"The most exciting phrase to hear in science, the one that heralds the most discoveries, is not 'Eureka!' (I found it!) but 'That's funny..."
Isaac Asimov

University of Alberta

Molecular Mechanisms Underlying the Regulation of Protein Phosphatase-1c by the Apoptotic Stimulating Proteins of p53

by

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To my husband Dan for all of his love and support

Abstract

Protein phosphatase-1c (PP-1c) is a ubiquitous serine/threonine protein phosphatase regulated in part by association with a large number of different regulatory subunits. Apoptotic Stimulating Proteins of p53 (ASPPs) are a family of proteins that regulate the apoptotic function of the tumour suppressor p53. In humans, there are three family members, ASPP1, ASPP2, and an inhibitory form, iASPP uniquely overexpressed in many cancers. Whilst iASPP lacks a tetrapeptide PP-1c RVXF binding motif, this work demonstrates that iASPP interacts with PP-1c via a previously uncharacterized"RARL" binding motif.

Molecular modeling and site-directed mutagenesis of PP-1c•ASPP protein complexes identified two novel modes of interaction between PP-1c and ASPP proteins. Firstly, a positively charged region of PP-1c, comprising residues Lys-260 and Arg-261, was found to be important for binding all three ASPP family members. Secondly, the PP-1c C-terminus was shown for the first time to contain a classic type-2 SH3 poly-proline binding motif (PxxPxR), which binds directly to the SH3 domain of ASPP proteins. Surface plasmon resonance (SPR) experiments further demonstrated that a peptide derived from the PP-1cα C-terminus alone binds with high affinity to iASPP (K=26 nM) and ASPP2 (123 nM).

The ability of the ASPP proteins to regulate PP-1c mediated dephosphorylation of p53 was examined. Gel filtration chromatography showed that binding between PP-1c, p53, and ASPP1/2 is mutually exclusive. In marked contrast, iASPP formed a stable PP-1c•iASPP•p53 trimeric complex. Dephosphorylation assays further demonstrated that formation of this complex facilitated site-directed dephosphorylation of p53 on residue Ser-15, but not Ser-37.

The results from this thesis provide support for the PP-1c•iASPP complex as a valuable drug target for cancer therapies. To investigate this, over 150 marine extract leads were tested for their ability to disrupt binding of PP-1c to iASPP, from which three marine compounds were isolated and identified: sokotrasterol sulphate

and two novel hippospongin-related compounds.

In conclusion, this PhD thesis provides for the first time a molecular explanation for how p53 may be regulated in part through interaction with iASPP and PP-1c. This study may aid in development of novel cancer therapies that specifically target the association between PP-1c and iASPP.

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ADP	adenosine diphosphate
AMP	adenosine monophosphate
AKAP149	A-kinase anchoring protein-149
APE-1	apoptotic protein enhancer -1
APP-BPI	amyloid precursor protein binding protein
Aq	aqueous
ASPP	apoptotic stimulating protein of p53
ATM	ataxia telangiectasia mutated protein
ATR	ataxia telangiectasia and rad3 related protein
ATP	adenosine 5' triphosphate
Bax	B-cell lymphoma-2 associated protein
BBP	Bcl-2 binding protein, shorter splice variant of ASPP2
BCAR-1	breast cancer anti-estrogen resistance protein -1
Bcl-2	B-cell lymphoma -2
BLAST	basic local alignment search tool
BRCA1	breast type 1 susceptibility protein
BSA	bovine serum albumin
C1P	ceramide-1-phosphate
Ca^{2+}	Calcium ion
CagA	cytotoxin-associated gene A protein from Helicobacter pylori
cAMP	cyclic adenosine monophosphate
CaN-A	calcineurin catalytic subunit A
CaN-B	calcineurin regulatory subunit B
CBP	CREB-binding protein, acetyl transferase that acetylates p53
CD45	cluster of differentiation - 45
cDNA	complementary deoxyribonucleic acid
CDC25	cell division cycle phosphatase - 25
cdk2	cyclin dependent kinase -2
cDNA	complementary DNA
Chk1/2	checkpoint kinases -1/2
CK2	casein kinase -2
CM5	carboxymethylated dextran-5
CReP	constitutive repressor of eukaryotic initiation factor-α phosphorylation
c-Src	cellular sarcoma protein
C-terminus	carboxy-terminus
DAG	diacylglycerol
DARPP-32	dopamine and cAMP-regulated phosphoprotein-32
DBD	DNA binding domain of p53
dCsk	C-terminal Src kinase
DDR	DNA damage response

Ddx42p	deadbox protein 42 protein
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	catalytic subunit of DNA-dependent protein kinase
DTT	dithiothreitol
E2F	E2 promoter binding factor
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylglycol-bis-(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immune sorbent assay
FCP	TFIIF-associating C-terminal domain phosphatase
Fe^{3+}	iron ion
FKBP-12	FK506 binding protein -12
FPLC	fast-performance liquid chromatography
GADD34	growth arrest and DNA damage protein -34
GADS	growth factor receptor bound protein-2 like adaptor protein
G_L	glycogen-binding subunit of PP-1 from liver
G1 phase	Growth phase -1, phase of the eukaryotic cell cycle in which growth of cell occurs
G_M	glycogen-binding subunit of PP-1 from skeletal muscle
Grb2	growth factor receptor bound protein -2
HCV	hepatitus C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus strain-1
iASPP	inhibitory apoptotic stimulating protein of p53
I-1	inhibitor-1; specific inhibitor of protein phosphatase-1c
I-2	inhibitor-2; specific inhibitor of protein phosphatase-1c
IC_{50}	concentration of inhibitor causing 50% inhibition of an enzyme activity
IPTG	isopropyl-b-D-thiogalactoside
IRS	insulin receptor sustrate -1
KAP1	kruppel-asskciated protein -1
Ku70/80	regulatory proteins that bind to DNA-dependent protein kinase
Lats2	large tumour suppressor protein -2
LB	Luria-Bertani broth
Lyso-PA	lysophosphatidic acid
Masp	D-erythro-β-methyl aspartic acid
Mdha	N-methyldehydroalanine
MC	microcystin
MC-Sepharose	microcystin-Sepharose
Mdha	N-methyldehydroalanine
mdm2	mouse double minute -2 protein
Mg^{2+}	magnesium ion

Mn^{2+}	manganese ion
M phase	mitotic phase, phase of the eukaryotic cell cycle in which mitosis occurs
MYPT1	myosin phosphatase targeting subunit 1
Nck1	non-catalytic region of tyrosine kinase adaptor protein -1
Nek2	never in mitosis A related kinase 2
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor -κB
NHEJ	non-homologous end joining
NHS	N-hydroxysuccinimide
Ni-NTA	nickel-nitriloacetic acid
NIMA	never in mitosis-A protein
NIPP1	nuclear inhibitor of protein phosphatase-1c
NMR	nuclear magnetic resonance
N-terminus	amino-terminus
NUAK	family of AMP-activated protein kinases
OA	okadaic acid
p53BP2	p53 binding protein-2; also known as ASPP2
p21	cyclin-dependent kinase inhibitor-1
p65	Rel A subunit of nuclear factor -κB
p300	acetyl transferase that acetylates p53
p53	tumour suppressor protein-53
p53BP1	p53 binding protein -1
p53BP2	p53 binding protein -2, also referred to as ASPP2
Par-3	partitioning defective -3
PCR	polymerase chain reaction
PA	phosphatidic acid or phosphatidate
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PIG3	p53-inducible gene -3
Pin-1	peptidyl-prolyl cis/trans isomerase never in mitosis-A-interacting -1
***p*NPP**	para-nitrophenolphosphate
PNUTS	phosphatase-1 nuclear targeting subunit
PP-1c	protein phosphatase-1 catalytic subunit
PP-2A	protein phosphatase-2A
PP-2Ac	protein phosphatase-2A catalytic subunit
PP-2B	protein phosphatase-2B
PP-4	protein phosphatase-4
PP-5	protein phosphatase-5
PP-6	protein phosphatase-6
PP-7	protein phosphatase-7
PPM	metal-dependent protein phosphatase
PPP	phosphoprotein phosphatase gene family
PMSF	phenylmethylsulfonyl fluoride

pRb	retinoblastoma protein
PRD	proline rich domain of p53
PR-enzyme	prosthetic-removing enzyme
PSI-BLAST	position specific iterated - basic local alignment search tool
PTP	phospho-tyrosine Phosphatase
pSer	phosphorylated serine
pThr	phosphorylated threonine
pTyr	phosphorylated tyrosine
PUMA	p53 unregulated modulator of apoptosis
Ras	rat sarcoma protein
REG	C-terminal regulatory domain of p53
RARL	non-classical tetra peptide RVXF PP-1c binding motif found in iASPP
RVXF	tetrapeptide PP-1c binding motif
RNA	ribonucleic acid
R-subunits	protein phosphatase-1c regulatory subunits
SCP	small C-terminal domain phosphatase
SCUBA	self-contained underwater breathing apparatus
SDS-PAGE	polyacrylamide gel electrophoresis performed in the presence of sodium dodecyl sulfate
SH3	Src homology domain -3
SHP1/2	src homology region 2 domain containing phosphatase - 1/2
SIPP1	splicing factor that interacts with PXBP-1 and PP-1
S phase	phase of the eukaryotic cell cycle in which DNA synthesis occurs
TAD 1/2	transactivation domains 1 and 2 of p53
TEAD	transcriptional enhancer activator domain
TET	tetramerization domain of p53
TFA	trifluoroacetic acid
TGF-β	transformating growth factor-β
TIMAP	transforming growth factor-β inhibited membrane associated protein
WIP1	wild-type p53-induced phosphatase-1
YAP1	yes-associated protein -1

Standard Amino Acids

Glycine	Gly	G
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Methionine	Met	M
Proline	Pro	P
Phenylalanine	Phe	F
Tryptophan	Trp	W
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y
Asparagine	Asn	N
Glutamine	Gln	Q
Cysteine	Cys	C
Lysine	Lys	K
Arginine	Arg	R
Histidine	His	H
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E

Units and Constants

$\mathring{A}$	Angstroms ($10^{-10} metres$)
°C	degrees Celsius
cpm	counts per minute
g	acceleration due to gravity
K_d	dissociation constant
kDa	kiloDaltons
M	molar (moles per litre)
ml	millilitres (10^{-3} litres)
mM	millimolar (10^{-3} moles per litre)
nM	nanomolar (10^{-9} moles per litre)
min	minutes
ng	nanogram (10^{-9} grams)
pH	-log of the concentration of H^+ in solution
RU	Resonance units
w/v	weight per volume
μl	microlitres (10^{-6} litres)
μM	micromolar (10^{-6} moles per litre)

Chapter 1

Introduction

1.1 Reversible Phosphorylation

Reversible protein phosphorylation is an important post-translational modification involved in the regulation of most eukaryotic cellular functions, including metabolism, contractibility, membrane transport, and cellular division. This highly dynamic process is regulated by the combined actions of protein kinases and phosphatases, which catalyze the addition or removal of phosphate groups from serine (Ser), threonine (Thr), tyrosine (Tyr), and histidine (His) residues. Phosphorylation or dephosphorylation of proteins can directly affect the stability of the protein, biological activity, subcellular localization, and affect its interaction with other proteins [47].

Recently, it has been shown that nearly 70% of all eukaryotic proteins are modified by phosphorylation during the cell cycle. [226]. The majority of protein kinases (more than 380 out of the ∼500 protein kinases in humans) phosphorylate Ser/Thr residues, while the remainder phosphorylate Tyr residues [66]. While there are greater than 300 Ser/Thr protein kinases within the cell, there are only a handful of Ser/Thr protein phosphatases (∼20 in humans). Due to the relatively low numbers of protein phosphatases and their ability to dephosphorylate multiple substrates, protein phosphatases were originally thought of as mere "house-keeping" proteins that opposed the actions of protein kinases with little to no specificity. Over the past two decades research has shown that protein phosphatases are highly regulated through multiple layers of control.

One example of reversible phosphorylation is the phosphorylation/dephosphorylation of glycogen phosphorylase (Figure 1.1). Glycogen phosphorylase, also referred to simply as phosphorylase, is the enzyme involved in catalyzing the breakdown to glycogen to produce glucose-6-phosphate. The enzyme was originally isolated by Carl and Gerty Cori in the late 1930s [92, 50] and was shown to exist in two forms: an active form (phosphorylase a) and inactive form (phosphorylase b). Two proteins were later isolated and characterized as the enzymes responsible for this conversion: a "Prosthetic-Removing"(PR) enzyme (later renamed protein phosphatase-1c) and a converting enzyme (later renamed phosphorylase kinase) [50, 142, 143]. These two enzymes were shown to interconvert phosphorylase a/b by the addition or removal of an inorganic phosphate. Since this discovery, researchers have identified many additional Ser/Thr protein kinases and a handful of Ser/Thr protein phosphatases

encoded by the human genome, which play important roles in almost every signalling pathway within the eukaryotic cell.

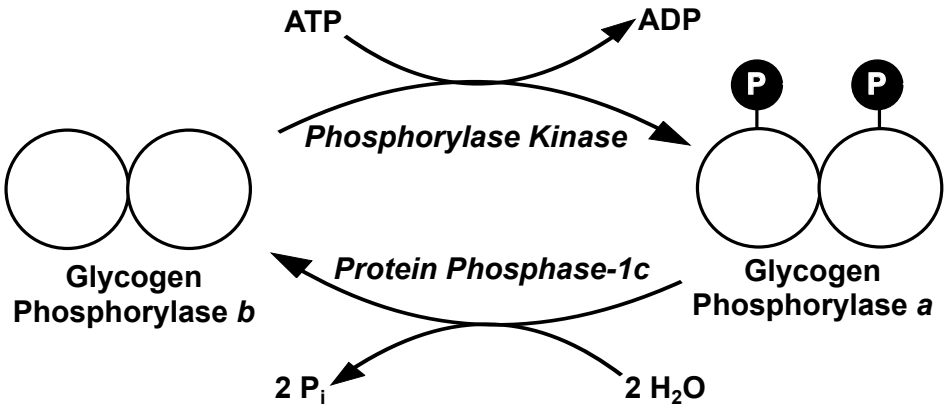

Figure 1.1: **Regulation of Glycogen Phosphorylase Activity by Reversible Phosphorylation**. Glycogen phosphorylase *b* is a homodimer activated in response to epinephrine/glucagon signalling via phosphorylation of residue Ser-14 by phosphorylase kinase. Insulin signalling promotes inactivation of glycogen phosphorylase through the dephosphorylation of phosphorylase *a* by protein phosphatase-1c forming the dephosphorylated form phosphorylase *b*.

1.2 Serine/Threonine Protein Phosphatases

Protein phosphatases catalyze the removal or hydrolysis of phosphate groups from phospho-serine (pSer), phospho-threonine (pThr), and phospho-tyrosine (pTyr) residues. Mammalian protein phosphatases are categorized into four main superfamilies: the Phospho-Tyrosine Phosphatase (PTP) family, the Phosphoprotein Phosphatase (PPP) family, the Metal-dependent Protein Phosphatase (PPM) family and the Aspartate-based family of Phosphatases (Table 1.1) [246, 210]. PTP family members dephosphorylate mainly pTyr residues (with the exception of the dual specificity phosphatases), while the remaining three phosphatase families dephosphorylate pSer and pThr residues. The PPP, PPM, and Aspartate-based phosphatases have unrelated gene sequences and are thought to have evolved independently from three ancestral genes.

The PPP phosphatase family is categorized further into four groups [PP-1c, PP-2A, calcineurin (CaN) and PP-2C] based on their sensitivity to endogenous inhibitor

Table 1.1: **Nomenclature of Eukaryotic Protein Phosphatases** CD45, Cluster of Differentiation Protein-45; CDC25, Cell Division Cycle Phosphatase-25; FCP, TFIIF-associating C-terminal Domain Phosphatase; SCP, Small C-terminal Domain Phosphatase; SHP1/2, Src Homology Region 2 Domain Containing Phosphatase 1/2; WIP1, Wild-type p53-Induced Phosphatase-1.

Phosphoprotein Phosphatase (PPP) Family
PP-1c
PP-2Ac Type Phosphatases – *PP-2Ac, PP-4, PP-6*
PP-2B (Calcineurin or PP3)
PP-5
PP-7
Metallo-Dependent Protein Phosphatase (PPM) Family
Pyruvate Dehydrogenase Phosphatase
PP2C Subfamily – *PPM1A, PPM1B, PPM1C, PPM1D (WIP1)*
Phospho-Tyrosine Phosphatase (PTP) Family
Tyrosine-specific Phosphatases – *PTP1B, SHP-1, SHP-2, CD45*
Dual Specificity Phosphatases – *CDC25, MAP kinase phosphatase-1*
Aspartate-Based Phosphatase Family
FCP/SCP phosphatases

proteins (inhibitor-1 and inhibitor-2), dependence on divalent cations, and substrate specificity in vitro (Tables 1.1 and 1.2) [46]. Since their original classification, other members of this gene family have been identified and include PP-4, PP-5, PP-6, and PP-7 [212, 283]. Structurally and functionally, PP-4 and PP-6 both closely resemble the PP-2A catalytic subunit (PP-2Ac) and form similar heterodimeric and heterotrimeric complexes required for their enzymatic activity and substrate targeting [212].

Table 1.2: **Characterization of the PPP Family Protein Phosphatase Catalytic Subunits**. IC_{50} refers to the concentration of inhibitor that results in 50% inhibition of the enzyme activity. MC, microcystin, OA, okadaic acid.

	PP-1c	**PP-2Ac**	**PP-2B**
Molecular Mass (kDa)	37	36	61
Divalent Cation Requirement	Mn^{2+}/Zn^{2+} $/Fe^{3+}$	Mn^{2+}/Zn^{2+} $/Fe^{3+}$	Ca^{2+} and Mn^{2+} $/Zn^{2+}/Fe^{3+}$
Inhibition by Inhibitor-1 and Inhibitor-2	yes	no	no
Inhibition by OA	yes IC_{50} = 20 nM	yes IC_{50} = 0.2 nM	weak
Inhibition by MC	yes IC_{50} = 0.2 nM	yes IC_{50} = 0.2 nM	weak

1.2.1 Protein Phosphatase-2A

Protein phosphatase-2A (PP-2Ac) is the most abundant protein phosphatase in cells and accounts for up to 2% of the total protein content in most tissues. Therefore, it is not surprising that PP-2A plays an important role within cells, and deregulation of PP-2A activity results in diseases such as cancer and Alzheimer's disease [64, 173]. PP-2A exists within cells as either a heterodimer or a heterotrimer complex. The heterodimeric core complex is made up of two subunits: a scaffold subunit (A or PR65 subunit) and a catalytic subunit (PP-2Ac or C subunit). The core heterodimeric complex binds to many regulatory subunits (B subunits) forming multiple heterotrimeric holoenzyme complexes (Figure 1.2).

In mammals, there are approximately 15 unique PP-2A B subunits, divided into four gene families (B, B', B", B"') based on sequence homology. By binding to a specific B subunit, PP-2Ac is targeted to specific locations and substrates [253]. Post-translational modification of PP-2Ac, including methylation and phosphorylation of the PP-2Ac C-terminus directly affects the ability of PP-2Ac to associate with specific B subunits [180, 64, 256]. The crystal structure of PP-2A holoen-

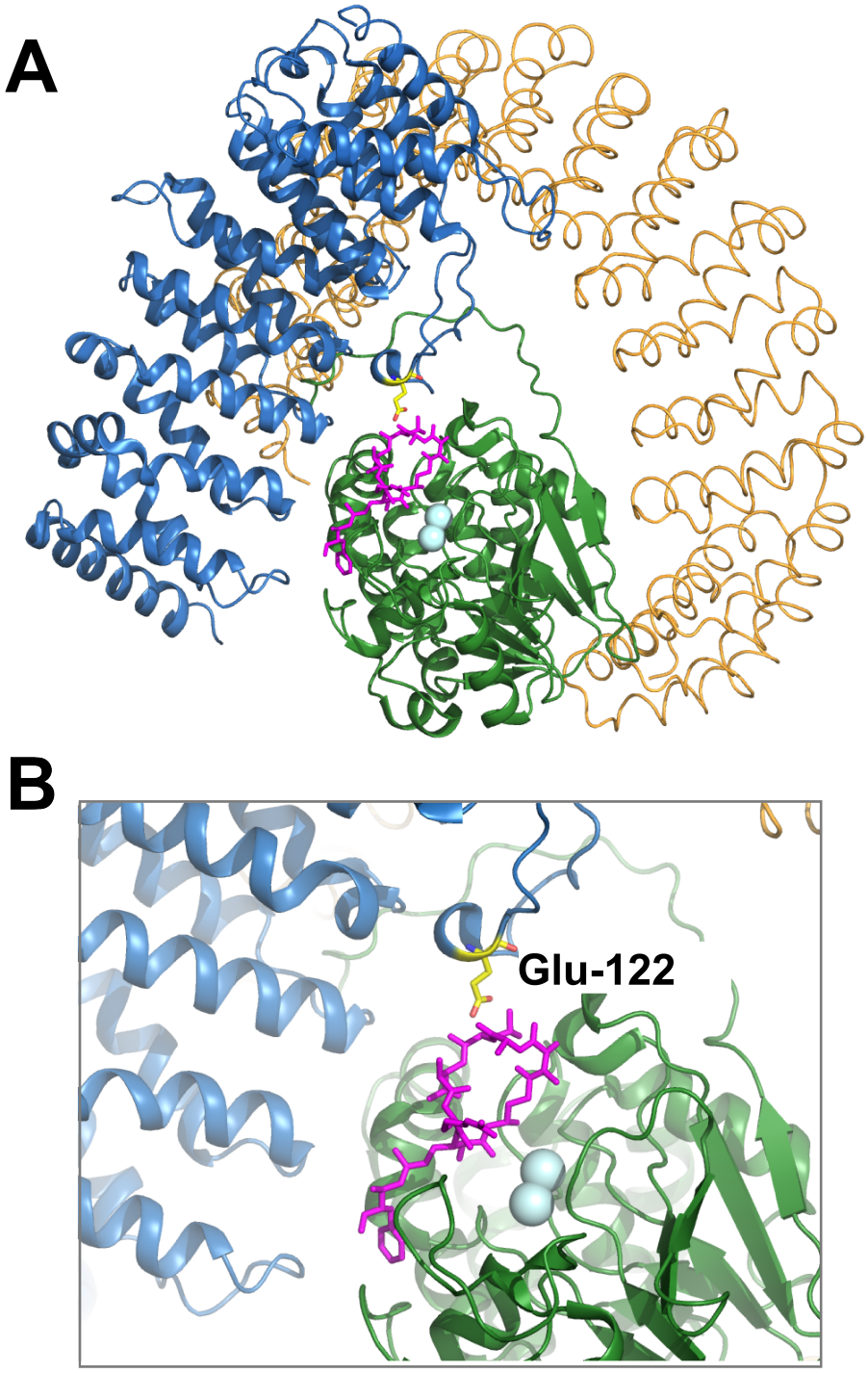

Figure 1.2: **The Structure of the PP-2A Trimeric Holoenzyme Complex**. *Panel A* depicts a cartoon representation of the crystal structure of recombinant human PP-2Ac (shown in blue, pdb# 2NPP) [299] bound to the PP-2A scaffolding A subunit (shown in orange) and the B'γ regulatory subunit (shown in green). The cyanotoxin microcystin-LR (shown in magenta sticks) is bound with the active site of PP-2Ac. *Panel B* depicts a close-up of the PP-2Ac active site, highlighting the position of residue Glu-122 (E122) from the B'γ subunit that is proposed to be important for substrate targeting.

zyme complex bound to the B'/B56 subunit, revealed direct interactions between the PP-2A catalytic subunit and a conserved loop of the B' subunit (Figure 1.2) [299]. Furthermore, mutational analysis has showed that residue Glu-153 within the loop of the B'β subunit (equivalent to residue Glu-122 in B'γ) is required for the dephosphorylation of the substrate tyrosine hydroxylase. It is hypothesized that positively charged residues upstream of the targeted phosphorylation site within target substrates may be part of a consensus sequence for B'-mediated PP-2A dephosphorylation [299, 240].

1.2.2 Calcineurin/Protein Phosphatase-2B

Unlike PP-1c and PP-2Ac, calcineurin (also referred to as PP-2B) is regulated in part by calcium (Ca^{2+}) and the Ca^{2+} binding protein calmodulin. The calcineurin core enzyme consists of a heterodimer comprising a catalytic A subunit (CaN-A) and a regulatory B subunit (CaN-B) (Figure 1.3). CaN-A contains four main domains: a catalytic domain, two C-terminal regulatory binding domains (for binding to CaN-B and calmodulin) and an auto-inhibitory domain at the extreme C-terminus. The catalytic domain of CaN-A is approximately 40 % identical to PP-1c, while the other regulatory domains are not present in either PP-1c or PP-2Ac. Calcineurin requires the binding of calmodulin in order to be fully active, which causes a conformational change of the CaN-A structure and induces the displacement of the CaN-A C-terminal auto-inhibitory domain (residues 469-486) from the CaN-A catalytic active site [164].

Calcineurin regulates many calcium-dependent signalling pathways, including hematopoiesis and the innate and adaptive immune response [80]. The most studied is its role in the dephosphorylation and activation of NFAT (Nuclear Factor of Activated T-cells) during the adaptive immune response. Extracellular events, such as the binding of an antigen to a T-cell receptor, induces the influx of Ca^{2+} into the cytoplasm from endoplasmic reticulum stores, which in turn promotes the binding of calmodulin and activation of calcineurin. Calcineurin interacts directly with NFAT via the calcineurin binding motif, PxIxIT (PRIEIT in NFAT1) ([165]. The dephosphorylation of NFAT by calcineurin activates NFAT, promoting its translocation from the cytoplasm into the nucleus. Within the nucleus, NFAT acts as a transcription factor, inducing the expression of various target genes involved in the immune response and hematopoiesis, such as interleukin-2 and interferon-γ [80].

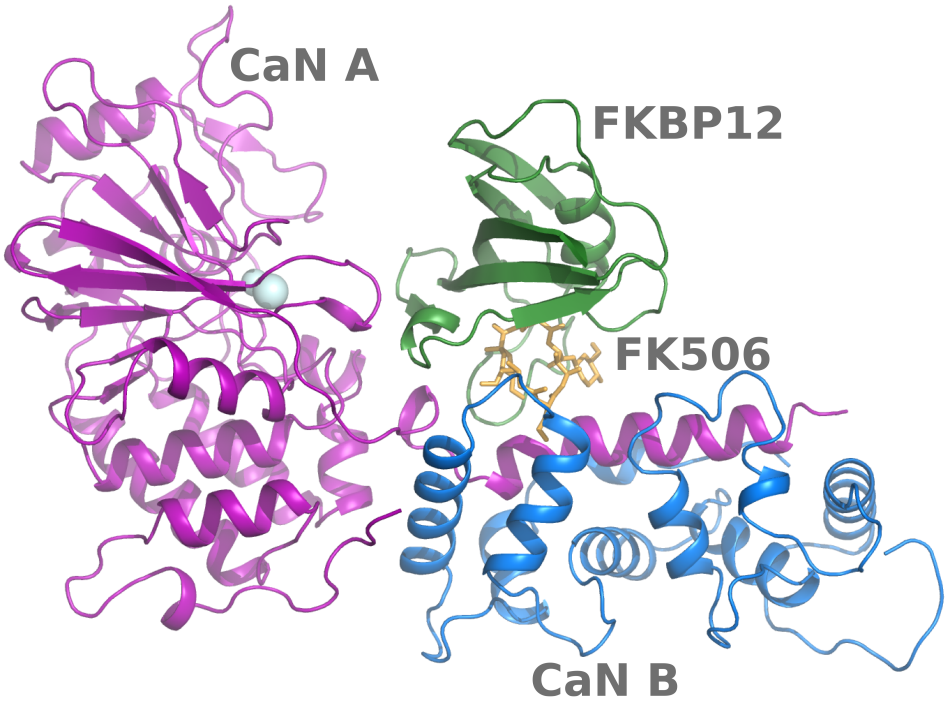

Figure 1.3: **The Structure of the Calcineurin Complex**. The figure depicts a ribbon representation of the crystal structure of the native bovine calcineurin catalytic A subunit (CaN-A, shown in magenta, pdb# 1TC0) [93], bound to the native bovine calcineurin regulatory B subunit (CaN-B, shown in blue), FK Binding Protein-12 (FKBP12) (shown in green) and the FK506 inhibitor (shown in pale orange).

Multiple studies have shown that dysregulation of calcineurin-NFAT signalling is involved in many diseases including cardiac hypertrophy, autoimmune diseases, osteoporosis, and cancer [207, 243, 265, 191]. Calcineurin is a billion dollar protein drug target for the immunosuppressive drugs cyclosporin and FK506, which have been used to prevent organ rejection during organ transplantation for over 20 years [268]. Cyclosporin and FK506 bind indirectly to calcineurin via the immunophilin proteins cyclophilin and FKBP-12 (FK506 Binding Protein-12), respectively (Figure 1.3) [122, 136]. Cyclosporin and FK506 inhibit calcineurin activity, which prevents the dephosphorylation of NFAT and blocks the innate immune response [245].

1.3 Protein Phosphatase-1c

PP-1c was first identified as prosthetic-removing (PR) protein that dephosphorylates glycogen phosphorylase in glycogen metabolism. In mammals, there are three protein phosphatase-1c isoforms, PP-1c α, β, and γ, which share 76 – 88 % sequence identity and differ mainly within their N and C-termini. Today, we now know that PP-1c and PP-2Ac carry out over 90% of all Ser/Thr protein dephosphorylation reactions in eukaryotes.

1.3.1 The 3-Dimensional Structure of the Catalytic Subunit of Protein Phosphatase-1c

All PPP family members share a similar structural fold and differ mainly within their solvent exposed loops [26]. The first x-ray crystal structure of the bacterially expressed PP-1 catalytic subunit (PP-1cα) was identified by Goldberg et al. in 1995 and was shown to adopt an α/β fold, comprising two alpha helical domains separated by a β sandwich [89, 246]. The boundaries of these domains create three shallow grooves (acidic, C-terminal, and hydrophobic grooves) that facilitate in the binding of ligands (Figure 1.4). The catalytic active site of PP-1c is located within the intersection of these three grooves and contains two metal ions (Mn^{2+} in the recombinant form of PP-1c) bound close together ($\sim 3\mathring{A}$ apart) that are directly involved in catalysis (described in detail below in Section 1.3.2). The identity of the metal ions is assumed to be Mn^{2+}, however this still remains controversial, as all crystal structures to date have used recombinant PP-1c and the occupancy of the metal binding sites is dependant on the type of metal added during bacterial expression and purification. Within the crystal structures of native bovine calcineurin [93]

and kidney bean purple acid phosphatase [259] Fe^{2+} and Zn^{2+} are present within the active site of each phosphatase. Additionally, Chu et al. (1996) [44] demonstrated that the addition of both $ZnCl_2$ and $FeCl_2$ to a solution of recombinant PP-1cα significantly activates the PP-1 catalytic subunit compared to when other metals are added including either $ZnCl_2$ and $FeCl_2$ added alone. Together this suggests that within the native protein phosphatase-1 catalytic subunit both Fe^{2+} and Zn^{2+} are bound within its active site.

1.3.2 Catalytic Mechanism of Protein Phosphatase-1c

Due to the similarities of the three-dimensional structures of PP-1c, PP-2Ac and calcineurin, all three phosphatases are thought to facilitate substrate hydrolysis via a similar catalytic mechanism. A schematic of the proposed mechanism for the PP-1c hydrolysis reaction is shown in Figure 1.6 [89, 62]. Metal binding is facilitated by a water molecule and an oxygen atom from a neighbouring Asp residue (Asp-92), which forms a bridge between the two ions. In addition, five PP-1c residues (His-66, Asp-64, Asn-124, His-173, and His-248) assist in the octahedral hexacoordination of the two metal ions. Hydrolysis of the phosphomonoester protein substrate is proposed to occur in one step, via a nucleophilic attack of a metal-activated water molecule [89, 15]. Two arginine residues (Arg-96 and Arg-221) position the phosphate group within the active site and are essential for catalysis. Mutation of either Arg-96 or Arg-221 results in a catalytically dead enzyme [112]. A salt bridge between Asp-95 and His-125 positions the His-125 residue such that it can donate a proton to the leaving oxygen group of the phosphate molecule. His-125 is conserved between PP-1c, PP-2Ac, and PP-2B and mutation of His-125 in PP-1c results in loss of its catalytic activity [308].

1.3.3 Native Versus Recombinant Protein Phosphatase-1

The biochemical properties of native and bacterially expressed recombinant PP-1c differ with respect to their substrate specificity, sensitivities to inhibitor-1 (a PP-1c regulatory protein that inhibits PP-1c activity), and dependance on metals for efficient expression and activity [4, 184]. While native and recombinant PP-1c have similar activities towards phosphorylase *a*, recombinant PP-1c has increased activity towards histone H1, casein, and pTyr substrates [4]. In addition, recombinant PP-1c requires the presence of 1 mM $MnCl_2$ for proper expression and activity and is less

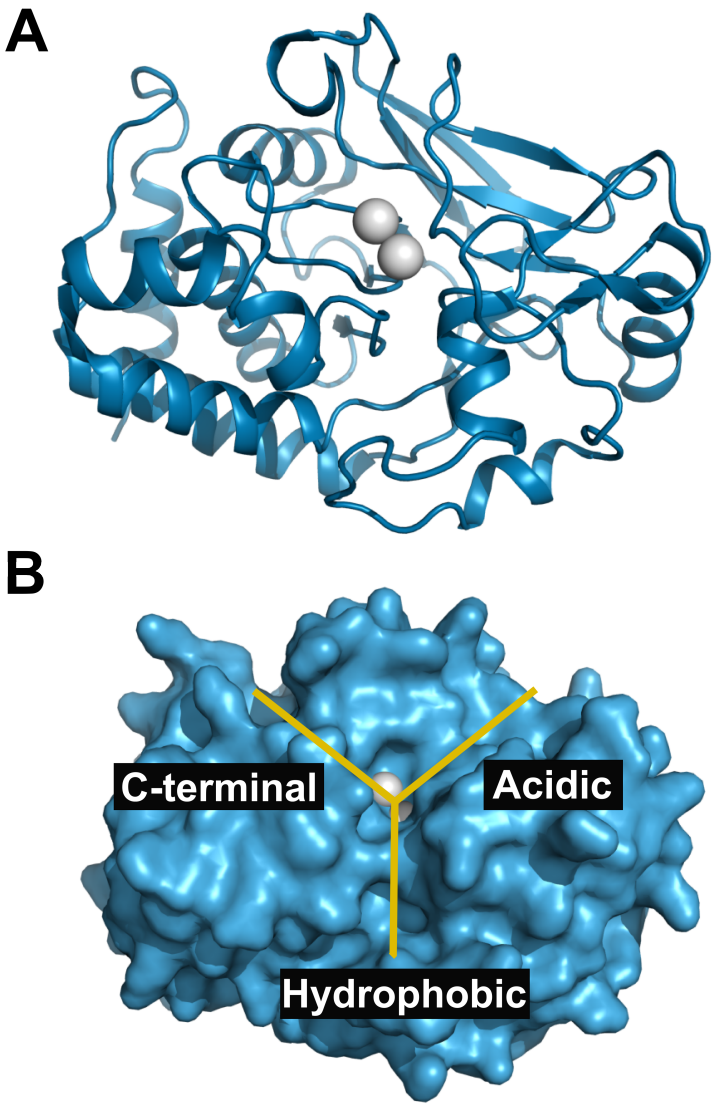

Figure 1.4: **The 3-Dimensional Structure of the Catalytic Subunit of Protein Phosphatase-1**. *Panel A* depicts a cartoon representation of the crystal structure of recombinant human protein phosphatase-1 catalytic subunit γ isoform (shown in blue, pdb# 2BDX) [195]. *Panel B* depicts a surface representation of protein phosphatase-1cγ, highlighting the three major grooves that surround that catalytic active site of the enzyme [195]. Light grey spheres represent the Mn^{2+} ions bound within the active site of PP-1c.

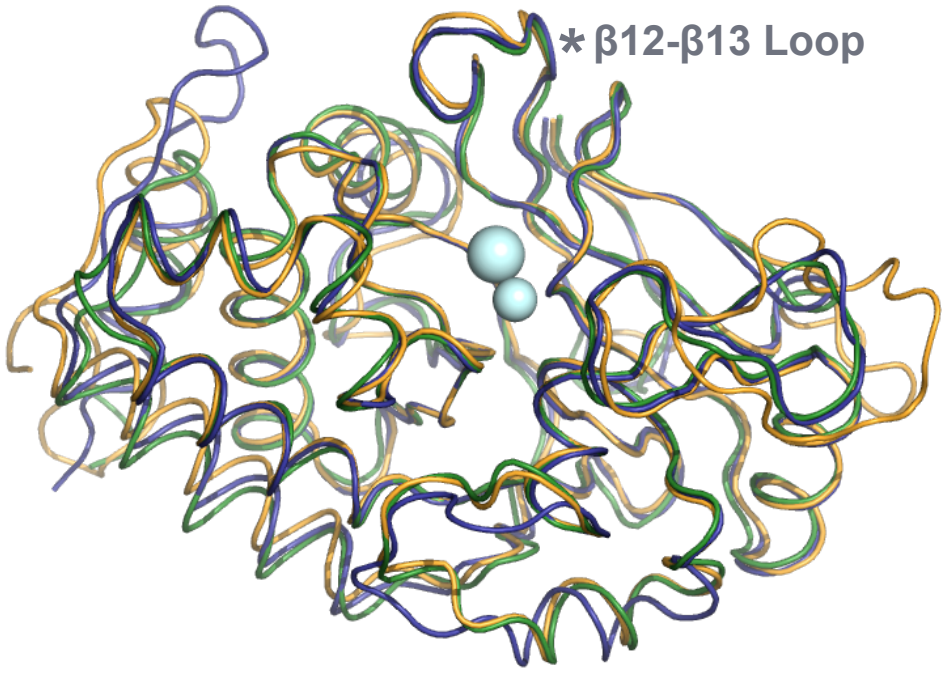

Figure 1.5: **Comparison of the Crystal Structures of PP-1c, PP-2Ac, and Calcineurin**. Figure depicts the alignment of recombinant human PP-1cγ (pdb#1JK7), recombinant human PP-2Ac (pdb#2IE4), and native bovine calcineurin (pdb#1AUI) [194, 298, 136]. The colour scheme is as follows: PP-1c, blue; PP-2Ac, green; calcineurin, orange. The β12–β13 loop of PP-1c and PP-2Ac (equivalent to the L7 loop in calcineurin) is labeled with an asterisk.

sensitive towards inhibitor-1 (by 100 to 1000 fold), okadaic acid and microcystin [4]. The mechanisms for these differences are not well understood.

Whilst to date there is no characterized structure of the native protein phosphatase-1c protein, structural comparison between the native calcineurin [93] and recombinant forms of calcineurin and PP-1c [136, 269, 194, 234, 115] reveal very few structural differences between native and recombinant forms of the protein phosphatases (Figure 1.7). The only significant difference observed is the positioning of PP-1c residue Arg-96 (equivalent to Arg-122 in calcineurin), whereas the positions of all other important residues within the active site remain unchanged. Since residue Arg-96 of PP-1c is critical for enzyme catalysis (see Section 1.3.2 above), increased flexibility of Arg-96 within the recombinant PP-1c protein may alter its ability to dephosphorylate substrates and increase or decrease its sensitivity towards small molecule inhibitors compared to the native PP-1c enzyme.

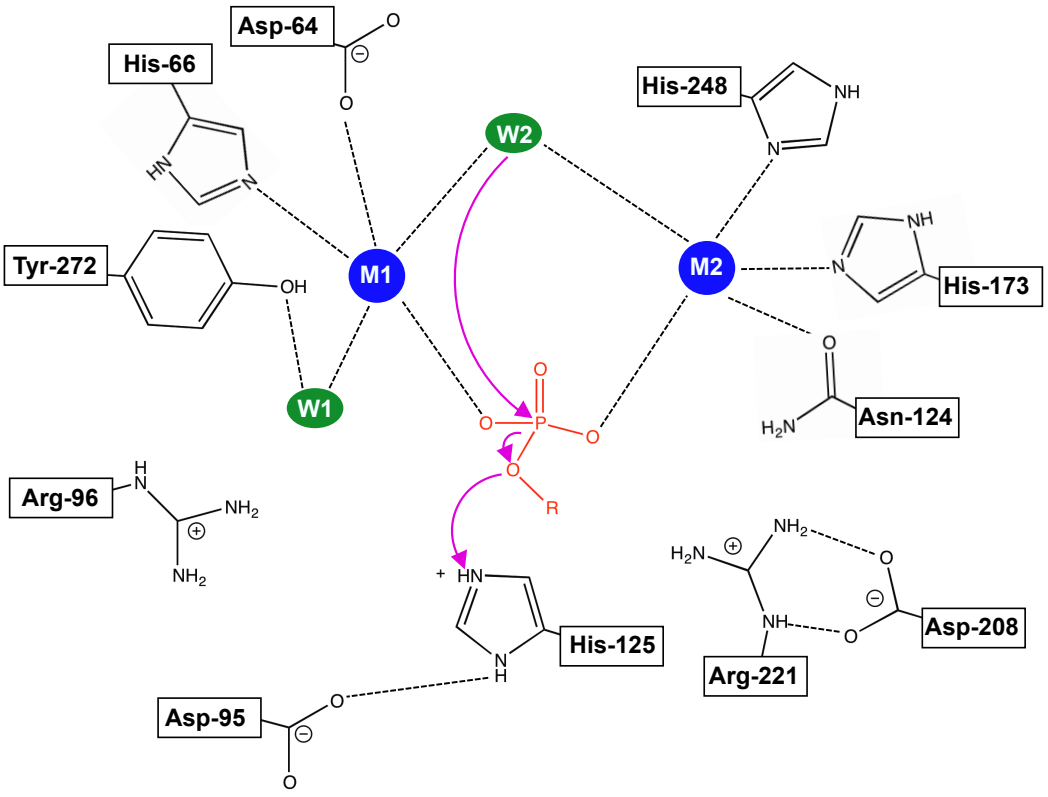

Figure 1.6: **A Schematic of the Proposed Mechanism for Substrate Hydrolysis by Protein Phosphatase-1c**. Figure depicts the coordination of metal ions and substrate phosphate group within the active site of PP-1c. The metal ions (M1 and M2) are coordinated within the active site via the interaction with five PP-1c residues (His-66, Asp-64, Asn-124, His-173, and His-248) and two water molecules (W1 and W2). Figure was adapted from [89, 62].

Previously, the crystal structure of recombinant human PP-1c alone revealed the presence of electron density within the acidic groove of PP-1c corresponding to 4–5 residues of the PP-1c C-terminal tail (Maynes et al., unpublished observation). The remainder of the tail was not visible and at the time it was not known what to make of this density. However, we now hypothesize that this provides structural evidence to suggest that the C-terminal tail of PP-1c can fold back towards the active site, interacting with negatively charged residues within the acidic groove. This could have a significant impact when investigating the PP-1c isoform specificity of various PP-1c regulatory subunits, as the sequences of the PP-1c α, β, and γ C-terminal

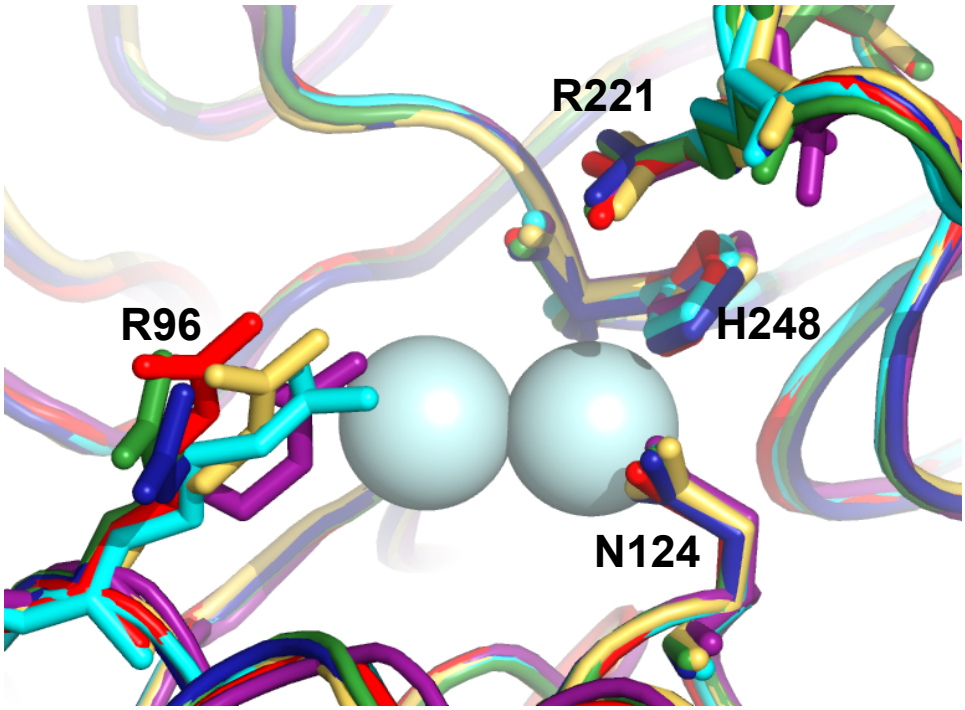

Figure 1.7: **A Comparision of the Active Sites of Calcineurin and Recombinant PP-1c Bound to Various Regulatory Proteins or Toxins**. The colour scheme is as follows: native bovine CN, purple (pdb# 1TCO) [93]; recombinant human PP-1cγ•Microcystin-LA, green (pdb# 2BDX) [195]; recombinant human PP-1cγ•okadaic acid, dark blue (pdb# 1JK7) [194]; recombinant chicken PP-1cβ•MYPT1, red (pdb# 1S70) [269]; recombinant human PP-1cα•spinophilin, cyan (pdb# 3EGG) [234]; and recombinant rat PP-1cγ•inhibitor-2, yellow (pdb# 2O8A) [115]. The two Mn^{2+} ions from the PP-1c•microcystin-LA structure are shown as light blue spheres.

tails differ greatly and are thought to mediate isoform specificity.

Binding of the PP-1c C-terminal tail within the acidic groove could explain several differences observed between the native and recombinant forms of PP-1c. For instance, recombinant PP-1c has been shown to be over 100 times less sensitive to inhibitor-1 [4]. Mutational studies have previously shown that inhibitor-1 binds within the acidic groove of PP-1c [112]. If the C-terminal tail of PP-1c folds back towards the active site and binds within the acidic groove then it would hinder the ability of inhibitor-1 to bind to PP-1c. This is supported by the fact that mutation of residues within the acidic groove (specifically Asp-220, Glu-256, and Glu-275) to either Ala or Arg increase the sensitivity to inhibitor-1 or DARPP-32 (Dopamine and cAMP-Regulated Phosphoprotein-32), making it in fact more like native PP-

1c [112]. While inhibitor-1 has been shown interact with PP-1c at multiple sites, mutation of the acidic groove of PP-1c would disrupt any intra-molecular interaction between the PP-1c C-terminal tail and acidic groove, thereby making it accessible for binding by inhibitor-1. In cells, the majority of native PP-1c is complexed with multiple regulatory subunits and this would likely prevent the C-terminal tail of PP-1c from folding back towards the active site.

1.3.4 Regulation of Protein Phosphatase-1c by Natural Product Toxins

The importance of Ser/Thr protein phosphatases in the regulation of cellular signaling pathways is highlighted by the fact that mulitiple organisms have evolved the ability to produce secondary metabolites that inhibit or block the function of these enzymes. Over the last two decades, many exogenous phosphatase inhibitors have been identified, including microcystins and nodularins (cyclic heptapeptides produced by cyanobacteria), calyculins (octamethyl polyhydroxylated fatty acids identified from marine sponge extracts), and okadaic acid (a polyether fatty acid produced by marine dinoflagellates, Figure 1.8) [107, 197]. PP-1c and PP-2Ac share over 40 % sequence homology and exhibit a shared sensitivity towards many natural product toxins, whereas CaN is typically resistant to the majority these toxins [197].

Exposure to protein phosphatase inhibitors poses a serious health threat to both humans and animals [43]. In 1996, approximately 50 patients undergoing kidney dialysis in Brazil died as a result of toxic hepatitis caused by exposure to microcystins produced by a cyanobacterial bloom in their water supply [123]. A study carried out by the American Water Works Asociation Research Foundation in 2000 revealed that over 80% of water samples collected from fresh water sources in Canada and the United States were positive for microcystins and approximately 4% of samples had microcystin concentrations above the World Health Organization's limit of 1.0 μg/L [292]. Microcystins are also considered a terrorist threat and in 2001 they were placed on the British Terrorist Watch List [47].

