems (Haber et al., 1993; Leone et al., 1991). Similarly, we observed that only 22% of the selected P2AβT clones we screened expressed levels of tagged PP2A-Aβ protein detectable by Western blot. We also observed a mild to dramatic decrease in expression of PP2A-Aβ protein in P2AβT cell lines with increasing passage number. This is consistent with a role for the PP2A-Aβ gene as a tumor suppressor. Also of note is that efforts to establish P2AβT stable cell lines by transfection of H1450 and H838 cell lines, both of which are null for the wild type PP2A-Aβ gene, were entirely unsuccessful, possibly due to a combination of the individual cell line's ease of transfection and selection pressure in cancer cell lines against tumor suppressor gene expression. The decreased percentage of G90D-P2AβT stable lines obtained compared to vector only lines

suggests that though the G90D mutant is deficient for binding of the B56γ subunit it is still able to bind other subunits and exert some influence on cell growth through pathways independent of B56γ. This is consistent with the observation that the G90D mutant does not affect PP2A-Aβ binding of the B'' PR72 subunit (Ruediger et al., 2001).

The PP2A-Aβ gene does not significantly influence the growth rate of H2009 stable cell lines. H2009 cells are heterozygous for the G90D alteration and the introduction of a wild type PP2A-Aβ gene into this potentially haploinsufficient state may not alter protein levels enough to impact cell proliferation. Absence of growth rate suppression, however, does not rule it out as a potential tumor suppressor. Other putative tumor suppressor genes exerting no detectable influence on growth rate have been shown to suppress anchorage independent growth and *in vivo* metastasis consistent with a tumor suppressor phenotype (Leone et al., 1991). The growth of the H2009 cell line is also influenced by other tumor suppressor genes and has been shown to be negative for RB expression and to carry a missense mutation in p53. It is possible that addition of the PP2A-Aβ gene is insufficient to down regulate cell proliferation in the presence of alterations in the activity of these tumor suppressors, suggesting it is a weaker tumor suppressor than RB or p53.

The inability of PP2A-Aβ gene expression to consistently suppress anchorage independent growth in stable cell lines raises similar questions about the sensitivity of our model system to detect changes in soft agar colony formation. The expression levels of the P2AβT constructs in stable cell lines appears to have been under constant down regulating selection pressure, making it difficult to overexpress PP2A-Aβ at the levels that may be necessary to visualize a change in colony formation. However, the dramatic suppression

demonstrated by the P2AβT-13 cell line compared to the P2AβT-1 cell line is additionally puzzling. P2AβT-1 expresses V5 tagged PP2A-Aβ protein at a higher level than does P2AβT-13, thus the tumor suppression observed in P2AβT-13 does not appear result from PP2A-Aβ gene expression. This could be due to stable integration of the P2AβT expression construct into a genomic site which results in the inactivation of an oncogene normally active in H2009. On the other hand, the P2AβT-13 cell line may represent an authentic PP2A-Aβ gene dependent non-tumorigenic phenotype. This could be the case if selection pressure against expression of the PP2A-Aβ gene in cell line P2AβT-1 combined with the need to express G418 resistance resulted in an accumulation of inactivating mutations in the endogenous or exogenous PP2A-Aβ gene coding region, abolishing the ability of PP2A-Aβ to suppress tumorigenesis in soft agar. These possibilities require further investigation.

The Wnt signaling pathway in humans is initiated as the Wnt glycoprotein bind its cell surface receptor. The Wnt signal is transduced through Dishevelled protein, which inactivates glycogen synthase kinase 3 (GSK3), a serine/threonine kinase. This allows cytoplasmic accumulation of β-catenin, which binds to Tcf/Lef family members, translocates to the nucleus and activates the transcription of targets such as cyclin D1 and c-*myc* (Bullions et al., 1998). In the absence of Wnt signal GSK3 is active and binds a complex containing APC, axin and β-catenin.. In this complex GSK3 phosphorylates β-catenin, which targets it for ubiquitination and subsequent degradation, decreasing overall levels of β-catenin (Seeling et al., 1999). Inactivating mutations of the tumor suppressor APC identified in human colon cancer that resulted in activation of β-catenin mediated transcription suggested a role for the