Mutational and structural studies have revealed that the vast majority of exogenous Ser/Thr protein phosphatase inhibitors exert their biological activity by binding directly to the phosphatase active site, blocking substrate binding and catalysis. Exceptions to this rule are the immunosuppressive inhibitors, cyclosporin and tacrolimus (FK506), which inhibit the enzymatic activity of calcineurin by

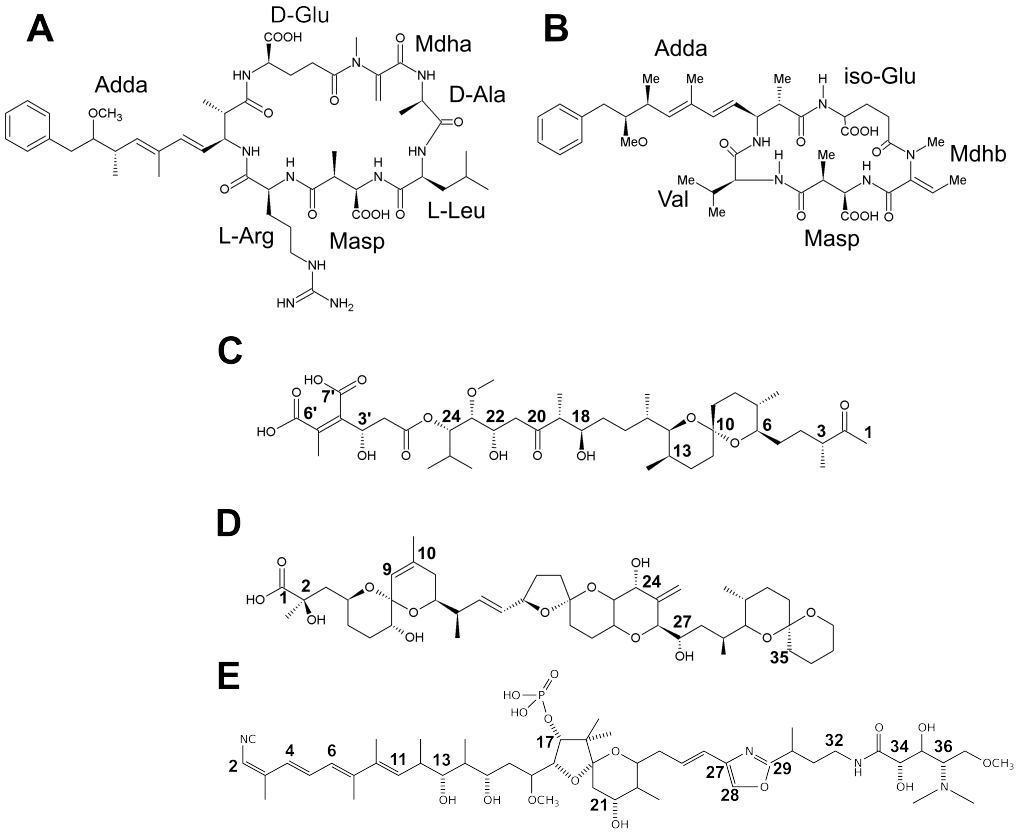

Figure 1.8: **Natural Product Inhibitors of Protein Phosphatase-1c**. The figure depicts the chemical structures of microcystin-LR, *panel A*; motuporin (nodularin-V), *panel B*; tautomycin, *panel C*; okadaic acid, *panel D*; and calyculin A, *panel E*. For microcystin and motuporin the amino acids are labeled on each structure. For tautomycin, okadaic acid, and calyculin A selected carbon atoms numbered on the structure.

binding indirectly via immunophilins [246]. The first crystal structure of PP1c bound to a natural toxin inhibitor (microcystin-LR) was characterized in 1995 by Goldberg et al [89]. Since then, more than ten additional structures of PP-1c and PP-2Ac bound to other natural toxin inhibitors have been determined [107, 196, 195, 298, 137, 132, 114, 234]. These structures revealed that almost all identified toxins share similar binding features, specifically: 1) interaction with the β12-β13 loop of PP-1c, 2) interaction of the toxins with the phosphatase hydrophobic groove, and 3) binding of the toxin directly to the active site of each enzyme. Direct comparison of these structures revealed that there are relatively few changes to the overall conformation of the PPP protein phosphatase upon toxin binding.

Despite the similarities in both sequence and structure (Figure 1.5), calcineurin is over 1,000 fold and 10,000 fold less sensitive to OA and MC, respectively [183, 197]. The mechanisms for the differential sensitivity has remained mostly a mystery up until recently.

Microcystins exert their effect by binding specifically to PP-1c and PP-2Ac, potently inhibiting each of them with an IC_{50} of 0.02 nM [197]. Structurally, microcystins are cyclic peptides comprised of cyclo (D-Ala-$^{1}{}_{L}$-X-$_{D}{}^{2}$-erytho-β-methylisoAsp^{3}-L-Y^{4}-Adda^{5}-$_{D}$-isoGlu^{6}-N-methyldehydroAla^{7}), where X and Y are variable amino acids (Figure 1.8). Over 60 different variants of microcystin have been identified, and typically differ with respect to their variable amino acids (X and Y), demethylation in amino acids 3 and/or 7, or specific modifications in the Adda residue (residue 5) [43]. Microcystin-LR (which contains a Leu and Arg in the variable X and Y positions) is the most commercially available variant and was first isolated from *Microcystis aeruginosa*. The crystal structure of the PP-1c•MC-LR complex [89] revealed that the N-methyl-dehydroalanine (Mdha) residue of microcystin forms a covalent bond with the sulphur of PP-1c residue Cys-273, through a proposed Michael addition reaction [209, 187]. However, this coupling reaction is slow and initial binding and inactivation of PP-1c by microcystin does not depend on the covalent modification step [51]. This is evident in the crystal structure of PP-1c bound to dihydromicrocystin-LA, a modified form of MC-LA where the Mhda group has been hydrogenated such that it can no longer covalently couple to the phosphatase [195].

1.3.5 Structural Insight from the Structures of Protein Phosphatase-1c Bound to Tautomycin and Protein Phosphatase-2A Bound to Okadaic Acid

Tautomycin is a polyketide antibiotic that was originally isolated from the soil bacterium *Streptomyces verticillatus* and is toxic to yeast, fungi, and animal cells [40]. It was later identified to be an inhibitor of both PP-1c and PP-2Ac; however, unlike all other characterized inhibitors, tautomycin inhibits PP-1c with a greater potency than PP-2Ac (IC_{50} of 0.16 nM for PP-1c and 0.4 nM for PP-2Ac) [185]. Tautomycin contains a rare 2,3–dialkylmaleic anhydride moiety that in solution exists as a tautomeric mixture of both the diacid and anhydride forms (Figure 1.8). Tautomycetin, a variant of tautomycin that lacks the spiroketal moiety, is even more selective for PP-1c, having an IC_{50} of 1.6 and 62 nM for PP-1c and PP-2Ac [106].

Due to its selectivity for PP-1c, tautomycin has become a valuable research tool for investigating the role of PP-1c and PP-2Ac signaling within cells [2, 309, 270].

The crystal structure of tautomycin bound to PP-1c was elucidated by Kelker et al. in 2009 [132]. Tautomycin binds to PP-1c in a similar manner as okadaic acid [194] and interacts in a pseudo-cyclic conformation, via an intramolecular interaction between the C22 hydroxyl and the C6 moiety of tautomycin (Figure 1.9). This contradicts an earlier computational model of the tautomycin•PP-1c complex by Colby et al [48]. The differences between the model and elucidated structure can be explained by the fact that the model allowed for flexibility of PP-1c active site residues. However, multiple crystal structures of either PP-1c alone [63], bound to other regulatory subunits [269, 115, 234] or inhibitors demonstrate the conserved rigidity of the PP-1c secondary structure and specifically the conserved orientation of PP-1c active site residues. Allowing significant changes in the orientation and positioning of PP-1c active site may produce a false binding model and hinder the search for specific PP-1c inhibitors through structure-based drug design.

One might also expect that the crystal structure of tautomycin bound to PP-1c would also explain why the toxin inhibits PP-1c more potently than PP-2Ac. Alignment of the tautomycin•PP-1c complex with the structure of PP-2Ac bound to okadaic acid (Figure 1.9) [298] reveals that very few structural differences between the phosphatases that could account for the differences in sensitivity. However, the hydrophobic groove of PP-2Ac contains a prominent hydrophobic cage, comprised in part by Gln-122 and His-191, that is less formed in PP-1c (the equivalent residues in PP-1 are Ser-129 and Asp-197, respectively) creating a longer but shallow hydrophobic groove (Figure 1.9). Differences in the PP-1c/PP-2Ac sensitivity towards okadaic acid have been attributed to the PP-2Ac hydrophobic cage better accommodating the hydrophobic end of okadaic acid compared to the hydrophobic groove of PP-1c [298].

Unlike okadaic acid, tautomycin is not predicted to be facilitated within the PP-2Ac hydrophobic cage due to steric clashes with PP-2Ac residues Gln-122 and His-191. However, tautomycin would not encounter similar hinderances when binding to the longer hydrophobic groove of PP-1c. This binding hypothesis is supported by experiments that demonstrate that PP-1c/PP-2Ac selectivity is not a function of the anhydride group of tautomycin (C1-C7), but the other half of tautomycin, containing the spiroketal moiety. It is most likely the length of the tautomycin

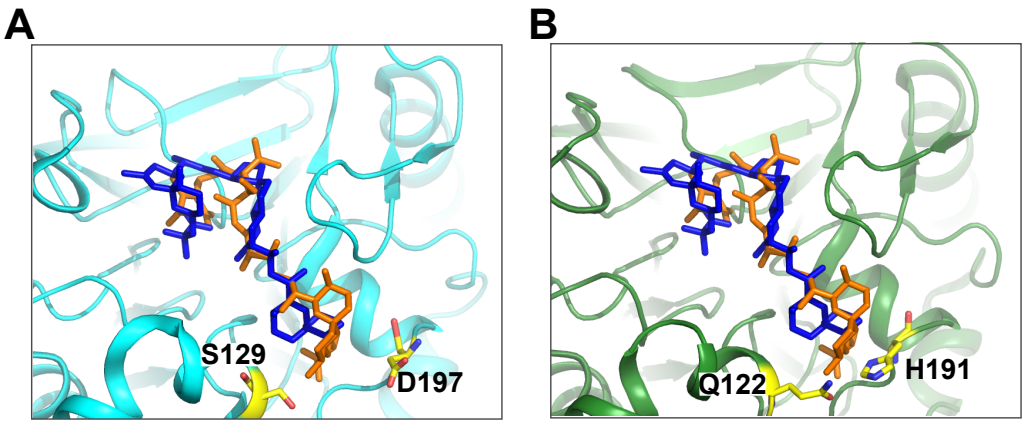

Figure 1.9: **Comparison of the Hydrophobic Grooves of PP-1c and PP-2Ac**. *Panel A* depicts the crystal structure of PP-1c (shown in cyan) bound to tautomycin (shown in orange, pdb# 3E7B) [132] aligned with the structure of okadaic acid from PP-2Ac structure (okadaic acid shown in dark blue, pdb# 2IE4) [298]. *Panel B* depicts the crystal structure of okadaic acid (shown in dark blue) bound to PP-2Ac (shown in green), aligned with the structure of tautomycin (shown in orange) from the PP-1c structure. Residues within the hydrophobic groove that differ between PP-1c and PP-2Ac are highlighted in yellow sticks.

toxin rather than the presence of the spiroketal moiety that confers selectivity; tautomycetin, which lacks the spiroketal moiety is even more selective for PP-1c than PP-2Ac than tautomycin [205]. To our knowledge, mutagenesis of the PP-1c Ser-129 and Asp-197 to the equivalent residues in PP-2A has not been carried out. Further studies are required to fully understand the differences observed in PP-1c/PP-2Ac sensitivity to tautomycin.

1.3.6 Structural Insight from the Protein Phosphatase-1c Structure Bound to Calyculin A

Calyculin A was originally isolated from a *Discodermia calyx* marine sponge extract that displayed anti-tumor properties [130]. It was later identified as specific inhibitor of PP-1c and PP-2Ac, inhibiting PP-2Ac only slightly more potently than PP-1c (IC_{50} of approximately 1.2 nM and 0.60 nM, respectively) [116, 106]. The crystal structure of protein phosphatase-1c bound to calyculin A was elucidated by Kita et al in 2002 [137]. While there are relatively few differences between the PPP protein phosphatase catalytic subunits, a detailed comparison of the PP-1c and PP-2Ac structures reveals several key differences within the active site and hydrophobic groove that may impact the binding of calyculin A to each phosphatase (Figure 1.10) [137]. Previously, mutation of PP-1c residue Val-223 to Ala, the equivalent residue

in PP-2Ac, was shown to result in a stronger inhibition of PP-1c by both calyculin A and two other structurally similar calyculin-like toxins, clavosine A and B [198]. This suggests that relatively small changes in the active site and hydrophobic residues of PP-1c and PP-2Ac can have drastic effects on the potency of naturally derived phosphatase inhibitors. Further mutational studies are required to identify other residue differences which contribute to the observed differences in sensitivity seen towards okadaic acid, tautomycin, and calyculin A.

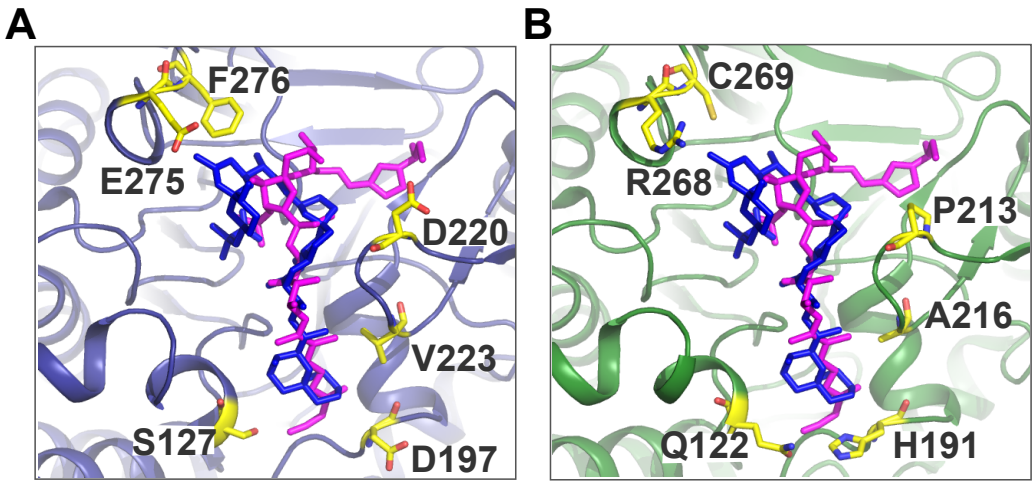

Figure 1.10: **Comparison of PP-1c and PP-2Ac Residues that May be Important for Binding to Calyculin A**. *Panel A* depicts PP-1c (shown in blue) bound to calyculin A (shown in magenta, pdb# 1IT6) [137] aligned with the structure of okadaic acid from the PP-2Ac structure (shown in blue, pdb# 2IE4) [298]. *Panel B* depicts the structure of PP-2Ac (shown in green) bound to okadaic acid (shown in blue), aligned with calyculin A (shown in magenta) from the PP-1c structure (pdb# 1IT6). Residues that differ between PP-1c and PP-2Ac in the active site and hydrophobic groove are highlighted in yellow sticks.

1.3.7 Regulation of Protein Phosphatase-1c by Association with Binding Subunits

A key biological question is how a small number of protein phosphatases can counteract the actions of many protein kinases to maintain spatial and temporal regulation of substrate dephosphorylation. Over the past twenty years research has shown that Ser/Thr protein phosphatases are dynamically regulated through post-translational modification and interaction with hundreds of regulatory binding proteins [100, 102]. Protein phosphatase-1c does not exist alone as a monomer within cells, instead it binds to over 180 identified regulatory subunits, forming

hundreds of mutually exclusive holoenzyme complexes that regulate enzyme activity and subcellular localization [100]. Alignment of several partial crystal structures of PP-1c bound to several regulatory proteins reveals that nearly the entire molecular surface of PP-1c can be used for binding to PP-1c regulatory proteins (Figure 1.11).

1.3.8 Protein Phosphatase-1c Regulatory Proteins Interact Via a Tetrapeptide RVXF Motif

The majority of PP-1c regulatory proteins interact with PP-1c via a conserved amino acid consensus sequence referred to as the RVXF binding motif ([K/R]-X0-1-[V/I/L]-X-[F/W], where X can be any amino acid except proline). The first RVXF motif was identified in the muscle glycogen targeting (G_M) subunit via the crystallization of PP-1c to a short 13 residue peptide derived from a conserved region of the G_M subunit. This crystal structure revealed that the RVXF motif of G_M (residues 64-69, RRVSFA) binds in an extended fashion within a hydrophobic pocket of PP-1c remote from the catalytic site [63, 287]. Multiple crystal structures of PP-1c bound to other regulatory proteins, such as MYPT1 and inhibitor-2, demonstrate that the interaction of RVXF motifs with PP-1c is highly conserved (Figure 1.12). Previously it was shown that mutation of hydrophobic Val and Phe positions in the RVXF motif abolish the ability of full-length R-subunits to bind to PP-1c [63, 279, 287].

Additionally, phosphorylation of Ser/Thr residues within or surrounding the PP-1c RVXF binding motif influence binding to PP-1c. In muscle cells, adrenalin signalling activates cAMP-activated protein kinase (PKA), which in turn phosphorylates the G_M subunit on Ser-67, which is located within the RVXF motif of the G_M subunit (RVSF, residues 65-68) [113]. Phosphorylation of this residue disrupts the binding of PP-1c to the G_M subunit and leads to the dissociation of PP-1c from the glycogen granule. Similarly, phosphorylation of residues flanking the RVXF motif can also alter binding to PP-1c. Phosphorylation of two Ser residues within the AKAP149 protein (A-Kinase Anchoring Protein-149) that surround the RVXF motif leads to the dissociation of PP-1c from AKAP149 [146]. Formation of the PP-1c•AKAP149 complex targets PP-1c to the nuclear envelope and promotes the dephosphorylation of B-type lamins during nuclear envelope reformation at the exit of mitosis (M to G1 phase). PP-1 is released from the nuclear envelope at the transition between G1 and S phases via the phosphorylation of AKAP149 [257].

Multiple studies have shown that short peptides derived from RVXF sequences

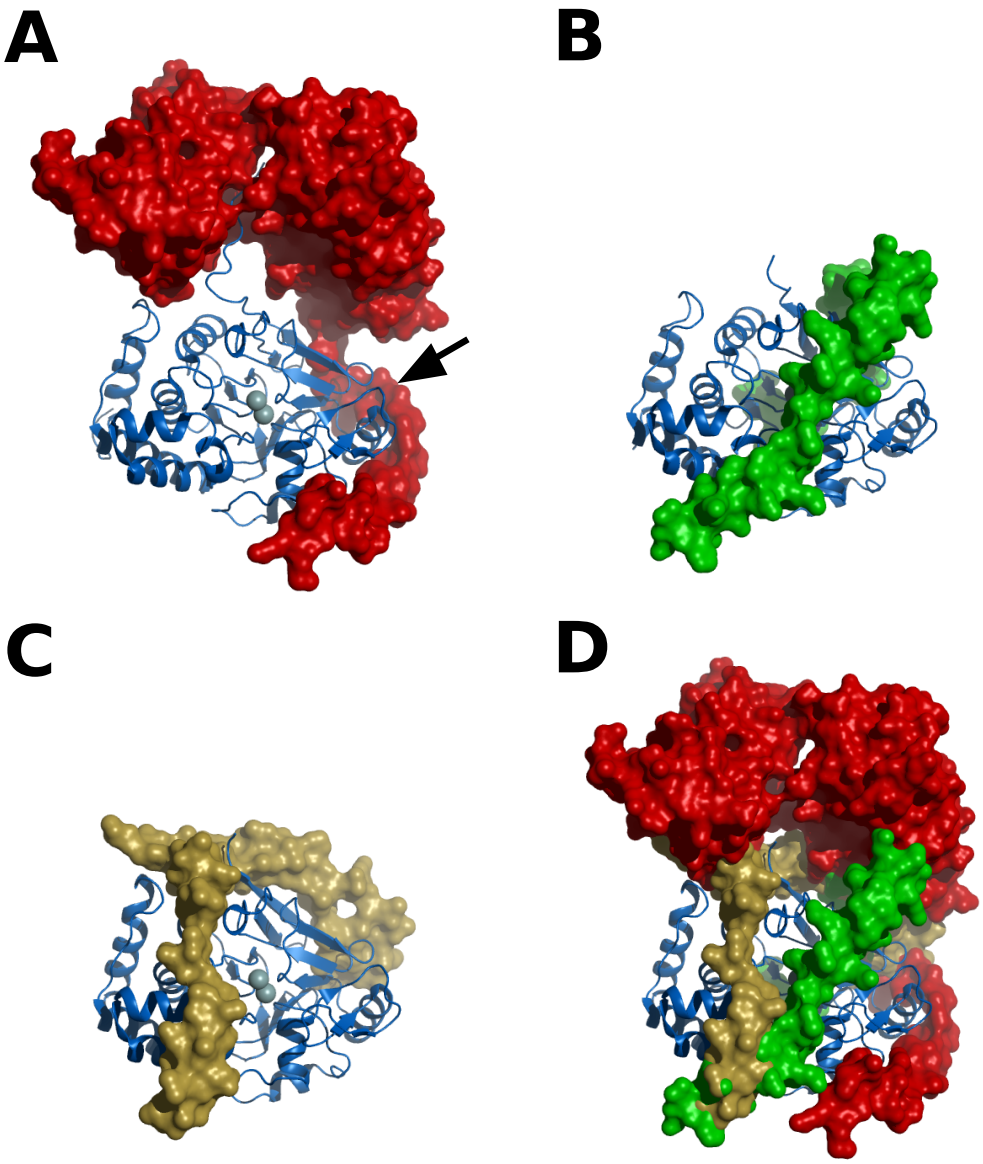

Figure 1.11: **The Crystal Structures of Protein Phosphatase-1c Bound to MYPT1, Inhibitor-2, and Spinophilin**. *Panel A* depicts the crystal structure of recombinant chicken PP-1cβ bound to the chicken myosin phosphatase targeting subunit (MYPT1, pdb# 1S70) [269]. *Panel B* depicts the partial structure of recombinant rat PP-1cγ bound to the rat inhibitor-2 protein (pdb# 2O8A) [115]. *Panel C* depicts the crystal structure of recombinant human PP-1cα bound to human spinophilin (pdb# 3EGG) [234]. *Panel D* depicts the hypothetical alignment of MYPT1, inhibitor-2, and spinophilin on the rat PP-1cβ structure. PP-1c (blue) is shown as a cartoon representation and MYPT1 (red), inhibitor-2 (green) and spinophilin (yellow) are shown as surface representations. The arrow highlights the location of the RVXF motif in MYPT1.

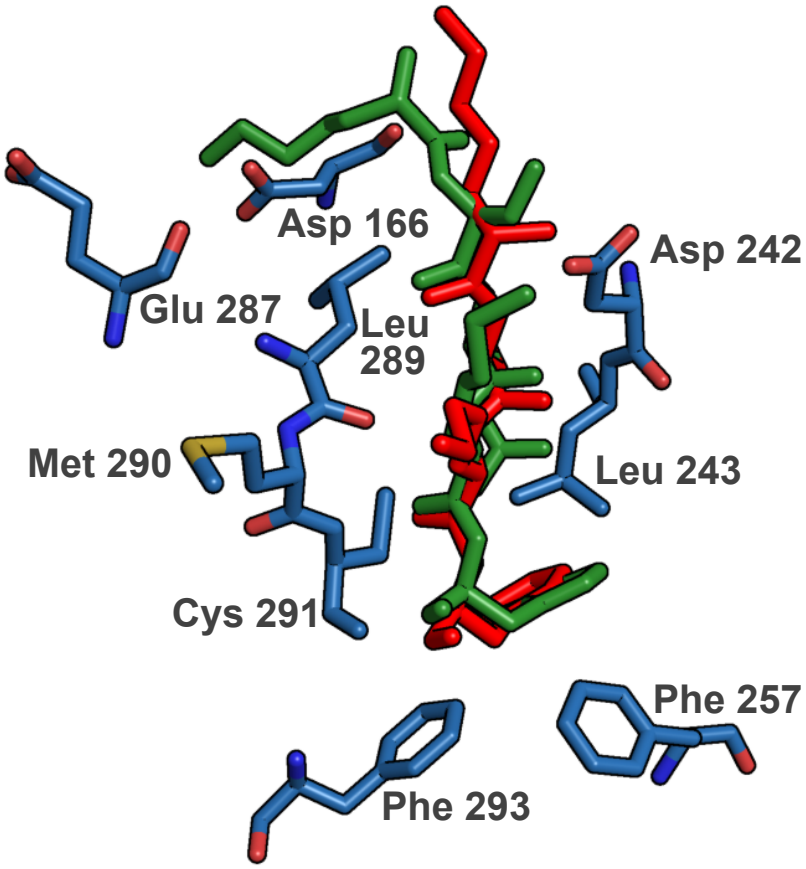

Figure 1.12: **The RVXF Motif Binds to a Hydrophobic RVXF-Binding Groove of PP-1c**. The figure depicts the interactions between the RVXF motifs of inhibitor-2 (shown in green, pdb# 2O8A) [115] and MYPT1 (shown in red, pdb# 1S70) [269] with the residues within the hydrophobic RVXF-binding groove of PP-1c (shown in blue sticks).

can disrupt the binding of PP-1c to RVXF–containing regulatory proteins [63, 287, 211, 250]. Synthetic RVXF-containing peptides have become a valuable research tool for investigating the interaction of PP-1c with various regulatory subunits [211, 250]. Short peptides or small molecules targeted to the PP-1c RVXF binding groove may also be medically valuable and have the potential to be used as a drug therapy for the treatment of diseases such as HIV. A small molecule, 1H4, targeted to the PP-1c RVXF binding groove was shown to inhibit HIV-1 transcription by over 80% by blocking the association of PP-1c with the HIV Tat transcription protein [8]. While 1H4 has no effect on the binding of NIPP (Nuclear Inhibitor of Protein Phosphatase-1c) or PNUTS (Phosphatase-1 Nuclear Targeting Subunit) to PP-1c [8], further testing is obviously required to investigate whether 1H4 affects the ability of PP-1c to bind to other regulatory proteins.

1.3.9 The GILK and MYPHONE Binding Motifs

Other motifs have also been identified as PP-1c recognition motifs, including the Myosin Phosphatase N-terminal Element (MyPhoNE) and the GILK ([G/S]ILK) motifs [269, 111, 287]. The GILK motif was first identified in human inhibitor-2 (IKGILK, residues 10-15) and was found to be essential for inhibition of PP-1c [111]. The GILK motif was also identified in other PP-1c regulatory proteins, such as SIPP1 (Splicing Factor that Interacts with PXBP-1 and PP-1) and gene products c1orf71 and sytl2. While this motif is typically located N-terminal to the RVXF motif [100, 179] and thought to work together with the RVXF motif to bind to PP-1c, there is some evidence to suggest that the GILK motifs of some PP-1c regulatory/binding proteins may also function independently from the RVXF motif. For instance, the NUAK protein, an AMP-activated protein kinase that is activated in response to cellular stress, has 3 GILK motifs but no RVXF motif [305]. It is not known whether having more than one GILK motif allows NUAK to bind to multiple PP-1c proteins simultaneously.

1.3.10 The Role of Protein Phosphatase-1c in the DNA Damage Response

The DNA damage response is an important cellular network that acts in response to DNA damage to promote DNA repair, cell cycle arrest and in some cases senescence or apoptosis. This process is largely regulated by reversible phosphorylation.

Quantitative mass spectrometry proteomic studies have identified over 500 phosphorylation sites on 200 proteins that are phosphorylated or dephosphorylated in response to DNA damage [19]. Several Ser/Thr protein kinases are directly activated in response to DNA damage, including ATM (ataxia-telangiectasia mutated), ATR (ataxia telangiectasia and Rad3 related) and DNA-dependent protein kinase. (DNA-PK) [247]. Together, ATM and ATR phosphorylate and activate two additional kinases, Chk1 and Chk2 (checkpoint 1 and checkpoint 2 kinases), which further amplify the ATM/ATR signalling. In addition, ATM phosphorylates hundreds of effector proteins, including histone variant γH2AX, BRCA1 (Breast Cancer type 1 susceptibility protein), CDC25 (Cell Division Cycle phosphatase), and p53 (Figure 1.13).

While the role of kinases in the DNA damage response have been well characterized [20, 19], less is known about how protein phosphatases regulate this pathway. Protein phosphatase-1c appears to have multiple and somewhat contradictory roles within the DNA damage response network. Many PP-1c regulatory proteins that bind directly to PP-1c are also involved in the DNA damage response (Table 1.3). In some instances PP-1c acts to enhance the DNA damage response, promoting cell cycle arrest and apopotosis. For example, the retinoblastoma protein (pRb) contains an RVXF-like binding motif (KLRF, residues 874-877) and is actively dephosphorylated by PP-1c [61, 280, 104]. When hypophosphorylated, pRb binds to the transcription factor E2F to block E2F-mediated transcription of protein involved in cell cycle progression [101]. During mitosis, pRb is inactivated via multiple site phosphorylation of pRb by the cyclin dependent kinases Cdk1 and Cdk2, which induces the release of pRb from the E2 transcription factor (E2F) and relieves the inhibition of E2F-mediated transcription [101]. Significantly, the PP-1c RVXF-like motif and cyclin binding motif (RXL) directly overlap on the pRb protein sequence. Previous studies indicate the PP-1c and Cdk2•cyclin A complexes directly compete with one another for binding to pRb. The expression of even catalytically inactive PP-1c has the ability to completely block Cdk2–mediated pRb phosphorylation in human Saos-2 (osteosarcoma) cells [104].

In contrast, PP-1c also functions as an inhibitor of the DNA damage response. The PP-1c regulatory protein Repo-man, localizes PP-1c onto mitotic chromatin during anaphase and targets PP-1c to directly dephosphorylate ATM [230, 275]. Dephosphorylation of ATM results in its inactivation and switches off the DNA

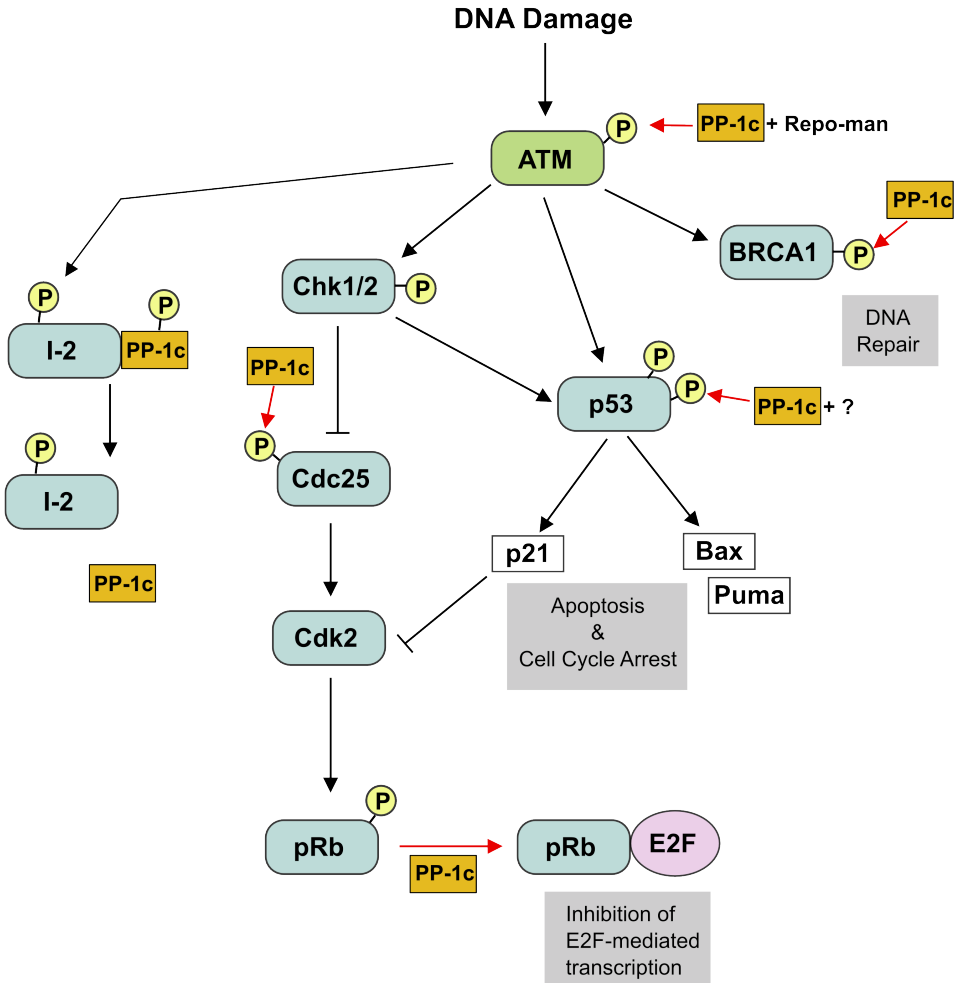

Figure 1.13: **The ATM DNA Damage Signaling Network**. The ATM kinase is activated upon DNA damage and it phosphorylates many key players in the DNA damage signalling network. ATM phosphorylation of the checkpoint kinases (Chk1 and Chk2) further amplifies ATM signalling. ATM also phosphorylate hundreds of effector proteins within this network including those involved in DNA damage repair (including BRCA-1) and Cell cycle arrest/Apoptosis (such as p53). Phosphorylation of p53 by both ATM and the Chk kinases leads to its stabilization and activation, promoting the expression of gene targets involved in cell cycle arrest (such as p21) and apoptosis (such as Bax and Puma) [237]. The Cdc25 phosphatase is also phosphorylated by ATM, which inactivates Cdc25 and promotes its proteosomal degradation. Inactivation of Cdc25 keeps the Cyclin-dependent kinase 2 (Cdk2) in a hyperphosphorylated inactive state and prevents cell cycle progression. Inhibition of Cdk2 also prevents the phosphorylation and inactivation of pRb (retinoblastoma protein). When hypophosphorylated pRb suppresses transcription of E2F target genes involved in cell growth and cell cycle progression).

Table 1.3: **Protein Phosphatase-1c Interacting Proteins Involved in the DNA Damage Response**. BRCA1, breast type 1 susceptibility protein; GADD34, growth arrest and DNA damage protein -34; KAP1, kruppel-asskciated protein -1; Ku70/80, regulatory subunits of DNA-dependent protein kinase; Nek2, never in mitosis A related kinase -2; PNUTS, phosphatase-1 nuclear targeting subunit; pRb, retinoblastoma protein.

PP-1c Regulatory Protein	**Functions in DNA Damage Response**	Targets PP-1c to	**References**
Repo-Man	Apoptosis and inhibition of DDR	ATM	[275, 230]
BRCA1	Promotes DNA repair and cell cycle arrest	BRCA1	[158, 110, 296]
GADD34	Promotes apoptotsis	?	[96]
Inhibitor-2	Inhibits cell cycle arrest	Inhibitor-2	[290]
pRb	Induces cell cycle arrest	pRb	[61, 280]
PNUTS	Controls cell cycle and promotion of Apoptosis	?	[151, 5]
Nek2	Separation of centrosomes	Nek2	[99]
KAP1	Transcriptional co-repressor of pro-apoptotic genes	KAP1	[170]
Taperin	Interacts with Ku70 and Ku80 at site of DNA damage	Taperin	[75]

damage response network, allowing cells to undergo mitosis. PP-1c also dephosphorylates many of ATM substrates including Cdc25, BRCA1, and the transcription factor p53 (Figure 1.13) [145, 224], by binding directly to the substrate or via the association with a RVXF-motif containing targeting subunit. In general, PP-1c mediated dephosphorylation of proteins involved in the DNA damage response is dictated not by the expression of the phosphatase itself, but rather the relative expression and localization of PP-1c regulatory proteins. Many PP-1c regulatory proteins that promote the suppression of the DNA damage response, such as Repoman, Nek2 (NIMA-related kinase 2), and KAP1 (Kruppel-associated protein 1), are over expressed in many forms of cancers [230, 276, 303].

1.4 The p53 Tumour Suppressor

p53 was first identified by Dr. David Lane in 1979 as a 53 kDa protein that bound to viral protein T antigen from the simian virus-40 transformed cells [152]. Further research revealed that p53 is an important tumour suppressor that is mutated or mis-regulated in over 50% of all human cancers. Mutation or improper regulation of p53 has been linked to other pathological disorders including Huntington's disease [14, 74], Parkinson's disease [28] and Alzheimer's disease [52]. p53 is one of the most studied proteins to date, described in over 65,000 scientific peer reviewed articles [1]. The p53 protein functions primarily as a transcription factor binding to DNA in a sequence-specific manner in response to many cellular distress signals, including DNA damage, oncogene activation, hypoxia, and heat shock [284, 203]. Activation of p53 leads to the induced expression of target proteins involved in promoting cell cycle arrest, senescence, or apoptosis. In addition, recent studies have shown that p53 has other important functions within cells, having a role in metabolism, development, autophagy, and aging [27, 10, 18, 285].

In normal unstressed cells, p53 levels are kept low via the constant proteosomal degradation of p53, which is regulated primarily by the E3 ubiquitin ligase mdm2 (mouse double minute 2). Ubiquitination of the p53 C-terminus promotes its translocation from the nucleus into the cytoplasm, where it is further ubiquitinated and targeted for proteosomal degradation [267, 84]. Upon phosphorylation by DNA-damage activated kinases, the p53 protein is stabilized within the nucleus, leading to its increased expression and enhanced transcriptional activity towards target genes involved in either cell cycle arrest and/or apoptosis.

1.4.1 The 3-Dimensional Structure of p53

The p53 protein contains five characterized domains: an N-terminal transactivation domain (residues 1–61, composed of two subdomains, TAD1 and TAD2), a proline rich domain (PRD, residues 62–97), a core DNA-binding domain (DBD, residues 98–292), and two C-terminal domains that are involved in tetramerization (TET, residues 324–356) and regulation (REG, residues 363–393) (Figure 1.16). Approximately 80% of all p53 deactivating missense mutations found in cancer occur within the p53 DNA binding domain [225]. p53 forms a tetramer via its TET domain and binds to DNA promoter regions in a sequence specific manner, interacting with two decameric binding sites, or four pentameric half-sites, that are typically separated by 0–13 nucleotides (RRRCWWGYYY, where R is an A or G, W is an A or T, and Y is a C or T) [190].

The first crystal structure of the p53 DNA-binding domain (residues 92-102) bound to a short sequence of double stranded DNA was determined by Cho et al. in 1994 [42]. The DBD consists of a β sandwich consisting of 4 and 5 strands and loop-sheet-helix motif packed towards one one end of the β-sandwich. The p53 DNA-binding domain interacts with the major and minor grooves of the DNA duplex primarily via two large loops (labelled L1 and L3) and an H2 helix (Figure 1.15). The structure of the p53•DNA complex revealed that many of the p53 residues that directly interact with DNA, within the H2 helix and the L1/L3 loops have a high frequency of mutation in cancers. The majority of these missense mutations have been shown to either disrupt the ability of p53 to bind to DNA or reduce the stability of the p53 DNA-binding domain [160].

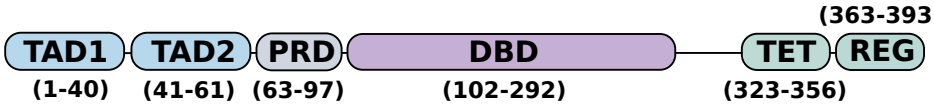

Figure 1.14: **Linear Domain Structure of the p53 Tumour Suppressor**. The p53 protein consists of five major domains: a N-terminal transactivation domain (comprising two subdomains, TAD1 and TAD2, residues 1–61), a proline rich domain (PRD, residues 62–97), a DNA-binding domain (DBD, residues 98–292), and two C-terminal domains involved in tetramerization (TET, residues 324–356) and regulation (REG, residues 363–393).

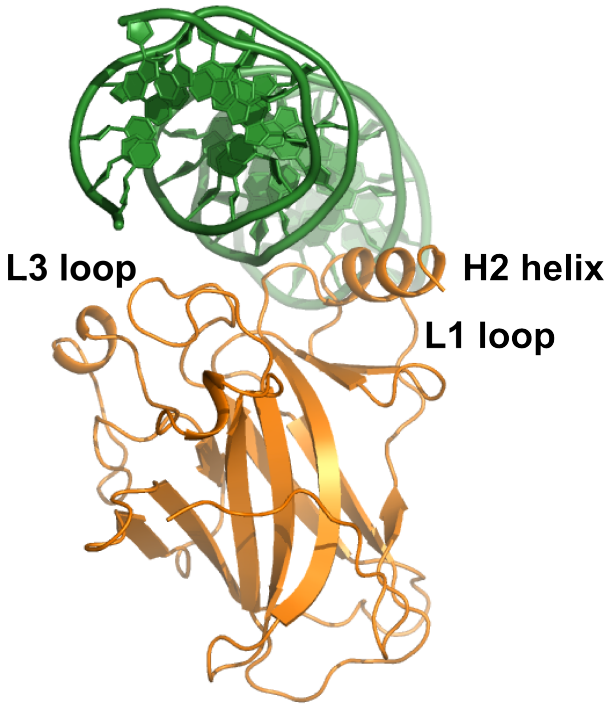

Figure 1.15: **Crystal Structure of p53 Bound to DNA**. The figure depicts the crystal structure of the DNA binding domain of p53 (shown in orange) bound to a DNA duplex (shown in green, pdb# 1TUP) [42].

1.4.2 Regulation of p53 Activity By Post-Translational Modifications

The p53 protein is highly regulated via post-translational modifications including phosphorylation, acetylation, mono and di-methylation, glycosylation, ubiquitylation, neddylation, sumoylation, and poly-ribosylation [199]. These modifications occur mainly within unstructured regions of p53, specifically the N-terminal transactivation and C-terminal domains (Figure 1.16 panel B). However, some phosphorylation and acetylation does occurs within the central DNA-binding domain. In general, phosphorylation within the transactivation domain and acetylation within the C-terminal domains acts to stabilize the p53 protein, whereas ubiquitination with the C-terminal region of p53 typically destabilizes p53 by promoting its proteosomal degradation. Multiple studies have shown that the post-translational modification of p53 is highly integrated, such that one form of modification will either promote or inhibit further modification of p53.

In normal non-stressed cells, p53 is hypophosphorylated and p53 levels are kept relatively low through the actions of ubiquitin ligases, primarily mdm-2. Poly-ubiquitination of p53 C-terminal lysines (Lys-370, Lys-372, Lys-373, Lys-381, Lys-382, and Lys-386) have been shown to promote the proteosomal degradation of p53 and inhibit p53-mediated transactivation [199]. DNA damage activates several kinases including ATM, ATR, and DNA-PK, which induce the phosphorylation of p53 within its N-terminal transactivation domain on multiple Ser/Thr residues. In particular, the phosphorylation of Ser-15 (by ATM, ATR, or DNA-PK) acts a nucleation event to prevent association with the ubiquitin ligase mdm-2 and promote the association of p53 with co-activators such as p300 and CBP (CREB-binding protein). p300 and CBP also function as acetyl transferases and acetylation of the C-terminus of p53 recruites additional transcriptional components to bind to p53 and prevents further ubiquitination within the C-terminus of p53, thus promoting the stability and accumulation of p53 within the cell.

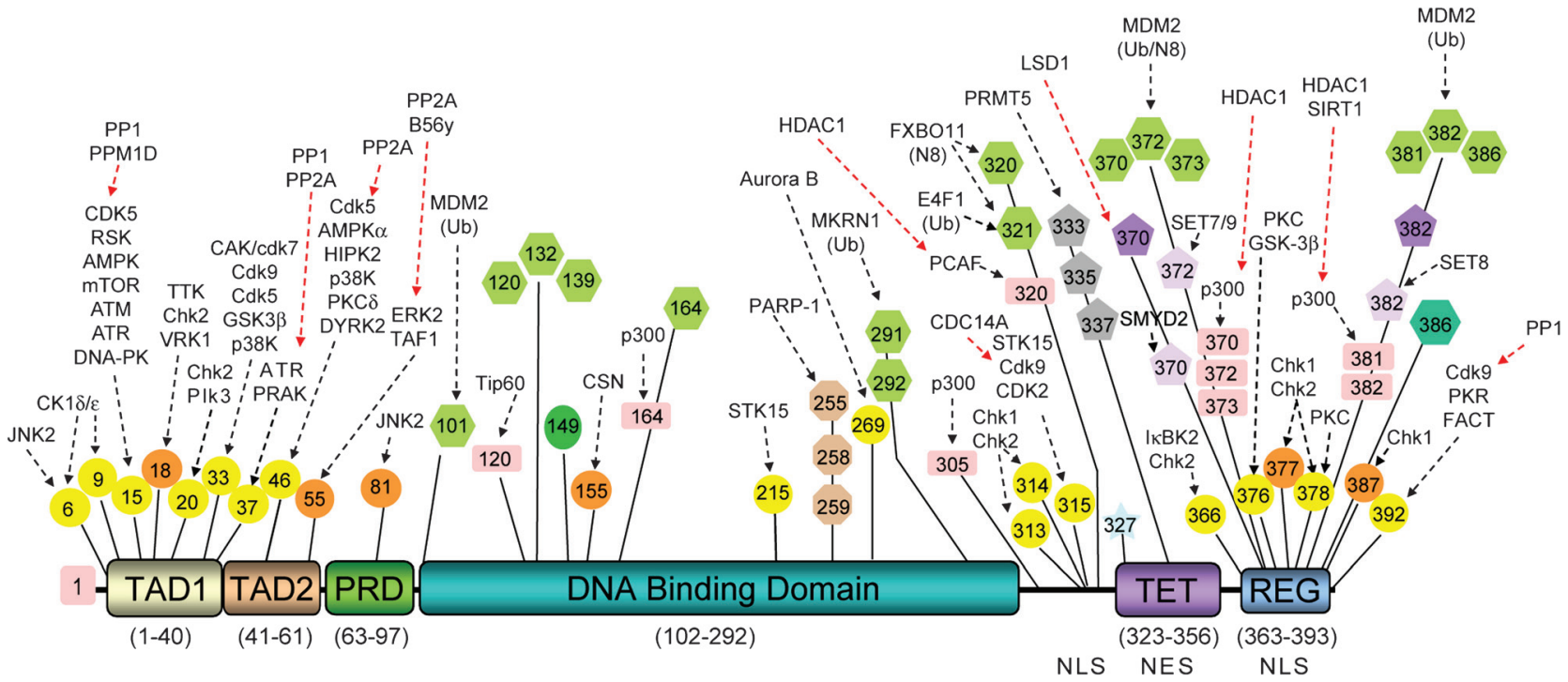

Figure 1.16: **p53 is Regulated by Multiple Post-translational Modifications**. The figure depicts sites on p53 that are modified post-translationally by phosphorylation of Ser/Thr residues (yellow and orange circles, respectively), ubiquitination (green hexagons), acetylation (pink squares), N-acetylglycosamine modification (dark green ovals), poly ADP-ribosylation (brown octagons), arginine or lysine mono/di methylation (gray, lavender, and violet pentagons), and nitration (blue star). Figure from Miller-Jenkins et al. (2012) [203].

1.5 Apoptotic Stimulating Proteins of p53

The Ankyrin-rich, SH3-domain and Proline-rich region containing Proteins (ASPPs) are a unique family of p53 binding proteins that modulate the activity of p53 [274]. Therefore, they are often referred to as the Apoptotic Stimulating Proteins of p53. The human genome encodes three principal ASPP family members: ASPP1, ASPP2 and an inhibitory ASPP (iASPP). All three members are homologous within their C-termini and contain proline-rich region, ankyrin-rich domain, and a SH3 domain (Src homology domain -3, Figure 1.17). Significantly, while ASPP1 and ASPP2 are known to enhance the apoptotic activity of p53, the inhibitory form iASPP inhibits p53-mediated apoptosis.

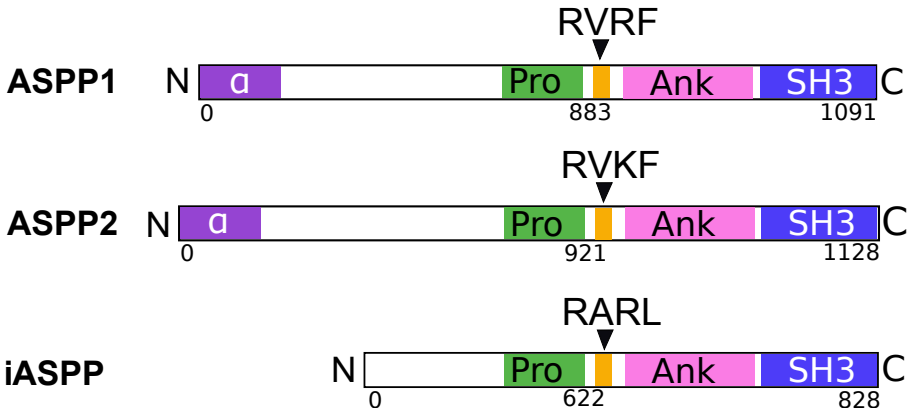

Figure 1.17: **Linear Domain Structures of the Ankyrin-repeat, SH3-domain and Proline-rich Region Containing Proteins**. All three ASPP proteins are homologous within the C-terminal regions, containing a proline-rich region (Pro), ankyrin-repeat domain (Ank), and a SH3 domain (SH3). ASPP1 and ASPP2 both contain a N-terminal alpha helical domain (α) and a canonical RVXF PP-1c binding motif (RVRF and RVKF respectively).

1.5.1 The Apoptotic Stimulating Protein of p53 Type-2 (ASPP2)

ASPP2 was the first ASPP protein to be characterized and was originally identified in a yeast two-hybrid screen as one of two p53-binding proteins, p53BP1 and p53BP2 (p53BP2 is now also referred to as ASPP2), respectively [117]. Whilst the p53BP1 and p53BP2/ASPP2 protein share no sequence similarity, the ankyrin repeat and SH3 domains of p53BP2/ASPP2 were found to be homologous to two other proteins, later named ASPP1 and iASPP [274]. Human cells produce two ASPP2

variants via alternative splicing of a single gene copy: $ASPP2_{1-1128}$ (referred to as simply ASPP2) and a shorter construct $ASPP2_{124-1128}$ (referred to as Bcl-2 binding protein, BBP) [274]. BBP, the shorter ASPP2 variant, is localized primarily to the nucleus, whereas full-length ASPP2 remains for the most part in the cytoplasm [239, 236]. In contrast, full length ASPP2 in Michigan Cancer Foundation-7 breast cancer cells is detected in the cytoplasm, nucleus, and at cell-cell junctions [254]. The reasons for ASPP2 cellular localization is still not well understood and likely is cell type specific.

Both ASPP1 and ASPP2 bind directly to p53 to enhance the apoptotic functions of p53 through selectively inducing the expression of pro-apoptotic genes such as Bax (B-cell lymphoma-2 associated protein) and PIG3 (p53-inducible gene -3) [239]. Significantly, ASPP1 and ASPP2 have no effect on p53-mediated cell cycle arrest and their expression does not effect the transcription of cell cycle arrest genes such as p21 (cyclin-dependent kinase inhibitor-1) [239]. The mechanism by which ASPP1 and ASPP2 enhance p53-mediated apoptosis is still not well understood. It has been proposed that the ASPP proteins may bind together with p53 to pro-apoptotic DNA promoters to enhance the expression of genes involved in apoptosis. However, electrophoretic mobility assay experiments showed that the interaction of p53 with DNA promoters and the ASPP proteins is mutually exclusive [229]. In addition, the crystal structure of the DNA-binding domain of p53 bound to the C-terminal region of ASPP2 shows that ASPP2 binds to p53 along the same interface that binds DNA [90, 42]. Together the evidence supports the notion that p53 and ASPPs do not bind together to DNA. Not surprisingly, mutants of p53 commonly found in cancer lack the ability to interact with DNA or ASPP2 [272].

ASPP2 may also have additional functions within the cell that are not modulated directly by p53 and it has been shown to bind to many other proteins including the p65 subunit (Rel A) of NF-κB (Nuclear-Factor-κB), the hepatitis C virus core (HCV) protein, Bcl-2, YAP (Yes-associated protein), Lats2 (Large Tumour Suppressor Protein 2), and PP-1c [33, 131, 69, 281, 13, 302, 98]. A comprehensive table of all identified ASPP protein interacting partners is shown in Table 1.4. To date, the majority ASPP2 interacting proteins bind to its C-terminal domains, primarily to the ankyrin repeat and SH3 domains. However, ASPP2 has been recently shown to interact directly with the Ras GTPase protein via its N-terminal β-GRASP Ras association domain (Figure 1.19) to influence the Ras/MAP/ERK pathway [291].

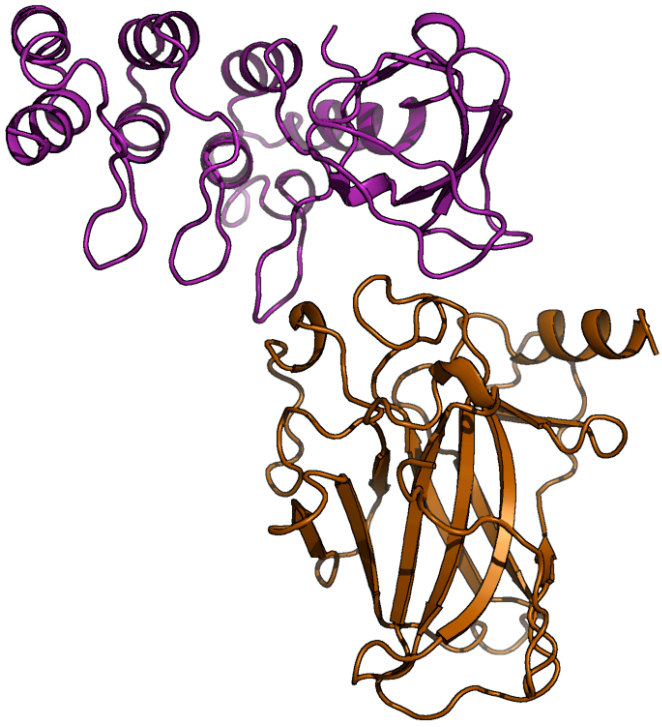

Figure 1.18: **The Crystal Stucture of ASPP2 Bound to the DNA-Binding Domain of p53**. The figure depicts the structure of the ASPP2 ankyrin repeat and SH3 domains (shown in magenta) bound to the DNA-binding domain of p53 (shown in orange, pdb# 1YCS) [90].

This corroborates with previous studies which show that ASPP2 overexpression can suppress Ras-mediated transformation of rat embryo fibroblasts [117, 22]. Together, the evidence suggests that ASPP2 carries out multiple functions within the cell and mediates both p53-dependent and independent functions.

1.5.2 The Apoptotic Stimulating Protein of p53 Type-1 (ASPP1)

While the ASPP1 protein shares over 75 % overall sequence identity with ASPP2, within their C-termini, the N-termini of ASPP1 and ASPP2 are much less conserved. The inability of ASPP1 to functional replace ASPP2 in mice [103, 128] suggests that these proteins may have different roles within cells. In addition to its role as a p53-mediated tumour suppressor protein, ASPP1 suppresses apoptosis and plays a role within the Hippo signalling pathway (Figure 1.20). In unstressed cells, ASPP1 remains primarily cytoplasmic and can bind directly to the YAP1 protein. YAP1 is a co-transcriptional activator that upon activation translocates from the cytoplasm

Table 1.4: **Interacting Partners of the ASPP Family**. APCL, Adenomatous Polyposis Coli Protein; APP-BPI, Amyloid Precursor Protein Binding Protein; Bcl-2, B-cell lymphoma -2; dCsk, C-terminal Src Kinase; CagA, cytotoxin-associated gene A protein; Ddx42p, Deadbox protein 42 protein; HCV, hepatitus C virus; IRS-1, Insulin Receptor Substrate-1; Lats2, large tumour suppressor -2 ; NF-κB, nuclear factor -κB; Par-3, partitioning defective -3; PP-1c, protein phosphatase-1c; Ras, rat sarcoma protein; YAP1, Yes associated protein

Interacting Partner	**Binding to ASPP2\ASPP1\iASPP**	**Functions**	**Reference**
p53	+\+\+	Modulates the Apoptotic Activity of p53	[117, 239, 21]
Bcl-2	+\?\?	Prevents Bcl-2 inhibition of apoptosis	[218, 264]
PP-1c	+\+\+	Modulates the activity of PP-1c towards p53	[98, 250]
IRS-1	+\?\?	Modulates insuling signaling	[95]
dCsk	+\?\?	Interacts with dCsk to regulate dSrk kinase	[153]
APP-BPI	+\?\?	Inhibits neddylation pathway	[39]
NF-κB/p65	+\?\+	Inhibits NF-βB apopototic pathways	[302, 264, 301]
HCV core protein	+\?\?	Inhibits apoptosis	[33]
Ddx42p	+\?\?	Inhibits apoptosis and cell growth	[278]
DDA3	+\?\?	Inhibits p53-mediated activation of BAX	[262]
YAP1	+\+\?	Modulates intracellular localization and gene expression	[69, 281]
APCL	+\?\?	Modulates intracellular localization of ASPP2	[216]
14-3-3s	+\?\+	Bind to 14-3-3s during interphase	[202]
CagA	+\?\?	association increases gastrointestinal cancers	[30]
Par-3	+\?\?	Involved in epithelial cell polarity	[49, 254]
Lats2 kinase	?\+\?	Nuclear translocation of ASPP1	[13]
p300	+\+\+	co-transactivation of p53?	[88]
Ras	+\?\?	Activation of MAPP kinase pathway	[291]

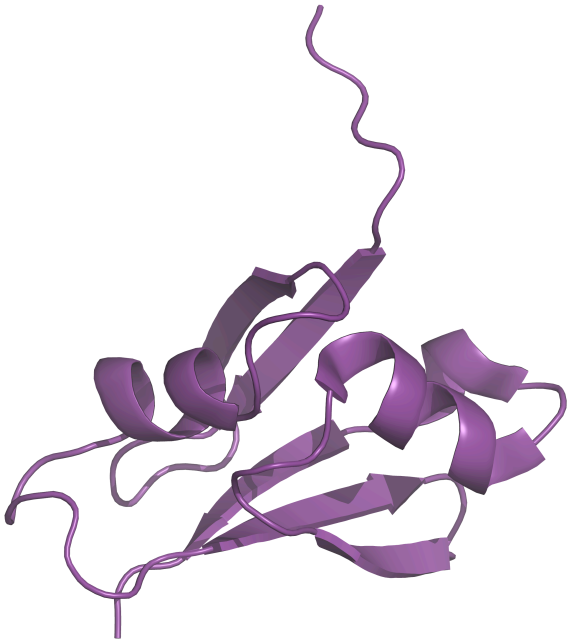

Figure 1.19: **The N-terminus of ASPP Contains an Ubiquitin-like Ras Association Domain**. The figure depicts the NMR solution structure of ASPP2 N-terminal resides 1-83 (shown in purple, pdb# 2UWQ) [273], which forms a β-GRASP ubiquitin-like domain. ASPP2 interacts directly with the Ras protein via this N-terminal domain.

to the nucleus to control organ development and cell proliferation [288]. The activity of YAP1 is inhibited by signalling through the Hippo kinase cascade pathway, via multiple phosphorylation of YAP1 by the Lats2 (large tumour suppressor 2) kinase. Phosphorylation of YAP1 promotes its association with 14-3-3 proteins, which prevents YAP1 from entering the nucleus and induces its proteosomal degradation. Overexpression of ASPP1 results in the decreased phosphorylation of YAP1 and YAP1 is translocated to the nucleus [281]. This suggests that in normal cells, ASPP1 may play a role in preventing the cell from unnecessarily undergoing apoptosis, by promoting the activation of YAP1.

The Lats2 kinase has also been shown to bind directly to the Ankryin repeat domain of ASPP1 and phosphorylate ASPP1 in response to oncogenic Ras activation [13]. ASPP1 contains a classical Lats2 kinase consensus sequence, HXRXXS (HGRSKS, ASPP1 residues 378 – 383) within its N-terminus, which is not conserved in either ASPP2 or iASPP. Overexpression of both Lats2 and ASPP1 promotes the translocation of ASPP1 from the cytoplasm into the nucleus and enhances the ability of p53 to induce the expression of pro-apoptotic genes such as CD95 and BAX [13].

In contrast, high levels of YAP1 override this protective mechanism and prevent ASPP1 translocation into the nucleus.

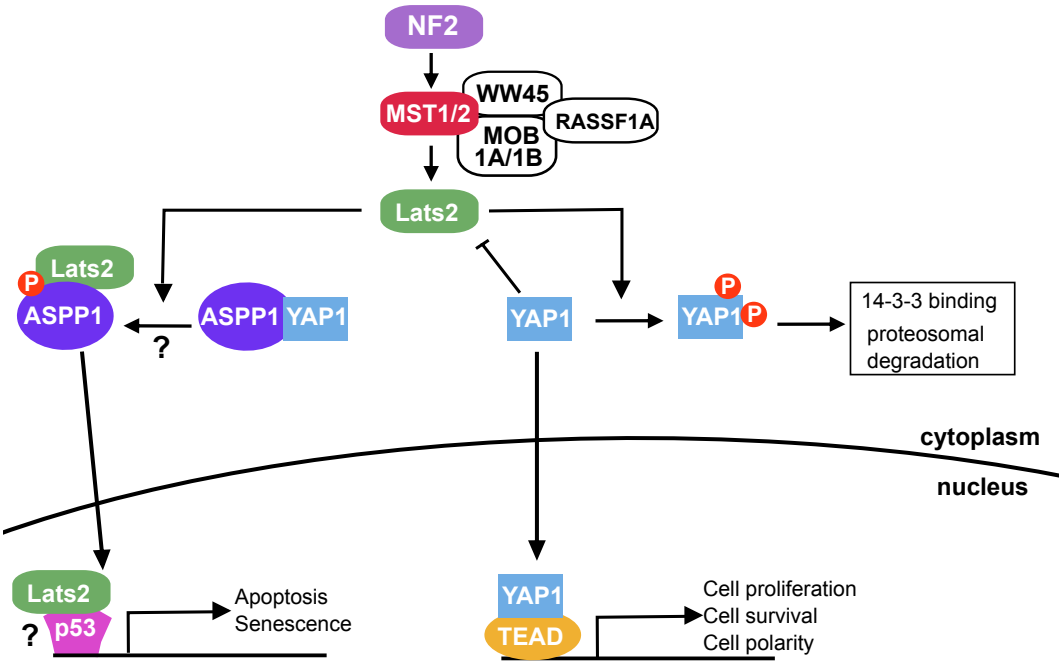

Figure 1.20: **The Importance of ASPP1 in the Hippo-YAP1-Lats2 Signalling Pathway**. A kinase signalling cascade, through the actions of NF2 (Neurofibromatosis type-2) and Mst1/2 (Mammalian Ste20-like serine/threonine 1/2) kinases, activates the Lats2 (Large Tumour Suppressor Protein -1) kinase. Lats2 phosphorylates YAP1 (Yes Associated Protein -1) on multiple sites, leading to its association with 14-3-3 proteins and eventual proteosomal degradation. Phosphorylation of YAP1 prevents its translocation from the cytoplasm into the nucleus, where it acts as transcriptional co-activator, regulating the expression of proteins involved in cell proliferation, cell survival, and cell polarity. YAP1 can bind to many transcription factors, including the TEAD (Transcriptional enhancer activator domain) proteins. Overexpression of YAP1 leads to the downregulation of Lats2 protein expression [282]. In response to oncogenic Ras activation, Lats2 can also bind directly to and phosphorylate ASPP1, leading to its translocation into the nucleus [13]. Within the nucleus ASPP1 acts to enhance the apoptotic activity of p53; however, it is not known whether ASPP1 and or Lats2 binds directly with p53 to DNA promoters. ASPP1 can also bind to YAP1 and prevent the phosphorylation of YAP1 by Lats2 [281].

1.5.3 The Inhibitory Apoptotic Protein of p53 (iASPP)

The inhibitory ASPP protein (iASPP) was originally identified as an inhibitor of the Rel A p65 subunit of NF-κB (RAI) [301]. Further studies demonstrated that

iASPP also functions as an inhibitor of p53 apoptotic activity [22]. The C-terminal ankryin repeat and SH3 domains of iASPP and ASPP2 share over 50 % sequence homology. In addition, analysis of iASPP and ASPP2 crystal structures [90, 235] reveals there are very few differences between the overall structures of iASPP and ASPP2 (Figure 1.21).

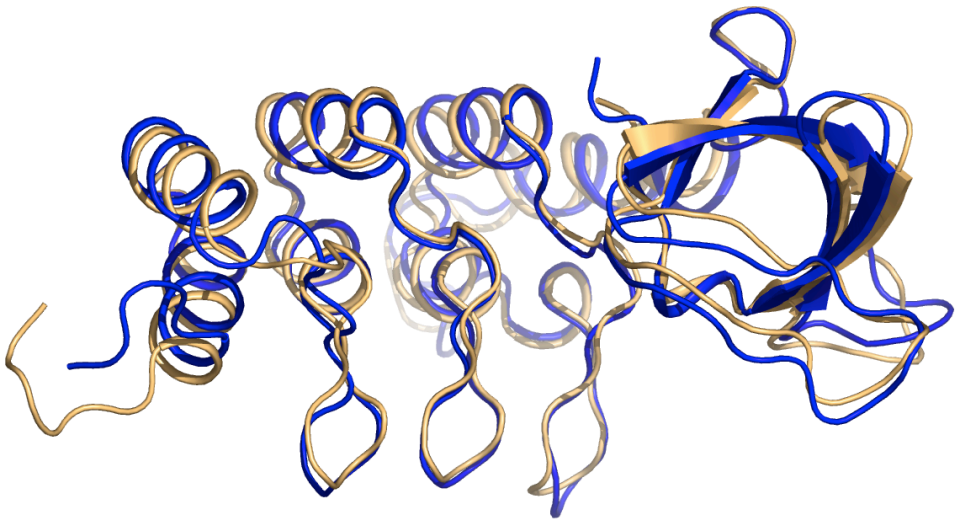

Figure 1.21: **The C-termini of ASPP2 and iASPP are Structurally Similar.** The figure depicts the alignment of the C-terminal ankryin repeat and SH3 domains of ASPP2 (residues 926–1128; shown in blue; pdb# 1YCS) and iASPP (residues 608-828; shown in light orange; pdb# 2VGE) [90, 235].

Found in all organisms from worms to humans, iASPP is one of the most evolutionary conserved inhibitors of p53 [22]. The genome from *Caenorhabditis elegans*, a non-parasitic unsegmented nematode, only contains one ASPP protein, APE-1 (Apoptotic Protein Enhancer-1). The *C. elegans* iASPP ortholog C-terminus shares approximately 45 % sequence homology with all three human ASPP protein family members. Inhibition of APE-1 via RNA interference results in an increased number of apopototic germ cell corpses. Therefore, APE-1 is considered to function similar to iASPP (not ASPP1 or ASPP2) and thus is also referred to as Ce-iASPP.

In humans, there are two splice variants of iASPP: a full-length protein (iASPP, residues 1–828) and a shorter C-terminal construct (Rel-A Inhibitor [RAI], residues 479–828) [252]. Similar to ASPP2, the shorter construct is localized primarily within the nucleus, whereas the longer form remains mostly cytoplasmic [252]. While the

C-terminus of iASPP does not contain a classical nuclear import signal, research has shown that the ankyrin repeat domain of ASPP2 may function as a non-classical nuclear import module [236]. Differences in sub-cellular localization of regulation via the N-terminal domain of iASPP may promote the cytoplasmic localization of full length iASPP.

Prior to this study, it was not well understood how iASPP functions as an inhibitor of p53, whereas ASPP1 and ASPP2 enhance p53-mediated apoptosis. The N-terminus of ASPP2 is important for mediating protein-protein interactions and subcellular localization [236, 239, 291]. While the N-terminus of iASPP also is important for conferring the sub-cellular localization of iASPP, the N-terminus of iASPP is not homologous to the N-termini of either ASPP1 and ASPP2. Therefore, there may be distinct differences in the regulation of iASPP and ASPP2 proteins.

iASPP binds preferentially to the proline-rich domain of p53 instead of the DNA-binding domain, whereas ASPP2 binds preferentially to the p53 DNA-binding domain [23]. Polymorphism of p53 residue 72 (Arg/Pro) within the proline-rich region of p53 greatly affects the ability of iASPP to bind to the p53 proline-rich domain, binding preferentially to p53Pro-72 versus p53Arg-72 (Figure 1.22) [23]. Additionally, phosphorylation within the proline-rich region of p53, on residues Ser-46 and Thr-81, induces the Pin-1 (petidyl-prolyl cis/trans isomerase NIMA-interacting-1 protein) mediated propyl isomerization of N-terminal p53 proline residues, promoting the dissociation of iASPP from p53 [193].

1.5.4 ASPP Protein Expression is Altered in Many Cancers

Inhibition of p53 activity by iASPP most likely evolved as a mechanism to turn off p53 signalling after a cellular stress has been dealt with. p53 directly promotes the expression of iASPP, generating a negative feedback loop to turn off p53 signalling [155]. In addition, inhibition of p53 by iASPP may also be important developmentally; iASPP expression is important for epithelial cell proliferation and epidermal differentiation [41, 223]. However, aberrant over expression of iASPP leaves cells unable to respond to cellular damage and has been linked to chemoresistance [121]. Significantly, iASPP is also over expressed in multiple forms of cancers (Table 1.5). Single polymorphisms within the iASPP gene (specifically A67T) have been identified in non-responsive cases of advanced non-small cell lung cancers [260]. It has been proposed that this the Thr-67 polymorphism may increase the stability of

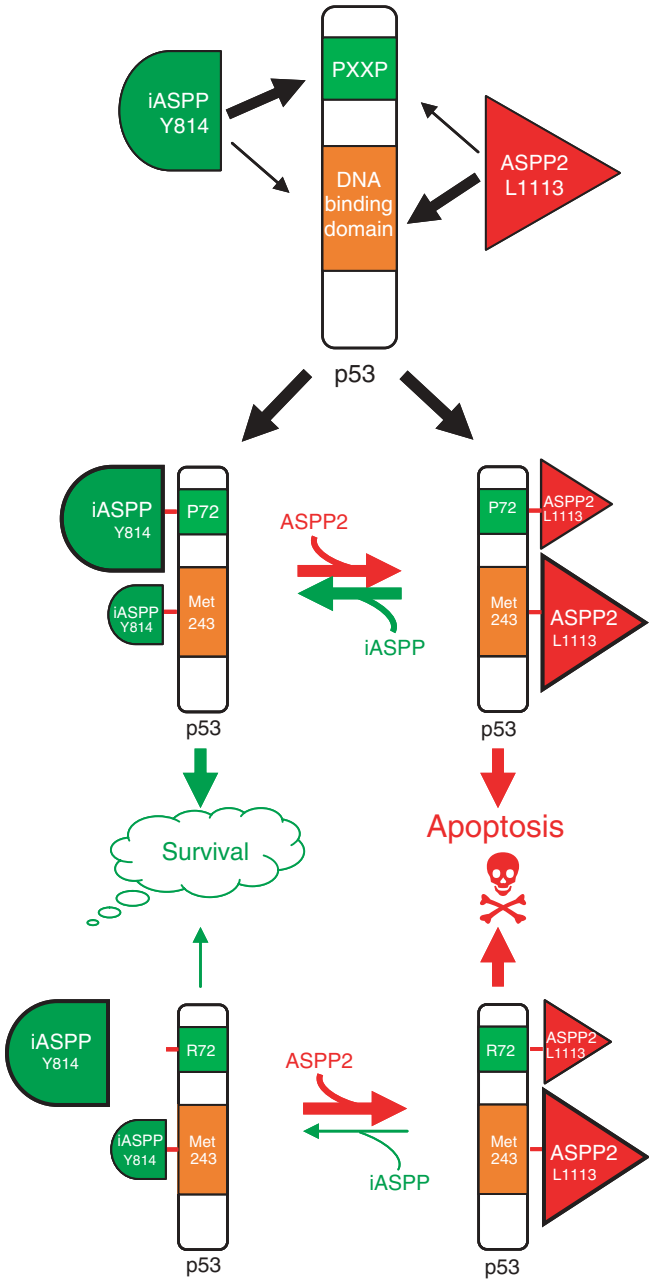

Figure 1.22: **iASPP Binds Preferentially to the Proline-Rich Domain of p53**. The figure depicts the preferential binding of iASPP to the proline-rich domain (PxxP) of p53 over the DNA-binding domain. In contrast, ASPP2 binds preferentially to the DNA-binding domain. Polymorphism within the proline-rich domain (p53 residue Arg/Pro-72) alters the ability of iASPP to bind to the proline-rich domain and iASPP binds preferentially to p53Pro-72 versus p53Arg-72. The presence of p53Arg-72 within a cell is proposed to promote the binding of ASPP2 over iASPP to p53 and shift cells from a pro-survival mode to a pro-apoptotic mode in response to cellular stress. Figure from [23].

iASPP mRNA and lead to the increased overall expression of the iASPP protein. Overexpression of iASPP has also been reported in cancers that have defective or mutated p53. Studies have shown that iASPP overexpression cancers that express defective p53 inhibits p53-independent apoptosis through the inhibition of p53 family members, p63 and p73 [31].

In contrast, the expression of ASPP1 and ASPP2 is decreased in some forms of cancer (Table 1.5). Multiple studies have shown that the promoters of ASPP1 and ASPP2 are hypermethylated in several forms of cancer, leading to a drastic decrease in ASPP1/2 expression levels [3, 188, 293]. In support of this, mice heterozygous for ASPP2 have decreased ASPP2 expression levels and increased incidence of spontaneous and γ-irradiation induced tumours [128]. Decreased levels of ASPP2 have also been linked to poor clinical outcome for patients with aggressive non-Hodgkin's lymphoma [181]. Overall, the relative expression levels of ASPP1/2 and iASPP appears to dictate to some degree whether or not a cancerous cell will undergo apoptosis.

1.5.5 The Role of the ASPP Proteins in Facilitating Site Specific Dephosphorylation of p53 by PP-1c

ASPP2 contains a principal PP-1c RVXF binding motif and has been shown to bind to PP-1c with a greater affinity than p53 [98]. ASPP1 also contains an equivalent RVXF motif and prior to this work we predicted it would also bind to PP-1c. Although iASPP retains its ability to bind to p53, it does not contain a classical RVXF motif, instead having a RARL motif (residues 622-625). Therefore, we initially predicted that iASPP would not interact directly with PP-1c. Nevertheless, our collaborators Llanos et al. (2011) demonstrated that iASPP does bind directly to PP-1c [178]. My initial research also confirmed that iASPP does in fact bind to PP-1c, despite lacking a canonical RVXF motif.

While the role of protein kinases in p53 signalling has been extensively studied, less is known about how Ser/Thr protein phosphatases are involved in the p53 pathway. In order for a cell to regain normal cellular functions after a cellular stress such as DNA damage has been alleviated, p53 signalling must be turned off (Figure 1.23). PP-1c has been shown to dephosphorylate two Ser residues within the p53 N-terminus, Ser-15 and Ser-37 [161]. While PP-1c does not directly interact with p53 [161], a PP-1c regulatory protein that can target PP-1c to dephosphorylate p53 has not been identified to date. Since all three ASPP proteins have the ability to

Table 1.5: **Aberrant Expression of the ASPP Proteins in Multiple Cancers**] ? indicates that status is not known.

Cancer Type	**p53 Status**	**ASPP1/2 Downregulated**	**iASPP Overexpression**	**References**
Bladder	?	?	YES	[174]
Breast	WT	YES	YES	[244, 97, 22, 177, 289]
Cervical	?	?	YES	[32]
Endometrial Adenocarcinoma	?	YES	YES	[175]
Gestational Trophoblastic Disease	?	YES	?	[188]
Glioblastoma	?	?	YES	[162]
Head and Neck Squamous Cell Carcinoma	?	?	YES	[176]
Hepatocellular Carcinoma	WT	?	YES	[312, 182, 227, 171]
Leukemia	WT	YES	YES	[3, 310, 172]
Lymphoma	?	YES	?	[181]
Medullablastoma	?	YES	?	[232]
Non-small Cell Lung Cancer	WT	YES	YES	[214, 37, 293, 169]
Ovarian	WT or Mutated	?	YES	[121]
Pituitary Tumors	?	?	YES	[231]
Prostate	Mutated	?	YES	[307]

bind to both p53 and PP-1c, I hypothesized that one or potentially all three human ASPP proteins form a trimeric complex with PP-1c and p53. If this is the case, then the formation of a trimeric PP-1c•ASPP•p53 complex may facilitate the site specific dephosphorylation of p53 by PP-1c.

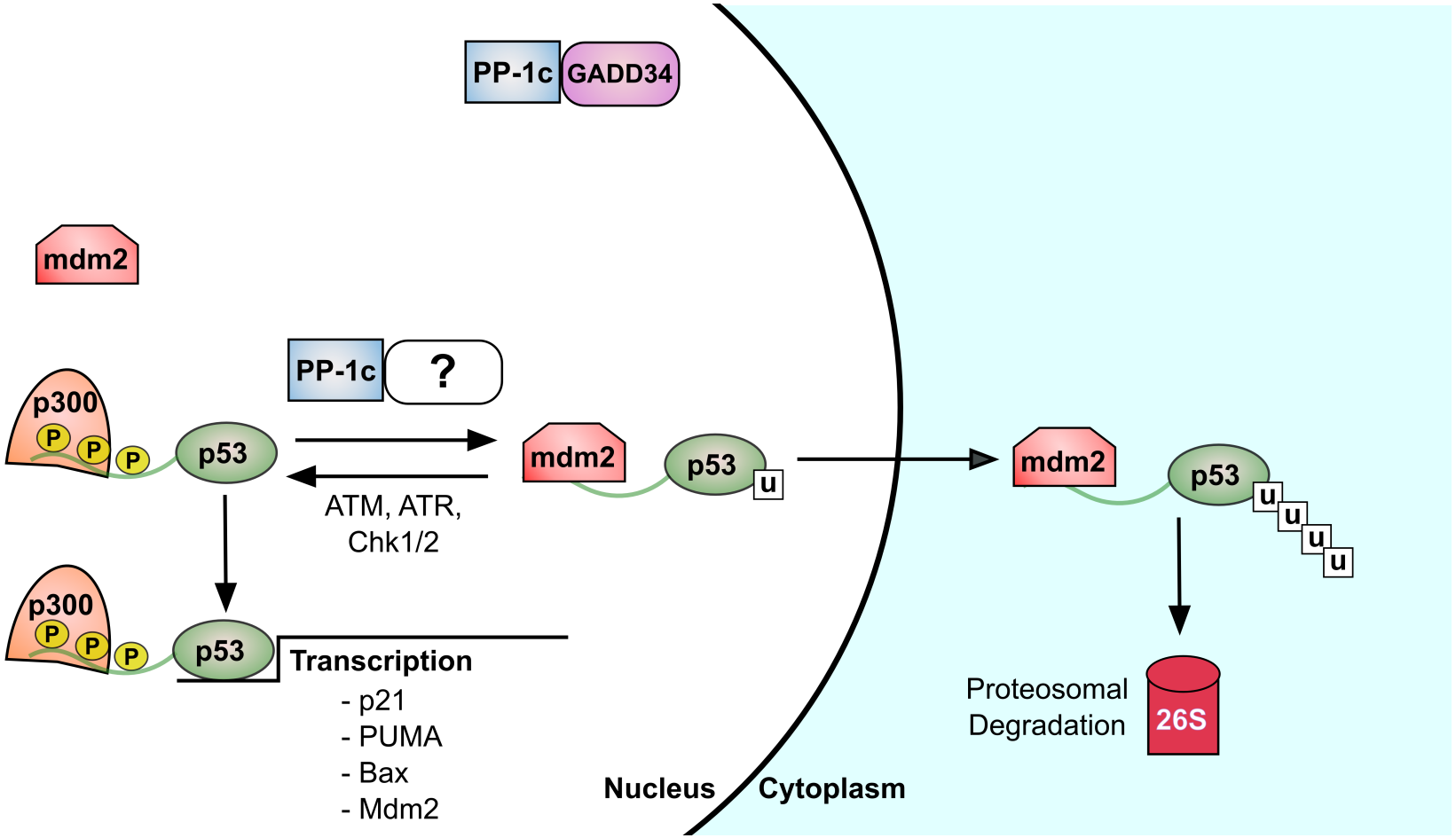
PP-1c
GADD34
mdm2
PP-1c
?
p300
P
P
P
p53
mdm2
p53
u
ATM, ATR,
Chk1/2
mdm2
p53
u
u
u
u
p300
P
P
P
p53
Transcription
- p21
- PUMA
- Bax
- Mdm2
Proteosomal
Degradation
26S
Nucleus
Cytoplasm

Figure 1.23: **Regulation of p53 Activity by Dephosphorylation** In normal functioning cells, p53 is kept at low levels, mainly via the actions of mdm2, an E3 ubiquitin ligase that promotes poly-ubiquitination of the p53 C-terminus. Ubiquitination of p53 leads to its translocation from the nucleus into the cytoplasm and targets p53 for proteosomal degradation. In the event of cellular stress, such as DNA damage, several Ser/Thr protein kinases are activated which phosphorylate the N-terminal transactivation domains of p53. This leads to the stabilization and activation of p53 as hyper-phosphorylation of the p53 N-terminus disrupts the binding of mdm2 and promotes the association of proteins that stablize p53 such as p300. Upon activation of p53, p53 binds to specific DNA promoters for gene targets involved in cell cycle arrest and apoptosis. In order to turn off p53 signalling after a cellular stress has been dealt with, the N-terminus of p53 must be dephosphorylated to turn off p53 signalling. PP-1c has been shown to directly dephosphorylate p53 [161], however a specific PP-1c regulatory protein that targets PP-1c to dephosphorylate p53 has been identified to date. However, some PP-1c regulatory proteins have been shown to inhibit the dephosphorylation of p53 by PP-1c [96].

1.6 Specific Aims of Thesis

1. **To elucidate the molecular interactions between protein phosphatase-1c and the apoptotic ASPP proteins**. Our first goal was to characterize the binding of ASPP1, ASPP2, and iASPP to protein phosphatase-1c and identify additional interaction sites between the ASPP proteins and PP-1c, besides the RVXF motif, that are critical for binding. The iASPP protein does not contain a classical RVXF motif; therefore, it was initially predicted that unlike ASPP1 and ASPP2, iASPP would not be able to directly bind to PP-1c. A central hypothesis was that the C-terminal tail of PP-1c contains a PxxPxR binding motif and interacts directly with the ankyrin repeat and SH3 domains of the ASPP proteins.

2. **To investigate the functional role of the ASPP proteins in regulation of PP-1c activity towards p53**. Our second goal was to investigate whether any of the ASPP proteins could bind in a heterotrimeric complex with both PP-1c and p53. A further hypothesis was that the putative trimeric complex may function to specifically dephosphorylate key phospho-Ser and phospho-Thr residues within the N-terminus of p53.

3. **To investigate the regulation of protein phosphatase-1c•iASPP complex by small molecules**. The overexpression of iASPP in many forms of cancer may be one method by which cancer cells disrupt the p53 apoptotic pathway and therefore the iASPP•PP-1c complex may be a potentially valuable molecular target for the development of cancer therapies. Our goal was to identify novel small molecular inhibitors that specifically disrupt the iASPP•PP-1c complex by the development of novel disruption screening assays.

Chapter 2

Interaction of Protein Phosphatase-1c with the ASPP Proteins

A portion of the research described in this chapter was originally published in Biochemical Journal. Skene-Arnold, T.D., Luu, H.A., Uhrig, R.G., De Wever, V., Nimick, M., Maynes, J., Fong, A., James, M.N., Trinkle-Mulcahy, L., Moorhead, G.B., and Holmes, C.F.B., Molecular Mechanisms underlying the interaction of protein phosphatase-1c with ASPP proteins. Biochemical Journal. 2013; 449: 649-659 Portland Press Limited.

2.1 Introduction

Nearly 70% of all eukaryotic proteins are modified by reversible phosphorylation reactions [226]. While the human genome encodes for approximately 400 Ser/Thr protein kinases, it encodes for substantially fewer Ser/Thr protein phosphatase catalytic subunits [36]. A key biological question is how a small number of protein phosphatases can counteract the actions of many protein kinases to maintain spatial and temporal regulation of substrate dephosphorylation. In mammals, there are three isoforms of the protein phosphatase-1c (PP-1c), α, β, and γ, that share 76-88% sequence identity and differ mainly within their N and C-termini. PP-1c associates with multiple regulatory subunits (R-subunits), which target PP-1c to specific substrates. To date, over 180 PP-1c R-subunits have been identified [100]. By binding to R-subunits, PP-1c exists in many mutually exclusive oligomeric states within a cell, altering not only its substrate specificity, but also targeting to specific subcellular locations.

While PP-1c regulatory subunits interact with PP-1c through multiple sites [6, 34, 269], the majority of PP-1c R-subunits contain a conserved amino acid consensus sequence referred to as the RVXF binding motif ([K/R] - X_{0-1} - [V/I/L] - X - [F/W], where X can be any amino acid except proline). This motif binds to PP-1c in an extended fashion within a hydrophobic pocket of PP-1c remote from the catalytic active site [63, 287]. Mutation of hydrophobic Val and Phe positions in the motif abolishes the ability of full-length R-subunits to bind to PP-1c [63, 287, 24, 94, 279]. Additonally, short synthetic RVXF-containing peptides competitively bind to PP-1c and are sufficient to disrupt binding of R-subunits that have RVXF motifs [63, 287, 125].

The Ankyrin-rich, SH3-domain and Proline-rich region containing Proteins (ASPPs) are a unique family of p53 binding proteins that modulate the activity of p53 [274]. Therefore, they are often referred to as the Apoptotic Stimulating Proteins of p53. The human genome encodes three principal ASPP proteins; ASPP1, ASPP2, and an inhibitory ASPP, iASPP. ASPP2 was originally identified in a yeast two-hybrid screen as one of two p53-binding proteins, p53BP1 and p53BP2 (p53BP2 is now referred to as ASPP2), respectively [117]. While the p53BP1 and p53BP2/ASPP2 protein share no sequence similarity, the ankyrin repeat and SH3 domains of p53BP2/ASPP2 were found to be homologous to two other proteins,

later named ASPP1 and iASPP [274]. ASPP1 and ASPP2 bind directly to the DNA-binding domain of wild-type p53 via their ankyrin-repeat and SH3 domains to enhance the apoptotic function p53 by increasing the p53-dependent transcription of pro-apoptotic genes [23, 239]. The molecular mechanism by which ASPP1 and ASPP2 activate p53 activity is currently not well understood [21]. The crystal structure of p53 bound to ASPP2 revealed that ASPP2 binds to the same region of p53 that interacts with DNA [90, 42]. Not surprisingly, mutants of p53 commonly found in cancer lack the ability to interact with DNA or ASPP2 [117, 272].

The inhibitory ASPP protein (iASPP) was originally identified as an inhibitor of the Rel A p65 subunit of nuclear factor κB (NF-κB) [302]. iASPP is conserved from worms to humans, and unlike ASPP1 and ASPP2, functions as an oncogene in cells by decreasing the apoptotic activity of p53. iASPP is over expressed in many cancers that typically have normal levels of ASPP2 and p53, including breast cell carcinomas and acute myeloid leukemias [22, 239, 310]. Additionally, iASPP and p53 form a negative feedback loop, as iASPP protein expression is induced directly through the accumulation and activation of p53 [155]. The C-terminus of iASPP (ankyrin repeat and SH3 domains) is homologous to the C-termini of ASPP1 and ASPP2 and iASPP has been shown to directly compete with ASPP1 and ASPP2 for binding to p53 [22].

ASPP2 was later shown to bind to other proteins, including the p65 subunit of NF-κB, the Hepatitis C virus (HCV) core protein, APCL (a homolog of the Adenomatous Polyposis Coli tumour suppressor), Bcl-2, YAP (Yes-associated protein), Lats2 (Large Tumour Suppressor Protein 2) and PP-1c [23, 33, 131, 69, 281, 13, 216, 302]. ASPP2 contains a principal RVXF binding motif and binds to PP-1c with a greater affinity than p53 [98]. ASPP1 also contains an equivalent RVXF motif and therefore was predicted to also bind to PP-1c. In contrast, although iASPP retains its ability to bind to p53, it does not contain a PP-1c RVXF motif and was not previously predicted to bind to PP-1c.

The objective of the work described in this chapter was to investigate the molecular interactions between the ASPP proteins and PP-1c. I demonstrate that both ASPP1 and unexpectedly iASPP bind to PP-1c in a stable 1:1 complex. Furthermore, determination of a modelled structure comprising the complex between PP-1c and ASPP2 has allowed us to identify additional regions of PP-1c that interact with the ASPP proteins and that are essential for binding.

2.1.1 Microcystin-Sepharose - a Capture Affinity Assay

As outlined in detail in Chapter 1, microcystin (MC) is a cyclic heptapeptide toxin (Figure 1.8) produced by cyanobacteria and exerts its inhibition of PP-1c by binding directly to its active site. Inhibition of PP-1c by microcystin can be permanent and irreversible due to its ability to covalently bind to PP-1c residue Cys-273 via its reactive N-methyldehydroalanine (Mdha) group. The reactive chemistry of the microcystin Mdha group can been exploited such that it can be linked synthetically to different matrices, including Sepharose and Biotin. Originally, Nishiwaki et al. (1991) generated a microcystin-Sepharose (MC-Sepharose) matrix to purify PP-2Ac from partially purified cellular lysates; however, the resin had limited binding capacity for PP-2Ac ($\sim$ 3 μg per ml of resin) and reportedly did not bind PP-1c at all [219]. Later, Moorhead et al. (1994) were successful in linking microcystin to Sepharose beads via an aminoethanethiol linkage, creating for the first time a stable high-affinity MC-Sepharose matrix with a high capacity for binding both PP-1c and PP-2Ac (1 mg of microcystin coupled to 6 ml of Sepharose yielded a resin with a binding capacity of approximately 1 mg of PP-1c per ml of resin) [209].

MC-Sepharose binds tightly to the phosphatase active site; but attached to Sepharose, microcystin is unable to covalently couple to the phosphatase and therefore the binding is reversible, allowing MC-Sepharose to be used to isolate and purify PP-1c and PP-2Ac from the resin. Bound to MC-Sepharose via the active site, the remainder of the protein phosphatase surface remains exposed and is still able to bind to regulatory subunits; therefore, it can also be used as a valuable tool for the purification of PP-1c/PP-2Ac regulatory proteins from complex mixtures including cellular lysates [211]. Previously, purification of phosphatase regulatory proteins has been challenging given that they are typically expressed in low concentrations within cells and they tend to dissociate from PP-1c/PP-2Ac during longer purification methods. MC-Sepharose has been used successfully to purify multiple PP-1c regulatory proteins from cellular lysates, including the Glycogen targeting subunit (G_L) [208], p99 [144], O-linked N-acetylglucosamine (O-GlcNAc) Transferase [294], the nuclear interacting protein ZAP [208] and the inhibitory ASPP protein (iASPP) [250]. Synthetic peptides containing an RVXF sequence (typically derived from a PP-1c regulatory protein) have also been used to elute regulatory proteins directly from PP-1c immobilized on MC-Sepharose. This has been extremely valuable in dif-

ferentiating regulatory proteins that specifically bind to PP-1c via an RVXF motif versus other modes of interaction. It also rules out the possibility that the particular binding protein is being pulled down via other MC sensitive phosphatases such as PP-2Ac, PP-4, and PP-6.

2.2 Materials and Methods

2.2.1 Materials

Microcystin-LR (MC-LR) was purified from *Microcystis aeruginosa* collected from Little Beaver Lake (Ferintosh, Alberta, Canada) as previously described [25]. Full-length human wild-type cDNA for ASPP2 and iASPP was used as described [23]. Full-length human wild-type cDNA (complementary deoxyribonucleic acid) for ASPP2 and iASPP was generously provided by Dr. Xin Lu, from the Ludwig Institute for Cancer Research, Oxford University, UK [23]. Full-length ASPP1 cDNA was provided by Dr. David Elliot from the Institute for Human Genetics at the University of Newcastle, UK. Human His-tagged inhibitor-2 was expressed and purified by Marcia Craig from our laboratory. DNA constructs for truncated PP-1cα_{1-306} was kindly provided by Greg Moorhead from the University of Calgary. A synthetic peptide containing the ASPP2 RVXF motif (RVKFNPLALLLDSS, residues 921-934) was purchased from the Alberta Peptide Institute at the University of Alberta. The iASPP RNYF peptide (GYVPRNYFGLFPRV, iASPP residues 807-821) was purchased from GLS Biochem (Shanghai, China). Unless otherwise stated, all other chemicals and reagents were purchased from Sigma Aldrich. Microcystin-Sepharose and control Sepharose preparations were generated as previously described by Ply Pasarj and Cindy Lee in our laboratory [209, 208].

2.2.2 Expression and Purification of Wild-type and Mutant Recombinant Protein Phosphatase-1c Isoforms

Human and rabbit recombinant PP-1c isoforms α, β, γ were expressed and purified to homogeneity as in [196], with the following modifications. A single colony of *E. coli* C41 DE3 cells transformed with either pKK223.3-hPP-1cα, pCW-rPP-1cβ or pCW-hPP-1cγ was used to inoculate a 400 ml overnight culture of Luria-Bertani (LB) media containing 1 mM $MnCl_2$ and 200 μg/ml Ampicillin (Amp). The overnight culture was then subcultured into 4 L of LB media containing 1 mM $MnCl_2$, 0.001% Vitamin B1 (w/v), and 200 μg/ml Amp. Once the culture reached

an optical density of approximately 0.5 at 600 nm, protein expression was induced with 0.1 to 0.5 mM IPTG for 18 hours. Cells were harvested by centrifugation at 6,000 × g and resuspended in approximately 130 ml of buffer A (50 mM imidazole pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 2.0 mM $MnCl_2$, 0.5 mM phenylmethane sulfonylfluoride (PMSF), 2.0 mM benzamidine, 3.0 mM dithiothreitol (DTT), 100 mM NaCl and 10% glycerol (v/v)) containing 2 μg/μl DNase A and one Sigma fast protease tablet. Cells were then lysed by three consecutive passes through an Emulsiflex-C3 high-pressure homogenizer (Avestin) and centrifuged for 45 min at 13,000 × g. Supernatant was then loaded onto a heparin-Sepharose CL-6B column (GE Healthcare) and PP-1c protein was eluted using a 400 ml linear gradient of buffer A containing 0.1 to 0.5 M NaCl. Fractions were assayed for phosphatase activity using the colourimetric p-nitrophenol phosphate (pNPP) assay described below [9, 198]. Active fractions were pooled and diluted 3.5 × in buffer B (50 mM imidazole pH 7.5, 0.5 mM EDTA, 2.0 mM $MnCl_2$, 2.0 mM DTT, 20% glycerol (v/v)), loaded onto a human inhibitor-2 affinity column and eluted in buffer B containing 1 M NaCl. Active fractions, confirmed to be homogeneously pure by SDS-PAGE analysis, were pooled and concentrated to 1 - 4.0 mg/ml, an equal volume of glycerol added and stored at $-20\,^{\circ}$C.

2.2.3 Mutagenesis of Protein Phosphatase-1c

Mutagenesis of PP-1cα (A259R, K260D, 261S) was carried out by Hue Anh Luu using QuikChange site-directed mutagenesis (Stratagene) as described previously [198]. All PP-1cα mutants were validated by DNA sequencing. PP-1c mutants were subsequently transformed into either DH5α cells for long-term storage or C41 DE3 cells (Lucigen) for protein expression. The PP-1cα mutants were purified as described for wild-type PP-1cα in section 2.2.2.

2.2.4 Expression, and Purification of the Wild-type and Mutant ASPP Proteins

N-terminally His-tagged human $ASPP1_{867-1090}$, $ASPP2_{905-1128}$, $iASPP_{608-828}$, and $iASPP_{626-828}$ constructs were generated using polymerase chain reaction (PCR) amplification, engineering unique 3′ and 5′ restriction enzyme cleavage sites (EcoRI and XhoI, respectively). The $iASPP_{608-828}$ L625A mutant was generated using QuikChange site-directed mutagenesis, as described for above PP-1cα mutants.

Each construct was cloned into pET28A vector and transformed into *E. coli* Rosetta pLys cells (Novagen). A single colony of *E. coli* Rosetta cells transformed with a plasmid construct was used to inoculate 400 ml of LB media containing chloramphenicol (34 μg/ml) and kanamycin (30 μg/ml). After cell growth overnight at 37 °C, the culture was used to inoculate 4 L of LB media containing chloramphenicol (34 μM) and kanamycin (30 μM) and were grown at 28 °C to an optical density of 0.5 at 600 nm. Protein expression was induced with 0.1 - 0.3 mM IPTG for 18 hours at 28 °C. Cells were harvested by centrifugation at 4 °C for 15 min at 6,000 $\times$ g. Pelleted cells were resuspended in 150 ml of buffer C (25 mM sodium phosphate pH 8.0, 125 mM NaCl, 10% (v/v) Tween-20) containing 15 mM imidazole, 2.7 mM KCl, 1 mM PMSF, 1.5 ml of protease inhibitor cocktail for His-tagged proteins (Sigma) and 5 mM β-mercaptoethanol (β-Me). Cells were lysed as described for PP-1c and centrifuged for 45 min at 13,000 $\times$ g. Supernatant was applied to a Ni-NTA (nickel-nitriloacetic acid) affinity His-prep FF 16/10 column (GE Healthcare) at 1.0 ml min^{-1}. Bound ASPP proteins were eluted in buffer C containing 250 mM imidazole. Elution fractions (5 ml) were pooled based on SDS-PAGE analysis and then separated on a Superdex 75 26/60 size exclusion column (GE Healthcare), at 0.9 ml min^{-1}. Protein was eluted with buffer D (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT) as previously described [235]. Fractions (3ml) were pooled based on SDS-PAGE analysis and concentrated to 5 mg/ml. An equal amount of glycerol was added and the solution stored at -20 °C.