Wnt signaling pathway in oncogenesis (Morin et al., 1997). PP2A has been implicated in the regulation of Wnt because of the ability of B' and C subunits to bind APC and axin, respectively, and due to the down regulation of cellular β-catenin levels by overexpression of B' family subunits (Janssens and Goris, 2001; Seeling et al., 1999). The ability of exogenous PP2A-Aβ gene expression in NSCLC cells to down regulate total β-catenin levels supports a role for PP2A-Aβ in suppression of tumorigenesis and suggests a mechanism by which it may occur. A model for tumorigenesis through inactivation of PP2A-Aβ could be as follows. B56γ targets the PP2A holoenzyme containing PP2A-Aβ and catalytic C subunits to the APC-axin-GSK3-β-catenin complex, where PP2A could contribute to complex stabilization and possibly dephosphorylation of one of the complex members, such as activation of GSK3 by dephosphorylation. This leads to β-catenin phosphorylation and degradation. PP2A-Aβ with a G90D inactivating mutation is still able to bind C subunit, but the core dimer is less efficiently targeted by B56γ to the Wnt signaling complex due to decreased affinity for the PP2A-Aβ subunit. This decreases association of PP2A with the signaling complex possibly leading to complex destabilization and might result in GSK3 hyperphosphorylation and inactivation, allowing β-catenin translocate to the nucleus and promote cell growth.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The correlation of inherited alterations of the PP2A-Aβ gene with cancer susceptibility, demonstration that cancer associated mutations in PP2A-Aβ result in loss of PP2A enzyme function and observation that exogenous PP2A-Aβ expression down regulates β-catenin levels all give support to a role for the PP2A-Aβ gene in cell growth and tumorigenesis. However, additional evidence is needed to confirm the ability of PP2A-Aβ to suppress some aspect of cell growth *in vivo* and there is much yet to be understood about the interactions of PP2A-Aβ in the circuitry of cell signaling.

In order to firmly establish a growth phenotype dependent upon PP2A-Aβ expression, derivation of stable lines might be done using a different tumor cell line, much higher selection for the expression construct or a construct with a stronger promoter. This might enable isolation of stable lines with a higher, more stable expression of PP2A-Aβ which could result in a more clearly defined growth phenotype as determined by growth rate, soft agar colony analysis and *in vivo* tumorigenesis in nude mice.

To better understand the level of functional redundancy between PP2A-Aβ and PP2A-Aα studies focusing on the characteristics of their distinguishing sequences should be done. For example, a yeast two hybrid screen comparing the N-terminal sequences of the α and β isoforms in an effort to identify binding partners or substrates which are specific for a single isoform. The Gly8Arg mutated PP2A-Aβ could be screened against the known B subunits of PP2A in order to identify any binding is effects. This could lend insight into

specific functions of PP2A-Aβ in cell growth that cannot be compensated by PP2A-Aα as this mutation occurs in the 13 amino acid N-terminal sequence unique to PP2A-Aβ.

A broad screening of the changes in cellular serine/threonine phosphorylation may identify targets of PP2A-Aβ specific PP2A dephosphorylation. This could be done by transient transfection of cells with PP2A-Aβ. Total cell lysate of transfected vs. non-transfected cell could be run on a one or two dimensional polyacrylamide gel and blotted using serine or threonine phospho-specific antibodies. Observed differences between transfected and non-transfected could be further characterized by Western blot or peptide sequencing.

The evidence suggests that additional subunits of PP2A act as tumor suppressor genes. Mutational analysis of PP2A subunits, especially those observed to fall into regions of LOH in human cancer, will become increasingly more feasible as all of the subunit genes are eventually identified and their locations are determined through the efforts of the Human Genome Project.

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VITAE

Edward D. Esplin was born in Saint George, Utah, on September 3, 1970, the son of Helen Harmon Esplin and D. Glen Esplin. He graduated from Cottonwood High School, Salt Lake City, Utah, in 1988. He started coursework at the University of Utah in the fall of 1988. In the summer of 1989 he interrupted his studies to serve a mission for the Church of Jesus Christ of Latter-day Saints in the Zuerich, Switzerland Mission. In the fall of 1991 he completed his mission service and enrolled in Dixie College. He completed his Associate of Arts at Dixie College in June 1992. He continued his education at the University of Utah in the fall of 1992 and graduated in June 1995 with a Bachelor of Arts in Biology with Genetics emphasis and a minor in German. He entered the M.D./Ph.D. program at the University of Texas Southwestern Medical Center at Dallas in July 1995 as a fellow of the Medical Scientist Training Program. He was awarded the degrees of Doctor of Medicine and Doctor of Philosophy in June 2005. In 2001, he married Michal Zahavi of Petah Tikva, Israel.

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