2.2.5 Microcystin-Sepharose Binding Experiments of PP-1c and the ASPP Proteins

MC-Sepharose was prepared by Ply Pasarj and Hue Anh Luu in our laboratory as previously described [209, 208]. PP-1cγ (10 μg) was diluted in buffer E (50 mM Tris-HCl pH 7.5, 0.1 mM EDTA, and 0.5 mM $MnCl_2$) containing 150 mM NaCl and bound to 25 μl of MC-Sepharose for our hour, end over end at 4 °C. Resin was washed buffer E containing 150 mM NaCl and subsequently incubated with approximately 10 μg of either $ASPP1_{867-1090}$, $ASPP2_{905-1128}$ or $iASPP_{608-828}$ overnight at 4 °C. After washing resin with buffer E containing 300 mM NaCl, bound protein was eluted by the addition of 2 $\times$ SDS-PAGE sample buffer (65 mM Tris-HCl pH 6.8, 26% glycerol (v/v), 2% SDS (w/v) and 0.1 % (w/v) bromophenol blue) and boiling at 100 °C for 5 min. Protein elutions were subsequently analyzed

by SDS-PAGE and Coomassie blue staining.

2.2.6 Gel Filtration Chromatography

Gel filtration chromatography was performed using fast protein liquid chromatography (FPLC) (Amersham Pharmacia Biotech). Highly purified preparations of PP-1cα and His-tagged constructs of either ASPP1$_{867-1090}$, ASPP2$_{905-1128}$, or iASPP$_{608-828}$ were incubated either alone or together at 30 °C for 45 min prior to resolution on a Superdex 75 10/30 gel filtration column (GE Healthcare). The proteins were resolved at a flow rate of 0.1 ml min^{-1} using buffer F (50mM imidazole pH 7.8, 0.3 M NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 2.0 mM DTT, 10% glycerol) containing 2 mM $MnCl_2$. Fractions (250 μL) were collected and analyzed by SDS-PAGE.

2.2.7 RVXF Peptide Disruption of the PP-1c•ASPP Protein Complexes

Peptide disruption of the PP-1c•ASPP protein complexes on MC-Sepharose was carried out as previously described in Section 2.2.5 with the following modifications. Proteins were eluted from the MC-Sepharose resin by incubating the resin for one hour at 4 °C with 100 μl of either buffer E or buffer E containing 0.5 mM of a synthetic RVXF peptide derived from the ASPP2 protein sequence (RVKFNPLALLLDSS, residues 921-934). Eluted protein was concentrated using a ultra-free durapore centrifugal filter (Millipore) and then analyzed by SDS-PAGE analysis and Coomassie blue staining.

2.2.8 Ni-NTA Binding Experiments of PP-1c and His-tagged ASPP Protein Constructs

Approximately 20 μl Ni-NTA agarose (Qiagen) was incubated end over end for one hour at 4 °C with 5 μg of wild-type or mutant ASPP protein and then reconstituted in 300 μl buffer G (25 mM Tris-HCl pH 7.5, 10 mM imidazole, 150 mM NaCl, 0.5 mM $MnCl_2$). Ni-NTA resin was washed with buffer H (25 mM Tris-HCl pH 7.5, 30 mM imidazole, 500 mM NaCl, 0.5 mM $MnCl_2$ and 0.1% Tween-20) until no further protein could be eluted. Wild-type or mutant PP-1c (5μg) was diluted in buffer H and incubated with bound Ni-NTA at 4 °C for 1 to 18 hours. After washing, protein was eluted from the resin by boiling samples in 30 μl of 2 × SDS-PAGE sample buffer at 100 °C for 5 min. Samples were then analyzed by SDS-PAGE.

2.2.9 RNYF Peptide Binding Experiments

To test whether the the RNYF motif located within the iASPP SH3 domain is a true RVXF motif that can bind directly to PP-1c, synthetic peptides containing either the RVXF motif of ASPP2 (RVKFNPLALLLDSS, residues 921-934) or the putative RVXF motif from iASPP (GYVPRNYFGLFPRV, residues 808-821) were coupled to NHS-Activated (N-hydroxysuccinimide) Sepharose (GE Healthcare) and the ability of PP-1c to bind to the peptide coupled resins was then tested. For each peptide, NHS-Activated Sepharose ($\sim$200 μl) was washed with 15 resin volumes of cold 1 mM HCl and 15 resin volumes of buffer I (0.1 M sodium bicarbonate pH 8.0 and 0.5 M NaCl). Approximately 0.5 mg of each peptide was dissolved in buffer I and the pH was adjusted to $\sim$ 8.5 with NaOH. The diluted peptide and NHS-Activated Sepharose were then incubated together overnight at 4 °C end over end. After sufficient washing with buffer I, resin was blocked for 1 hour at room temperature with buffer I containing 0.1 M Tris-HCl, pH 8.0 and 0.5 M NaCl. Resin was then washed with an acidic buffer (50 mM sodium acetate pH 4.0 and 0.5 M NaCl), followed by washing with a basic buffer (50 mM Tris-HCl pH 8.0 and 0.5 M NaCl). The concentration of the each peptide was measured before and after coupling to determine the percentage of peptide bound to the resin and was calculated to be approximately 85 and 93 % for the ASPP2 and iASPP peptides, respectively.

The binding of PP-1cγ to the ASPP2 or iASPP peptide resins was carried out as follows. Each peptide-coupled resin ($\sim$25μl) was washed with 40 resin volumes of H_2O, followed by 40 resin volumes of buffer E containing 150 mM NaCl. PP-1cγ (5 μg) was diluted in buffer E containing 150 mM NaCl and incubated together with each resin end over end at 4 °C overnight. Resin was then washed with 50 resin volumes of buffer E containing 150 mM NaCl and bound protein was eluted by the addition of SDS-PAGE sample buffer and boiling at 100 °C for 5 min. Samples were then analyzed by SDS-PAGE and Coomassie blue staining.

2.2.10 Phosphorylase *a* Phosphatase Activity Assays

Phosphorylase *a* phosphatase 30 μl assays were carried as previously described [105] with the following modifications. PP-1cβ and PP-1cγ (1 μg from a 1 mg/ml glycerol stock) were further diluted approximately 10,000 fold in 10 μl of buffer J (50 mM Tris-HCl pH 7.0, 0.1 mM EDTA, 1 mg/ml BSA, 1 mM $MnCl_2$, and 0.2 %

β-mercaptoethanol). PP-1c was incubated with increasing amounts of His-tagged $ASPP2_{905-1128}$ or $iASPP_{608-828}$ for 10 min at 30 °C. Dephosphorylation reactions were initiated by the addition of 10 μl of ^{32}P-phosphorylase *a* diluted in buffer K (50 mM Tris-HCl pH 7.0, 0.1 mM EDTA, 1 mg/ml BSA, 0.2 % β-mercaptoethanol, and 3.75 mM caffeine). ^{32}P-phosphorylase *a* was prepared as previously described [186]. Samples were incubated for 10 min at 30 °C, after which the reaction was stopped by the addition of 200 μl of cold 20 % trichloroacetic acid. After two minutes on ice, samples were centrifuged to separate the protein pellet from the supernatant containing the free ^{32}P-labelled phosphate. The amount of ^{32}P-labelled phosphate released was then measured by adding 200 μl of the supernatant to 1 ml of aqueous scintillation fluid and measuring the counts per minute (cpm) in a Wallac 1209 RackBeta scintillation counter. Reactions were carried out in duplicate and repeated 3 times.

The protein phosphatase inhibition curve for the phosphorylase assay is approximately linear within the 30 - 60% PP-1c activity range [150]. Percent PP-1c activity for all tested samples was calculated using the following equation:

$$\%\, PP1c\,, activity\; = \frac{100 - (sample - blank)}{(control - blank)} \times 100\%$$

where the control represents the maximum PP-1c activity (100%) when tested in the absence of any inhibitor, the blank represents the background cpm (the free phosphate in the phosphorylase *a* solution), and the sample represents the cpm of a sample containing PP-1c and a known amount of protein/inhibitor.

The level of inhibition seen by any given inhibitor is affected by the concentration of phosphatase within the assay, which can be calculated within the phosphorylase *a* assay by calculating the percent release of ^{32}P from phosphorylase *a* during the assay:

$$\%\, ^{32}P\,, released\; = \frac{control\, cpm - blank\, cpm}{total\, (cpm - blank\, cpm)} \times 1.15 \times 100$$

where the control cpm is the released ^{32}P with a known amount of PP-1c enzyme, the blank cpm is the amount of free ^{32}P in the phosphorylase substrate, total cpm is

the amount of ^{32}P in the phosphorylase *a* substrate, and 1.15 is the dilution factor. The assay was standardized such that the % release of ^{32}P from phosphorylase *a* was 15%.

2.2.11 pNPP Phosphatase Activity Assays

The colourimetric phosphatase activity assays using paranitrophenol (pNPP) as a small chemical substrate were carried out as follows. ASPP2, iASPP, or inhibitor-2 glycerol stock samples ($\sim$ 1 mg/ml) were diluted in Buffer L (50 mM Tris-HCl pH 8.3, 1.0 mM EGTA, 30 mM $MgCl_2$, 1 mg/ml BSA, and 0.2 % β-mercaptoethanol) to a final concentration of 10, 20, 50, 100, and 500 nM within the 60 μl assay. PP-1cα was diluted (approximately 1/1000) in Buffer L such that the control sample (lacking any protein or inhibitor) reached an absorbance of 0.4 - 0.6 at 405 nm after 1 hour incubation at 37 °C. ASPP proteins and PP-1c were incubated together for 10 min at 37 °C prior to the addition of pNPP (5 mM final concentration). Samples were incubated for an additional 45 min at 37 °C, after which the absorbance at 405 nm was measured to determine phosphatase activity for each sample.

2.2.12 Multiple Sequence Alignments

Protein sequences were identified from the National Center for Biotechnology Information (NCBI) database [11] and CLUSTALW2 program from the European Bioinformatics Institute was used to perform the multiple alignment [154, 91].

2.2.13 Molecular Computer Modeling of the ASPP-PP-1c Complex

Molecular computer modelling of the ASPP-PP-1c complex was carried out by Dr. Jason Maynes from the James laboratory at the University of Alberta (currently at the University of Toronto, Departments of Anaesthesia and Pain Medicine). The coordinates for PP-1cβ were taken from the complex PP-1cβ and MYPT-1 (PDB #1S70) [269] and the coordinates for the p53-ASPP2 complex were taken from PDB # 1YCS [90]. The ankyrin repeat domains of ASPP2 were aligned with equivalent regions of MYPT-1 using the program DALI (http://protein.hbu.cn:83/fssp/www.ebi.ac.uk/dali/index.html) to give a probable method of interaction between PP-1c and ASPP2. The model was then submitted to simulated annealing to remove bias and rigid body refinement using the program CNS [29]. The stereochemistry was confirmed using the programs WHATCHECK [108] and PROCHECK [156]. All figures

were composed using the program PYMOL (PyMOL Molecular Graphics System, Version 1.2rpre, Schrodinger, LLC).

2.3 Results

2.3.1 Identification of ASPP1 and iASPP as Novel Protein Phosphatase-1c Binding Proteins

The majority of PP-1c R-subunits interact with PP-1c via a conserved RVXF motif and mutation of either the Val and Phe positions of this motif abolishes the interaction [63, 287, 24, 94, 279]. All three ASPP family members (ASPP1, ASPP2, and iASPP) show significant sequence homology within their C-terminal domains (Table 2.1). ASPP1 and ASPP2 both contain a RVXF motif (RVRF, residues 883-886 and RVKF, residues 921-924, respectively), whereas iASPP does not (RARL, residues 622-625). ASPP2 has previously been shown to bind to protein phosphatase-1c with a higher affinity than p53 [98]. We predicted that the ASPP1 protein would interact with PP-1c via its classical RVXF motif in a similar manner as ASPP2. Due to the importance of the Val and Phe positions within the motif, we initially hypothesized that the iASPP protein would not be able to bind to PP-1c because it does not have a functional RVXF motif.

To investigate this hypothesis, a His-tagged truncation construct of iASPP ($iASPP_{608-828}$) was created by PCR amplification. The equivalent ASPP1 and ASPP2 constructs ($ASPP1_{867-1090}$ and $ASPP2_{905-1128}$, respectively) were also made. These constructs were designed to include the PP-1c RVXF motif of ASPP1 and ASPP2 (Table 2.1:; RVKF, residues 921-924) and the corresponding region in iASPP (RARL, residues 622-625). We then utilized microcystin-Sepharose (MC-Sepharose) affinity chromatography to identify novel PP-1c regulatory subunits [211]. MC-Sepharose immobilizes PP-1c via its active site, leaving the hydrophobic RVXF-interacting groove exposed; therefore, MC-Sepharose can pull-down many different PP-1c multi-protein complexes. Our results show that both $ASPP1_{867-1090}$ and $ASPP2_{905-1128}$ bind in a stable complex to PP-1cα on MC-Sepharose (Figure 2.1). Surprisingly, the MC-Sepharose binding experiments show that $iASPP_{608-828}$ also binds directly to PP-1cα.

Gel filtration chromatography was then used to confirm the interactions between PP-1c and the ASPP proteins. Purified preparations of PP-1cα and either His-tagged $ASPP1_{867-1090}$, $ASPP2_{905-1128}$, or $iASPP_{608-828}$ were incubated alone or together at 30 °C for 45 min prior to resolution on a Superdex 75 10/30 gel filtration column. Fractions were then analyzed by SDS-PAGE and Coomassie blue staining.

Alone, PP-1cα and ASPP2$_{905-1128}$ elute as a 37 kDa and 28 kDa monomer, respectively (Figure 2.2, panels A and B). After incubating PP-1cα with ASPP2$_{905-1128}$ at 30 °C for 45 min, they elute together as a ∼60 kDa heterodimeric complex (Figure 2.2, panel C). Similar results were also seen for the binding of ASPP1$_{867-1090}$ to PP-1cα (Figure 2.3).

When iASPP$_{608-828}$ is incubated alone, it is eluted from the gel filtration column as a ∼28 kDa monomer (Figure 2.3.1, panel B). There is a small absorbance peak (fractions 36-38) that initially suggested that there is a small amount of aggregated iASPP$_{608-828}$ within the protein sample. However, SDS-PAGE analysis of the gel filtration fractions reveal that there is little to no iASPP protein within these fractions and the majority exists as a monomer. Upon incubation with PP-1c, there is a dramatic shift in the elution of iASPP$_{608-828}$ and it elutes with PP-1c as a 60 kDa heterodimeric complex (Figure 2.3.1, panel C).

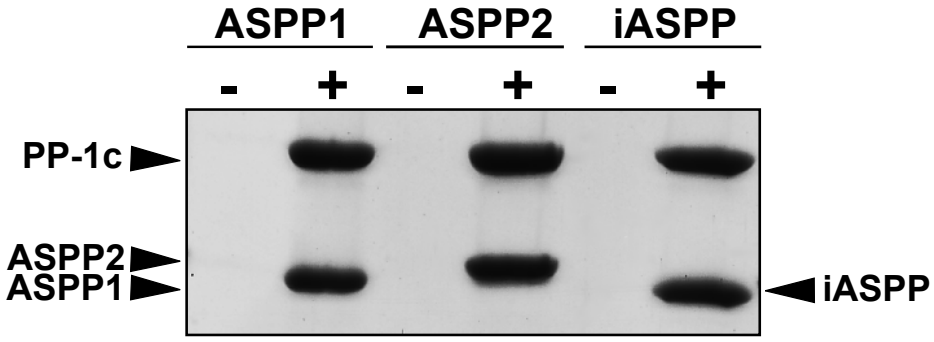

Figure 2.1: **ASPP1, ASPP2 and iASPP Bind to PP-1c Immobilized on Microcystin-Sepharose**. The figure depicts an *in vitro* MC-Sepharose binding experiment, comparing the binding of ASPP1$_{867-1090}$, ASPP2$_{905-1128}$ and iASPP$_{608-828}$ with PP-1cα. PP-1cα (∼7.5 μg) was bound end over end to MC-Sepharose for one hour at 4 °C. Resin was washed and 10 μg of either ASPP2$_{868-1090}$, ASPP2$_{905-1128}$ or iASPP$_{608-828}$ was incubated with the resin for an additional hour at 4 °C. After sufficient washing, bound protein was eluted by the addition of 2X SDS-PAGE sample buffer, heated at 100 °C for 5 mins and analyzed by SDS-PAGE with coomassie blue staining.

Table 2.1: **Multiple Sequence Alignment of the Human ASPP Protein Family (1 of 2)**. The table depicts the multiple sequence alignment of ASPP1, ASPP2, and iASPP using the CLUSTALW2 alignment algorithm [154, 91].

```
iASPP   ----------MDSEAFQ.........SARDFLDM.........NF........QSLAMKHM.....................................    25
ASPP1   MMPMILTVFLSNNEQILTEVPITPETTCRDVVEFCKEPGEGSCHLAEVWRGNERPIPFDHMMYEHLQIWGPRREEVKFFLRHEDSPTENSEQGGRQTQEQ   100
ASPP2   MMPMFLTVYLSNNEQHFTEVPVTPETICRDVVDLCKEPGESDCHLAEVWCGSERPVADNERMFDVLQRFGSQRNEVRFFLRHERPPGRDIV.SGPRSQDP    99


iASPP   ................................DL.....................................................................    27
ASPP1   RTQRNVINVPG.DKRTEYGVGNPRVELTLSELQDMAARQQQQIENQQQMLVAKEQRLHFLKQQERRQQQSISENEKLQKLKERVEAQENKLKKIRAMRGQ   199
ASPP2   SLKRNGVKVPGEYRRKENGVNSPRMDLTLAELQEMASRQQQQIEAQQQLLATKEQRLKFLKQQDQRQQQQVAEQEKLKRLKEIAENQEAKLKKVRALKGH   199


iASPP   ......................KQMELDTAAAKVDELTKQLESLWSDSPA..PP..GPQAGPPS..RPPR.YSSSSIPEP.................    78
ASPP1   VDYSKIMNGNLSAEIERFSAMFQEKKQEVQTAILRVDQLSQQLEDLKKGKLNGFQSYNGKLTGPAAV.ELKRLYQELQIRNQLNQEQNSKLQQQKELLNK   298
ASPP2   VEQKRLSNGKLVEEIEQMNNLFQQKQRELVLAVSKVEELTRQLEMLKNGRIDS......HHDNQSAVAELDRLYKELQLRNKLNQEQNAKLQQQRECLNK   293


iASPP   ..................FGSRGSPRKAAT.....DGADT.PFGRSESAPTLHPYSPLSPKGR..........PSS.................PRTPL.   125
ASPP1   RNMEVAMMDKRISELRERLYGKKIQLN..RV.....NGTSS.PQ............SPLSTSGRVAAVGPYIQVPSA.................GSFPVL   361
ASPP2   RNSEVAVMDKRVNELRDRLWKKKAALQ..QKENLPVSSDGNLPQ............QAASAPSRVAAVGPYIQSSTMPRMPSRPELLVKPALPDGSLVIQ   379


iASPP   .........YL.....................................................................................QPD.AYG   133
ASPP1   GD..PIKPQSLSI....ASNAAHGRSKSANDGNWPTLKQNSSSSVKPVQVAGADW...........KDPSVE.......GSVKQGTVS....SQPV.PFS   432
ASPP2   ASEGPMKIQTLPNMRSGAASQTKGSK...................IHPVGPDWSPSNADLFPSQGSASVPQSTGNALDQVDDGEVPLREKEKKVRPFS   458


iASPP    SLDRATS....PRPRAFDGAGS..............SLGRAPSPRPGPGPLRQQGPPTPF.DFLGRAGSPRGSPLAEGPQAFFPER..GPSPRPPATAY   21
ASPP1    AL..GPT....EKPGI...................EIGKVPPPIPGVG..KQL.PPSYG.TYP....SP..TPLGPGSTSSLERRKEGSLPRPSA...   49
ASPP2    MF..DAVDQSNAPPSFGTLRKNQSSEDILRDAQVANKNVAKVPPPVPTKPK.QIN.LPYFGQTNQ....PP..SDIKPDG...................   52


iASPP   DAPASAFGSSLLGSGGSAFAPPLRAQDDLTLRRRPP..KAW...........NESDLDVA.YEK.KPSQ.TASYE............RLDVF.....ARP   278
ASPP1   ..........................GLPSRQRPTLLPATGSTPQPGSSQQIQQRISVPPSPTYPPAG.PPAFPAGDSKPELPLTVAIRPFLADKGSRP   564
ASPP2   ............................SSQQLSTVVPSMGTKPKPAG.A..QQPRVLL..SPSIPSVGQDQTLSPGSKQESPPAAAVRPFTPQ.PSKD   592


iASPP   AS..PSLQLLPWRESSLDGLGGTGKDNLTSATLPRNYKVSPLASDRRSDAGSYRRSLGSAGPSGTLPRSWQPVS......R......I......PMPPS.   357
ASPP1   QS..PRK..............GPQTVNSSSI...................YSMYLQQATP....PKNYQPAAHSALNKS......VKAVYGKPVLPS.   616
ASPP2   TLLPPFR..............KPQTVAASSI...................YSMYTQQQAP....GKNFQQAVQSALTKTHTRGPHFSSVYGKPVIAAA   653
```

Table 2.2: **Multiple Sequence Alignment of the Human ASPP Protein Family (continued, 2 of 2)**. The table depicts the multiple sequence alignment of ASPP1, ASPP2, and iASPP using the CLUSTALW2 alignment algorithm [154, 91]. The RVXF motifs of ASPP1 and ASPP2 (as well as the corresponding RARL motif from iASPP) is highlighted by a grey box. The alternative RNYF binding motif of iASPP (residues 812-815), is highlighted by a black outlined box.

```
iASPP   ..SPQPRGAPRQRPIPLSMIFKLQNAFWEHGASRAMLPGSPLFTRAPPPKLQPQPQPQPQPQSQPQPQLPPQPQTQPQTPTPAPQHPQQTWPPVNEGPPK   455
ASPP1   ..GSTS.......PSPLPFLH.........GSLS...................TGTPQPQPPSE..............ST...................   645
ASPP2   QNQQQH.......PENI.YSN.........SQGK...................PGSPEPETE.....................................   679


iASPP   PPTELEPEPEIEGLLTPVLEAGDVD.EGPVARPLSPTRLQP..ALPPEAQSVPELEEVARVLAEIPRPLKRRGSMEQAPAVALPPTHKKQYQQIISRLFH   552
ASPP1   .....EKEPEQDG...PAAPADGST.VESLPRPLSPTKLTPIVHSPLRYQSDADLEALRRKLANAPRPLKKRSSIT.......................   712
ASPP2   ................PVSSVQENHENERIPRPLSPTKLLPFLSNPYRNQSDADLEALRKKLSNAPRPLKKRSSIT.......................   739


iASPP   RHGGPGPGGPEPEL.....................................................................................   566
ASPP1   ..EPEGPGGPNIQKLLYQRFN.................TLAGGMEG.....TPFYQPSPSQDFMGTLA......DVDNGNTNANGNLEELPPAQPTAPLP   782
ASPP2   ..EPEGPNGPNIQKLLYQRTTIAAMETISVPSYPSKSASVTASSESPVEIQNPYLHVEPEKEVVSLVPESLSPEDVGNASTEN.................   820


iASPP   ..................................................SPITEGSE.....AR...AGPPAPAPPA...........PIPPPAPSQ   595
ASPP1   AEPAPSSDANDNELPSPEPEELICPQTTHQTAEPAEDNNNNVATVPTTEQIPSPVAEAPS....PGEE..QVPPAPLPPA...........SHPPA....   861
ASPP2   ...........SDMPAPSPG......LDYEPEG.VPDNSPNLQNNP.....EEPNPEAPHVLDVYLEEYPPYPPPPYPSGEPEGPGEDSVSMRPPE....   893


iASPP   SSPPEQPQSM......EMRSVLRKAGSPRKAR..RARLNPLVLLLDAALTGELEVVQQAVKEMNDPSQPNEEGITALHNAICGANYSIVDFLITAGANVN   687
ASPP1   .......TST......NKRTNLKKPNSERTGHGLRVRFNPLALLLDASLEGEFDLVQRIIYEVEDPSKPNDEGITPLHNAVCAGHHHIVKFLLDFGVNVN   948
ASPP2   .......ITGQVSLPPGKRTNLRKTGSERIAHGMRVKFNPLALLLDSSLEGEFDLVQRIIYEVDDPSLPNDEGITALHNAVCAGHTEIVKFLVQFGVNVN   986


iASPP   SPDSHGWTPLHCAASCNDTVICMALVQHGAAIFATTLSDGATAFEKCDPYREGYADCATYLADVEQSMGLMNSGAVYALWDYSAEFGDELSFREGESVTV   787
ASPP1   AADSDGWTPLHCAASCNSVHLCKQLVESGAAIFASTISDIETAADKCEEMEEGYIQCSQFLYGVQEKLGVMNKGVAYALWDYEAQNSDELSFHEGDALTI  1048
ASPP2   AADSDGWTPLHCAASCNNVQVCKFLVESGAAVFAMTYSDMQTAADKCEEMEEGYTQCSQFLYGVQEKMGIMNKGVIYALWDYEPQNDDELPMKEGDCMTI  1086

---
iASPP   LRRDGPEETDWWWAALHGQEGYVPRNYFGLFPRVKPQRSKV-   828
ASPP1   LRRKDESETEWWWARLGDREGYVPKNLLGLYPRIKPRQRTLA  1090
ASPP2   IHREDEDEIEWWWARLNDKEGYVPRNLLGLYPRIKPRQRSLA  1128
```

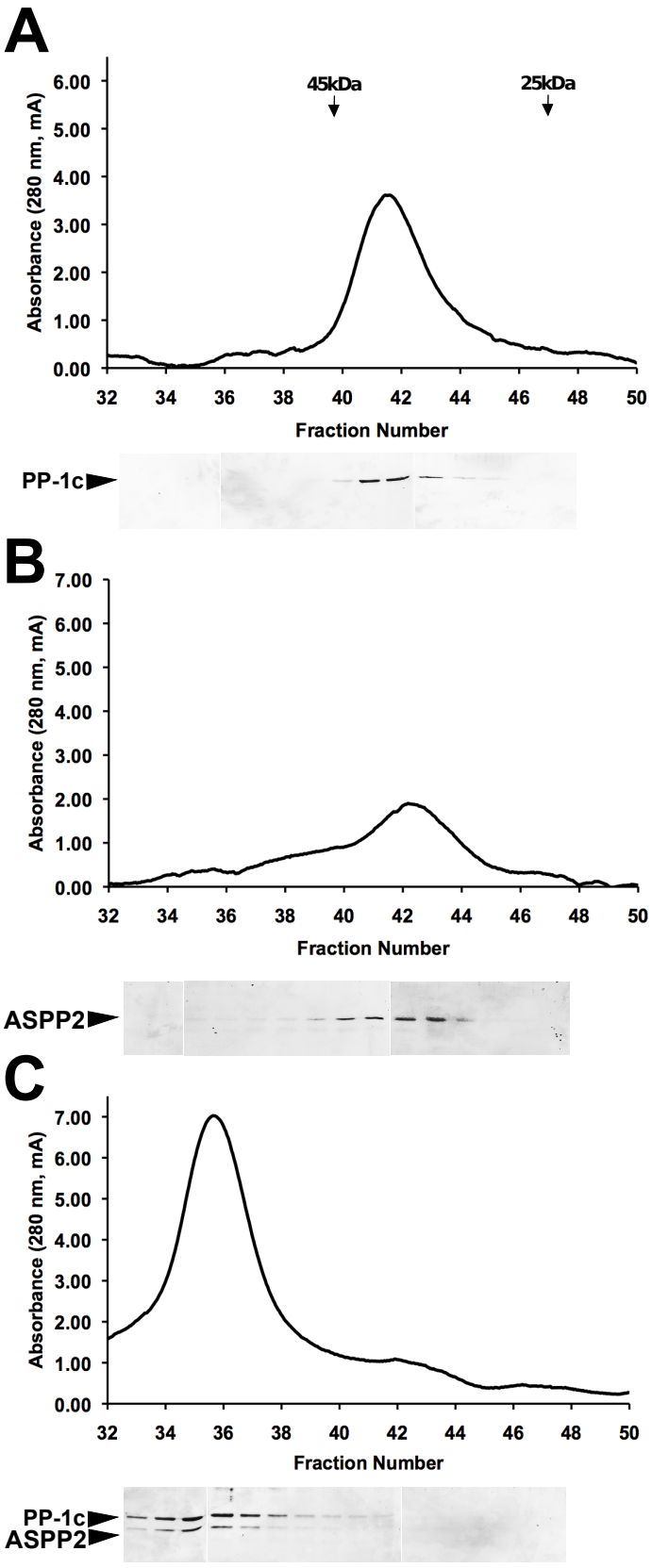
A
45kDa
25kDa
Absorbance (280 nm, mA)
6.00
5.00
4.00
3.00
2.00
1.00
0.00
32
34
36
38
40
42
44
46
48
50
Fraction Number
PP-1c
B
Absorbance (280 nm, mA)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Fraction Number
ASPP2
C
Absorbance (280 nm, mA)
Fraction Number
PP-1c
ASPP2

Figure 2.2: **Gel Filtration Chromatography of the ASPP2•PP-1cα Complex**. Panels A-C depict the gel filtration chromatography of PP-1cα alone (*panel A*), ASPP2$_{905-1128}$ alone (*panel B*), and the PP-1cα•ASPP2$_{905-1128}$ complex (*panel C*). Highly purified preparations of the PP-1cα and His-tagged ASPP2$_{905-1128}$ were incubated either alone or together at 30 °C for 45 min prior to resolution on a Superdex 75 (10/30) gel filtration column. The proteins were resolved at a flow rate of 0.1 mL min^{-1} using Buffer F and 0.25 mL fractions were collected for analysis by SDS-PAGE. SDS-PAGE gels of fractions 33 to 49 (8.5 to 12.25 mL) are shown below each chromatograph.

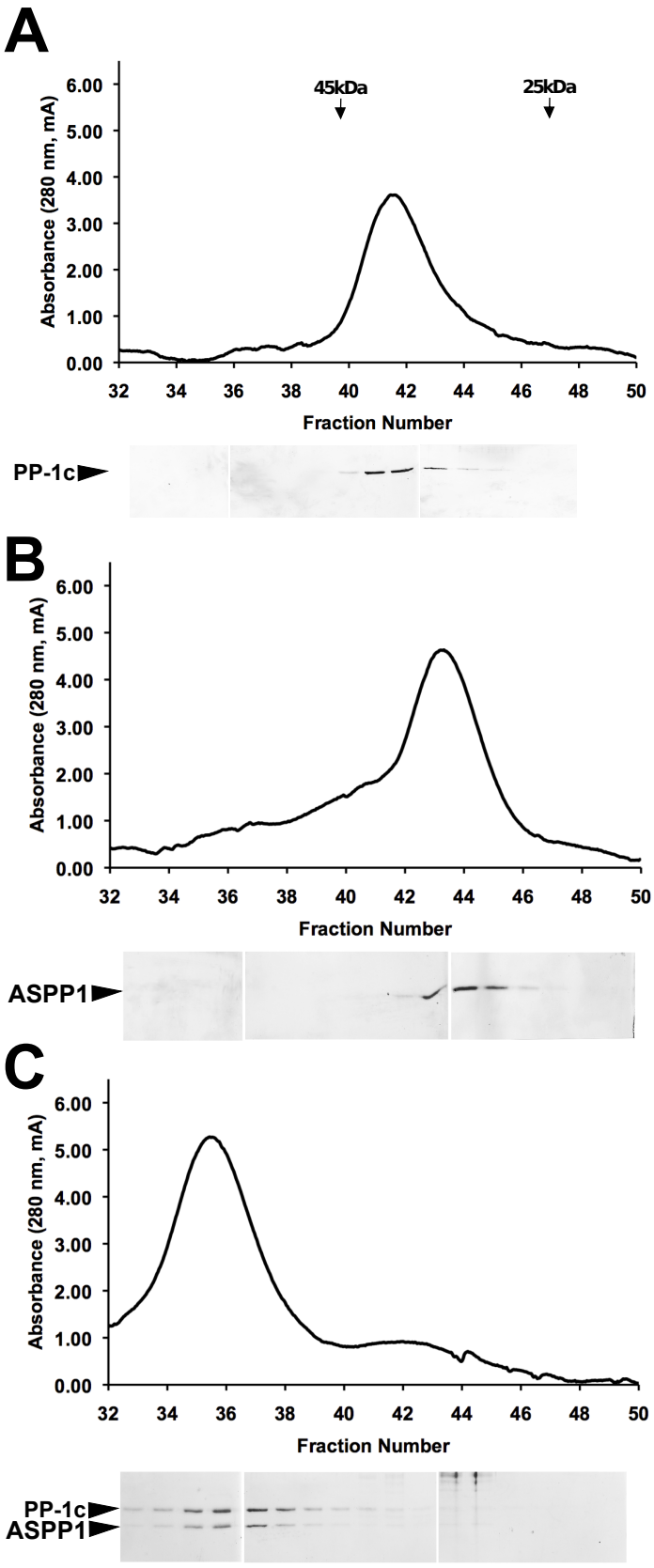
A
45kDa
25kDa
Absorbance (280 nm, mA)
6.00
5.00
4.00
3.00
2.00
1.00
0.00
32
34
36
38
40
42
44
46
48
50
Fraction Number
PP-1c
B
Absorbance (280 nm, mA)
Fraction Number
ASPP1
C
Absorbance (280 nm, mA)
Fraction Number
PP-1c
ASPP1

Figure 2.3: **Gel Filtration Chromatography of the ASPP1•PP-1cα Complex**. Panels A-C depict the gel filtration chromatography of PP-1cα alone (*panel A*), ASPP1$_{867-1090}$ alone (*panel B*), and the PP-1cα•ASPP1$_{876-1090}$ complex (*panel C*). Highly purified preparations of the PP-1cα and His-tagged ASPP1$_{867-1090}$ were incubated either alone or together at 30 °C for 45 min prior to resolution on a Superdex 75 (10/30) gel filtration column. The proteins were resolved at a flow rate of 0.1 mL min^{-1} using Buffer F and 0.25 mL fractions were collected for analysis by SDS-PAGE. SDS-PAGE gels of fractions 33 to 49 (8.5 to 12.25 mL) are shown below each chromatograph.

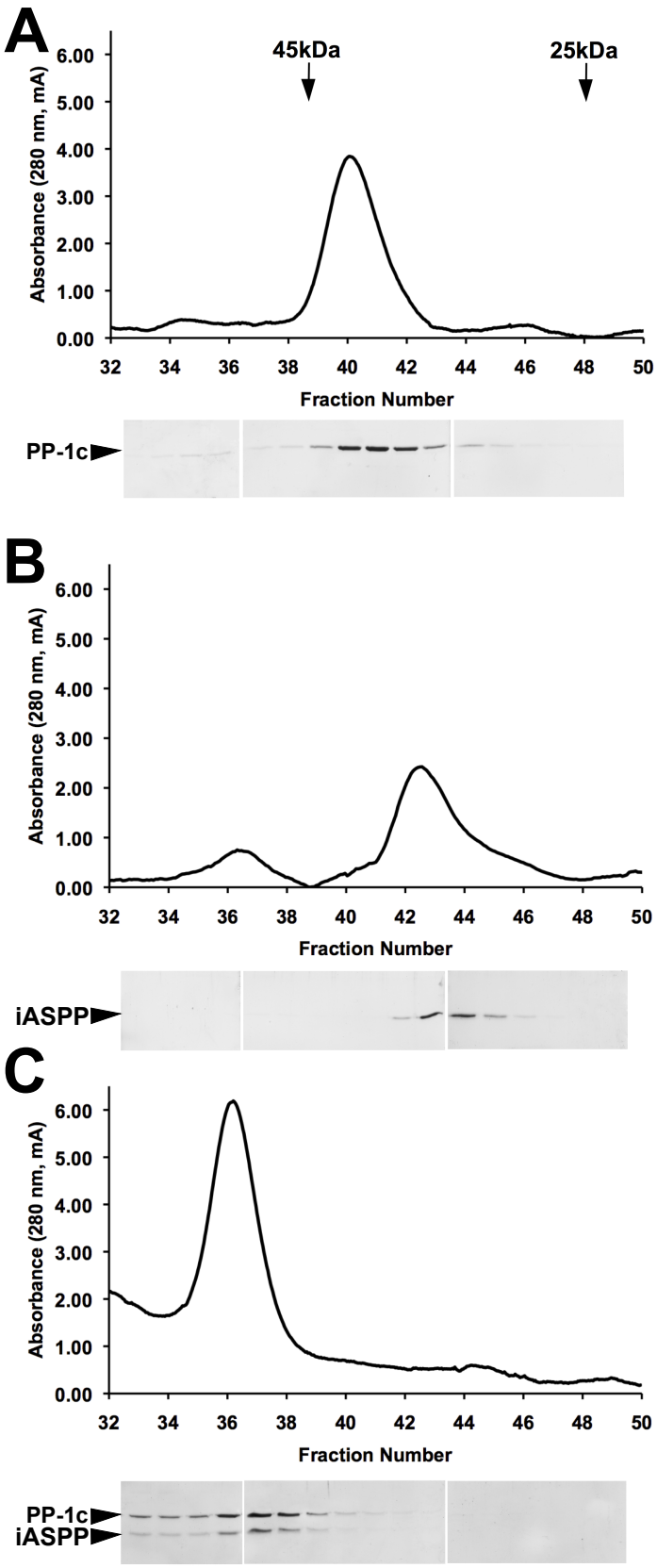
A
45kDa
25kDa
Absorbance (280 nm, mA)
6.00
5.00
4.00
3.00
2.00
1.00
0.00
32
34
36
38
40
42
44
46
48
50
Fraction Number
PP-1c
B
Absorbance (280 nm, mA)
Fraction Number
iASPP
C
Absorbance (280 nm, mA)
Fraction Number
PP-1c
iASPP

Figure 2.4: **Gel Filtration Chromatography of the iASPP•PP-1cα Complex**. Panels A-C depict the gel filtration chromatography of PP-1cα alone (*panel A*), iASPP$_{608-828}$ alone (*panel B*), and the PP-1cα•iASPP$_{608-828}$ complex (*panel C*). Highly purified preparations of the PP-1cα and His-tagged iASPP$_{608-828}$ were incubated either alone or together at 30 °C for 45 min prior to resolution on a Superdex 75 (10/30) gel filtration column. The proteins were resolved at a flow rate of 0.1 mL min^{-1} using Buffer F and 0.25 mL fractions were collected for analysis by SDS-PAGE. SDS-PAGE gels of fractions 33 to 49 (8.5 to 12.25 mL) are shown below each chromatograph.

2.3.2 ASPP2 and iASPP Modulate PP-1cβ and PP-1cγ Phosphorylase *a* and pNPP Activity

Many protein phosphatase-1c R-subunits are thought to direct PP-1c to specific substrates within the cell and also inhibit the activity of PP-1c towards other substrates [63]. To understand the role of ASPP proteins in modulating the activity of protein phosphatase-1c, I examined the effect of $ASPP2_{905-1128}$ and $iASPP_{608-828}$ on the enzymatic activity wild-type PP-1c isoforms β and γ, using ^{32}P-labelled phosphorylase *a* protein. Only weak inhibition of the PP-1c β isoform was observed in the presence of high nanomolar levels of $ASPP2_{905-1128}$ and $iASPP_{608-828}$ (Figure 2.5, panel A). There was a more pronounced effect on the enzymatic activity of PP-1cγ, as both $ASPP2_{905-1128}$ and $iASPP_{608-828}$ inhibited PP-1c with an IC_{50} (concentration of ASPP protein that results in 50% inhibition) of $\sim$180 and 700 nM, respectively (Figure 2.5, panel B).

The effect of iASPP and ASPP2 on the activity of protein phosphatase-1cγ towards para-nitrophenylphosphate (pNPP), small molecule substrate, was also tested using a colourimetric pNPP assay. Our results show that while ASPP2 and iASPP both inhibit the activity of PP-1c towards phosphorylase *a*, they do not inhibit the activity towards a smaller chemical substrate. In fact, ASPP2 and iASPP slightly activate PP-1cγ towards pNPP (Figure 2.6).

2.3.3 iASPP Binds to PP-1c Via a Non-Classical RVXF Motif

We originally hypothesized that iASPP (lacking an RVXF motif) would not bind to PP-1c; however, our results show that iASPP does in fact bind to PP-1c in a 1:1 stable complex, raising the idea that iASPP may have a hitherto unidentified RVXF motif. To further investigate this hypothesis, we first tested whether a synthetic peptide containing the RVXF motif from ASPP2 (RVKFNPLALLLDSS, residues 921-924) could elute $iASPP_{608-828}$ bound to PP-1cα on MC-Sepharose. An RVXF-containing peptide (derived from the ZAP protein) from MC-Sepharose has been shown previously to disrupt the binding of PP-1c to many regulatory subunits that contain a functional RVXF motif [211]. PP-1c (7 μg) was bound to MC-Sepharose for one hour at 4 °C. Bound MC-Sepharose samples were further incubated with either $iASPP_{608-828}$ or $ASPP2_{905-1128}$ for an additional hour at 4 °C. After washing to remove any unbound ASPP protein, samples were incubated either with or without 0.5 mM of a synthetic peptide derived from the ASPP2 RVXF motif

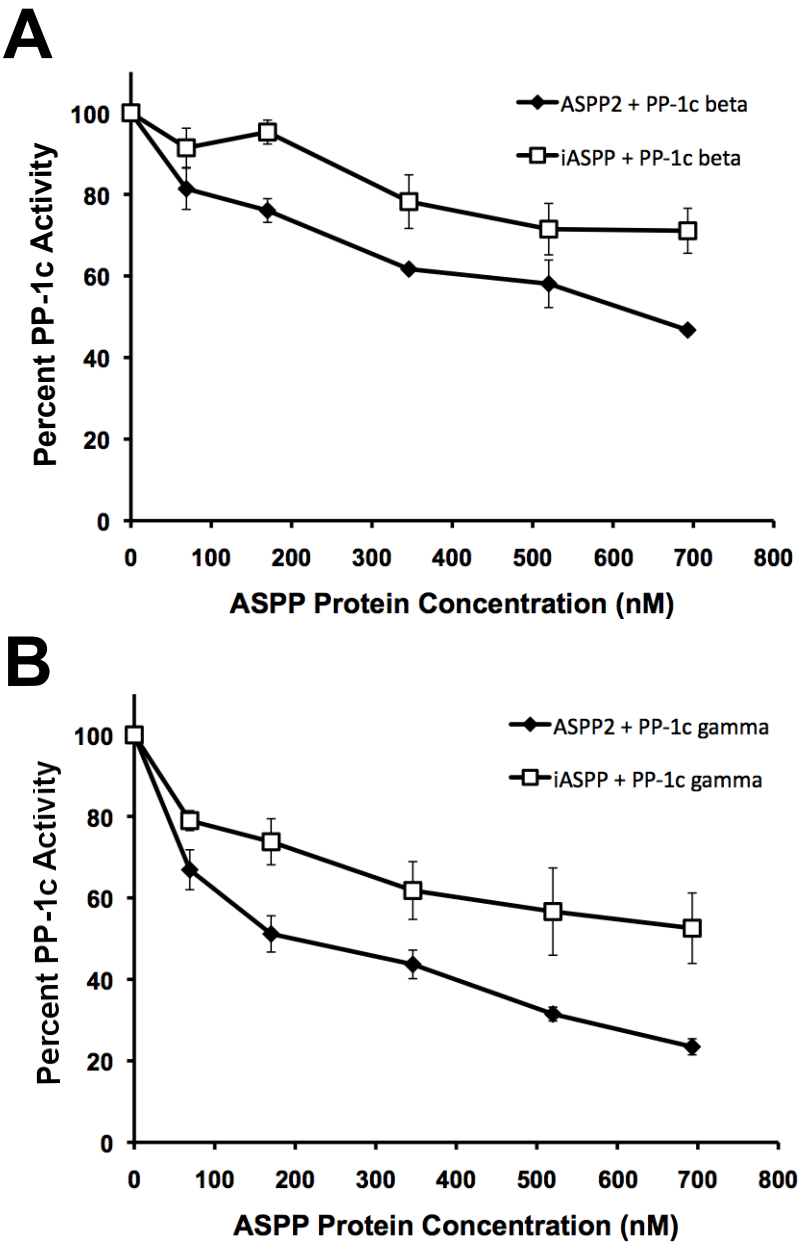

Figure 2.5: **ASPP2 and iASPP Inhibit the Activity of Protein Phosphatase-1c Towards Phosphorylase *a***. The figure depicts the effect of increasing concentrations of iASPP and ASPP2 on PP-1c β (panel A) and γ (panel B) phosphorylase a phosphatase activity. Increasing amounts of either His-tagged $\text{iASPP}_{608-828}$ or $\text{ASPP}_{905-1128}$ were incubated with PP-1c for 10 min prior to adding ^{32}P-labelled phosphorylase a as a substrate. Inhibition data are expressed as a percentage of total activity of PP-1c. Results shown are a representative of at least two triplicate assays.

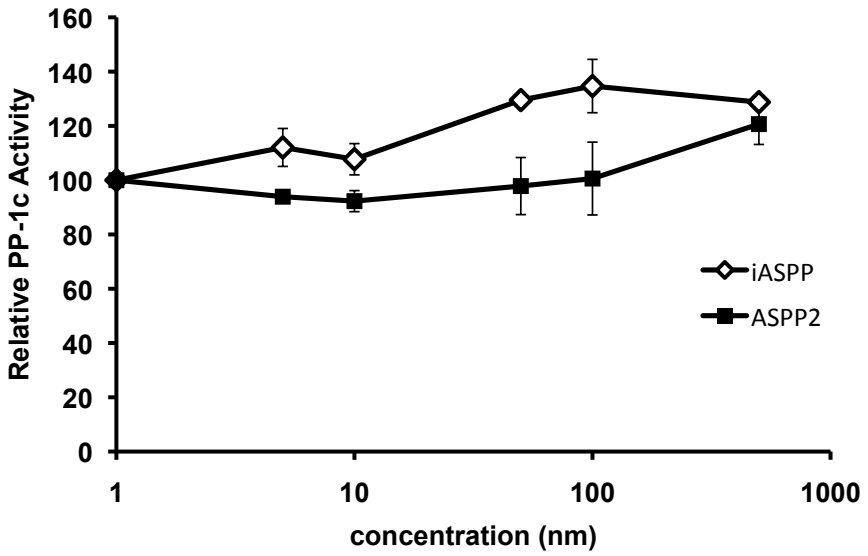

Figure 2.6: **ASPP2 and iASPP Do Not Inhibit Protein Phosphatase-1cγ Activity Towards pNPP, a Small Chemical Substrate**. The figure depicts the effect of increasing concentrations of iASPP$_{608-828}$ and ASPP2$_{905-1128}$ on PP-1cγ phosphatase activity towards pNPP. Increasing amounts of either His-tagged iASPP$_{608-828}$ or ASPP$_{905-1128}$ were incubated with PP-1c for 10 min prior to adding 5 mM pNPP (final concentration). Inhibition data are expressed as a percentage of total activity of PP-1c. Results shown are a representative of at least two duplicate assays.

(RVKFNPLALLLDSS) for 30 min at 4 °C. Results show that both iASPP$_{608-828}$ and ASPP2$_{905-1128}$ can be eluted using an RVXF-containing peptide, however only a small portion of the total bound ASPP protein was eluted (2.7.

To further test whether iASPP contains a functional RVXF motif, we created two additional His-tagged iASPP constructs: a N-terminal truncation, iASPP$_{626-828}$, that lacks the RARL motif and a single point mutant iASPP$_{608-828}$ L625A. iASPP$_{608-828}$ Leu-625 was mutated to Ala to create the equivalent of a non-functional RARA motif in iASPP. Ni-NTA affinity binding experiments were carried out by comparing the binding of PP-1cα to wild-type iASPP$_{608-828}$, truncated iASPP$_{626-828}$ or mutated iASPP$_{608-828}$ L625A. Both deletion of the iASPP residues 608-625 or point mutation of L625A disrupted the ability of iASPP to bind to PP-1cα (Figure 2.8), indicating that RARL is a determinant for PP-1c docking.

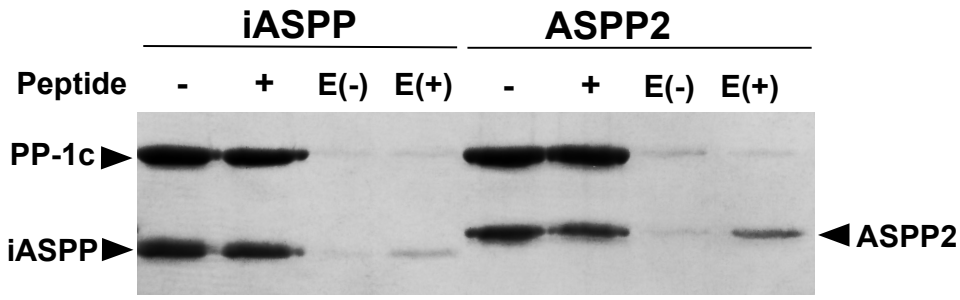

Figure 2.7: **iASPP is Eluted from PP-1cα on MC-Sepharose Using a Synthetic RVXF-Containing Peptide.** PP-1cα (7 μg) was diluted in Buffer E and incubated with $\sim$20 μl of MC-Sepharose for 1 hour at 4 °C. Resin was washed and then incubated with 7 μg of either $ASPP2_{905-1128}$ or $iASPP_{608-828}$ for an additional hour at 4 °C. After subsequent washing, resin was incubated with (+) or without (-) a synthetic peptide containing the RVXF motif from ASPP2 (RVKFNPLALLLDSS) for 30 min at 4 °C. Remaining protein left on the MC-Sepharose was eluted by boiling in 2 × SDS-PAGE sample buffer at 100 °C for 5 min. Peptide elute was concentrated and also analyzed by SDS-PAGE. Elute in the absence of peptide is labelled as E(-) and elute containing the ASPP RVXF peptide is labelled as E(+).

2.3.4 An Alternative RVXF Motif Within the SH3 Domain of iASPP

In parallel to our study, Llanos et al. (2011) have shown that mutations within the iASPP SH3 domain disrupt binding of iASPP to PP-1c [178]; in particular, mutation of iASPP residue Phe-815 to Ala causes a complete loss of binding to PP-1c. They identify a RNYF motif (residues 812-815) within the iASPP SH3 domain that is proposed to be critical for interaction with PP-1c. In order to further test whether the RNYF motif is in fact, a true RVXF binding motif, we tested whether a synthetic peptide containing the iASPP RNYF motif (GYVPRNYFGLFPRV, iASPP residues 807-821) could bind directly to protein phosphatase-1cα. To test this model, we covalently attached the RNYF peptide to NHS-Activated Sepharose (GE Healthcare) according to the manufacturer's instructions and tested the binding of protein phosphatase-1cα. Our results show that PP-1cα readily binds to the RNYF peptide similar to the ASPP2 RVXF peptide (Figure 2.9).

2.3.5 ASPP Proteins Bind Preferentially to the Protein Phosphatase-1cα Isoform

Several other key PP-1c regulatory proteins have been shown to exhibit selective binding to one PP-1c isoform (α, β, or γ) over the others [6, 34, 275]. In order to

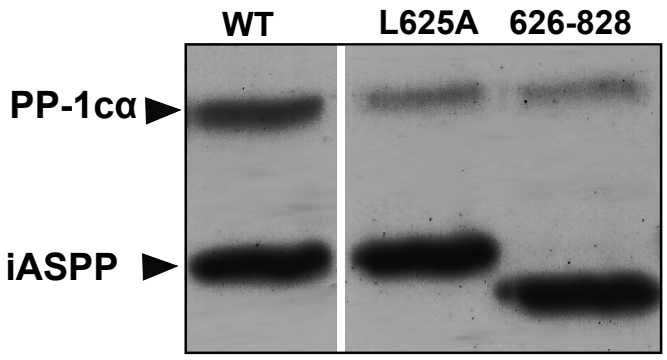

Figure 2.8: **iASPP Binds to PP-1c in Part Via Non-Classical RARL Motif**. The figure depicts an *in vitro* Ni-NTA agarose pull-down experiment, comparing the binding of wild-type PP-1cα to wild-type iASPP$_{608-828}$, a point mutant iASPP$_{608-828}$ L625A, and truncation construct iASPP$_{626-828}$, which lacks the RARL motif. Each iASPP construct (5 μg) was bound to Ni NTA resin for 1 hour at 4 °C end over end. Resin was washed and 5 μ of PP-1cα was incubated with the resin for an additional hour at 4 °C. After sufficient washing, bound protein was eluted by the addition of 2 × SDS-PAGE sample buffer, heated to 100 °C for 5 mins and analyzed by SDS-PAGE with Coomassie blue staining.

test whether the ASPP proteins bind preferentially to PP-1c α, β, or γ, I performed Ni NTA pull-downs, comparing the binding of all three PP-1c isoforms to each of the His-tagged ASPP protein constructs (ASPP1$_{867-1090}$, ASPP2$_{905-1128}$, and iASPP$_{608-828}$). My results show that all three ASPP proteins bind preferentially to PP-1cα (Figure 2.10). In this set of Ni NTA binding experiments, each phosphatase isoform was incubated with the ASPP1, ASPP2, and iASPP constructs for 45 min. When employing longer incubation times (over 5 hours), all three PP-1c isoforms were capable of binding to each ASPP protein.

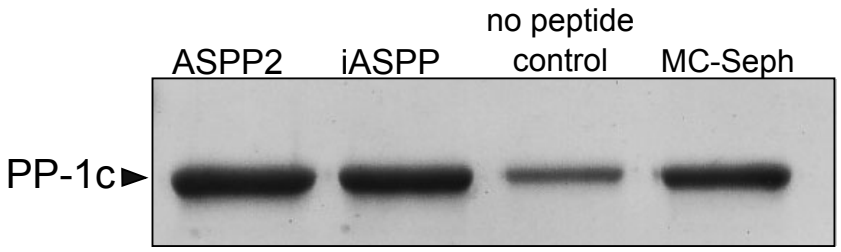

Figure 2.9: **Protein Phosphatase-1c Binds to a Synthetic Peptide Containing the RNYF Sequence**. Peptides containing the iASPP RNYF motif (GYVPRNYFGLFPRV, residues 808-821) or the ASPP2 RVXF motif (RVKFNPLALLDSS, residues 921-934) were coupled to NHS-Activated Sepharose and binding of PP-1cγ to each peptide resin was tested. PP-1cγ (5 μg) was diluted in buffer E and incubated with 25 μl of each peptide resin at 4 °C overnight. After washing with buffer E containing 150 mM NaCl, bound protein was eluted by adding 2 × SDS-PAGE sample buffer and boiling at 100 °C for 5 min. Samples were then analyzed by SDS-PAGE and Coomassie blue staining.

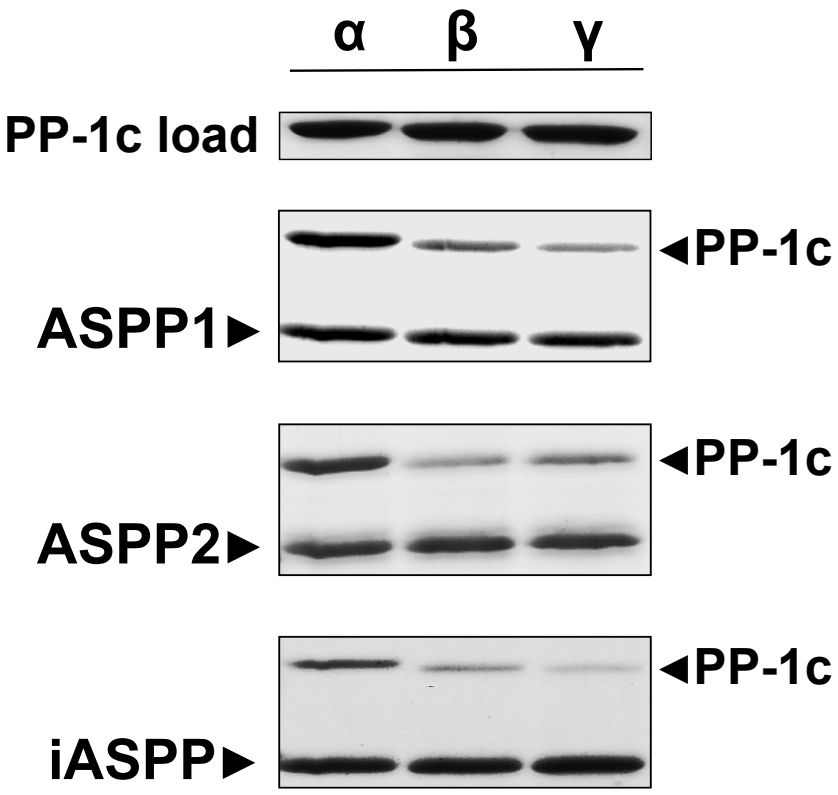

Figure 2.10: **ASPP Proteins Preferentially Bind to the Protein Phosphatase-1c α Isoform**. The figure depicts an *in vitro* Ni-NTA agarose pull-down experiment comparing the binding of $ASPP1_{867-1090}$, $ASPP2_{905-1128}$ and $iASPP_{608-828}$ to PP-1c isoforms α, β, and γ. Each ASPP construct (5 μg) was bound to Ni NTA for 1 hour at 4 °C. Resin was washed and 5 μg of PP-1c was incubated end over end for 45 min at 4 °C. Bound protein was eluted by boiling samples in 2 × SDS-PAGE sample buffer and analyzed by SDS-PAGE with coomassie staining.

2.3.6 A Structural Model of the ASPP•PP-1c Complex Predicts Multiple Sites of Interaction

To better understand the interaction between PP-1c and the ASPP proteins, we created, with the help of our collaborator Jason Maynes, a structural model of the ASPP•PP-1c dimeric complex (Figure 2.11) using the coordinates from the separately determined crystal structure of PP-1cβ (bound to MYPT1) and ASPP2 crystallized in complex with p53 [269, 90]. Our model of the ASPP•PP-1c complex predicts that in addition to the RVXF motif, there are several additional interactions between the phosphatase catalytic subunit and the ASPP proteins. One potential interaction involves a positively charged patch on the surface of PP-1c (residues Lys-260 and Arg-261) and an acidic region of ASPP2 located within its first ankyrin loop

(residues Glu-938 and Asp-940; Figure 2.12). The acidic residues of ASPP2, located 14 residues C-terminal to the RVXF motif, are conserved in both ASPP1 and iASPP. Sequence alignment of several key PP-1c regulatory proteins including the ASPP proteins (Table (2.3) reveals the presence of a common and conserved acidic motif 12 to 15 residues downstream of the RVXF motif.

In addition, our model of the ASPP•PP-1c complex also suggests that there could be extensive interactions between the C-terminal tail of PP-1c and the ankyrin-repeat domain of the ASPP proteins. Within the crystal structure of PP-1c used in this model [269], the C-terminal tail is actually partially disordered and only residues 300 to 309 of the tail are actually visible. Therefore, it is possible that the PP-1c C-terminus may also interact with the SH3 domain of the ASPP proteins. This interaction will be discussed in detail in Chapter 3 of this thesis.

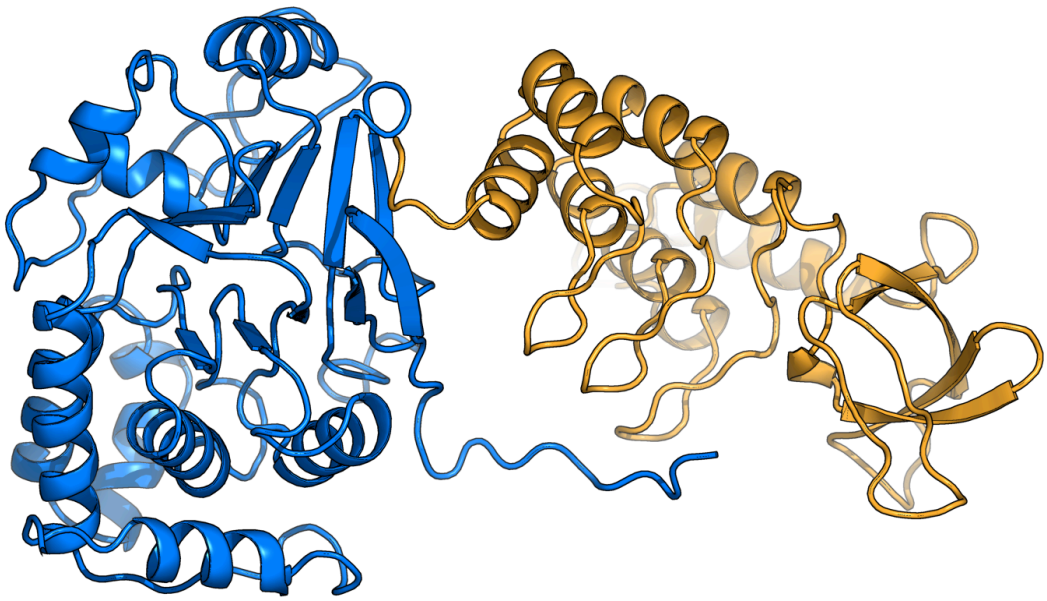

Figure 2.11: **Structural Model of the ASPP•Protein Phosphatase-1c Complex**. The model, describing predicted interactions within this complex, was systematically constructed using coordinates from the separately determined crystal structures of PP-1cβ [269] (pdb accession number 1S70) and a complex between p53 and ASPP2 (pdb accession number 1YCS) [90]. Ribbon representation: PP-1cβ (blue) and ASPP2 (orange).

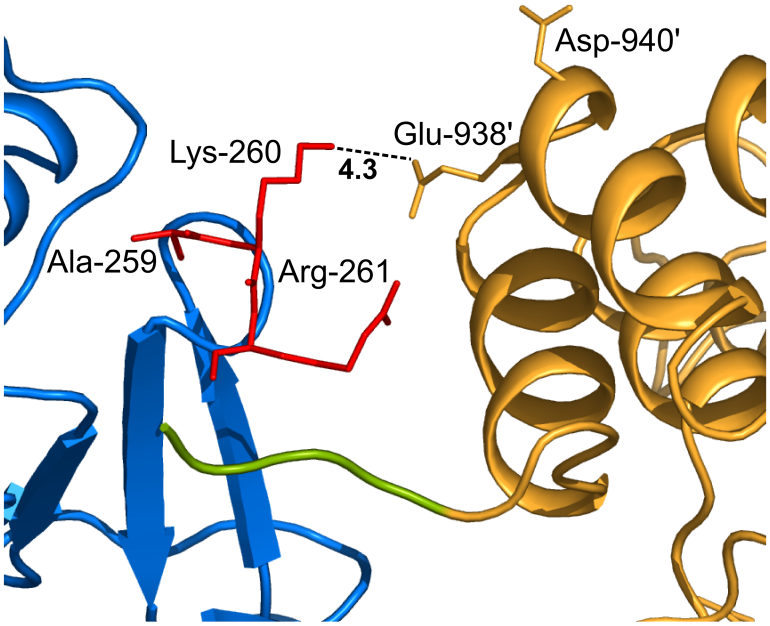

Figure 2.12: **Protein Phosphatase-1c Contains a Positively Charged Region That is Important for Binding to the ASPP Proteins**. Ribbon representation: PP-1cβ (green) and ASPP2 (blue). Highlighted basic residues of PP-1cβ and acidic residues of ASPP2 are shown in red and yellow, respectively. Figure highlights residues of ASPP2 Glu-938' and Asp-940' that are predicted to interact with Lys-260 and Arg-261 of PP-1c. The distance between PP-1c residue Lys-260 and ASPP2 residue Glu-938 is shown in Angstroms.

2.3.7 Protein Phosphatase-1c Residues A259, K260, and R261 Form a Binding Interface With the ASPP Proteins

In order to test whether PP-1c residues Ala-259, Lys-260, and Arg-261 interact directly with the ASPP proteins, we created three PP-1cα mutants (A259R, K260D, and R261S) and tested their ability to bind to $\text{ASPP1}_{867-1090}$, $\text{ASPP2}_{905-1128}$, and $\text{iASPP}_{608-828}$ via Ni-NTA binding experiments. Mutation of the PP-1cα residue A259R resulted in a robust increase in the amount of ASPP1, ASPP2, and iASPP that could bind to the phosphatase (Figure 2.13). However, mutation of either K260D or R261S reduced the ability of the ASPP proteins to bind by approximately 40% and 70%, respectively.

Table 2.3: **Many Protein Phosphatase-1c R-subunits Contain an Acidic Motif C-terminal to the RVXF Motif**. The table shows the multiple sequence alignment of PP-1c regulatory subunit sequences surrounding the canonical RVXF motif. The RVXF motif of each PP-1c regulatory protein is outlined in black. The shaded grey box highlights a cluster of acids residues C-terminal to the RVXF motif previously shown to be important for binding to PP-1c isoforms [34, 35].

Protein Name	Amino Acid Sequence	Residues
Spinophilin	EIPGLSEEEDPAPSRKIHFSTAP-IQVFSTYSNEDYDRRNEDVDPMAASAE	433-482
Neurabin	EIVGLPEEEEPANSRKIKFSSAP-IKVFNTYSNEDYDRRNDEVDPVAASAE	441-490
GL	PTVQEKKVKKRVSFADNQGLALTMVKVSEFDDPLDIPFNITELLDNIVS	51-100
GM	DIYLDTPSSGTRRVSFADS---FGFNLVSVKEFDCWELPSASTTFDLGTDIF	51-99
MYPT1	ETDLEPPVVKRQKTKVKFDD--GAVFLAACSSGDTDEVLKLLHRGADINY	21-68
MYPT2	AGRQPLTRRGSPRVRFED--GAVFLAACSSGDTDEVRKLLARG	41-81
ASPP1	NSERTGHGLRVRFNPL--ALLLDASLEGEFDLVQRIIYEVEDPSKPNDEGIT	874-923
ASPP2	TGSERIAHGMRVKFNPL--ALLLDSSLEGEFDLVQRIIYEVDDPSLPNDE	911-958
iASPP	SPRKA--RRARLNPL--VLLLDAALTGELEVVQQAVKEMNDPSQPNEE	616-659
Inhibitor-2	GNVDEELSKKSQKWDEMNILATYHPADKDYGLMKIDEPSTPYHSMMGDDE	34-83
Inhibitor-1	MEQDNPRKIQFTVPLLEPHLDEAAEQIRRRRPTPATLVLTGDQSSPEI	1-60

2.3.8 Protein Phosphatase-1c Residues A259R and R261 Are Important for the Interaction with Inhibitor-2

The next question was whether PP-1c residues Ala-259, Lys-260, and Arg-261 were also involved in binding to other PP-1c regulatory proteins. As described above, alignment of several key PP-1c regulatory proteins including the ASPP proteins (Table 2.3), reveals the presence of a common and conserved acidic motif 12 to 15 residues downstream to the RVXF motif. The partial crystal structure of the PP-1c•inhibitor-2 complex (Chapter 1, Sections 1.3.7 and 1.3.8) indicates that PP-1c Arg-261 interacts directly with Glu-49 of inhibitor-2 [115] ; however, because it is only a partial structure of inhibitor-2, it is unclear if PP-1c residues Ala-259 and Lys-260 play a role in the interaction (Figure 2.14, panels A and B). In order to test whether PP-1c residues A259, K260, and R261 are important for binding to inhibitor-2, we carried out Ni NTA binding experiments testing the effect of the PP-1cα A259R, K260S, and R261D mutants on binding to His-tagged inhibitor-2. Our results show that mutation of either A259R or R261S disrupted the ability of His-tagged inhibitor-2 to bind to PP-1cα, whereas the PP-1c K260S point mutant did not (Figure 2.14, panel C). Both PP-1cα A259R and R261S also decrease the sensitivity of PP-1c to inhibition by inhibitor-2 (Figure 2.15).

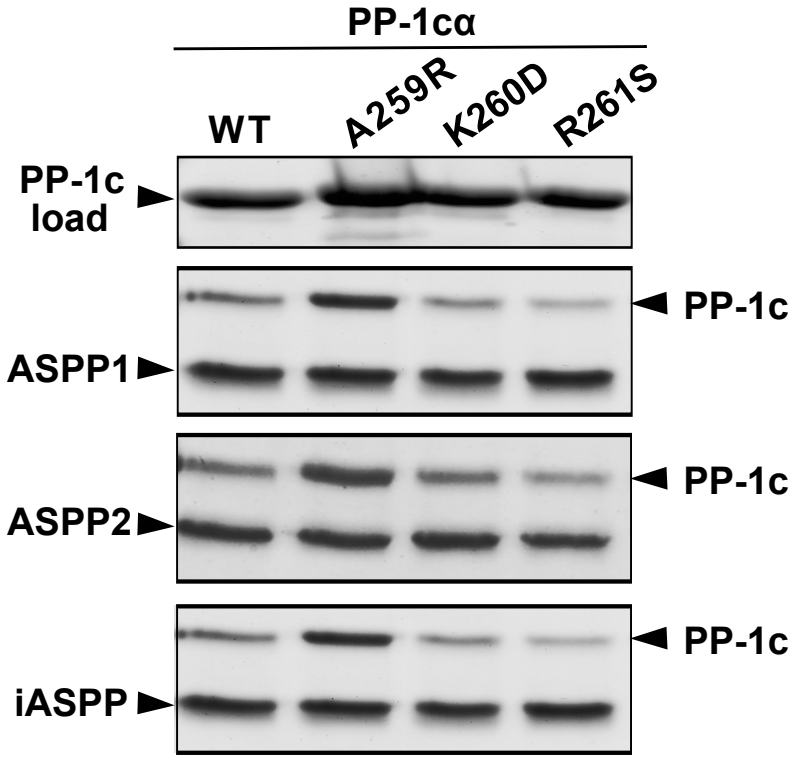

Figure 2.13: **A Basic Region of PP-1c is Important for Binding to the ASPP Proteins**. The figure depicts an *in vitro* Ni-NTA agarose binding experiment, comparing the binding of wild-type PP-1cα (WT), PP-1cα A259R, PP-1cα K260D, and PP-1cα R261S to His-tagged ASPP1$_{867-1090}$, ASPP2$_{905-1128}$, and iASPP$_{608-828}$. Each ASPP protein (5 μg) was bound to Ni-NTA for one hours at 4 °C. end over end. Resin was washed and wild-type PP-1cα or mutant PP-1cα (5 μg each) was incubated for an additional hour at 4 °C. After washing, bound protein was eluted by the addition 2 × SDS-PAGE sample buffer, heated to 100 °C for 5 min and analyzed by SDS-PAGE with Coomassie blue staining.

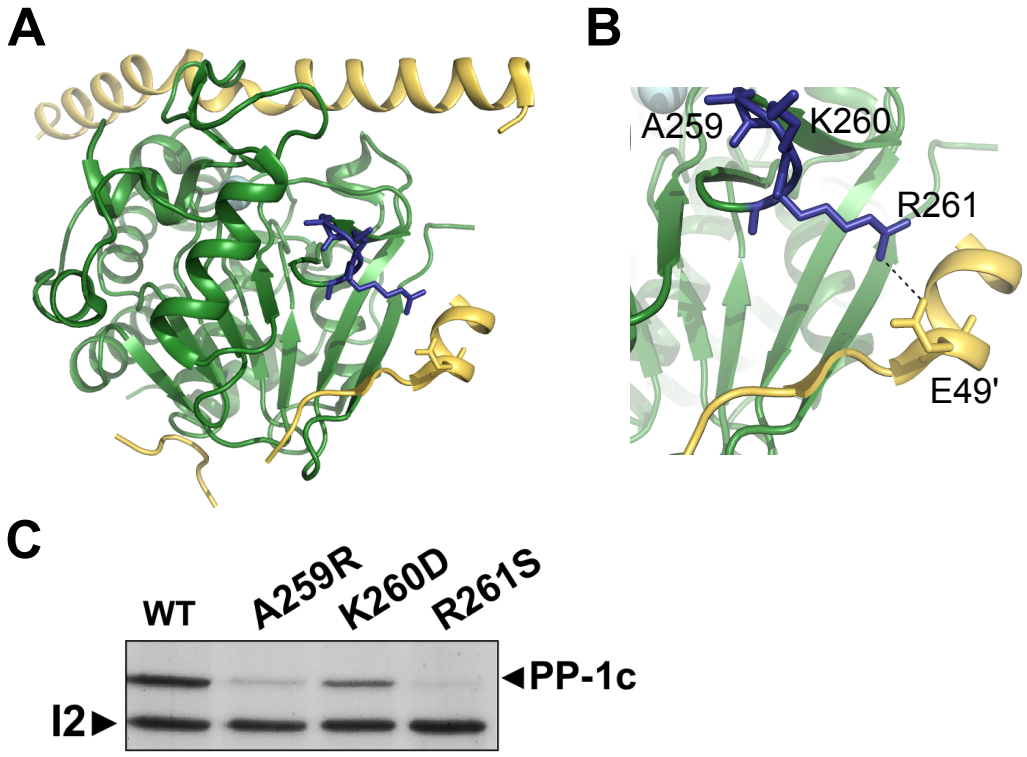

Figure 2.14: **Inhibitor-2 Interacts with PP-1cα Residues Ala-259 and Arg-261**. *Panel A* - crystal structure of PP-1cα bound to inhibitor-2 (pdb accession number 2O8A) [115] shows that PP-1c residue Arg-261 interacts directly with Glu-49 of inhibitor-2. Colour scheme is as follows: PP-1, green; inhibitor-2, yellow. PP-1c residues Ala-259, Lys-260, and Arg-261 are shown as blue sticks. *Panel B* shows a closeup of this region within the PP-1c•I-2 partial crystal structure. *Panel C* depicts an *in vitro* Ni-NTA agarose binding experiment comparing the binding of His-tagged inhibitor-2 to PP-1cα A259R, K260D, or R261S point mutants. Inhibitor-2 was incubated with Ni-NTA for 1 hour at 4 °C end over end. Resin was washed with binding buffer and 5 μg of each PP-1c construct was added and incubated end over end for an additional hour at 4 °C. After washing, bound protein was eluted with 2× SDS-PAGE sample buffer and boiled at 100 °C for 5 min. Eluted protein was analyzed by SDS-PAGE with Coomassie blue staining.

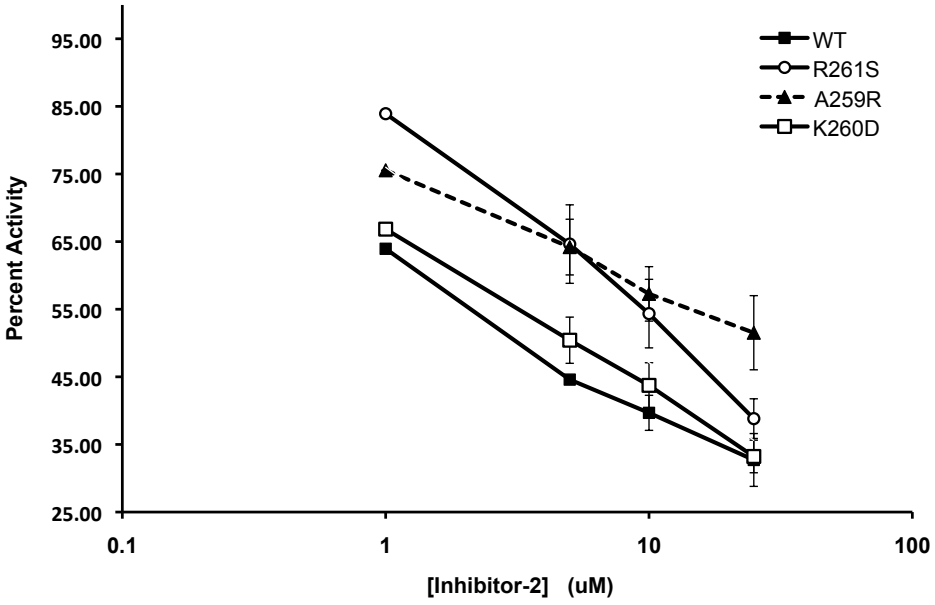

Figure 2.15: **PP-1c Point Mutants A259R, K260D, and R261S Decrease the Sensitivity of PP-1c to Inhibition by Inhibitor-2**. The figure depicts the inhibition of wild-type PP-1cα and point mutant A259R, K260D, and R261S by wild-type recombinant inhibitor-2. Increasing amounts of inhibitor-2 were incubated with either wild-type or mutant PP-1cα for 10 min prior to the addition of 5 mM pNPP (final concentration). Results shown are a representative of three duplicate assays.

2.4 Discussion

2.4.1 iASPP is Novel PP-1c Binding Protein that Interacts Via an RARL Motif

In the experiments outlined in this Chapter, I sought to identify the molecular mechanisms underlying the interactions of PP-1c with the ASPP proteins and have shown that both proteins bind directly to PP-1c, forming stable 1:1 complexes. Similar to ASPP2, ASPP1 contains an RVXF binding motif RVRF (residues 883-886) and forms a stable 1:1 complex with PP-1c. These interactions have also been shown to occur *in vivo*, as demonstrated using SILAC (stable isotope labelling by amino acid in cell culture) and co-immunoprecipitation experiments [250, 178].

ASPP2 has previously been shown to bind to PP-1c in a stable 1:1 complex using a classical RVXF motif [98, 63]. While ASPP1 and ASPP2 both contain classical RVXF motifs, the equivalent sequence in iASPP is RARL (residues 622-625). Previous mutation studies [63, 287] have demonstrated the importance of the Val and Phe residues of the RVXF motif for binding to PP-1c. However our initial MC-Sepharose binding experiments revealed that iASPP was also a direct PP-1c binding protein (Figure 2.1). Furthermore, interaction between iASPP and PP-1c is disrupted by the addition of a synthetic RVXF containing peptide, indicating that iASPP binds to or near the hydrophobic RVXF-binding groove of PP-1c (Figure 2.7). The fact that neither ASPP2 or iASPP are completely disrupted from PP-1c on MC-Sepharose using an RVXF peptide suggested that there are additional sites of interaction between PP-1c and the ASPP proteins that are important for stabilizing complex formation.

To further investigate the interaction between iASPP and PP-1c, we created two constructs, $iASPP_{626-828}$ containing a deletion and iASPP L625A containing a mutation within the RARL motif. Ni-NTA binding experiments demonstrated that deletion or mutation of the iASPP RARL motif partially disrupted the binding of PP-1c to iASPP (Figure 2.8). When combined with the fact that the iASPP•PP-1c complex is disrupted by the addition of a synthetic RVXF-containing peptide, this suggests that the iASPP RARL motif may be a functional, degenerative RVXF motif and additional interactions between iASPP and PP-1c must be sufficient to override degeneracy within the iASPP RARL motif.

Crystal packing of the iASPP$_{608-828}$ crystal structure revealed that residues 616-623 of one iASPP molecule (SPRKARRA) interact with the SH3 domain of another neighbouring iASPP molecule [235]. This suggests that alone iASPP$_{608-828}$ may exist as a dimer and may impede binding to protein phosphatase-1c. However, gel filtration chromatography experiments revealed that all three ASPP proteins exist alone as 28 kDa monomers (Figures 2.2, 4.7, and 2.3.1, panels B). Bound to PP-1c, the ASPP proteins form heterodimers approximately 60 kDa in size (Figures 2.2, 4.7, and 2.3.1, panels C). Whether full-length iASPP forms a homo-dimer *in vivo* has not been determined. However, our collaborator Dr. Greg Moorhead has shown that all three full-length ASPP proteins can bind directly to endogenous PP-1c in HeLa cells and be pulled-down on MC-Sepharose [250]. This demonstrates that the C-terminal ASPP protein constructs designed in this study function *in vitro* in a manner similar to full-length ASPP proteins *in vivo*.

2.4.2 iASPP and ASPP2 Inhibit the Activity of Protein Phosphatase-1c Towards Phosphorylase *a*, But Not pNPP

The binding of a regulatory protein to PP-1c is thought to enhance or promote the activity of PP-1c towards specific protein substrates, while inhibiting its activity towards other substrates. For example, MYPT1 (myosin phosphatase targeting subunit-1) increases the activity of PP-1c towards phosphorylated myosin light chain and the retinoblastoma protein (pRb), while inhibiting the activity of PP-1c towards glycogen synthase and phosphorylase *a* [125, 135]. As an initial step towards understanding how the ASPP proteins regulate PP-1c activity, the effect of ASPP2$_{905-1128}$ and iASPP$_{608-828}$ on the activity of PP-1cβ and PP-1cγ towards phosphorylase *a* was measured using a ^{32}P-labelled phosphorylase *a* radioactive assay. Our results show that both ASPP2$_{905-1128}$ and iASPP$_{608-828}$ inhibited PP1-c towards phosphorylase *a*, with an IC$_{50}$ in the high nano molar range for both iASPP and ASPP2 (greater than 700 nM, see Figure 2.5).

In contrast, ASPP2$_{905-1128}$ and iASPP$_{608-828}$ do not inhibit the activity of PP-1cγ towards para-nitrophenylphosphatate (pNPP, Figure 2.6). Comparing the effect of the ASPP proteins on the activity towards phosphorylase *a*, a large protein substrate, and pNPP, a small chemical substrate, allows speculation as to where ASPP2$_{905-1128}$ and iASPP$_{608-828}$ may bind to PP-1c. While pNPP interacts only directly with the active site of PP-1c, phosphorylase *a* makes additional interac-

tions with PP-1c. Previous research has shown that mutation of key residues within the RVXF hydrophobic groove of PP-1c (PP-1cγ residues Cys-127S and Ile-130A) greatly impair the ability of PP-1c to dephosphorylate phosphorylase *a* while having a lesser effect on the dephosphorylation of pNPP [112]. Therefore, our results suggest that both ASPP2 and iASPP may interact with PP-1c residues within the RVXF hydrophobic groove.

2.4.3 An Alternative RVXF Motif within the SH3 Domain of iASPP is Inaccessible

Llanos et al (2011) proposed that there is an alternative iASPP RVXF PP-1c binding motif, located within the iASPP SH3 domain (RNYF, residues 812–815) [178]. However, analysis of the crystal structure of iASPP alone (Figure 2.16) reveals that Phe-815 of iASPP is fully buried within the iASPP SH3 domain [235]. Furthermore, previous NMR studies have shown that mutation of the equivalent residue in the Fyn SH3 domain causes destabilization and misfolding of the entire SH3 domain [222]. I have shown that a peptide containing the RNYF motif and the region surrounding it (GYVPRNYFGLFPRV, residues 807-821) can bind directly to PP-1c (Figure 2.9). However, it is unlikely that these residues within the iASPP SH3 domain function as a true RVXF-like PP-1c binding motif since they are not accessible to the phosphatase for binding.

2.4.4 The ASPP Proteins Preferentially Bind to the α Isoform of Protein Phosphatase-1c

Previous research has shown that many PP-1c regulatory proteins bind preferentially to one PP-1c isoform over the others [275, 35, 34]. For example, the myosin phosphatase targeting subunit (MYPT1) protein has been shown to bind preferentially to PP-1cβ, while spinophilin binds with a higher affinity to PP-1cγ [269, 35, 34]. Our results indicate that the ASPP proteins also bind preferentially to PP-1cα versus the β and γ isoforms. Although all three PP-1c isoforms share over 50% sequence homology, they differ mainly within their N and C-termini. We hypothesize that the C-terminal tail of PP-1c isoforms is important for binding to the ankyrin repeat and SH3 domains of all three ASPP proteins. Differences in the C-terminal sequences of PP-1c α, β, and γ affects the ability of each PP1-c isoform to bind to the ASPP proteins. In Chapter 3, interactions between the C-terminus of PP-1cα and PP-1cγ and the ASPP proteins are discussed in detail.

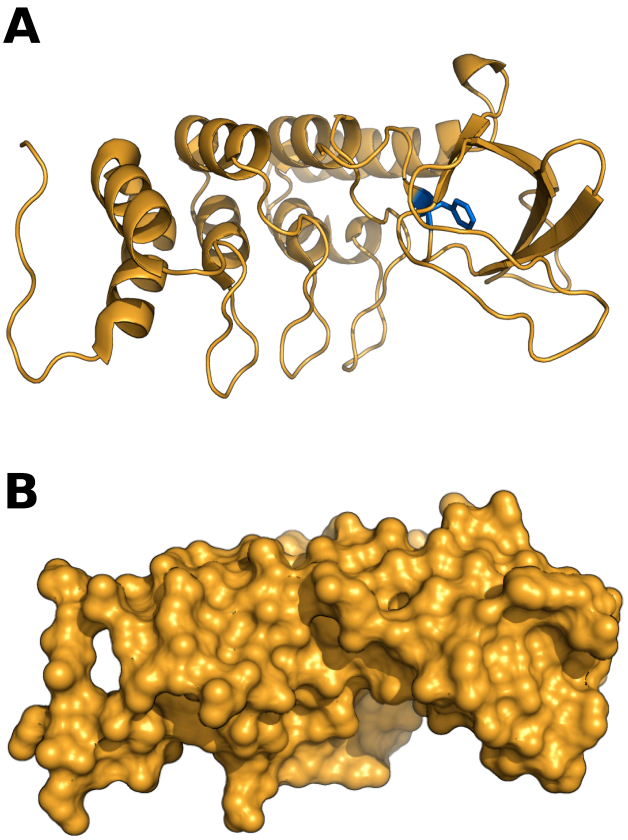

Figure 2.16: **iASPP Residue Phe-815 is 100% Buried Within the SH3 Domain**. *Panel A* depicts the crystal structure of iASPP alone (pdf accession number 2VGE) [235] in orange, highlighting the Phe-815 Residue in blue sticks. *Panel B* depicts the surface of the iASPP protein.

2.4.5 A basic region on PP-1c Is Important for Binding to the ASPP Proteins and Inhibitor-2

Carmody et al. (2008) demonstrated that the PP-1c regulatory protein spinophilin contains an acidic region located only 13 residues C-terminal to the RVXF motif [34]. Alignment of several well-characterized PP-1c regulatory proteins reveals that many PP-1c regulatory subunits (Table 2.3), including all three ASPP proteins, also contain an acidic motif located 10 to 13 residues downstream of their RVXF motifs. Our model of the ASPP•PP-1c heterodimeric complex (Figure 2.11) shows that the acidic region in ASPP2 (centered around residues Glu-938 and Asp-940 of ASPP2) is located within the first ankyrin-repeat (13 residues from its RVXF motif) and is predicted to interact with a basic region of PP-1c (residues Lys-260 and Arg-261

of PP-1c). Our results show that mutation of PP-1cα residues K260D and R261S caused a partial or almost complete loss of binding to the ASPP proteins, respectively (Figure 2.13). On the other hand, mutation of PP-1cα A259R, creating a more basic interface, resulted in a robust increase in the binding of ASPP1, ASPP2, and iASPP. These results suggest that the PP-1c residues Lys-260 and Arg-261 form a positively charged interface that binds directly to the ASPP proteins and this interaction is essential to stabilize the formation of the ASPP•PP-1c complex. PP-1c residues Lys-260 and Arg-261 are conserved in all three PP-1c isoforms, which suggests that this PP-1c basic region may not be directly involved in the isoform specificity displayed by many PP-1c R-subunits.

I have shown that residues Ala-259 and Arg-261 of PP-1c are also important for interaction with another PP-1c R-subunit, inhibitor-2. Mutation of PP-1cα A259R or R261S disrupted the ability of His-tagged inhibitor-2 to bind to PP-1c and also disrupted the ability of inhibitor-2 to inhibit PP-1c (Figures 2.14 and 2.15). Crystal structures of PP-1c bound to the MYPT1 or spinophilin suggest that PP-1c residue Arg-261 also interacts directly with Glu-54 and Glu-438 of MYPT1 and spinophilin, respectively (Figure 2.17). This region also appears to be important for interaction with substrates such as phosphorylase *a* and interaction with modifying enzymes such as cdk2•cyclinA (Andrea Fong, unpublished observations). Therefore the formation of the ASPP•PP-1c complex would likely block access to specific substrates, thus inhibiting their specific dephosphorylation. It would also be predicted to prevent the binding of cdk2•cyclinA complexes, therefore decreasing C-terminal phosphorylation of PP-1c.

Overall, the results from this Chapter demonstrate in addition to ASPP1 and ASPP2, iASPP is a PP-1c regulatory protein that binds to PP-1c via a non-classical RVXF motif, RARL. The interaction between PP-1c and the ASPP proteins is further stabilized by additional interactions and a positively charged region of PP-1c, comprising residues Lys-260 and Arg-261, was identified as important for PP-1c•ASPP complex formation. Our model of the PP-1c•ASPP complex also suggests that there may be additional sites of interaction between PP-1c and the ASPP proteins. Chapter 3 of this thesis will discuss the specific interaction between the C-terminal tail of PP-1c with the ankyrin-repeat and SH3 domains of the ASPP proteins.

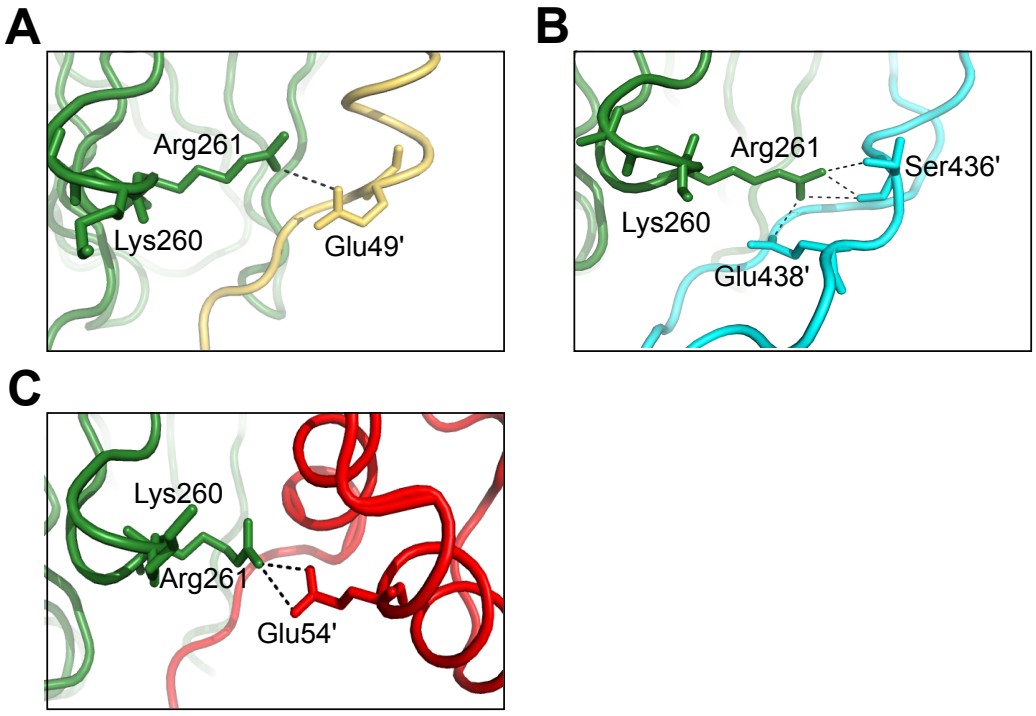

Figure 2.17: **The PP-1c Basic Region (Residues K260 and R261) May be Important for Binding to Other PP-1c Regulatory Proteins**. *Panels A - C* depict the crystal structures of PP-1c (green) bound to inhibitor-2 (yellow, pdb accession number 2O8A), spinophilin (blue, pdb accession number 3EGG) and MYPT1 (red, pdb accession number 17S0). These crystal structures reveal that PP-1c residues, Lys-260 and Arg-261, interact directly with acidic residues of all three regulatory proteins.

Chapter 3

Elucidation of a A Novel Interaction Between the PP-1c C-terminus and the ASPP Proteins

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A portion of the research described in this chapter was originally published in Biochemical Journal. Skene-Arnold, T.D., Luu, H.A., Uhrig, R.G., De Wever, V., Nimick, M., Maynes, J., Fong, A., James, M.N., Trinkle-Mulcahy, L., Moorhead, G.B., and Holmes, C.F.B., Molecular Mechanisms underlying the interaction of protein phosphatase-1c with ASPP proteins. Biochemical Journal. 2013; 449: 649-659 Portland Press Limited.

3.1 Introduction

In Chapter 2 of this thesis, the identification of iASPP as a novel PP-1c regulatory protein that interacts with protein phosphatase-1c (PP-1c) via a non-canonical RVXF-like motif (RARL, residues 622 – 625) is described. Our model of the PP-1c•ASPP2 complex (Figure 2.11) suggested that there may be additional interaction sites between PP-1c and the ASPP proteins that help to stabilize the formation of the heterodimeric complex. Furthermore, a positively charged surface of PP-1c (comprised of residues Ala-259, Lys-260, and Arg-261) was shown to be important for the binding to all three ASPP proteins and also inhibitor-2 (Section 2.3.7). The model also predicted that the C-terminal tail of PP-1c may bind directly to the ankyrin repeat or SH3 domains of the ASPP proteins (Figure 2.11). The PP-1c C-terminus or C-terminal tail (PP-1cα residues 301 – 330) is a flexible and intrinsically disordered region of PP-1c thought to mediate specific interactions with several regulatory proteins, including the myosin phosphatase targeting subunit (MYPT1) [269]. In the majority of PP-1c crystal structures, the last 25 – 30 residues of the PP-1c C-terminus are disordered and are not visible within the crystal structure [16, 62, 194, 234]. However, analysis of the PP-1c•MYPT1 crystal structure revealed that the ankyrin repeats of MYPT1 make extensive contacts with residues 301 – 309 of the PP-1c C-terminus [269].

The PP-1c C-terminus is phosphorylated by cdk2•cyclin A complexes *in vitro* and *in vivo* on residues Thr-320 and Thr-311 in PP-1cα and γ, respectively [149, 161]. Phosphorylation on this residue allows the C-terminal tail of PP-1c to fold back into the active site to inhibit the catalytic activity of PP-1c [58, 149]. During mitosis, the inhibition of PP-1c via C-terminal phosphorylation is necessary for the increased phophorylation of mitotic proteins [233, 297]. However, PP-1c is slowly reactivated through autodephosphorylation and PP-1c activity is required in order for cells to exit mitosis [251, 297]. Andrea Fong, a graduate student from our laboratory, has shown previously that mutation of PP-1cγ Thr-311 to Asp (T311D) abolished the ability of cdk2•cyclinA to phosphorylate PP-1cγ. Surprisingly, the phospho-mimic T311D PP-1cγ mutation does not cause auto-inhibition of PP-1c [250].

3.1.1 The PP-1c C-terminus Contains a Proline-rich PxxPxR Motif

The Src-homology 3 (SH3) domains are small protein interaction modules (55 – 70 amino acids) that bind to proline-rich peptide ligands and mediate protein-protein interactions in multiple signalling pathways [238]. The minimum SH3 binding consensus sequence is six amino acids and falls within two categories, type I (RxPxxP) and type II (PxxPxR), depending on the relative location of a positively charged residue (typically Arg) two residues from the PxxP core motif [53]. SH3 proline-rich binding motifs form an extended left handed poly-proline helix (PP-II helix) and the proline residues bind within two aromatic xP dipeptide binding pockets [73] (Figure 3.1). The Arg residue within the motif binds within a negatively charged binding cleft, often referred to as a specificity pocket [73, 304, 53]. Residues surrounding the core RxPxxP/PxxPxR motif are thought to confer specificity by binding to variable and unique regions of a particular SH3 domain [238].

Multiple sequence alignment of the C-termini of PP-1c α, β, and γ isoforms (Table 3.1) reveals that all three PP-1c isoforms contain a canonical type II SH3 binding motif (PVTPPR in PP-1cγ, residues 309-314). As described above, the PP-1c C-terminal tail is phosphorylated by cdk2•cyclin A complexes, resulting in autoinhibition of the PP-1 catalytic subunit. Interestingly, this cdk2•cyclinA phosphorylation site is located directly within the putative SH3 PxxP binding motif. Therefore, if the C-terminus of PP-1c is important for its ability to bind to the ASPP proteins, I hypothesized that phosphorylation of the PP-1c C-terminus would disrupt the formation of the PP-1c•ASPP complex entirely. The objective of the work described in this chapter was to investigate the role of the PP-1c C-terminal tail for binding to the ASPP proteins and how phosphorylation of the PP-1c C-terminus may regulate PP-1c•ASPP complex formation.

3.2 Materials and Methods

3.2.1 Materials

The PP-1cα_{1-306} truncation construct was generously provided by Dr. Greg Moorhead from the University of Calgary and was purified as previously described in Chapter 2, Section 2.2.2. A peptide of the C-terminus of PP-1cα (residues 301 –

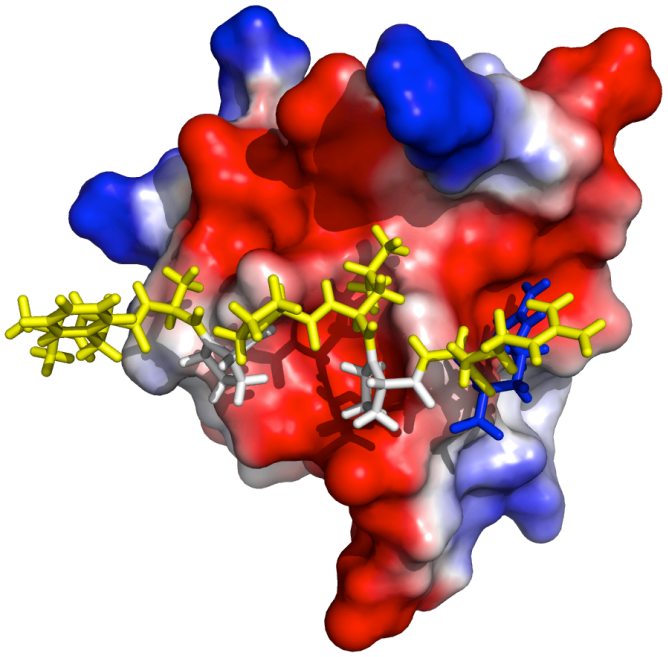

Figure 3.1: **PxxP SH3 Binding Motifs Bind to SH3 Domains**. The figure depicts the NMR structure of the second SH3 domain from Src bound to a PxxPxR containing peptide [73]. The electrostatic surface of Src was generated using PYMOL [56] and negative and positives charges are coloured red and blue, respectively. The PxxPxR peptide (AFAPPLPRR) is shown in sticks, with the Pro and Arg residues of the PxxPxR motif are highlighted in white and blue. SH3 proline-rich motifs form an extended left handed poly-proline helix (PP-II helix). The two prolines that form the PxxP core motif bind within two aromatic xP dipeptide binding pockets. The charged Arg residue binds within a negatively charged binding cleft, often referred to as a specificity pocket.

Table 3.1: **Multiple Sequence Alignment of the C-termini of PP-1c Isoforms α, β, and γ.** Table depicts a multiple sequence alignment of the C-terminal of PP-1c isoforms α, β, and γ.The shaded box highlights the canonical type-II SH3 binding motif PxxPxR, found in all three isoforms. The arrow denotes the location of residue 300 in PP-1cγ, at which the PP-1cγ (1-300) construct was truncated.

Protein Name	Amino Acid Sequence	Residues
PP-1c alpha	MCSFQILKPADKNKGKYGQFSGLNPGGRPITPPRN--SAKAKK	290-330
PP-1c beta	MCSFQILKPSEK-KAKY-QYGGLN-SGRPVTPPRTANPPK-KR	289-327
PP-1c gamma	MCSFQILKPAEKKKPN---------ATRPVTPPRGMITKQAKK	290-323

300

330 of PP-1cα) was generously provided by Jon Pan from the Sykes laboratory, University of Alberta, Canada. Unless otherwise specified, all other materials and reagents were purchased from Sigma or Fisher Scientific.

3.2.2 Mutagenesis of Protein Phosphatase-1cγ T311D and Truncation of PP-1cγ_{1-300}

Expression and purification of PP-1cγ was carried out as previously described for PP-1cα in Chapter 2, Section 2.2.2. Mutagenesis of PP-1cγ was carried out using QuikChange site-directed mutagenesis (Stratagene) as previously described [198] and the DNA sequence of the T311D construct was validated by DNA sequencing. The PP-1cγ T311D mutant was transformed into either DH5α cells for long-term storage or C41 DE3 cells (Lucigen) for protein expression. While the PP-1cα phospho-mimic mutant (T320D) was very poorly expressed, the PP-1cγ (T311D) expressed well and yielded milligram quantities of enzyme. The specific activities of wild-type PP-1cγ and the T311D mutant were equivalent (25.7 and 27.1 U/mg, respectively) [250]. PP-1cγ T311D was purified as previously described in Section 2.2.2.

The sequences of PP-1c isoforms α, β, and γ were aligned using the multiple sequence alignment program ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2) (Table 3.1). After analysis of this alignment and the crystal structure of the PP-1cβ•MYPT1 complex [269], we decided to truncate PP-1cγ at residue 300 (PP-1cγ_{1-300}), removing the entire C-terminal tail from the catalytic subunit of PP-1. Truncation of PP-1cγ_{1-300} was carried out as previously described in Section 2.2.4.

3.2.3 Microcystin-Sepharose and Ni-NTA Chromatography Binding Experiments of PP-1c and the ASPP Proteins

Microcystin-Sepharose and Ni NTA binding experiments were carried out as described in Sections 2.2.5 and 2.2.8.

3.2.4 Interaction of the ASPP Proteins With a PP-1cα C-terminal Peptide

The PP-1c C-terminal peptide, corresponding to residues 300 – 330 of PP-1cα ($\sim$250 μg), was covalently attached to 250 μl NHS-Activated Sepharose as described in Section 2.2.9. A control resin, deactivated with 0.1 M Tris-HCl, pH 8.0, was also made to serve as a negative binding control to ensure that the ASPP proteins were not binding non-specifically to the NHS-Sepharose itself.

The binding of the ASPP1$_{867-1090}$, ASPP2$_{905-1128}$, and iASPP$_{608-828}$ to PP-1c C-terminal peptide linked resin was carried out as follows. The C-terminal PP-1c peptide and negative control resins ($\sim$25 μl each) were washed with 40 resin volumes of H_2O, followed by 40 resin volumes of buffer E containing 150 mM NaCl. Approximately 7 μg of each ASPP protein (from a 1 mg/ml 50 % glycerol stock) was further diluted in buffer E containing 150 mM NaCl and incubated with each resin overnight at 4 °C end over end. The samples were then washed with 50 resin volumes of buffer E containing 150 mM NaCl and bound protein was eluted by the addition of 2$\times$ SDS-PAGE sample buffer and boiling at 100 °C for 5 min. Samples were then analyzed by SDS-PAGE and Coomassie blue staining.

3.2.5 Surface Plasmon Resonance Binding Experiments Between PP-1cα, iASPP, and ASPP2

Interactions between PP-1cα and the ASPP proteins (iASPP$_{608-828}$ and ASPP2$_{905-1128}$) were monitored by surface plasmon resonance using a Biacore 3000 biosensor instrument. A CM5 (carboxymethylated dextran-5) sensor chip (containing 4 lanes) was activated by injecting 35 μl of a 1:1 mixture of N-hydroxysuccinimide and N-ethyl-N-(3-dimethylaminopropyl)-carbodiimide hydrochloride at 5 μl min^{-1}. ASPP2 and iASPP constructs (35 μM) were diluted in 10 mM sodium acetate, pH 5 and immobilized on lanes 2 and 4, at 5 μl min^{-1} until protein was well coupled (indicated by an increase of 1300 to 1500 resonance units [RU]). Lanes 1 and 3 of the sensor chip were not coupled with protein and were instead used as control lanes. All lanes were subsequently blocked with 1 M ethanolamine, pH 8.5 (35 μl) at 5 μl

min^{-1}. Kinetic analyses were performed at 25 °C using Buffer M (10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5), 150 mM NaCl, 0.005 % Tween-20, and 0.5 mM $MnCl_2$) at a flowrate of 30 μl min^{-1}. Full-length WT PP-1cα or the PP-1cα C-terminal peptide (PP-1cα residues 301 – 330) was used as the analyte in a titration curve of 78 nM to 5000 nM, performed in triplicate. Each sample was injected at 30 μl min^{-1}, 90 μl total volume, followed by a 15 min dissociation time. Lane of the sensor chip were regenerated by injecting 15 μl of Buffer M containing 0.2 % SDS, followed by a 2 min stabilization time. PP-1cα or the PP-1c C-terminal peptide was simultaneously passed over the control lane to determine non-specific binding. The baseline from the control lane was subtracted from the lane containing either immobilized $iASPP_{608-828}$ or $ASPP2_{905-1128}$. The association/dissociation curve for each sample injection was fitted using BIAevaluation 3.2 analysis program (BIAcore control software, 3.2; Biacore, Inc) using the kinetic assay result wizard and a drifting baseline (1:1 langmuir binding) model.

3.2.6 BLAST and Multiple Sequence Alignments of PP-1c Isoforms and iASPP

The sequences of the iASPP SH3 domain (residues 756 – 828) and the C-terminal tail of PP-1cα (residues 301 – 330) were used as an initial target sequence to initiate a PSI-BLAST (Position Specific Iterated - Basic Local Alignment Search Tool) search on non-redundant sequences from the NCBI (National Center for Biotechnology Information) human protein database [11, 7]. Identified protein sequences were then aligned using the ClustalW tool [271].

3.3 Results

3.3.1 Deletion or Mutation of the PP-1c C-terminus Disrupts Binding to the ASPP Proteins

Our model of the PP-1c•ASPP2 complex suggests that the PP-1c C-terminal tail may interact directly with the ankyrin repeat and SH3 domains of the ASPP proteins. While the PP-1c isoforms (α, β, and γ) are over 75% identical, they differ mainly within their N and C-termini. Sequence alignment of all three PP-1c isoforms reveals that they share high sequence homology until residue 300, after which their sequences diverge greatly (Table 3.1). Differences within the PP-1c C-terminus may partly explain why all three ASPP proteins bind preferentially to PP-1cα over

the other isoforms.

To further investigate the importance of the C-terminal tail of PP-1c for binding to the ASPP proteins, Ni NTA agarose binding experiments were performed to test the ability of a PP-1cα_{1-306} truncation construct to bind to His-tagged ASPP1$_{867-1090}$, ASPP2$_{905-1128}$, and iASPP$_{608-828}$. Results show that deletion of the PP-1cα C-terminus results in its inability to bind to iASPP and ASPP2 (Figure 3.2).

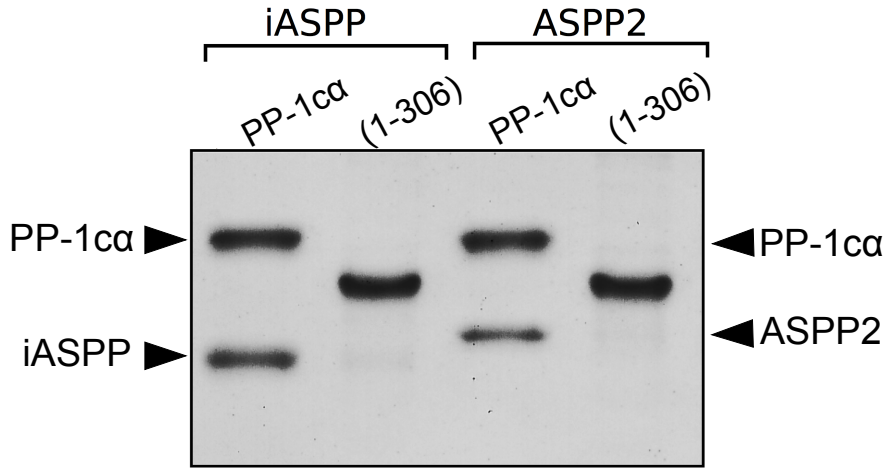

Figure 3.2: **Deletion of the C-terminus of PP-1cα Abolishes Binding to ASPP Proteins**. The figure depicts an *in vitro* MC-Sepharose binding experiment comparing the binding of iASPP and ASPP2 to full-length PP-1cα and truncated PP-1cα_{1-306} [labelled as (1-306)]. Approximately 5 μg of each PP-1cα construct was incubated with $\sim$20 μl of MC-Sepharose for 2 hours end over end at 4 °C. Sepharose was washed and incubated with 5 μg of iASPP$_{608-828}$ or ASPP2$_{905-1128}$ for an additional hour at 4 °C. Resin was washed and eluted by adding 2× SDS-PAGE sample buffer, heating at 100 °C for 5 min. Eluted protein was analyzed by SDS-PAGE with Coomassie blue staining.

Despite extensive sequence differences within the C-termini of the α, β, and γ PP-1c isoforms, all three PP-1c isoforms contain a putative type II SH3 binding motif (PVTPPR in PP-1cγ, residues 309 – 314). This suggests that PP-1c may interact directly with the ASPP SH3 domains. In addition, phosphorylation of PP-1c by cdk2•cyclin A complexes within this PxxPxR motif (PP-1cγ residues Thr-311) may disrupt the ability of PP-1c to interact with the ASPP proteins. The PP-1cγ

T311D phospho-mimic mutant was an ideal choice to test the effect of a negative charge within the type II SH3 binding motif, since it does not inhibit the catalytic activity of PP-1c. Unlike the case for phosphorylated WT PP-1cγ, the C-terminus of the PP-1cγ T311D mutant would not be expected to fold back into the active site of PP-1c and would be accessible to interact with the ASPP proteins. This allowed us to investigate the likely effect of phosphorylation within the putative PxxPxR SH3 binding motif, without the complicating risk of competing autodephosphorylation of the C-terminal tail of PP-1c itself. To investigate this hypothesis, Ni-NTA agarose binding experiments characterizing the binding of His-tagged ASPP proteins to PP-1c$\gamma 1-300$ and the PP-1cγ T311D mutant were carried out. These results show that deletion or mutation of the PP-1cγ C-terminal tail completely abolish the ability of all three ASPP proteins to bind to PP-1cγ (Figure 3.4).

3.3.2 A PP-1c C-terminal Peptide Alone Binds Directly to the ASPP Proteins

The truncation of the PP-1c C-terminal tail completely disrupts the ability of PP-1c to bind with the ASPP proteins; this begs the question of whether the C-terminal tail alone is sufficient to bind ASPP proteins. A PP-1cα C-terminal peptide, comprising resides 301 – 330 from PP-1cα, was covalently linked to NHS-Activated Sepharose and tested for its ability to pull down iASPP, ASPP1, and ASPP2. The results from these binding experiments (Figure 3.5) show that the PP-1cα C-terminal peptide binds to all three ASPP protein constructs, preferentially binding to iASPP over ASPP1 and ASPP2.

Surface plasmon resonance experiments (Figure 3.6) also demonstrate the ability of the PP-1c C-terminus to preferentially bind to iASPP; the C-terminal PP-1c peptide bound to $\text{iASPP}_{608-828}$ and $\text{ASPP2}_{905-1128}$ with a K_D equal to 53 +/- 7 nM and 136 +/- 8 nM, respectively. Similarly, full-length WT PP-1cα binds with a higher affinity to $\text{iASPP}_{608-828}$ (K_D = 26 +/- 0.4 nM), compared to $\text{ASPP2}_{905-1128}$ (K_D = 123 +/- 8 nM).

3.3.3 Protein Phosphatase-1c Does Not Bind to the SH3 Domain of the Cellular Sarcoma Protein

The presence of a classical type-II SH3 binding motif within the C-termini of all three human PP-1c isoforms raised the question of whether PP-1c can directly bind to other SH3 domains. A BLAST (Basic Local Alignment Search Tool) search using

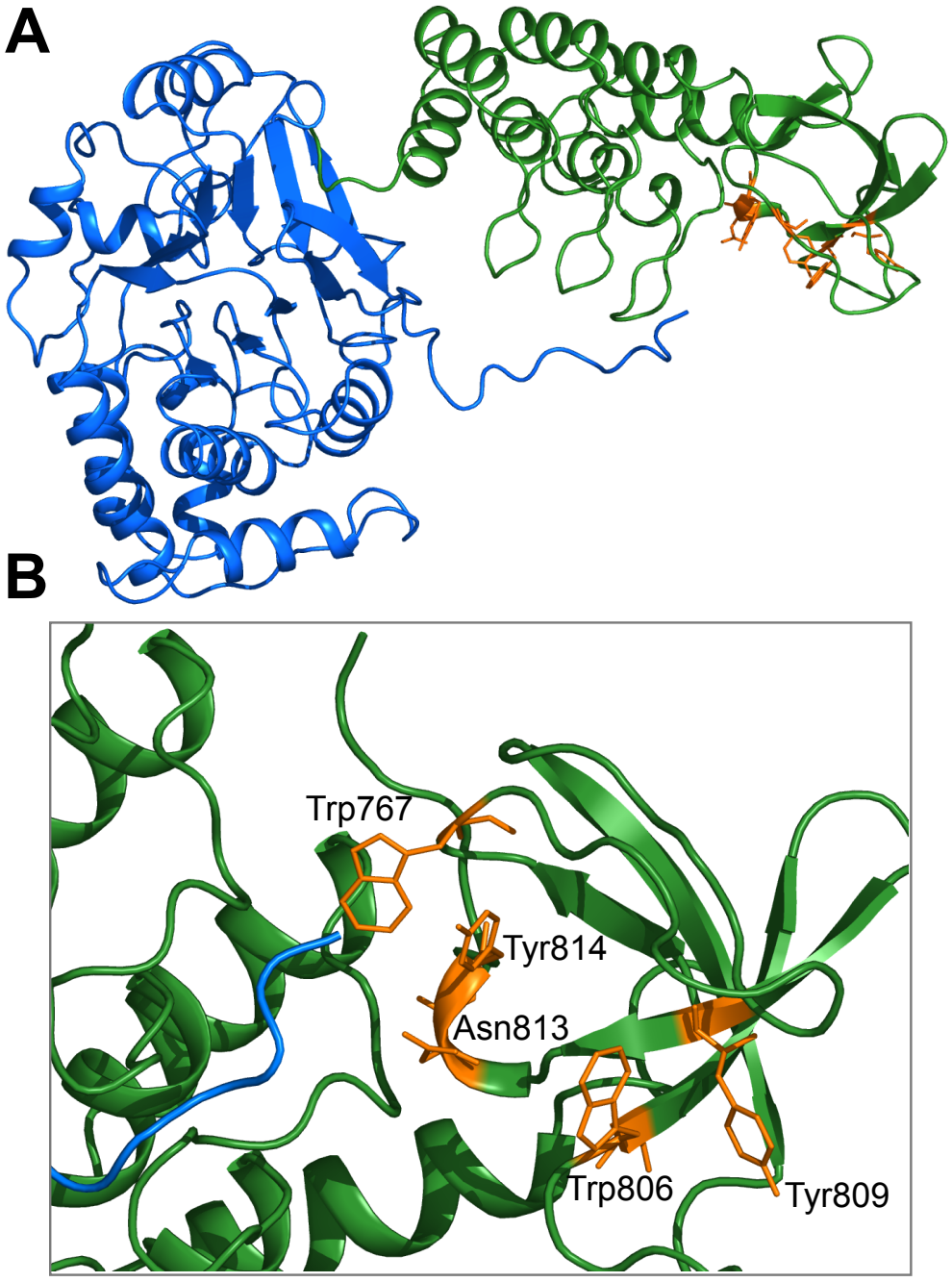

Figure 3.3: **Structural Model of the PP-1c•iASPP Complex**. The figure depicts a close-up view of the predicted interaction between the SH3 domain of iASPP (shown in blue) and the C-terminus of PP-1c (shown in green) [235, 269]. Highlighted in orange are conserved residues within the iASPP SH3 domain that are predicted to be important for binding to PxxPxR sequences. Within the PP-1cβ•MYPT1 crystal structure (Figure 1.11), which was used for our model, the majority of the PP-1cβ C-terminal tail is disordered (residues 309 – 323), therefore the PxxPxR motif is not visible within our model. The PP-1c•iASPP model was generated by aligning the iASPP crystal structure alone with the model of PP-1c bound to ASPP2 (PP-1c•ASPP model, Figure 2.11).

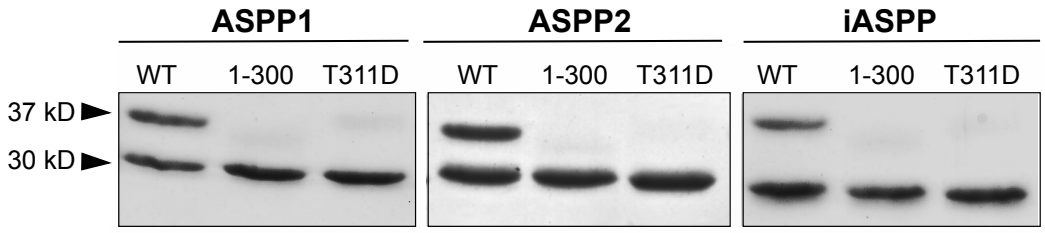

Figure 3.4: **Mutation or Deletion of the PP-1cγ C-terminus Disrupts the Ability of PP-1cγ to Bind to the ASPP Proteins**. The figure depicts an *in vitro* Ni-NTA agarose binding experiment comparing the binding of $ASPP1_{867-1090}$, $ASPP2_{905-1128}$, and $iASPP_{608-828}$ with full-length PP-1cγ, truncated PP-1cγ (1-300) and mutated PP-1cγ T311D. Each ASPP protein construct (5 μg) was bound to Ni-NTA resin for one hour at 4 °C end over end. Resin was washed and 5 μg of each PP-1cγ construct was incubated with the ASPP bound resin for an additional hour at 4 °C. After washing, bound protein was eluted by the addition of 2× SDS-PAGE sample buffer, heated at 100 °C for 5 min and analyzed by SDS-PAGE with Coomassie blue staining.

the SH3 domain from iASPP as a target sequence (Table 3.2) revealed that the iASPP SH3 domain is similar to several SH3 domains including the SH3 domains from c-Src (cellular Sarcoma protein), BCAR-1 (Breast Cancer Anti-Estrogen Resistance Protein -1), Grb2 (Growth Factor Receptor-bound Protein 2) and Nck1 (Non-catalytic Region of Tyrosine Kinase Adaptor Protein 1). Next, MC-Sepharose binding experiments were carried out to test whether full-length PP-1cα can bind directly to the SH3 domain from c-Src (residues, 84-145). Significantly, the SH3 domain from c-Src does not bind to PP-1cα (Figure 3.8).

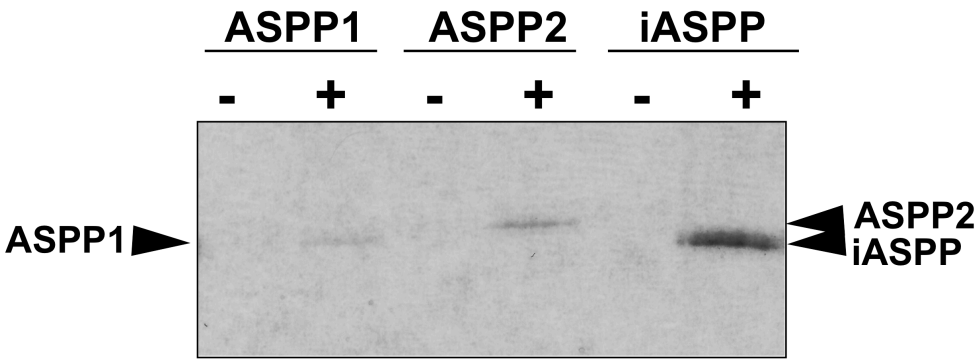

Figure 3.5: **A PP-1cα C-terminal Peptide Binds Directly to ASPP1, ASPP2, and iASPP**. A PP-1cα C-terminal peptide (residues 301 – 330) was covalently attached to NHS-activated Sepharose according to the manufacturer's protocol. Resin was washed and equilibrated with Buffer E containing 0.15 M NaCl and approximately 7 μg of either $\text{ASPP1}_{867-1090}$, $\text{ASPP2}_{905-1128}$, or $\text{iASPP}_{608-828}$ was incubated end over end at 4 °C for 1 hour. After washing, bound protein was eluted by adding 2× SDS-PAGE sample buffer and boiling at 100 °C for 5 mins. Eluted protein was analyzed by SDS-PAGE with Coomassie blue staining. A negative control lane (-) is shown where no ASPP protein was added to the peptide Sepharose.

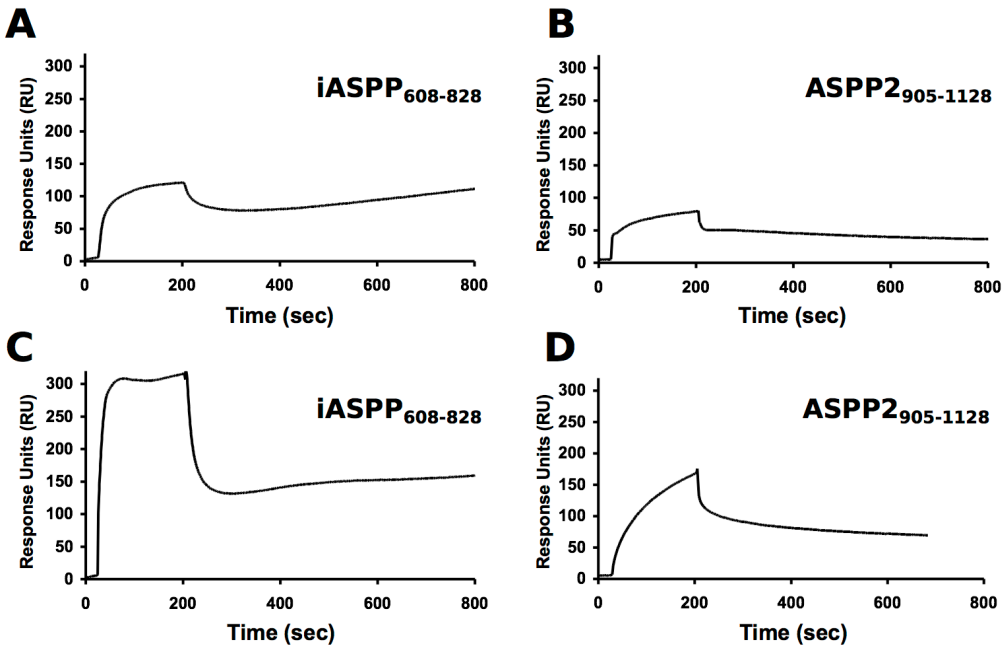

Figure 3.6: **Interaction of iASPP and ASPP2 with Full-length WT PP-1cα and a C-terminal PP-1cα Peptide (residues 301 – 330).** The figure depicts surface plasmon resonance sensograms of the interaction of iASPP$_{608-828}$ and ASPP2$_{905-1128}$, with full-length WT PP-1cα (panels A and B) and a C-terminal PP-1cα peptide corresponding to PP-1cα residues 301 – 330 (panels C and D). Approximately 0.3 μM solutions of either iASPP$_{608-828}$ and ASPP2$_{905-1128}$ were immobilized on a CM5 sensor chip at 5 μl min^{-1} for 7 minutes. All lanes were blocked with 1 M ethanolamine. Kinetic analyses were performed by injecting either full-length PP-1cα or a PP-1cα C-terminal peptide as an analyte in a titration curve of 78.125 nM to 5000 nM, performed in triplicate. The association/dissociation curve for each sample injection was fitted using BIAevaluation 3.2 analysis software using the kinetic analysis result wizard and a drifting baseline (1:1 langmuir binding) model. A representative curve at 5000 nM is shown for each kinetic analysis.

Table 3.2: **Multiple Sequence Alignment of the iASPP SH3 Domain with Homologous SH3 Domains**. The iASPP SH3 domain was used as a target sequence to initiate a PSI-BLAST search of the non-redundant human protein sequences from the NCBI database. Similar sequences were aligned using ClustalW. Conserved residues important for binding to proline motifs are shown in bold font. It has been shown previously that Nck1 interacts with PP-1c [157] and the proposed RVXF binding domain (YVKF, residues 112 – 115) is underlined within the Nck1 sequence.

Accession No.	Protein Name	Sequence	Residues
CAC83011	ASPP1	AYAL**W**DYEAQNSDELSFHEGDALTILRRKDESETE**WW**WARLGDR---EG**Y**VPKN**LL**GLYPR	1024-1081
CAC83012	ASPP2	IYAL**W**DYEPQNDDELPMKEGDCMTIIHREDEDEIE**WW**WARLNDK---EG**Y**VPRN**LL**GLYPR	1052-1119
AAW51146.1	iASPP	VYAL**W**DYSAEFGDELSFREGESVTVLRRDGPEETD**WW**WAALHGQ---EG**Y**VPRN**YF**GLFPR	762-819
ABV24867	Intersectin-1	FIAM**Y**TYESSEQGDLTFQQGD-V-ILVTKK--DGD**WW**TG-TVGD--KAG**V**FPSN**YV**RLKDS	1002-1051
AAF59904.1	Intersectin-2	YIAL**Y**PYSSVEPGDLTFTEGE--EILVTQK--DGE**WW**TG-SIGD--RSG**I**FPSN**YV**KPKDQ	985-1038
NP_001156002	c-Src	FVAL**Y**DYESRTETDLSFKKGERLQIVNNT---EGD**WW**LAHSLSTGQT-G**Y**IPSN**YV**APSDS	89-145
XP_001716809	BCAR-1	AKAL**Y**DNVAESPDELSFRKGDIMTVLEQDTQGLDG**WW**LCSLHGR---QG**I**MPGNH**L**KILVG	8-65
CAC00655.1	HEF-like protein	ARAL**Y**DNCPDCSDELAFSRGDILTILEQHVPESEG**WW**KCLLHGR---QG**L**APANR**L**QILTE	16-73
CAG29359.1	Grb2	VQAL**F**DFDPQEDGELGFRRGDFIHVMDNSGPN---**WW**KGACHGQ--TGM**F-**PRN**YV**TPVNR	161-216
NP_006144	Nck1	A<u>YVK**F**</u>NYMAEREDELSLIKGTKV-IVMEKCS--DG**WW**RGSYNGQ--VGW**F-**PSN**YV**TEEGD	111-165

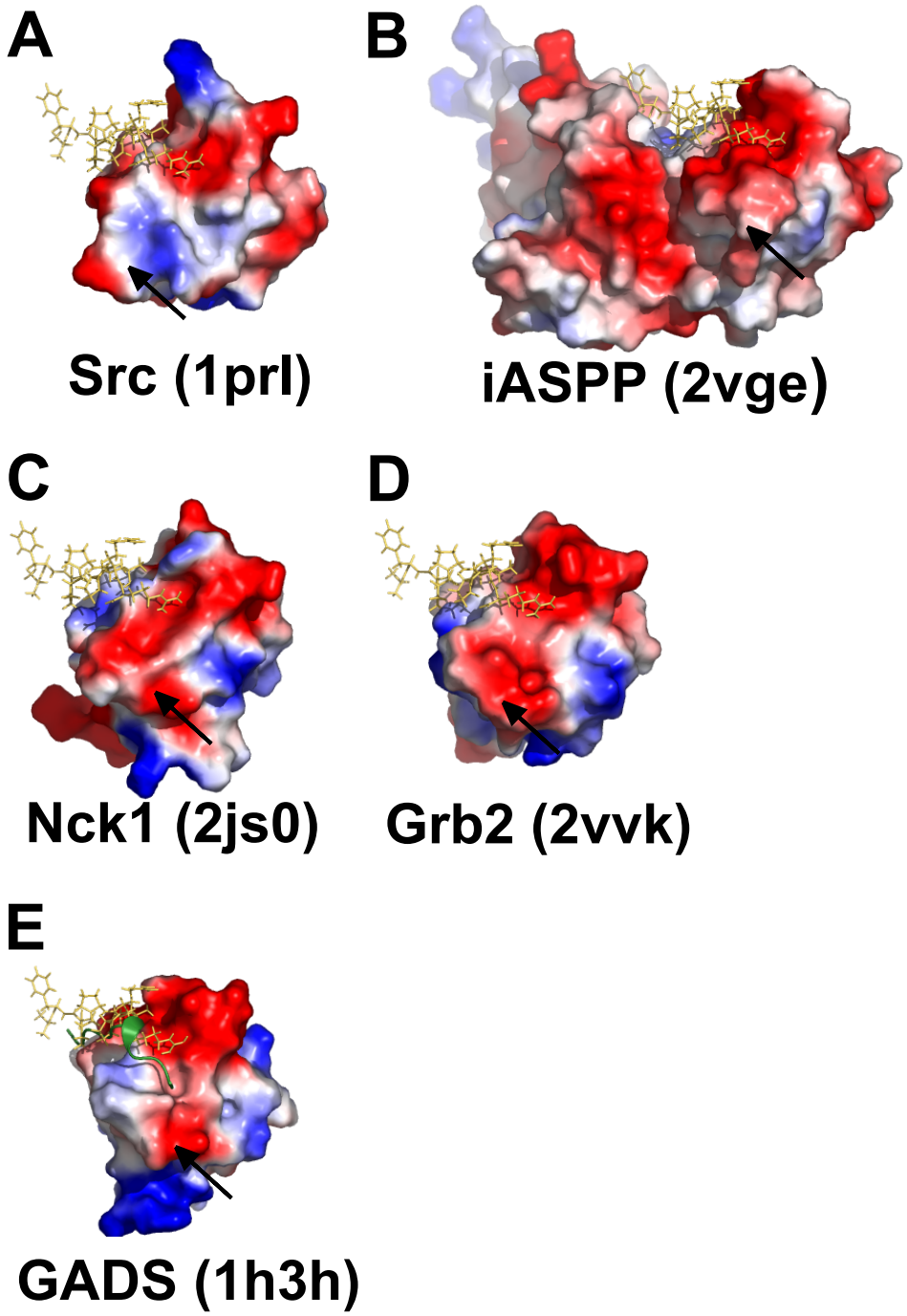

Figure 3.7: **Structural Comparison of the ASPP Ankyrin/SH3 Domains with other SH3 Domain Containg Proteins**. The figure depicts the ankyrin repeat and SH3 domains from iASPP SH3 domain from c-Src (*panel B*, pdb 2VGE), and the SH3 domains from c-Src (*panel A*, pdb 1PRL), Nck1 (*panel C*, pdb 2JS0), Grb2 (*panel D*, pdb 2VVK), and GADS (growth factor receptor bound protein-2 like adaptor protein, *panel E*, pdb 1H3H). Blue and red colouring represents positive and negative surface charge, respectively. The poly-proline peptide bound to the c-Src domain is overlaid on each SH3 domain in yellow sticks to serve as a reference. Arrow highlights the N-Src loop of each SH3 domain.

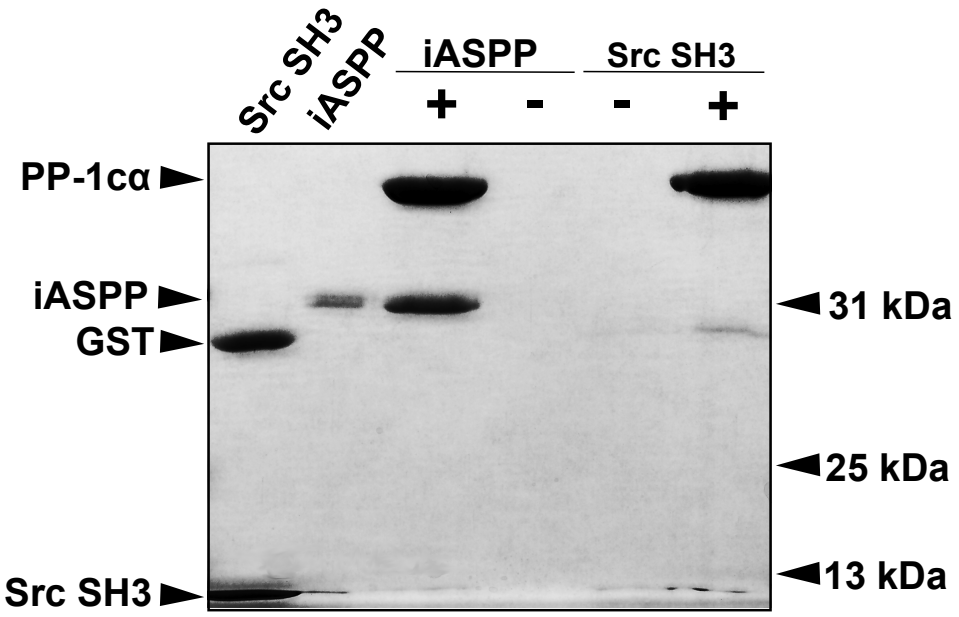

Figure 3.8: **The Src SH3 Domain Does Not Bind Directly to PP-1c**. The figure depicts an *in vitro* MC-Sepharose affinity pull-down experiment, comparing the binding of PP-1cα to iASPP$_{608-828}$ and the second SH3 domain of human c-Src (residues 84-145) to PP-1cα. PP-1cα ($\sim$ 5 μg) was incubated with 20 μl of MC-Sepharose resin end over end for 1 hr at 4 °C. Resin was washed and then incubated further with either iASPP$_{608-828}$ or Src SH3 (5 μg each) for 18 hours end over end at 4 °C. After sufficient washing, bound protein was eluted from MC-Sepharose by adding 2× SDS-PAGE sample buffer and boiling samples at 100 °C for 5 min. Samples were then analyzed by SDS-PAGE and Coomassie blue staining. The SH3 domain of c-Src binds a small amount to MC-Sepharose in the absence of PP-1cα.

3.4 Discussion

Our model of the PP-1c•ASPP2 complex (Figure 2.11) suggested that there may be extensive interactions between the C-terminal tail of PP-1c and ankyrin repeat and SH3 domains of the ASPP proteins. In this study, I have demonstrated the importance of the PP-1c C-terminus for binding to the ASPP proteins and truncation of either PP-1cα_{1-306} or PP-1cγ_{1-300} completely abolished the ability of PP-1c to bind to ASPP1, ASPP2, and iASPP (Figures 3.2 and 3.4).

Sequence analysis of the C-termini of PP-1c α, β, and γ isoforms revealed that all three isoforms contain a classical type II SH3 proline-rich binding motif, PxxPxR (Table 3.1). Andrea Fong, from our laboratory, has previously confirmed that PP-1cγ residue Thr-311, within this PxxPxR motif, is robustly phosphorylated by cdk2•cyclinA resulting in auto-inhibition of PP-1c [250]. Mutation of PP-1cγ T311D completely abolished the ability of PP-1cγ to bind to all three ASPP proteins (Figure 3.4), suggesting that prior phosphorylation of PP-1c Thr-311 within the PxxPxR motif would prevent the subsequent association of PP-1c with the ASPP proteins. It has been shown previously that when PP-1c is bound to the TGF-β inhibited membrane associated protein (TIMAP), a protein similar to MYPT1, cdk2•cyclin A complexes are unable to phosphorylate the C-terminus of PP-1c [249]. Similarly, a preformed PP-1c•ASPP protein complex would be predicted to also block the phosphorylation of PP-1cγ C-terminal tail by cdk2•cyclin A.

Despite the importance of the RVXF motif in the binding of PP-1c to multiple regulatory proteins, it is becoming increasingly clear that the interaction between PP-1c and the ASPP proteins requires multiple binding sites. The C-terminus of PP-1c binds directly to the ASPP proteins, as a peptide derived from the PP-1cα C-terminus (residues 301 – 330) was sufficient to bind to all three ASPP proteins (Figure 3.5). Surface plasmon resonance experiments, confirmed that the C-terminus of PP-1c alone was sufficient for binding to ASPP2 and iASPP (Figure 3.6). Llanos et al. (2011) have previously shown that mutations of a hydrophobic core residue within the iASPP SH3 domain (Phe-815) disrupts the ability of iASPP to bind to PP-1c [178]. Furthermore, they hypothesized that the iASPP SH3 domain contains a putative RVXF motif, RNYF (residues 812 – 815). However iASPP residue Phe-815 is completely buried within the SH3 domain and mutation of this residue likely

causes the structural collapse of the entire SH3 domain. Based on the results presented in this chapter, a structural collapse of the SH3 domain would prevent the association of iASPP with the PP-1c C-terminal PxxPxR motif and thus, not bind to PP-1c.

The interaction between the PP-1c C-terminus and the SH3 domain of the ASPP proteins appears to be very specific. PP-1c was unable to directly bind to the second SH3 domain from c-Src (Figure 3.8). This result is not unexpected, ASPP2 was found to bind to an optimal SH3 proline-rich peptide sequence (RPXψPψRSXP, where X and ψ are any amino acid and aliphatic residues, respectively), which does not bind to a number of other SH3 domains, including c-Src [255]. Additionally, ASPP proteins preference for binding to an optimal proline-rich sequence may also explain why the ASPP proteins preferentially bind to PP-1cα over the β or γ isoforms; the C-terminal tail of PP-1cα most closely resembles the optimal proline-rich binding sequence, as it contains a Ser residue two residues C-terminal to the PxxPxR motif (Table 3.1).

Structural comparison of the SH3 domains of iASPP with other homologous SH3 domains reveals significant differences in the electrostatic surfaces between these proteins, suggesting charged residues surrounding the proline-rich PXXP motif may be important for conferring specificity to a particular SH3 domain. While the c-Src SH3 domain does not bind directly to PP-1c, this does not rule out the possibility of that other SH3 domains more closely resembling the ASPP SH3 domain may interact with PP-1c. Interesting, the second SH3 domain from the Nck1 adaptor protein binds to a multi-protein complex containing both PP-1c and the PP-1c regulatory subunit CReP (Constitutive Repressor of eukaryotic initiation factor-α Phosphorylation) [157]. The second SH3 domain of Nck1 contains a putative RVXF motif (YVKF, residues 112-115). However, it is unclear Nck1 binds directly to PP-1c or via the CReP regulatory subunit. PP-1c may instead interact directly with the Nck1 SH3 domain via its C-terminal PxxPxR motif.

Our results show that the iASPP protein binds with a higher affinity to the PP-1c C-terminus than ASPP2, results which suggest that iASPP and ASPP2 may differentially interact with the PP-1c C-terminus. Mutations of a core hydrophobic residues, F815A, disrupted the ability of iASPP to bind to PP-1c in cultured cells [178]. Comparison of the crystal structures of iASPP and ASPP2 [235, 90] reveals there are subtle differences in the electrostatic surfaces that could influence binding

of charged residues surrounding the PP-1c PxxPxR motif (Figure 3.9). The SH3 domains of iASPP and ASPP2 are unique among SH3 domains in that they are tightly linked to an ankyrin-repeat domain and the interface between the ankyrin-repeat and SH3 domain forms a narrow deep groove that may facilitate protein-protein interactions with PP-1c and other regulatory proteins. Repeated attempts at purifying the SH3 domain of iASPP or ASPP2 alone have been unsuccessful because the majority of expressed protein is aggregated [Gronenborn et al., personal communication], suggesting that both the ankyrin-repeat and SH3 domains of the ASPP proteins are required for proper protein folding and function. The crystal structure of the ASPP2•p53 complex and models of ASPP2 bound to NF-κB and Bcl-2 reveal that these proteins bind to different sites along the same molecular surface [90, 235]. Therefore, the PP-1c C-terminal tail may differentially bind to iASPP and ASPP2, due to the differences in their electrostatic surface.

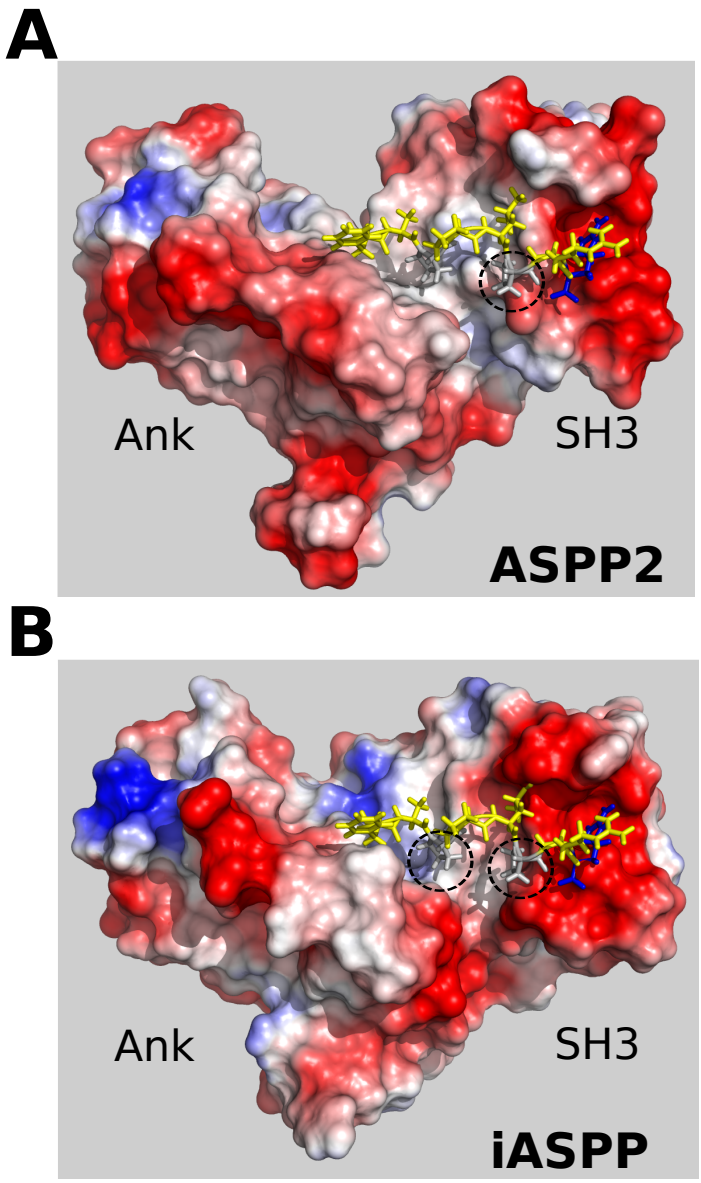

Figure 3.9: **Electrostatic Differences Between the Binding Surfaces of ASPP2 and iASPP**. The figure depicts the electrostatic surfaces of the ankyrin repeat and SH3 domains of ASPP2 (*Panel A*, pdb #1YCS) and iASPP (*Panel B*, pdb # 2VGE) [90, 235]). The electrostatic surface of each ASPP protein was generated using PYMOL [56] and negative and positives charges are coloured red and blue, respectively. The class II peptide bound to the c-Src SH3 domain (AFAPPLPRR) is overlaid onto the ASPP2 and iASPP SH3 domains and shown in sticks as binding reference [73]. The proline residues of the PxxPxR motif are shown light grey (circled) and the arginine residue is shown in dark blue.

Chapter 4

Molecular Mechanisms Underlying the Regulation of PP-1c and p53 by the ASPP Proteins

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4.1 Introduction

4.1.1 Multi-site Phosphorylation of p53

Often referred to as the "guardian of the genome", p53 is an important tumour suppressor that responds to many cellular stresses including DNA damage, ribosomal stress, loss of cell to cell adhesion, and hypoxia [109]. Upon activation, p53 functions primarily as a transcription factor that promotes the expression of genes involved in cell cycle arrest and apoptosis, including p21 (cyclin dependent kinase inhibitor-2), Bax (Bcl-2 associated protein) and PUMA (p53 unregulated modulator of apoptosis) [65, 206, 217]. p53 is mutated in 50% of all cancers, and is inactivated in almost all others. Due to its critical importance in the regulation of cell survival pathways, p53 must be tightly regulated within cells and only be appropriately activated upon cellular stress. In wild-type unstressed cells, the activity of p53 is down regulated largely in part by the association with the mdm2 (mouse double minute 2) protein, an E3 ubiquitin ligase that targets p53 for proteasomal degradation by inducing poly-ubiquitination [258]. Mdm2 has also been shown to promote the mono-ubiquitination of p53, inducing nucleocytoplasmic shuttling of p53 [204, 38]. Upon cellular stress, such as DNA damage, the levels of p53 increase and there is an increase in the transcriptional activity of p53.

Structurally, p53 contains five major domains, including an acidic N-terminal transactivation domain (composed of two subdomains, TAD1 and TAD2, residues 1-40 and 41-61 respectively), a proline-rich domain (PRD, residues 62-97), a central DNA-binding domain (DBD, residues 98-292), a tetramerization domain (TET, residues 324-356) and a disordered C-terminal regulation domain (REG, residues 363-393) (See Section 1.4.2 and Figure 1.8). As described in detail in Chapter 1, p53 is regulated in part by a multitude of post-translational modifications (Figure 1.16). Reversible phosphorylation reactions play an important role in the activation/deactivation of p53, which is mainly phosphorylated within its N-terminal transactivation domain.

Phosphorylation within the p53 transactivation domain, an intrinsically disordered and flexibly region of the p53 protein, plays an important role in the activation of p53 in response to DNA damage. Phosphorylation of Ser-15 is carried out by several kinases including ATM (ataxia-telangietasia mutated protein kinase), ATR (Rad3-related kinase), and DNA-PK (DNA-dependent protein kinase) in response

to DNA damage and is an important step in the activation of p53 for several reasons [200, 199]. Firstly, phosphorylated Ser-15 acts a nucleation site for the sequential phosphorylation of Thr-18 by casein kinase-2 (CK2) which disrupts the association of p53 with the E3 ubiquitin ligase mdm2 [286]. Secondly, phosphorylation of Ser-15 (as well as Thr-18, Ser-20, Ser-37, and Ser-46) increases the affinity of p53 for the histone/lysine acetyltransferases CBP and p300 [76, 72]. This in turn promotes the acetylation of C-terminal lysine residues within the p53 REG domain on the same lysine residues that are ubiquitinated (via association with mdm2) to suppress p53 activity.

The crystal structure of mdm2 bound to a p53 TAD1 peptide (residues 17 – 29) reveals that the TAD1 domain folds into an amphipathic helix upon binding to mdm2 and binds within a deep hydrophobic cleft on mdm2 [148]. Residue Thr-18 of p53 binds within an acidic region at one side of the binding cleft (Figure 4.1, panel A). In contrast, the NMR structure of the Taz2 domain of p300 bound to a longer p53 peptide (residues 2-39) [72] shows that p53 binds across an extended basic surface of p300 and both Ser-15 and Thr-18 bind within basic regions of the p300 binding groove (Figure 4.1, panel B). These structures help explain why phosphorylation of p53 residue Thr-18 (and Ser-15 in the case of p300) promotes the binding to mdm2 and p300, respectively.

4.1.2 Phosphorylation of p53 by DNA-PK

DNA-dependent protein kinase (DNA-PK) is a Ser/Thr protein kinase that is activated upon binding to double-stranded DNA during the DNA damage response in a sequence-independent manner. DNA-PK is a member of the phosphoinositol-3-related kinase family and consists of two subunits: a 350 kDa catalytic subunit (DNA-PKcs) and a targeting subunit, Ku 70/80 (a dimer consisting of a 70 kDa and 80 kDa polypeptide, respectively). One of the main functions of DNA-PK is to promote double strand break repair. DNA-PK is thought to mediate repair through the phosphorylation of key players in the non-homologous end joining (NHEJ) process [201]. DNA-PK preferentially phosphorylates protein substrates containing the consensus sequence S/T-Q and the majority of the key protein players in NHEJ, including XRCC4, Ku 70/80, and DNA Ligase IV are targets of DNA-PK. However, the autophosphorylation of the catalytic subunit of DNA-PK itself remains one of the only functionally relevant targets for the role of DNA-PKcs in DSB repair [60].

DNA-PK has also been shown to be involved in the regulation of several other cellular processes including innate immunity, apoptosis, and cell cycle arrest [201]. DNA double-strand breaks is the main form of DNA damages that activates p53 mediated apoptosis and DNA-PK has been shown to phosphorylate p53 on Ser-15 and Ser-37 following DNA damage [159].

4.1.3 Regulation of p53 by Ser/Thr Protein Phosphatases

Whilst the phosphorylation of the p53 N-terminus has been extensively studied, much less is known about the specific dephosphorylation of p53 by Ser/Thr protein phosphatases. Exposure of cells to PP-1c/PP-2A inhibitors, such as microcystin and okadaic acid, induces apopotosis through the upregulation of p53 expression and activity [96, 81, 163]. This may appear contradictory, since microcystin and okadaic acid are both known tumour promoters [261, 82, 220], however, it does suggest that both play PP-1c and PP-2Ac multiple roles within the DNA damage and stress response pathways.

Multiple phosphatases have been shown to directly dephosphorylate p53, including PP-1c, PP-5, PP-2Ac, Cdc14 [168, 161, 313, 266, 241, 166]. Previously, protein phosphatase-2Ac has been shown to dephosphorylate several pSer/pThr residues, notably Ser-37 and Thr-55 [59, 166]. Specific dephosphorylation of p53 by PP-2Ac is thought to be mediated by the interaction of PP-2Ac with specific regulatory B subunits. For instance, PP-2Ac bound to the B56γ subunit mediates the specific dephosphorylation of p53 on Thr-55 [166]. Whereas most Ser/Thr residues within the p53 N-terminal transactivation domain have been shown to be phosphorylated in response to cellular stress, pThr-55 is rapidly dephosphorylated in response to DNA damage [167]. PP-1c has also been shown to specifically dephosphorylate p53 on pSer-15 and pSer-37 residues [96, 161]. Whether or not PP-1c dephosphorylates additional Ser/Thr residues has not been extensively studied. We hypothesize that similar to PP-2Ac, the association of PP-1c with iASPP may target PP-1c to specifically dephosphorylate key p53 Ser/Thr residues.

Helps et al. (1995) have shown that GST-tagged p53BP2 (ASPP2) does not interact with p53 and PP-1c simultaneously [98]. In order to understand the significance of the novel iASPP•PP-1c interaction, a key question was whether iASPP or ASPP1 could interact with both PP-1c and p53 in a trimeric complex. The ability of the ASPP proteins to form a trimeric complex with PP-1c and p53 was tested

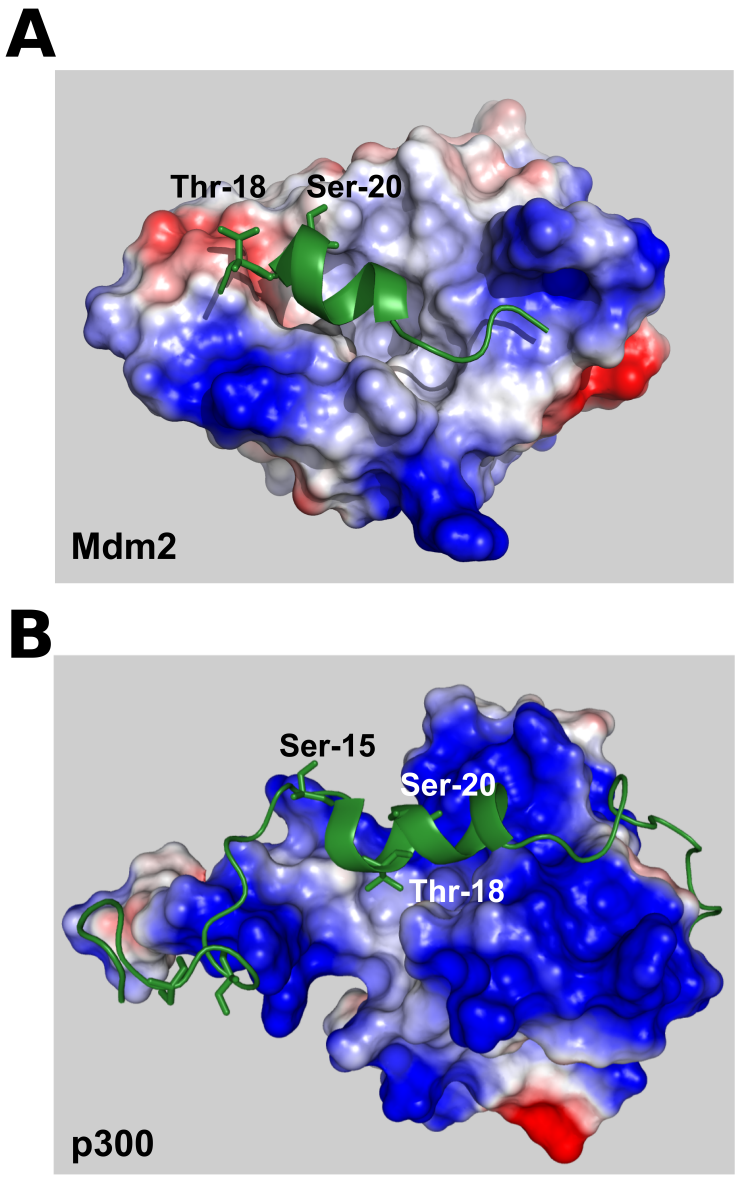

Figure 4.1: **Structural Explanations of the Effects of p53 N-terminal Phosphorylation on the Binding of p53 to Mdm2 and p300**. The structures of the binding domain of mdm2 (*Panel A*) and the Taz2 domain of p300 (*Panel B*) bound to short peptides derived from the N-terminal p53 transactivation domain provides evidence to explain why phosphorylation of Thr-18 (and Ser-15 in the case of p300) disrupts the association of p53 with mdm2 and promotes the association of p53 with p300. The electrostatic charge of the mdm2 and p300 surfaces is shown in red (negative charge) and blue (positive charge).

using gel filtration chromatography and MC-Sepharose binding experiments. Furthermore, p53 *in vitro* dephosphorylation assays were carried out with PP-1cα in the presence or absence of iASPP and ASPP2 to test the effect of the ASPP proteins on the specific dephosphorylation of p53 on the S15 and S37 phosphorylation sites.

4.2 Materials and Methods

4.2.1 Materials

DNA-PKcs and Ku 70/80 were kindly provided by Dr. Susan Lees-Miller from the University of Calgary or were purchased from Promega. The full-length cDNA construct for p53 was purchased from B-Bridge International (RZPD, Germany). Unless otherwise specified, all other materials and reagents were purchased from Sigma Aldrich or Fisher Scientific.

4.2.2 Expression and Purification of p53(2-293)

The His-tagged $p53_{2-293}$ construct was generated from full-length human p53 cDNA using PCR amplification as previously described for the ASPP protein constructs in Section 2.2.4. Constructs were expressed and purified as previously described for the ASPP proteins (see section 2.2.4).

4.2.3 Superdex 75 Gel Filtration Chromatography of PP-1cα, p53 and the ASPP Proteins

Gel filtration chromatography binding experiments were carried out as described in Section 2.2.6, with the following modifications. Highly purified preparations of PP-1cα and His-tagged constructs of either $ASPP1_{867-1090}$, $ASPP2_{905-1128}$, or $iASPP_{608-828}$ were incubated at 30 °C for 45 min in the presence or absence of an equimolar amount of His-tagged $p53_{2-293}$. Protein complexes were then resolved on a Superdex 75 10/30 column (GE Healthcare) at a flow rate of 0.1 ml min^{-1} using Buffer F (50 mM imidazole pH 7.8, 0.3 M NaCl, 0.5 mM EDTA, 2.0 mM DTT) containing 2 mM $MnCl_2$. Fractions (0.25 ml) were collected and analyzed by SDS-PAGE and Coomassie blue staining. Since the ASPP proteins do not inhibit the activity of PP-1c towards para-nitrophenylphosphate (pNPP, see Section 2.3.2), the elution of PP-1c can be traced by monitoring the activity of PP-1c towards pNPP in the gel filtration fractions. The colourimetric phosphatase activity assays using pNPP was carried out as previously described in Section 2.2.11. As a control, PP-

1cα was incubated together with His-tagged $p53_{2-293}$ to establish whether PP-1cα and p53 bind directly.

4.2.4 Superdex 200 Gel Filtration Chromatography of the PP-1cα•iASPP•p53 Complex

Superdex 200 gel filtration chromatography was carried out as described for the Superdex 75 chromatography (see Section 4.2.3), with the following changes. PP-1cα and $iASPP_{608-828}$ were diluted in buffer N (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 % glycerol, and 2 mM $MnCl_2$) and incubated together or together in the presence of an equimolar amount of $p53_{2-293}$ at 30 °C for 45 min. Protein samples were then loaded onto a Superdex 200 10/30 column (GE Healthcare) with a flow rate of 0.9 ml min^{-1} and eluted with buffer N, collecting 0.3 ml fractions. Fractions were analyzed by both SDS-PAGE with Coomassie blue staining and pNPP phosphatase activity assays were carried out in order to determine which fractions contained PP-1cα. As a control, PP-1cα was incubated together with only $p53_{2-293}$ to test whether PP-1c binds directly to the p53 protein.

4.2.5 Microcystin-Sepharose Binding Experiments of PP-1cα, p53, and the ASPP Proteins

Microcystin-Sepharose and Ni NTA binding experiments were carried out as described in Sections 2.2.5 and 2.2.8, with the following modifications. An equimolar amount of PP-1cα, $p53_{2-293}$, and either $ASPP1_{867-1090}$, $ASPP2_{905-1128}$, or $iASPP_{608-828}$ were incubated together at 30 °C for 45 min. Samples were then incubated with MC-Sepharose ($\sim$25 μl) for an additional hr at 4 °C end over end. After sufficient washing, bound protein was eluted by adding 2× SDS-PAGE sample buffer and boiling at 100 °C for 5 min. Samples were then analyzed by SDS-PAGE and Coomassie blue staining.

4.2.6 Phosphorylation of $p53_{2-293}$ by DNA-Dependent Protein Kinase

DNA-dependent protein kinase (DNA-PKcs) and Ku 70/80 were diluted in buffer O (50 mM Tris-HCl pH 8.0, 5 % glycerol, 0.2 mM EDTA, 100 mM KCl, 0.2 mM DTT, 0.2 mM PMSF). Reactions were carried out as follows. Approximately 1 μg of $p53_{2-293}$ (from a 0.5 mg/ml glycerol stock) was further diluted in buffer P (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM EGTA, 0.5 mM $MnCl_2$,

75 mM KCl, 0.5 mM ATP, 10 μg/ml sonicated calf thymus DNA) and 100 ng of both DNA-PKcs and Ku 70/80 were added to initiate the reaction. Reactions were carried out at 30 °C for 10 – 45 min and then stopped by the addition of 20 μl of 2× SDS-PAGE sample buffer for SDS-PAGE analysis or by the addition of the 1 mM LY249002, a DNA-PK ATP competitive inhibitor (Calbiochem) [118], so that the samples could be used in subsequent dephosphorylation assays. Phosphorylation at Ser-15 and Ser-37 was analyzed by SDS-PAGE and western blotting using antibodies specific for p53 phosphorylated on Ser-15 or Ser-37.

4.2.7 Dephosphorylation of $p53_{2-293}$ by Protein Phosphatase-1c in the Presence of iASPP and ASPP2

The dephosphorylation of $p53_{2-293}$ by PP-1cα in the presence or absence of $iASPP_{608-828}$ and $ASPP2_{905-1128}$ was carried out as follows. An aliquot containing 250 ng of $p53_{2-293}$ phosphorylated at sites Ser-15 and Ser-37 was diluted in buffer P containing 0.5 mM $MnCl_2$ and incubated with an equimolar amount of PP-1cα alone or in the presence of $iASPP_{608-828}$ or $ASPP2_{905-1128}$. Samples were incubated for 10 - 45 min at 30 °C and reactions were stopped by the addition of 10 μl of 2× SDS-PAGE sample buffer and boiled at 100 °C. Reactions were analyzed by SDS-PAGE and western blotting using p53 phospho-specific antibodies specific for phosphorylated S15 and S37 (Cell Signaling).

4.2.8 Western Blot Analysis

Phosphorylation and dephosphorylation reactions were fractionated by 12% SDS-PAGE and transferred onto nitrocellulose using a Mini-Blot Module (Biorad). Nitrocellulose blots were first stained with Memcode Reversible Protein Stain kit (Pierce Scientific) and then scanned to analyze total protein input in each dephosphorylation reaction. Blots were blocked using 10% milk in TBST buffer (Tris-HCl pH 7.5, 150 mM NaCl, and 0.1 % Tween-20) for 1 hr at room temperature and subsequently incubated at 4 °C overnight with rabbit polyclonal antibodies specific for either p53 pSer-15 or p53 pSer-37 (Cell Signaling), diluted to 1:1500 and 1:3000 in 0.1% gelatin in TBST buffer. After washing with TBST, blots were further incubated with a secondary antibody consisting of a horseradish peroxidase-conjugated anti-rabbit immunoglobin-G (Cell Signaling) for 30 min at room temperature. Blots were developed for 1 min in enhanced chemiluminescence solution (100 mM Tris-HCl pH 8.5, 0.75 mM luminol, 0.2 mM p-coumaric acid, and and 4.2% hydrogen

peroxide) and exposed to Fuji Medical X-Ray Film (Fujifilm) for 2 – 120 sec.

4.3 Results

4.3.1 Protein Phosphatase-1c and p53 Do Not Directly Interact

It has been shown previously that p53 and PP-1c do not directly bind [98]; however, they do indirectly interact since co-immunoprecipitation experiments show that PP-1c can be pulled down using antibodies specific for the DNA-binding domain of p53 [161]. In order to test whether that PP-1c and p53 do not directly bind, gel filtration chromatography of PP-1c incubated either alone or in the presence of $p53_{2-293}$ was performed using a Superdex 75 gel filtration column. As previously described in Section 2.3.1, PP-1c elutes as a ∼37 kDa monomer (Figure 4.7, panel A and Figure 4.2, panel A).

Incubated alone, $p53_{2-293}$ behaves abnormally on gel filtration chromatography. This was not entirely unexpected, since SDS-PAGE analysis of $p53_{2-293}$ reveals it has an apparent molecular weight of 45 kDa on SDS-PAGE and the predicted molecular weight of $p53_{2-293}$ from the protein sequence is only 33 kDa. The N-terminus of p53 (residues 2-93) is natively unfolded and adopts an extended structure in solution [54], which may explain the discrepancy in the predicted and apparent molecular weights of the $p53_{2-293}$ construct. When PP-1cα is incubated with an equimolar amount of $p53_{2-293}$, they elute as two separate peaks on gel filtration chromatography (Figure 4.2, panel B). We hypothesized that the dephosphorylation of p53 by PP-1c may be facilitated by the association of PP-1c and p53 with the ASPP proteins.

4.3.2 ASPP2 Does Not Form a Trimeric Complex with p53 and PP-1c

Previously, it was shown that the binding of ASPP2 to PP-1c and p53 is mutually exclusive [98], but it is not known whether ASPP1 or iASPP can bind simultaneously to PP-1c and p53. PP-1c was incubated with each ASPP construct in the presence or absence of p53 and the mixtures were analyzed by gel filtration chromatography on a Superdex 75 column to detect the formation of dimeric or trimeric complexes. Fractions (0.25 ml) were collected and analyzed by both SDS-PAGE and for phosphatase activity using the pNPP phosphatase activity assay. When incubated together, ASPP2 and PP-1cα elute together as a 60 kDa dimer and PP-

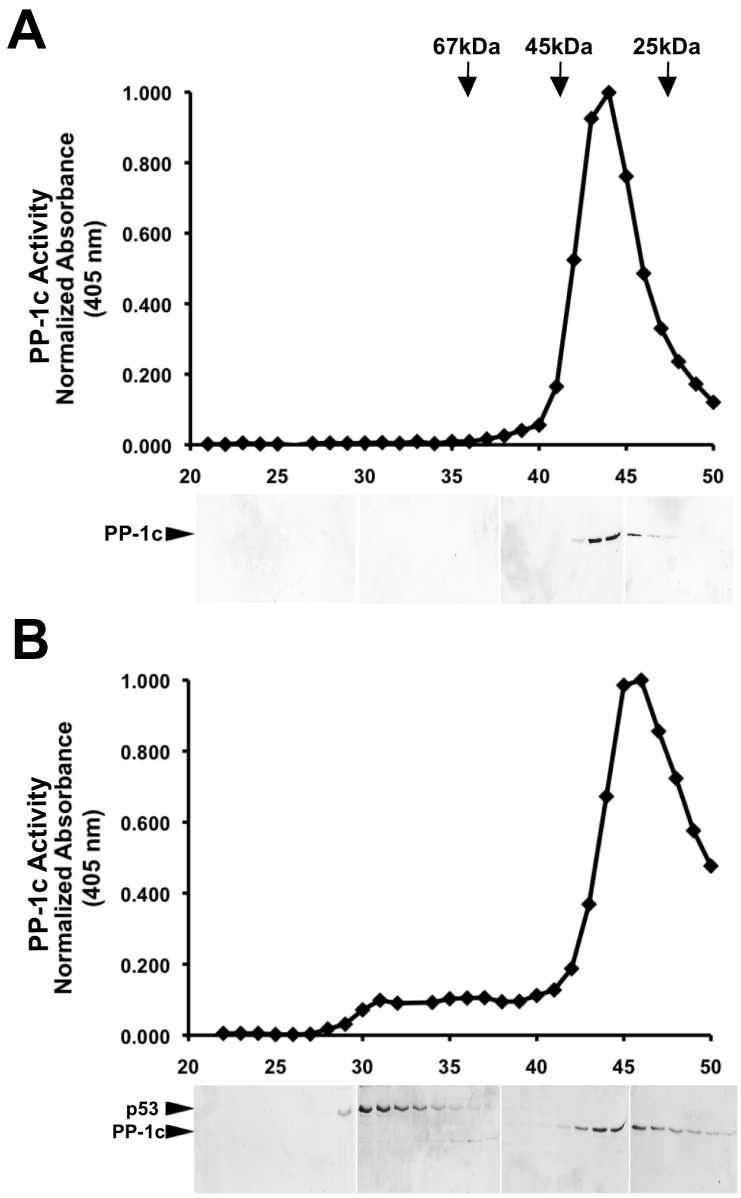

Figure 4.2: **Protein Phosphatase-1c Does Not Bind Directly to p53$_{2-293}$.** PP-1cα was incubated either alone (*panel A*) or together with p53$_{2-293}$ (*panel B*) for 45 min at 30 °C and then loaded onto a pre-equilibrated Superdex 75 10/30 gel filtration column. Protein was eluted at 0.1 ml min^{-1} and 250 μl fractions were collected. Fractions from each gel filtration run were measured for phosphatase activity using pNPP activity assay to determine when PP-1c eluted from the Superdex column.

1c remains fully active within the dimeric complex (Figure 4.3, panel A). When $ASPP2_{905-1128}$ is incubated together with PP-1cα and $p53_{2-293}$, the majority of PP-1c and $ASPP2_{905-1128}$ remain bound together as a 1:1 dimeric complex and do not associate with $p53_{2-293}$ (Figure 4.3, panel B).

4.3.3 ASPP1 Partially Forms a Trimeric Complex with p53 and PP-1c

Gel filtration chromatography of a equimolar mixture of $ASPP1_{867-1090}$ and PP-1cα results in the elution of a ~60 kDa dimeric protein complex (Figure 4.4, panel A), similar to $ASPP2_{905-1128}$. However, when $ASPP1_{867-1090}$ is incubated with both PP-1cα and $p53_{2-293}$, a small amount of $ASPP1_{867-1090}$ and PP-1cα eluted with $p53_{2-293}$ (Figure 4.4, panel B). The majority of $ASPP1_{867-1090}$ and PP-1cα remain in together as a dimeric complex.

4.3.4 iASPP Forms a Trimeric Complex with Protein Phosphatase-1c and p53

Incubation of $iASPP_{608-828}$ and PP-1cα results in the formation of a stable 1:1 dimeric complex, as shown by Superdex 75 gel filtration chromatography (Figure 4.5, panel A). In contrast to $ASPP2_{905-1128}$, when $iASPP_{608-828}$ is incubated in the presence of both PP-1cα and $p53_{2-293}$, all three are eluted together as a ternary complex (Figure 4.5, panel B). The Superdex 75 gel filtration column has a separation range of 3 to 70 kDa; therefore, it was difficult to determine the exact molecular weight of the ternary complex. However, SDS-PAGE analysis of the gel filtration fractions suggests that the ternary complex is a 1:1:1 trimeric complex.

Gel filtration chromatography analyses of the PP-1cα•iASPP•p53 complex was also carried out using a Superdex 200 10/30 column. When run alone, PP-1cα eluted from the Superdex 200 column as a ~37 kDa monomer (Figure 4.7, panel A). Incubation of PP-1cα and $iASPP_{608-828}$ results in the formation of a 1:1 heterodimeric complex (Figure 4.7, panel B). The addition of $p53_{2-293}$ results in a dramatic shift in the elution of both PP-1cα and iASPP, which eluted together with p53 in fractions 20 - 27 (Figure 4.7, panel C). It was confirmed that PP-1cα and $p53_{2-293}$ do not bind directly (Figure 4.6). Incubation of $p53_{2-293}$ alone reveals that it also runs abnormally high on the Superdex 200 column, eluting as a protein greater than 97 kDa.

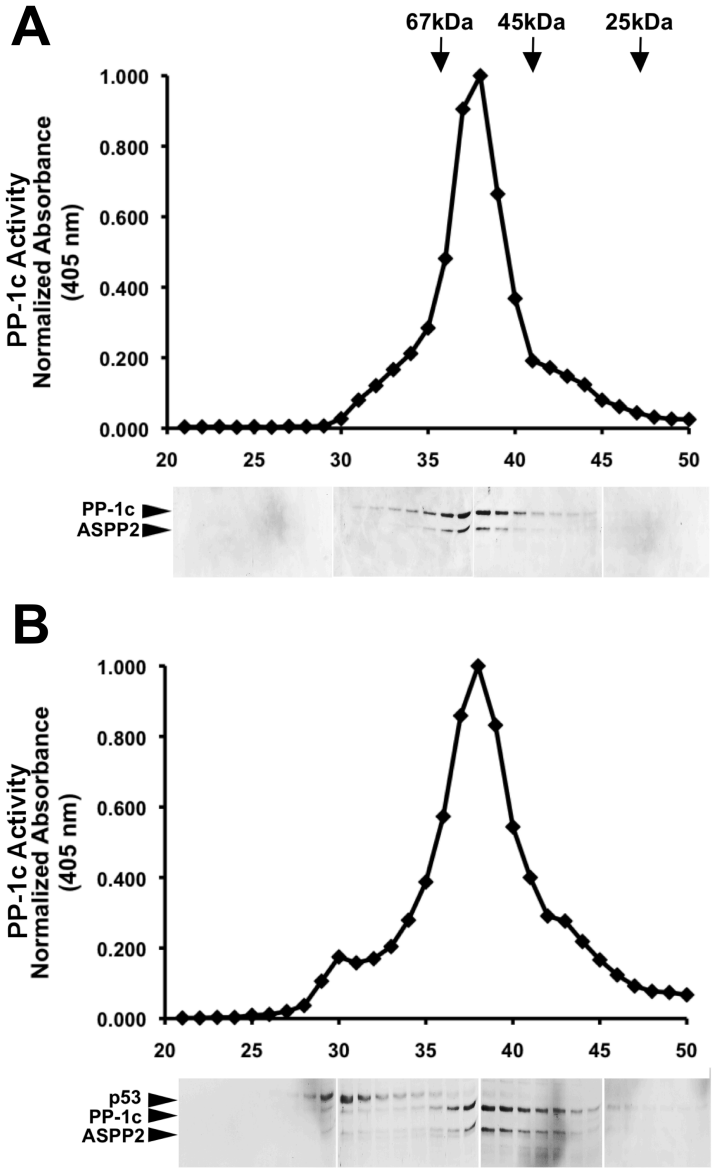

Figure 4.3: **ASPP2, PP-1c and p53 Do Not Form a Trimeric Complex**. PP-1cα was incubated with ASPP2, or with both $ASPP2_{905-1128}$ and $p53_{2-293}$ for 45 min at 30 °C. Samples were loaded onto a pre-equilibrated Superdex 75 10/30 gel filtration column. Protein was eluted at 0.1 ml min^{-1} and 250 μl fractions were collected. Fractions from each gel filtration run were measured for phosphatase activity using the pNPP phosphatase assay to determine when PP-1c eluted from the column. PP-1cα formed a dimeric complex with ASPP2 (*panel A*) but did not form a trimeric complex with both ASPP2 and p53 (*panel B*).

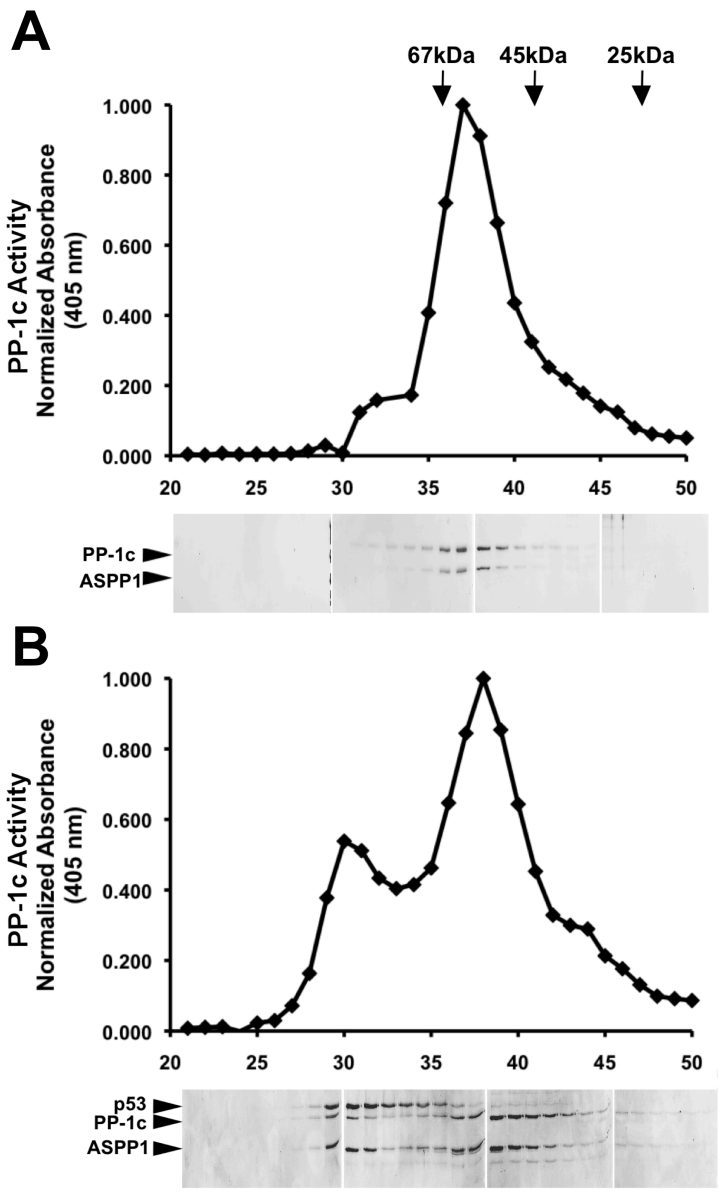

Figure 4.4: **ASPP1, PP-1c and p53 Partially Form a Trimeric Complex**. PP-1cα was incubated with $\text{ASPP1}_{867-1090}$, or with both $\text{ASPP1}_{867-1090}$ and p53_{2-293} for 45 min at 30 °C. Samples were loaded onto a pre-equilibrated Superdex 75 10/30 gel filtration column. Protein was eluted at 0.1 ml min^{-1} and 250 μl fractions were collected. Fractions from each gel filtration run were measured for phosphatase activity using the pNPP phosphatase assay to determine when PP-1c eluted from the column. PP-1cα formed a dimeric complex with ASPP1 (*panel A*) but partially formed a trimeric complex with both ASPP1 and p53 (*panel B*).

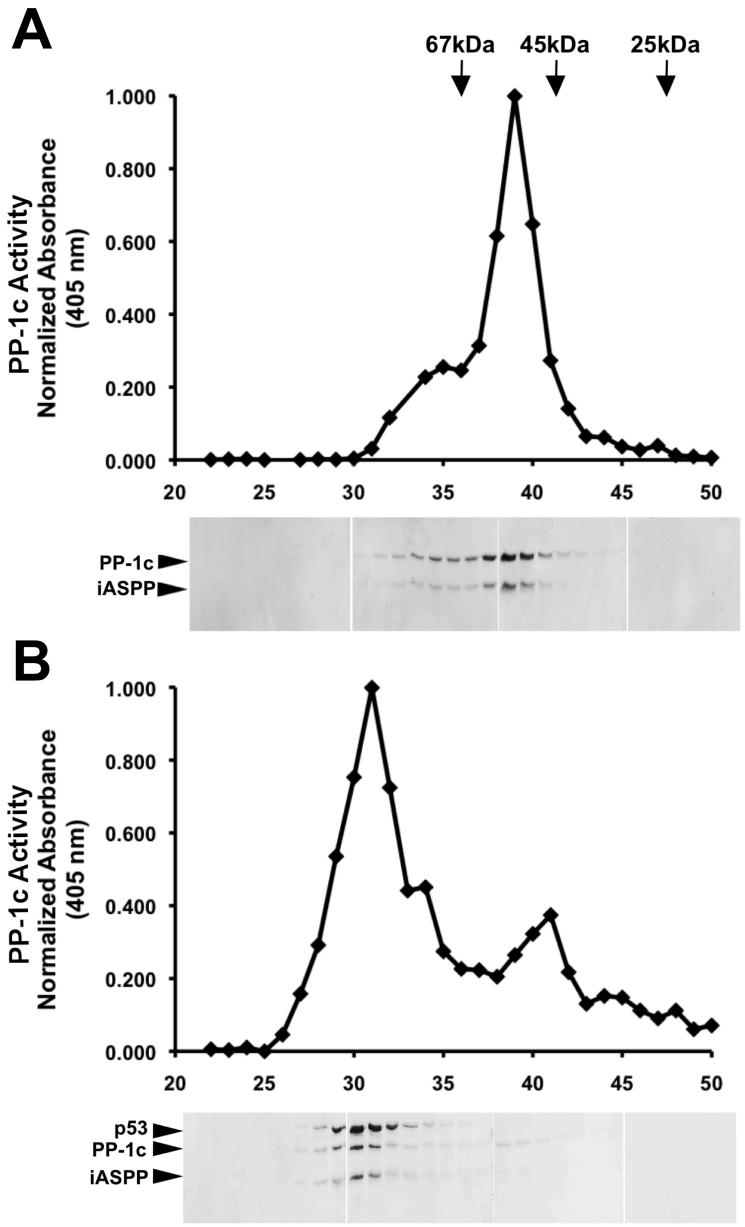

Figure 4.5: **iASPP Forms a Stable Complex with p53 and PP-1c**. PP-1cα was incubated with iASPP, or with both iASPP$_{608-828}$ and p53$_{2-293}$ for 45 min at 30 °C. Samples were loaded onto a pre-equilibrated Superdex 75 10/30 gel filtration column. Protein was eluted at 0.1 ml min^{-1} and 250 μl fractions were collected. Fractions from each gel filtration run were measured for phosphatase activity using the pNPP phosphatase assay to determine when PP-1c eluted from the column. iASPP readily formed a dimeric complex with PP-1cα (*panel A*) and also formed a trimeric complex with both p53 and PP-1cα (*panel B*).

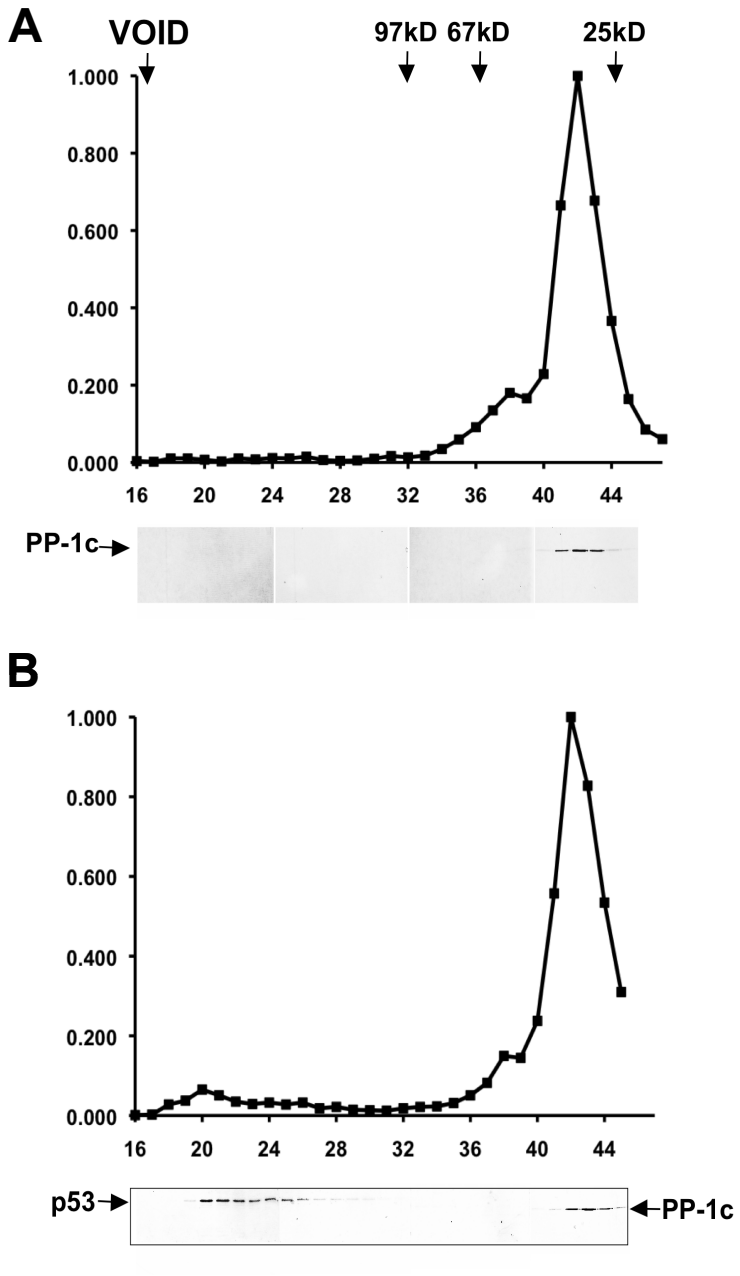

Figure 4.6: **Superdex 200 Gel Filtration Chromatography Demonstrates that Protein Phosphatase-1c Does Not Bind Directly to p53$_{2-293}$.** PP-1cα was incubated either alone (*panel A*) or together with p53$_{2-293}$ (*panel B*) for 45 min at 30 °C and then loaded onto a pre-equilibrated Superdex 200 10/30 gel filtration column. Protein was eluted at 0.1 ml min^{-1} and 250 μl fractions were collected. Fractions from each gel filtration run were measured for phosphatase activity using pNPP phosphatase assay to determine when PP-1c eluted from the Superdex column.

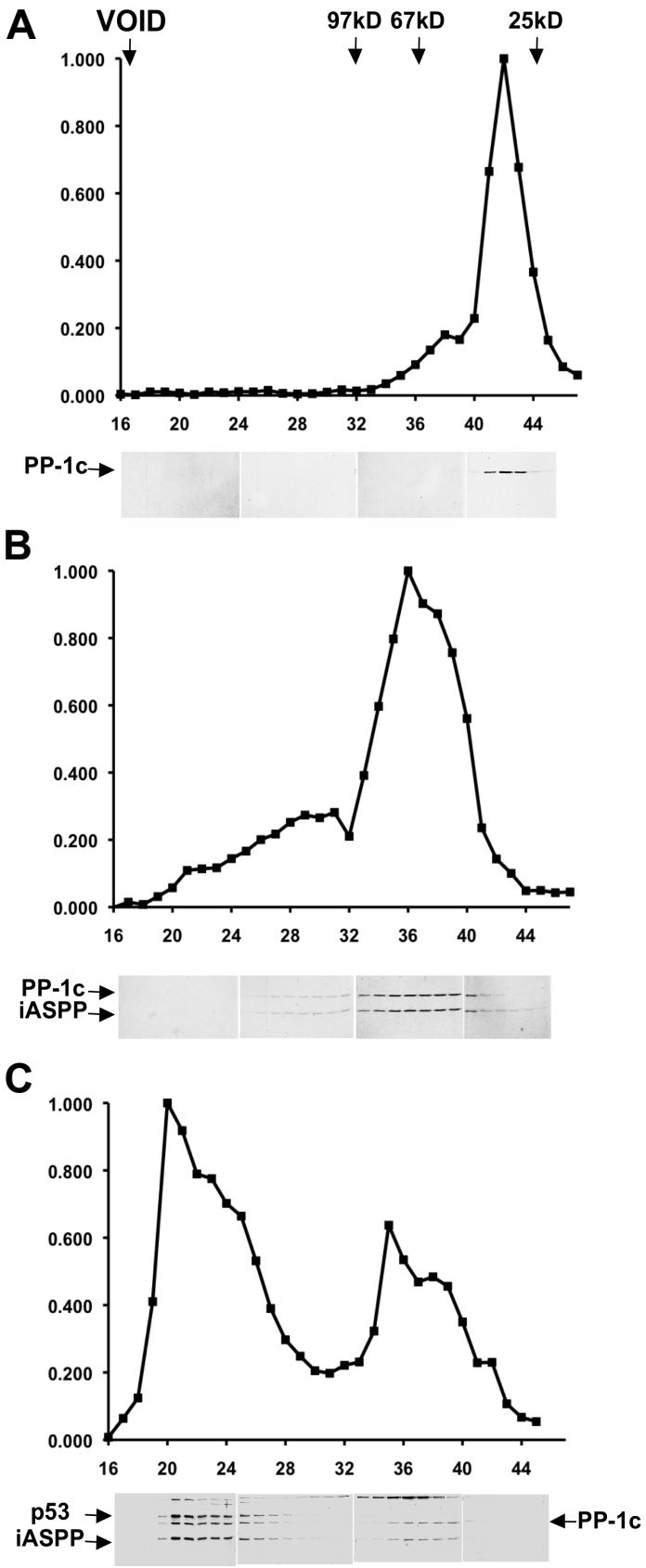
A
VOID
97kD 67kD
25kD
1.000
0.800
0.600
0.400
0.200
0.000
16 20 24 28 32 36 40 44
PP-1c
B
1.000
0.800
0.600
0.400
0.200
0.000
16 20 24 28 32 36 40 44
PP-1c
iASPP
C
1.000
0.800
0.600
0.400
0.200
0.000
16 20 24 28 32 36 40 44
p53
iASPP
PP-1c

Figure 4.7: **Analysis of the PP-1c•iASPP•p53 Complex Via Superdex 200 Gel Filtration Chromatography**. PP-1cα was incubated with iASPP in the presence or absence of $p53_{2-293}$ for 45 min at 30 °C. Samples were loaded onto a pre-equilibrated Superdex 200 10/30 gel filtration column. Protein was eluted at 0.9 ml min^{-1} and 250 μl fractions were collected. The phosphatase activity of each fraction was measured using the pNPP activity assay to determine when PP-1c eluted from the column. Alone PP-1cα elutes as a monomer at approximately 37 kDa (*panel A*). When incubated with iASPP, PP-1c and iASPP form a stable dimeric complex (*panel B*). The incubation of PP-1cα, iASPP, and $p53_{2-293}$ results in the formation of a stable trimeric complex (*panel C*). The high molecular weight protein shown gel filtration fractions containing p53, was identified as DnaK, a heat shock protein homologous to the mammalian heat shock protein Hsp70. During the bacterial expression of p53, the bacterial DnaK protein is co-purified as reported previously [45].

4.3.5 Microcystin-Sepharose Pull-down of the PP-1•iASPP•p53 Trimeric Complex

The ability of $ASPP1_{867-1090}$, $ASPP2_{905-1128}$ and $iASPP_{608-828}$ to form a trimeric complex with both PP-1c and p53 was also analyzed using MC-Sepharose binding experiments. Each ASPP proteins was incubated with an equimolar amount of PP-1cα and $p53_{2-293}$ and incubated with MC-Sepharose to pull down PP-1c multimeric protein complexes. Our results show that $iASPP_{608-828}$ and $ASPP1_{867-1090}$ (albeit partially) are able to interact simultaneously with both PP-1c and p53 (Figure 4.8, panels A and C). In contrast, $ASPP2_{905-1128}$ is unable to bind to $p53_{2-293}$ when bound to PP-1cα (Figure 4.8).

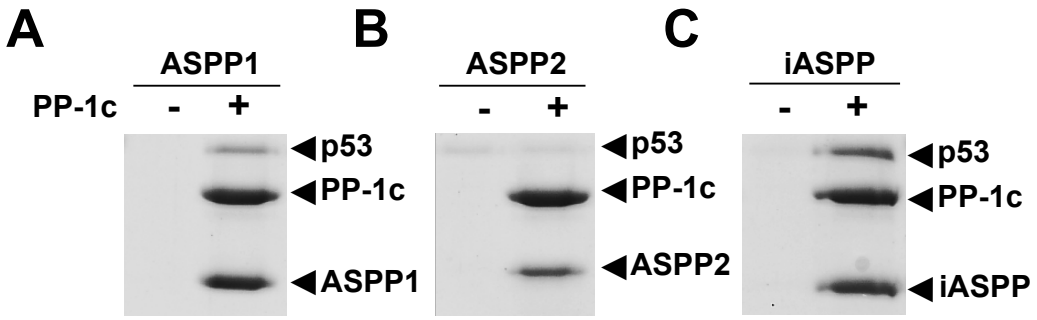

Figure 4.8: **iASPP and ASPP1, but not ASPP2 Forms a Trimeric Complex with PP-1cα and p53**. Approximately 5 μg of PP-1c, $p53_{2-293}$, and either $ASPP1_{867-1090}$ (*panel A*), $ASPP2_{905-1128}$ (*panel B*), or $iASPP_{608-828}$ (*panel C*) were incubated together at 30 °C for 45 min. Proteins were then incubated with 20 μl of MC-Sepharose end over end for 1 hr at 4 °C. After sufficient washing, bound protein was eluted using 2× SDS-PAGE sample buffer and boiling for 5 min at 100 °C. Proteins were analzyed by SDS-PAGE with Coomassie blue staining.

4.3.6 The Formation of a PP-1c•iASPP•p53 Trimeric Complex Increases the Dephosphorylation of p53 on Ser-15

Previously, PP-1c has been shown to dephosphorylate p53 on phosphorylated Ser-15 and Ser-37 residues [96, 161]. We hypothesized that the formation of the PP-1c•iASPP•p53 trimeric complex may direct PP-1c to specifically dephosphorylate key residues within the p53 N-terminus. In order to answer this question, p53 was phosphorylated by DNA-PK on Ser-15 and Ser-37 and p53 *in vitro* dephosphorylation experiments were carried out with PP-1cα in the presence of either

iASPP$_{608-828}$ or ASPP2$_{905-1128}$. The level of phosphorylation on Ser-15 and Ser-37 p53 residues was monitored by using antibodies specific for phosphorylated Ser-15 or Ser-37. Our results show p53 is readily phosphorylated *in vitro* on Ser-15 and Ser-15 by DNA-PK, as previously described [159]. The phosphorylation reaction was almost completely inhibited by the addition of an ATP competitive inhibitor, LY249002. (Figures 4.9 and 4.10). When p53 is incubated with PP-1c for a limited time (10 – 30 min), p53 is partially dephosphorylated at both Ser-15 and Ser-37.

There were no observed differences in the phosphorylation levels of Ser-37 when p53 was dephosphorylated with PP-1cα alone or in the presence of either iASPP$_{608-828}$ or ASPP2$_{905-1128}$ (Figure 4.9, panel A - lanes 4 to 6). In contrast, there was a drastic decrease in the phosphorylation state of Ser-15 in the presence of PP-1cα and iASPP$_{608-828}$, compared to PP-1cα alone (Figure 4.10, panel A - lanes 4 and 5). Increased dephosphorylation of phosphorylated Ser-15 was not seen when p53 was incubated with PP-1cα and ASPP2$_{905-1128}$ (Figure 4.10 panel A - lane 6). This suggests that the formation of the PP-1•iASPP•p53 complex directs the specific dephosphorylation of p53 on Ser-15.

4.4 Discussion

In this study, the role of the ASPP proteins in the specific dephosphorylation of p53 by PP-1c was investigated. Previous experiments have shown that ASPP2 is unable to bind trimerically to both PP-1c and p53 [98]. Gel filtration chromatography and MC-Sepharose binding experiments were carried out to investigate whether ASPP1 or iASPP could interact with both p53 and PP-1c in a trimeric complex. These binding experiments revealed that while ASPP2 is unable to interact with PP-1c and p53, ASPP1 partially binds to PP-1cα and p53$_{2-293}$ simultaneously. Significantly, iASPP forms a stable 1:1:1 heterotrimeric complex, binding to both PP-1cα and p53$_{2-293}$ at the same time.

Protein phosphatase-1c dephosphorylates two N-terminal p53 serine residues, Ser-15 and Ser-37 [96, 161]. We hypothesized that the formation of the PP-1c•iASPP •p53 trimeric complex may specifically alter the phosphorylation state of p53 on one or both of these phosphorylation sites. *In vitro* dephosphorylation assays of p53 by PP-1c showed that when bound within a PP-1c•iASPP•p53 trimeric complex with iASPP and p53, PP-1c specifically dephosphoryates Ser-15 (Figure 4.10). The PP-1c•iASPP•p53 complex does not affect the dephosphorylation of Ser-37 (Figure

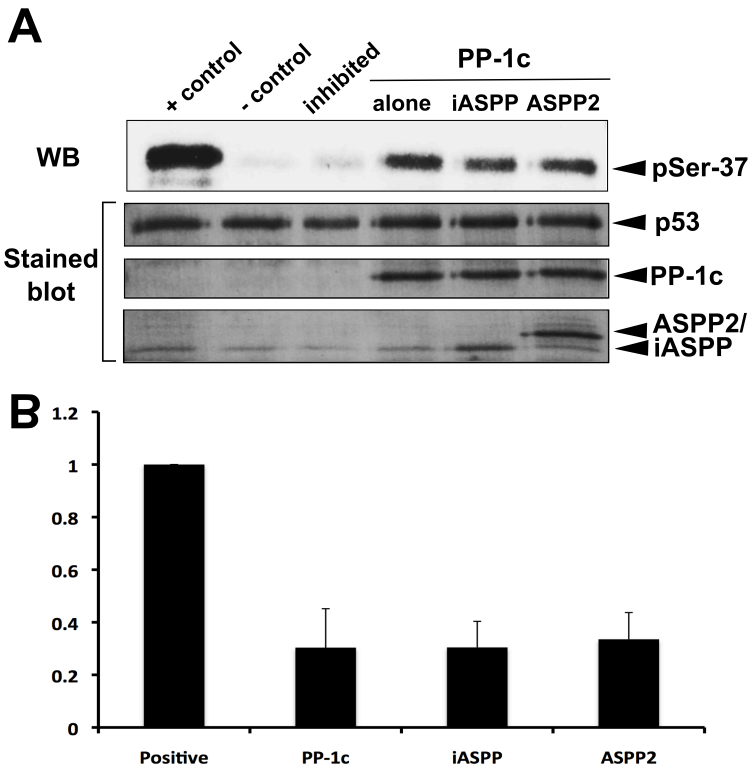

Figure 4.9: **iASPP and ASPP2 Do Not Affect Dephosphorylation of p53 on Ser-37**. A $p53_{2-293}$ glycerol stock (0.5 mg/ml, total amount 1 μg) was further diluted in Buffer P and phosphorylated with 100 ng of DNA-PK for 30 min at 30 °C. The phosphorylation reaction was stopped by the addition of 1 mM LY249002, a DNA-PK ATP competitive inhibitor (final concentration). An aliquot of the phosphorylation reaction was diluted further in buffer P and incubated with PP-1cα with either $iASPP_{608-828}$ or $ASPP2_{905-1128}$. Samples were separated by SDS-PAGE and analyzed by western blotting. Nitrocellulose membranes were stained to visualize total protein and blotted with a primary antibody specific for p53 phosphorylated on residue Ser-37. The negative control lane (– control) was a sample of p53 that was not phosphorylated by DNA-PK (no DNA-PK or ATP added to phosphorylation reaction). The lane labelled "inhibited" represents a sample that contained DNA-PK, however phosphorylation of Ser-15 and Ser-37 was inhibited by the addition of 1 mM LY249002, an ATP competitive inhibitor of DNA-PK.

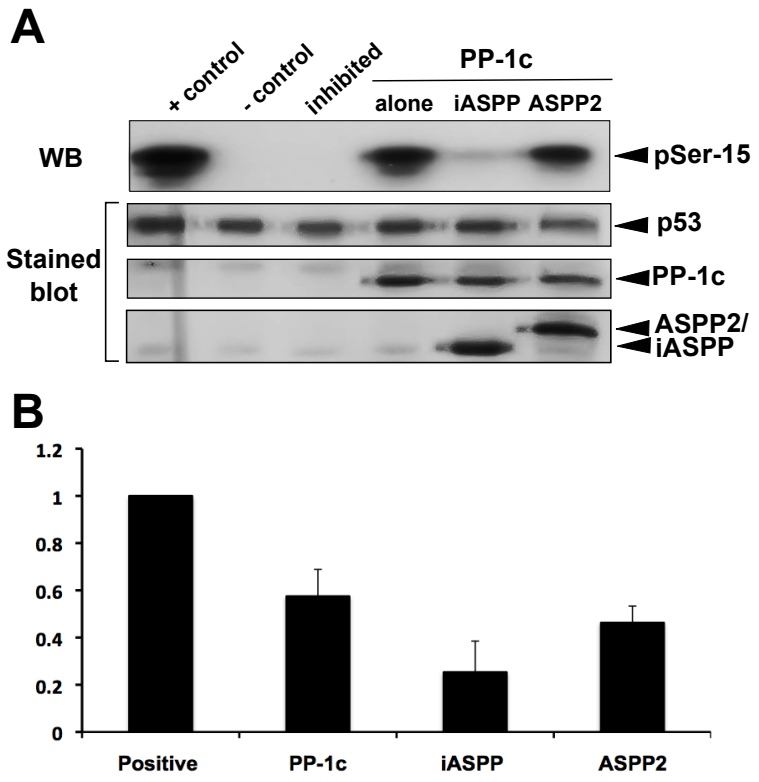

Figure 4.10: **iASPP Enhances the Specific Dephosphorylation of p53 by PP-1c**. A $p53_{2-293}$ glycerol stock (0.5 mg/ml, total amount 1 μg) was further diluted in Buffer P and phosphorylated with 100 ng of DNA-PK for 30 min at 30 °C. The phosphorylation reaction was stopped by the addition of an DNA-PK ATP competitive inhibitor, LY249002 (1 mM final concentration). An aliquot of the phosphorylation reaction was diluted further in kinase buffer and incubated with PP-1cα with either $iASPP_{608-828}$ or $ASPP2_{905-1128}$. Samples were separated by SDS-PAGE and analyzed by western blotting. Nitrocellulose membranes were stained to visualize total protein and blotted with a primary antibody specific for p53 phosphorylated on residue Ser-15.The negative control lane (– control) was a sample of p53 that was not phosphorylated by DNA-PK (no DNA-PK or ATP added to phosphorylation reaction). The lane labelled "inhibited" represents a sample that contained DNA-PK, however phosphorylation of Ser-15 and Ser-37 was inhibited by the addition of 1 mM LY249002, an ATP competitive inhibitor of DNA-PK.

4.9).

The phosphorylation state of p53 Ser-15 has a dramatic effect on the stability and activation of p53. Phosphorylation of p53 residue Ser-15 is absolutely necessary for the stabilization of p53 within cells [17]. Since prior phosphorylation of Ser-15 is required for the subsequent phosphorylation of Thr-18 by CK1, decreased phosphorylation on Ser-15 would likely lead decreased phosphorylation of Thr-18. This would lead to the increased association of p53 with mdm2 and degradation or inactivation of p53. Furthermore, decreased phosphorylation of Ser-15 would also decrease the affinity of p53 towards p300 and prevent the stabilization and activation of p53. When Ser-15 is mutated to alanine the expression of p53 is not upregulated in response to cellular stress and there is a decreased ability of p53 to induce cell cycle arrest [17, 77]. Amplification of the mdm2 protein alone is not sufficient to initiate malignancy in epithelial cells, and is typically found in combination with defective p53 phosphorylation in malignant epithelial cancers [138]. Overall, this suggests that the phosphorylation of residue Ser-15 of p53 is an important and required step in the activation of p53. Impaired phosphorylation or constitutive dephosphorylation of at this site would promote the association of p53 with mdm2 and lead to the inactivation or proteosomal degradation of p53 via ubiquitylation.

The iASPP protein inhibits the activity of p53 and is over expressed in many forms of cancers [172, 310, 121, 169]. The mechanism by which iASPP inhibits the apoptotic activity of p53 is not well understood. Expression of iASPP is directly induced by p53 in the response to DNA damage [155], suggesting that in normal cells iASPP protein functions to attenuate p53 signalling after a cellular stress, allowing a repaired cell to regain normal cellular function. However, over-expression of iASPP would prevent the proper activation of p53 during the cellular stress response, by targeting the specific dephosphorylation of pS15 by PP-1c. This would ultimately lead to the inactivation and degradation of p53 and allow cancerous or pre-cancerous cells to evade apoptosis and accumulate additional malignant mutations.

Further investigation is required to fully understand the role of iASPP in site specific dephosphorylation of p53 by PP-1c. To date, it not known whether PP-1c can specifically dephosphorylate other p53 Ser/Thr residues in addition to Ser-15 and Ser-37. It is possible that the the association of PP-1c with iASPP targets PP-1c to specifically dephosphorylate several residues within the N-terminus of p53. This can be tested by carrying out further *in vitro* p53 dephosphorylation assays, similar

to the experiments described in this chapter. The next step will be to investigate the role of the PP-1c•iASPP•p53 trimeric complex *in vivo*, specifically to look at how the overexpression of iASPP in cancer cells affects the dephosphorylation state of p53. The formation of the PP-1c•iASPP•p53 trimeric complex, leading to the targeted dephosphorylation of p53 on Ser-15 by PP-1c may be one mechanism by which iASPP inhibits p53 activity.

Chapter 5

Identification of Small Molecules that Disrupt the iASPP-PP-1c Heterodimeric Complex

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5.1 Part I: Regulation of PP-1c by Phosphatidic Acid

5.1.1 Introduction

Protein phosphatase-1c binds to multiple small molecule compounds, including toxins such as microcystin and okadaic acid, polyamines such as spermine and lipids including phosphatidic acid and ceramide [183, 116, 277, 126]. Extensive structural studies have demonstrated that the majority of small molecule toxins bind directly to the PP-1c active site to inhibit enzymatic activity (discussed in detail in Chapter 1, Section 1.3.4). However, much less is known about the interactions between PP-1c and other small molecules such as phosphatidic acid.

Ceramide and phosphatidic acid (PA) (Figure 5.1) are important bioactive lipids that regulate a multitude of cellular functions, including cell survival/apoptosis, vesicle trafficking, and cytoskeletal reorganization [87, 86, 85, 213, 306]. While similar in structure, ceramide and PA appear to have opposing roles within cells. Ceramide plays an important role in promoting apoptosis, while PA enhances cell proliferation [68, 67]. Phosphorylation of ceramide produces ceramide-1-phosphate (C1P), which also opposes ceramide signalling to block apoptosis and induce mitosis.

Previous experiments have shown that ceramide and ceramide-1-phosphate (Figure 5.1) directly modulate the activity of protein phosphatase-1c. Ceramide activates the activities of PP-1c and PP-2A, while ceramide-1-phosphate inhibits the activity of PP-1c towards phosphorylase *a* with an IC_{50} of 150 nM (Perreault et al., unpublished observations). Kathleen Perreault, a previous graduate student from our laboratory, proposed that ceramide-1-phosphate inhibits the activity of PP-1c by binding directly to the active site of PP-1c in a similar manner as calyculin A [Perreault et al., unpublished observations]. Calyculin A, a marine toxin originally purified from the marine sponge *Discodermia calyx*, consists of a dipeptide moiety and a polyketide moiety that contains a phosphate group (Figure 5.1, panel C). The crystal structure of the PP-1c•calyculin A complex reveals that the phosphate group of calyculin A binds directly within the active site, thereby blocking substrate access to the PP-1c active site (Figure 5.3) [137].

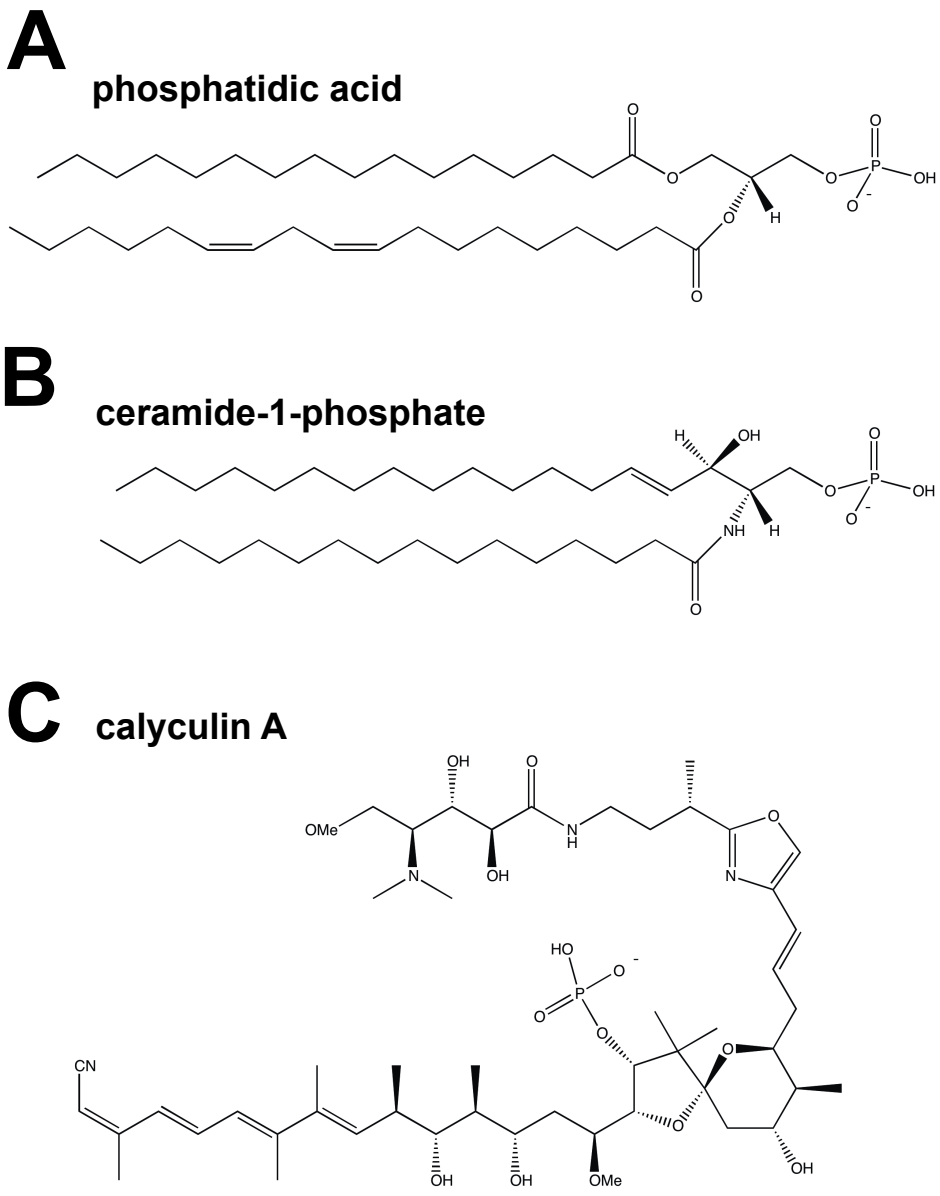

Figure 5.1: **The Chemical Structures of Phosphatidic Acid (PA), Ceramide-1-phosphate, and Calyculin A.** Phosphatidic acid (PA or 1,2-diacyl-sn-glycero-3-phosphate; *Panel A*) is a glycerophospholipid consisting of a glycerol backbone with two acyl chains in positions 1 and 2 and a phosphate group in position 3. Ceramide-1-phosphate (*Panel B*) is a spingolipid consisting of a spingolipid base connected to a a fatty acid chain via an amide bond. Calyculin A (*Panel C*) is a marine toxin consisting of a octamethyl polyhydroxylated C_{28} fatty acid (polyketide) and a dipeptide containing an esterified phosphate group.

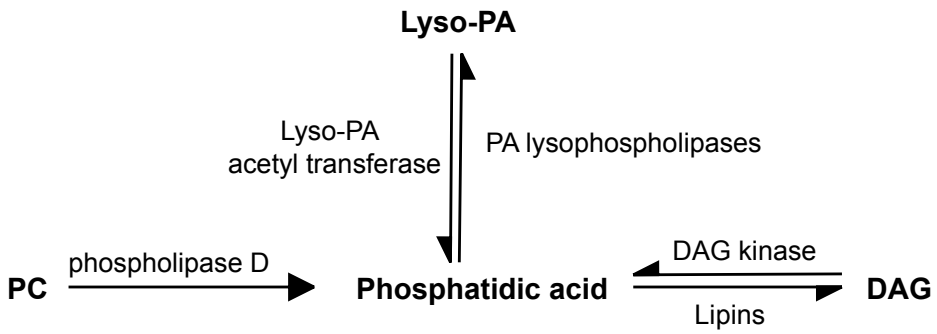

Figure 5.2: **Phosphatidic Acid is Produced by Multiple Pathways.** Phosphatidic acid is produced by three main pathways: 1) via hydrolysis of phosphatidylcholine (PC) by phospholipase D 2) phosphorylation of diacylglycerol (DAG) by DAG kinase and 3) acetylation of lysophosphatidic acid (Lyso-PA). DAG can be regenerated from phosphatidic acid through the actions of the Lipin family of lipid phosphatases. Lyso-PA can also be produced directly from PA via the actions of PA lysophospholipases.

5.1.1.1 Phosphatidic Acid Binds and Inhibits the Activity of Protein Phosphatase-1c

While PA comprises only 1% of mammalian membranes, PA is an important node in lipid metabolism. PA is produced via three main pathways: 1) via hydrolysis of PC via phospholipase D, 2) phosphorylation of diacylglycerol (DAG) by DAG kinase, 3) acetylation of lysophosphatidic acid (Lyso-PA) via Lyso-PA acetyl transferase (Figure 5.2). PA is an important second messenger and regulates many cellular functions such as cell proliferation, cytoskeletal reorganization and vesicle trafficking [119]. PA can bind directly to many eukaryotic proteins, including kinases such as PI4P5K [120], Raf1 [87, 86, 85], and mTor [70], GTP binding proteins such as ARF1 and ARF6 [192] and also protein phosphatases including SHP-1 [79] and PP-1c [134, 127].

The established model for PA-protein interactions, known as the "electrostatic/ hydrogen bond switch" model, involves hydrogen bonding between the anionic phosphate head group of PA and key Arg and/or Lys residues within a PA binding domain of the binding protein. This interaction increases the charge of the PA head group, which further stabilizes protein-lipid interactions [141, 140]. PA-protein binding does not always involve basic amino acids within the binding protein; experimental evidence suggests that the interaction of PA with PP-1c involves mainly hydrophobic interactions [127]. Jones et al. (2005) have identified a putative PA-binding region within PP-1c, residues 286 – 299, that form a minimal high-affinity

binding PA binding domain at the base of the flexible C-terminal tail (Figure 5.3). While PP-1c residues 286 – 296 are sufficient for binding to PA alone, authors hypothesized that the rest of the PP-1c C-terminal tail, that contains multiple Lys residues, folds back to form a more complete PA binding site [127].

Similar to ceramide-1-phosphase, PA also inhibits the activity of PP-1c with an apparent IC_{50} of 15 nM. Other glycerolipids, such as diacylglycerol (DAG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) have no effect on the activity of PP-1c [126]. Due to similarities between the chemical structures of ceramide-1-phosphate and PA (Figure 5.1), we hypothesize that PA also inhibits PP-1c by binding to PP-1c in a manner similar to calyculin A (Figure 5.3). Previous PA inhibition studies have only tested the effects of PA on the activity of PP-1c towards phosphorylated protein substrates. It is not known whether PA can inhibit the activity of PP-1c towards a smaller chemical substrate, such as p-nitrophenylphosphate (pNPP). If PA inhibited the activity of protein phosphatase-1c towards a small molecular substrate such as pNPP, this would suggest that PA does in fact bind directly to the active site site and not to the binding site predicted by Jones et al. (Figure 5.3) [127]. In order to investigate this hypothesis, the effect of PA on the enzymatic activity of PP-1c using the pNPP PP-1c activity assay was tested. In addition, MC-Sepharose and Ni-NTA binding experiments were carried out to test the effect of PA on the binding of PP-1c to microcystin and iASPP.

5.1.2 Materials and Methods

Phosphatidic acid (PA) and phosphatidylcholine (PC) were generously provided by Dr. Bern Kok from the laboratory of Dr. David Brindley (Department of Biochemistry, University of Alberta). Unless otherwise specified, all other materials and reagents were purchased from Sigma or Fisher Scientific.

5.1.2.1 Colourimetric pNPP Phosphatase Activity Assay

The colourimetric *p*-nitrophenylphosphate (pNPP) PP-1c activity assay was carried out as previously described in Chapter 2 (Section 2.2.11), with the following modifications. PA and PC were aliquoted from a chloroform:methanol stock, dried under nitrogen stream and resuspended in Buffer L (50 mM Tris-HCl pH 8.3, 1.0 mM EGTA, 30 mM $MgCl_2$, 1 mg/ml BSA, and 0.2 % β-mercaptoethanol) by sonicating for 20 min. PA is known to aggregate in the presence of divalent cations [228, 263];

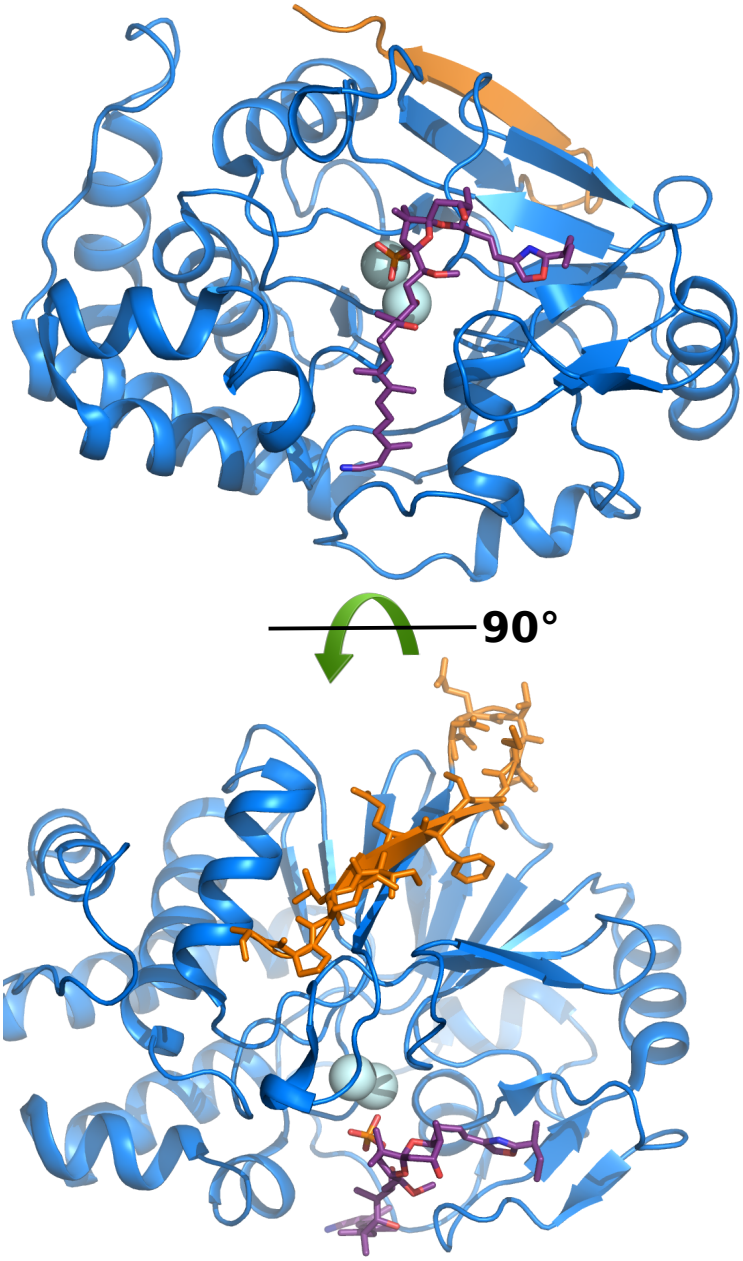

Figure 5.3: **PP-1c Residues 286 – 296 Are Important for Binding to Phosphatidic Acid**. The figure depicts the crystal structure of PP-1c (shown in blue) bound to calyculin A (shown in purple sticks) [137]. Highlighted in orange are PP-1c residues 286 – 296, a region that Jones et al. (2005) have identified as important for PA binding. In the lower panel, the PP-1c•calyculin A structure is rotated 90 degrees towards the page and the proposed PA binding domain is highlighted in orange sticks.

therefore, to avoid this this issue, assays were carried out in the absence of $MgCl_2$, $MnCl_2$, and EDTA. PC does not inhibit the catalytic activity of PP-1c [127] and so PC was used as a negative control with the pNPP assay. Samples containing PP-1c and glycerophospholipids were incubated together at room temperature for 10 min, prior to the addition of the pNPP substrate (final concentration 5 mM). Samples were further incubated for an additional hour at 37 °C and the absorbance at 405 nm was read using a plate reader. Phosphatase activity was expressed as a percentage relative to a PP-1c control sample that did not contain any glycerophospholipid.

5.1.2.2 Microcystin-Sepharose and Ni NTA Binding Disruption Experiments

MC-Sepharose binding experiments were carried out as previously described, with the following modifications. PP-1cα ($\sim$7.5 μg) was incubated with either 0.3 mM PA or PC diluted in Buffer Q (50 mM Tris-HCl pH 7.5 and 150 mM NaCl) for 20 min at 30 °C, prior to incubation with 25 μl of MC-Sepharose for 1 hour at room temperature. After sufficient washing with Buffer Q, bound protein was eluted with the addition of 30 μl of 2 $\times$ SDS-PAGE sample buffer and boiling at 100 °C for 5 min.

Ni-NTA binding experiments were carried out as previously described in Section 2.2.8, with the following modifications. In order to avoid the aggregation of PA, which occurs in the presence of divalent cations, PA was diluted in buffer containing no $MnCl_2$, $MgCl_2$, or EDTA. Samples were then incubated with Ni NTA agarose at room temperature for 1 hr. Bound protein was eluted from the Ni NTA resin by the addition of 30 μl 2$\times$ SDS-PAGE sample buffer and boiled at 100 °C for 5 min. Samples were then analyzed by SDS-PAGE and Coomassie blue staining.

5.1.3 Results

5.1.3.1 Inhibition of Protein Phosphatase-1cα by Phosphatidic Acid

Previous inhibition studies tested the effect of PA on PP-1c activity towards large phosphorylated protein substrates, including myelin basic protein and histone [134]. However, it is not known whether PA can inhibit PP-1c towards *p*-nitrophenyl-phosphate (*p*NPP), a small chemical substrate. The activity of PP-1c towards *p*NPP was measured using the pNPP PP-1c activity assay, in the presence of increasing amounts of either PA or PC. Results show that while PC had no effect on PP-1c

pNPP activity, PA inhibited PP-1c activity towards pNPP, with an IC_{50} of $\sim$10 μM (Figure 5.4).

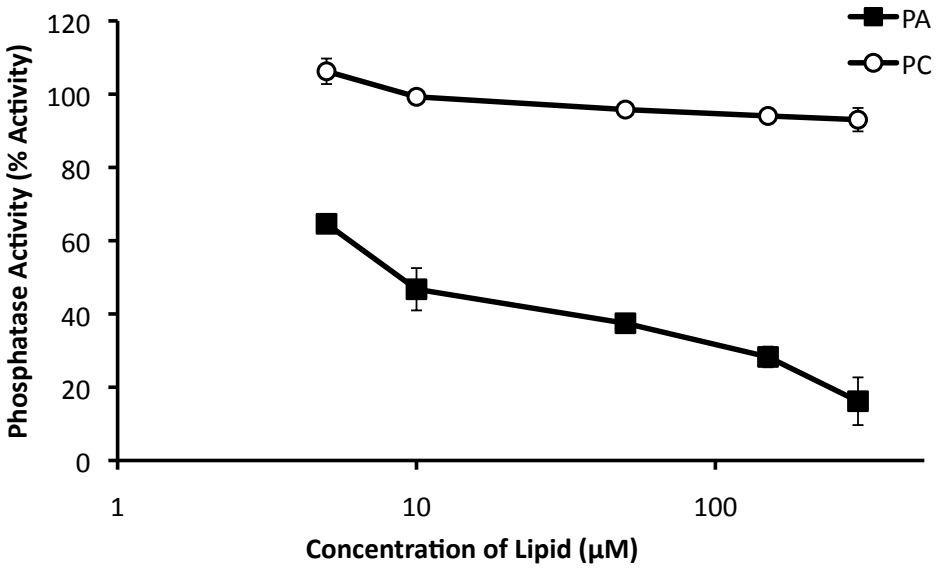

Figure 5.4: **Phosphatidic Acid Inhibits the Activity of Protein Phosphatase-1c Towards pNPP**. The figure depicts the standard curve for the inhibition of PP-1cα by phosphatidic acid (PA). Phosphatidylcholine (PC) was used as a negative control within the assay. Phosphatase activity is expressed as a percentage relative to a PP-1c control containing no glycerophospholipid.

5.1.3.2 Phosphatidic Acid Disrupts the Binding of PP-1c to Microcystin-Sepharose

The results show that PA inhibits the activity of PP-1c towards pNPP, a small chemical substrate. This suggests that PA is a competitive PP-1c inhibitor that binds close to or near the active site of PP-1c. Based on this result, I hypothesized that PA would also disrupt the ability of PP-1c to bind to other small inhibitors such as microcystin. In order to test this hypothesis, MC-Sepharose binding experiments were carried out to test the effect of PA or PC on the ability of PP-1c to bind to microcystin. While PC had no affect on the binding of PP-1c to MC-Sepharose, PA clearly disrupted the binding of PP-1c to MC-Sepharose (Figure 5.5).

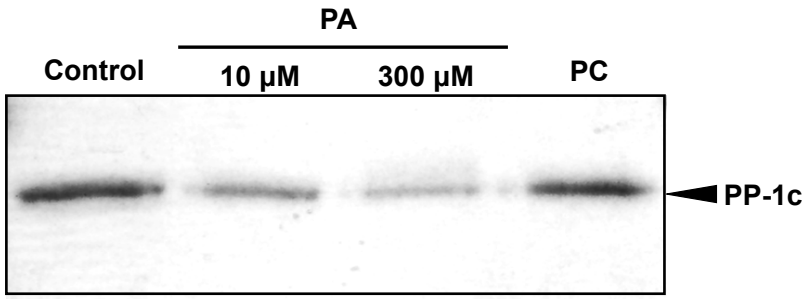

Figure 5.5: **Phosphatidic Acid Partially Disrupts the Binding of Protein Phosphatase-1c to Microcystin-Sepharose**. The figure depicts the MC-Sepharose binding experiment of PP-1cα alone alone and in the presence of phosphatidic acid (PA) or phosphatidylcholine (PC). Approximately 5 μg of PP-1cα was incubated with 10 μM PA, 300 μM PA or 300 μM PC for 20 min at 30 °C and subsequently incubated with 20 μl of MC-Sepharose for 1 hr at room temperature. Resin was washed with Buffer Q, eluted with 30 μg of 2X SDS-PAGE sample buffer, and analyzed by SDS-PAGE with Coomassie blue staining.

5.1.3.3 Disruption of the iASPP•PP-1cα Complex by Phosphatidic Acid

In order to test the effect of PA on the ability of PP-1c to bind to the iASPP protein, Ni NTA agarose experiments were carried out, using His-tagged iASPP. The MC-Sepharose binding experiment could not be used for these experiments, since PA disrupts the ability of PP-1c to bind to MC-Sepharose (Figure 5.5). Results from the Ni-NTA agarose binding experiments reveal that pre-incubation of PP-1c with PA drastically reduces the ability of PP-1c to bind to iASPP by approximately 50% (Figure 5.6). In contrast, incubation of PP-1c with PC had no affect on the formation of the PP-1c•iASPP complex.

5.1.4 Discussion

The work described in this study demonstrates that PA inhibits the activity of PP-1c towards pNPP, a small non-physiological chemical substrate (Figure 5.4). In addition, PA disrupts the binding of PP-1c to microcystin (Figure 5.5). Together, these results suggest that PA binds directly to the active site of PP-1c, thereby blocking the binding of substrates and small molecular PP-1c inhibitors to the PP-1c active site.

The results described in this study contradict previous kinetic studies that show that PA is a non-competitive inhibitor of PP-1c that binds to a region of PP-1c remote from the active site [126]. However, Jones et al. (2002) carried out their

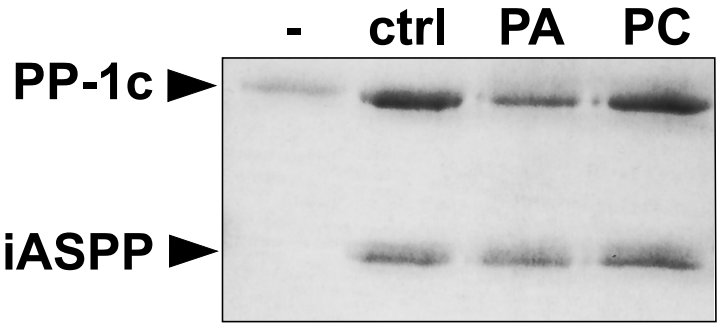

Figure 5.6: **Phosphatidic Acid Disrupts the Formation of the PP-1c•iASPP Complex**. His-tagged $iASPP_{608-828}$ was incubated with Ni NTA resin for 1 hr at 4 °C. PP-1cα ($\sim$ 7.5 μg) was incubated with 0.3 mM PA or PC for 20 minutes at 30 °C and subsequently incubated with His-tagged iASPP immobilized onto Ni-NTA agarose resin for an additional 1 hr at room temperature. Resin was washed with buffer G containing no $MnCl_2$, eluted with 30 μg of 2X SDS-PAGE sample buffer, and analyzed by SDS-PAGE.

PP-1c kinetic studies in the presence of $MnCl_2$, which is required for the basal activity of PP-1c [126]. Divalent cations, including $MnCl_2$, are also known to cause aggregation of PA in solution [71]. Aggregation of PA and Mn^{2+} ions may also have induced the chelation of the manganese ions from the PP-1c active site and lead to decreased enzymes activity or efficiency. Therefore, kinetic analysis of the effect of PA on PP-1c activity is challenging, albeit impossible, since the amount of free PA in solution can not be properly measured in the presence of $MnCl_2$.

In addition, this study has demonstrated for the first time that the binding of PA to PP-1c disrupts the formation of a PP-1c holoenzyme complex, specifically the PP-1c•iASPP complex. The specific physiological significance of this result is still unclear and it is not known whether PA disrupts the binding of PP1-c to other PP-1c regulatory proteins. Levels of phospholipase D, which catalyzes the production of PA from PC, are elevated in many human cancers, including breast, lung, bladder, pancreatic, and kidney cancers [78]. The effect of phospholipase D overexpression are thought to be mediated mainly by the accumulation of PA . Based on the results described in this chapter, the accumulation of PA in cancer cells may cause the inhibition/disruption of specific PP-1c holoenzyme complexes within the cell. Further investigation is required to determine the role of PA in the formation of PP-1c holoenzyme complexes and how this may impact cell proliferation and cancer progression. Overall, the results from these experiments suggested that other small compounds, such as those produced by marine organisms, may also have

the potential to disrupt the PP-1c•iASPP complex and this is described in detail in part II of this Chapter.

5.2 Part II: Disruption of the iASPP•Protein Phosphatase-1c Complex using Natural Marine Compounds

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5.2.1 Introduction

Until recently, modern drug design focused primarily on targeting the activity of a single enzyme or receptor protein. A new emerging area of drug design is to instead target a specific protein-protein interaction. The majority of protein-protein interactions occur over a large surface area, making the identification of small cell-permeable molecules or peptides that disrupt complex formation challenging. However, it is now well understood that many protein-protein interactions involve concentrated binding "hot-spots", small regions of specific high-affinity binding [295]. It is thought that these smaller "hot-spot" binding regions could be successfully targeted by small drug compounds.

Due to their importance in cellular signalling, Ser/Thr protein kinases are one of the most exploited classes of drug targets, comprising approximately 30% of all pharmaceutical drug programs [47]. While the regulation of Ser/Thr protein phosphatases is also critical for proper cell function, to date there are only two FDA approved drugs that target protein phosphatases: cyclosporin and FK506. Cyclosporin and FK506 are immunosuppressant drugs that non-competitively inhibit calcineurin activity by binding together with a specific cyclophilin protein (cyclophilin A and FK506 binding protein, respectively) [147]. Other known Ser/Thr protein phosphatase inhibitors, such as microcystin and okadaic acid, inhibit phosphatase activity by binding directly to the catalytic active site of PP-1c and PP-2Ac. Inhibition of PP-1c is generally cytotoxic to cells; exposure to microcystin has been shown to induce the collapse of the hepatocyte cytoskeleton, leading to hepatic hemorrhaging and liver failure [55].

One approach to generate a more specific PP-1c inhibitor would be to target and disrupt a specific PP-1c holoenzyme complex. Multiple studies have identified short synthetic peptides that disrupt the ability of PP-1c to bind to key regulatory proteins [12, 211, 133, 8]. These disruption experiments have focused solely on the

disruption of the PP-1c RVXF motif interaction. While effective, disruption of the RVXF motif interaction will likely not generate an inhibitor specific for a particular PP-1c holoenzyme complex; it is predicted that a RVXF disrupting peptide would prevent PP-1c from interacting with all PP-1c regulatory proteins that contain an RVXF motif.

The PP-1c•iASPP complex is an ideal candidate for the design of novel protein-protein disrupting compounds for several reasons. Firstly, the structure of both PP-1c and iASPP has been elucidated [194, 235]. Secondly, the binding interactions between PP-1c and iASPP have been well characterized, as described in Chapters 2 and 3 of this thesis. In addition, there are several known interaction "hot-spots" between PP-1c and iASPP. These include the interaction of PP-1c with the RVXF-like motif of iASPP (RARL, residues 622 – 625) and the interaction between the C-terminal tail of PP-1c and the SH3 domain of iASPP. Both interactions appear to the essential for PP-1c•iASPP complex formation; however, unlike the RVXF interaction, the interaction between the C-terminal tail of PP-1c and the SH3 domain of iASPP appears to be specific for the ASPP proteins (as described in Chapter 3).

5.2.1.1 Marine Organisms Produce Novel Chemically Diverse Lead Drug Compounds

The marine environment is the most significant source of novel bioactive molecules in the world. Benthic and sessile, soft-bodied marine organisms (such as marine sponges or starfish) are prone to predation and produce a multitude of chemically diverse bioactive compounds for chemical communication and defense. These same compounds are also molecular leads for drug design. Many compounds identified from marine sources have passed FDA clinical trials, including Ara-A, for the treatment of herpes and Ara-C, for the treatment of leukemia [221]. Drug discovery within the marine environment is still largely untapped. The majority of bioactive compounds have been identified from marine organisms living on the ocean fringes, which represents only 1% of the total ocean surface. Since the 1970's over 20,000 novel compounds have been discovered from marine sources and approximately 1,000 new drug leads are discovered each year [221].

The waters of Papua New Guinea comprise a coral triangle between Papua New Guinea, the Phillipines and Indonesia. These waters are a rich and collectable source of marine organisms, particularly marine sponges, that are still largely unexplored. Significantly, many of the natural products extracted from marine sponges are not

actually produced by sponge itself, rather they are produced by microorganisms that exist symbiotically or parasitically within the marine sponge [221]. Microbial symbionts can comprise as much as 40% of the total sponge tissue volume [242] and it is often difficult to discern whether it is in fact the microbe or the marine organism that produces a specific metabolite.

Dr. Ray Andersen, our collaborator from the University of British Columbia (British Columbia, Canada) is a member of the"Papua New Guinea International Cooperative Biodiversity Group" and together with members of his laboratory participates in the collection of marine organisms from Papua New Guinea. For over a decade, the Holmes laboratory has been working in collaboration with the Andersen laboratory to identify novel marine compounds that modify Ser/Thr protein phosphatase activity.

Multiple studies have shown that iASPP is over expressed in many different forms of cancer [310, 121, 169]. Chapter 4 of this thesis describes the role of the PP-1c•iASPP complex in the regulation of p53 dephosphorylation. These results suggested that the PP-1c•iASPP complex is a valuable drug target and the identification of novel compounds that disrupt the ability of PP-1c to bind to iASPP could be extremely beneficial towards the treatment of cancers that overexpress the iASPP protein. Chapter 5 of this thesis describes the development of two novel PP-1c protein-protein disruption assays: the His-tagged solid-phase disruption assay and the MC-Sepharose PP-1c disruption assay. In collaboration with the Andersen laboratory at the University of British Columbia, I have identified three marine compounds that disrupt the PP-1c•iASPP complex, sokotrasterol sulphate from marine crude extract 08-35 and two hippospongin-related compounds from marine crude extract 08-39.

5.2.2 Materials and Methods

5.2.2.1 Materials

A synthetic peptide containing the ASPP2 RVXF motif (RVKFNPLALLLDSS, residues 921 – 934) was purchased from the Alberta Peptide Institute at the University of Alberta. Microcystin and MC-Sepharose were produced as described in Section 2.2.1. All other chemicals, unless otherwise specified were purchased from Sigma or Fisher Scientific.

5.2.3 Preparation and Fractionation of Marine Crude Extracts Collected From Papua New Guinea

Marine extracts were provided by Dr. Ray Andersen from the University of British Columbia (Vancouver, BC). Marine specimens were collected by hand using SCUBA (Self-Contained Underwater Breathing Apparatus) near Keviang, Papua New Guinea and were frozen on site for transport to Vancouver, British Columbia. Samples were thawed and extracted with MeOH to yield a crude MeOH extract. Lyophilized marine crude extracts were shipped to our laboratory, where they were dissolved in 100 % MeOH, to a final concentration of 2 mg of crude extract per ml of MeOH. Extracts were then screened both for their ability to inhibit PP-1c activity towards pNPP and also their ability to disrupt the PP-1c•iASPP complex using the solid-phase disruption and MC-Sepharose PP-1c disruption assays, described in detail below in Section 5.2.4.2.

5.2.4 Assay Guided Purification of Natural Products From Marine Crude Extracts

The marine crude extracts 08-35 and 08-39 were partitioned into ethyl acetate (EtOAc) and water soluble fractions. The water partition was subsequently extracted with butanol (BuOH). The EtOAc and BuOH fractions were lyophilized and sent to our laboratory to be tested for the ability to disrupt the formation of the PP-1c•iASPP complex using the MC-Sepharose PP-1c disruption assay.

The 08-35 aqueous fraction purified via solvent partition into aqueous-aqueous (Aq-Aq) and butanol (BuOH) sub-fractions. The 08-35 BuOH and 08-39 EtOAc fractions were further purified by Sephadex LH20 chromatography using 100% MeOH or 80% MeOH/CH_2Cl_2 for the 08-35 aqueous and 08-39 EtOAc fractions, respectively. This yielded six total fractions for 08-35 (Aq-Aq, BuOH, BuOH-A, BuOH-B, BuOH-C, and BuOH-D) and four fractions for 08-39 (EtOH-A to EtOH-D). Lyophilized samples were delivered to our laboratory and resuspended in 100% MeOH to a final concentration of 2 –10 mg/ml. A summary of the purification of marine crude extracts 08-35 and 08-39 is shown in Figures 5.7 and 5.7, respectively.

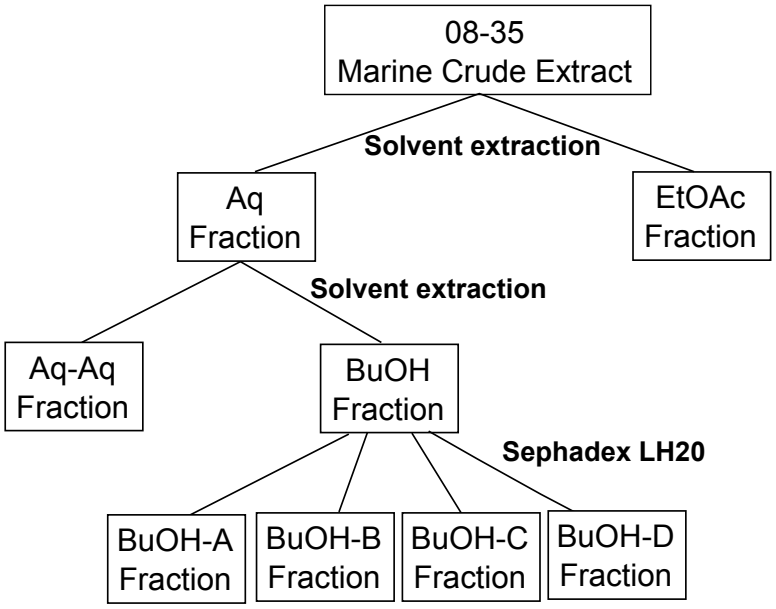


Figure 5.7: **Summary of the Assay Guided Purification of Marine Crude Extract 08-35**. The 08-35 marine crude extract was separated into aqueous (Aq) and ethyl acetate (EtOAc) fractions via solvent partitioning. Based on the MC-Sepharose disruption assay results, the Aq fraction was further partitioned into aqueous-aqueous (Aq-Aq) and BuOH fractions. Sephadex LH20 chromatography was carried out on the BuOH fraction to yield four BuOH fractions, BuOH-A, BuOH-B, BuOH-C, and BuOH-D.

5.2.4.1 Colourimetric pNPP Phosphatase Activity Assay

The pNPP PP-1c activity assays were carried out as previously described in Section 2.2.11 with the following modifications. PP-1cα was diluted in Buffer L and incubated with 2 –10 μl of each marine extract (0.33 mg/ml final concentration within assay) for 10 min at 37 °C prior to the addition of pNPP (final concentration of 5 mM). Samples were incubated for an additional 1 hr at 37 °C and the absorbance at 405 nm in each well was measured.

5.2.4.2 Solid-phase Disruption Assay

Nickel (Ni) coated 96-well plates (HisGrab, Pierce Scientific) were washed with buffer R (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05 mM $MnCl_2$) containing 0.05% Tween-20. His-tagged iASPP$_{608-828}$ was further diluted to a final concentration of 0.58 μM in buffer R and immobilized onto the Ni coated plates by incubating overnight at 4 °C. Wells were washed with buffer R containing 0.05% Tween-20 and subsequently incubated with 0.6 μM PP-1cα for 1 to 2 hours at 4 °C. After sufficent

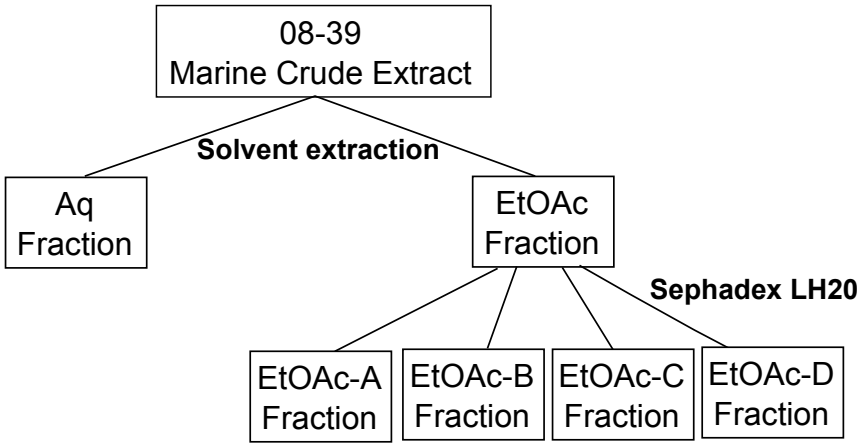


Figure 5.8: **Summary of the Assay Guided Purification of Marine Crude Extract 08-39**. The 08-39 marine crude extract was separated into aqueous (Aq) and ethyl acetate (EtOAc) fractions via solvent partitioning. Based on the MC-Sepharose disruption assay results, the BuOH fraction was further separated by Sephadex LH20 chromatography into four BuOH fractions, BuOH-A, BuOH-B, BuOH-C, and BuOH-D.

washing with buffer R containing 0.05 % Tween-20, 100 μl of 10 mM pNPP was added to each well and the plate was incubated for one hour at room temperature and the optical density at 405 nm was measured every 5 to 10 min for 1 hr. A summary of the solid-phase disruption experiment is shown in Figure 5.9.

5.2.4.3 Microcystin-Sepharose Marine Extract Disruption Assay

MC-Sepharose disruption assays were carried as previously described in Section 2.2.5, with the following modifications. Marine crude extracts were diluted in 100% methanol and approximately 20 μg of each extract was added to each dephosphorylation reaction. In order to ensure that the MeOH from the marine crude extracts did not affect the binding of PP-1c to iASPP within the assay, an equivalent amount of methanol was added to a PP-1c•iASPP positive control sample no marine crude extract.

5.2.5 Results

5.2.5.1 Majority of Marine Extracts Do Not Inhibit Protein Phosphatase-1c pNPP Activity

With the help of Ply Pasarj, a graduate student from our laboratory, marine crude extracts collected from Papua New Guinea in 2008 were screened for inhibition of

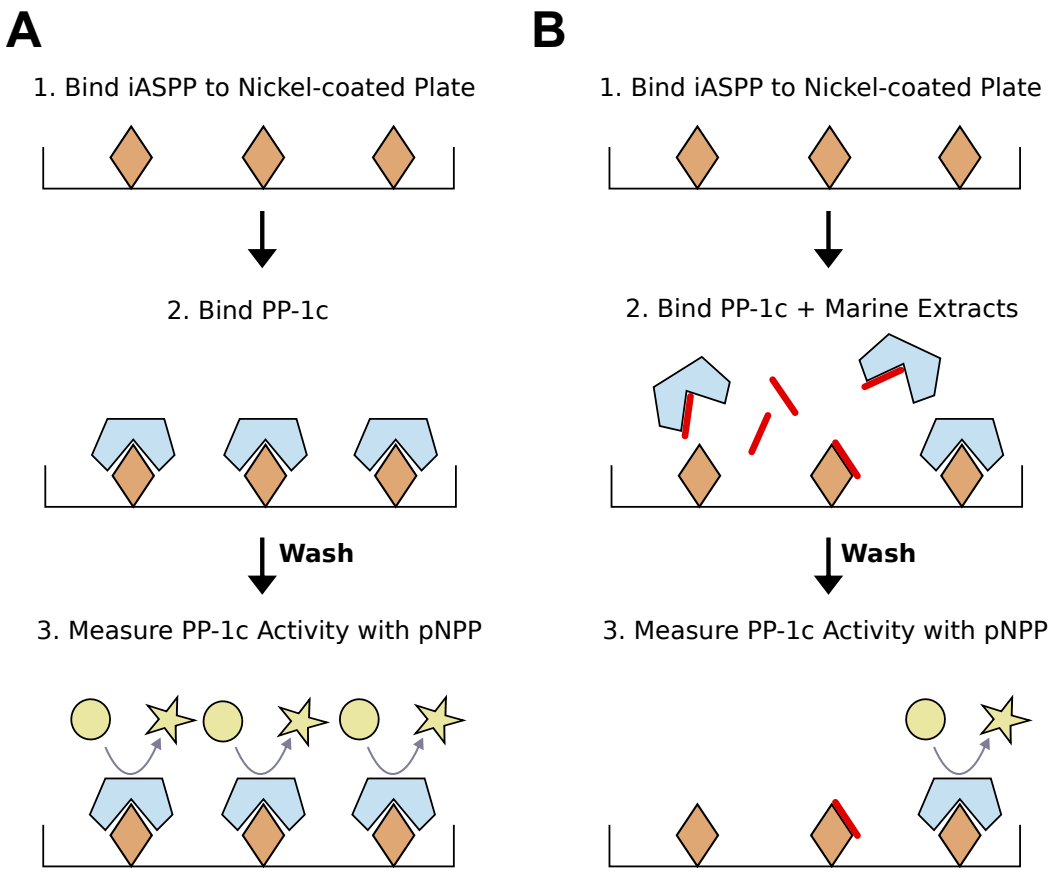


Figure 5.9: **Development of a Novel Solid-Phase Disruption Assay**. The figure outlines the basic steps of the solid-phase disruption assay in the absence (*Panel A*) or presence (*Panel B*) of a marine crude extract. His-tagged iASPP was bound to the Ni-coated plate (1) and then each well was incubated with either PP-1c alone or a mixture of PP-1c and a marine crude extract (2). After sufficient washing, amount of PP-1c in each lane was determined by measuring the relative pNPP phosphatase activity in each well (3).

PP-1c activity using the pNPP activity assay. The results show that the majority of the marine crude extracts do no inhibit the activity of PP-1c towards pNPP (Figure 5.10). Out of 142 marine crude extracts, 18 were identified as inhibitory towards PP-1c *p*NPP activity and inhibited PP-1c greater than 10% (Table 5.1).

Table 5.1: **Identification of Marine Crude Extracts that Inhibit Protein Phosphatase-1c Activity Towards pNPP.** Table lists marine crude extracts that inhibit the enzymatic activity of PP-1c towards pNPP greater than 10 %.

Extract No.	% PP-1c Inhibition
08-20	57
08-35	49
08-37	13
08-39	26
08-44	50
08-45	38
08-48	76
08-71	12
08-94	11
08-101	23
08-114	39
08-115	23
08-116	39
08-124	53
08-133	94
Anz A	34

5.2.5.2 Development of a Novel Solid-Phase PP-1c•iASPP Disruption Assay

In order to screen hundreds of marine crude extracts for the ability to disrupt the binding of PP-1c to iASPP, a novel solid-phase disruption assay (similar to an ELISA or enzyme-linked immune sorbent assay) was developed using Ni coated 96-well plates. As outlined in Figure 5.9, His-tagged iASPP$_{608-828}$ was immobilized onto each well and the sample was further incubated with PP-1c either alone or in the presence of a specific marine crude extract. The amount of PP-1c that remained bound to iASPP after sufficient washing was determined by measuring the pNPP PP-1c activity in each sample. Marine crude extracts were pre-screened

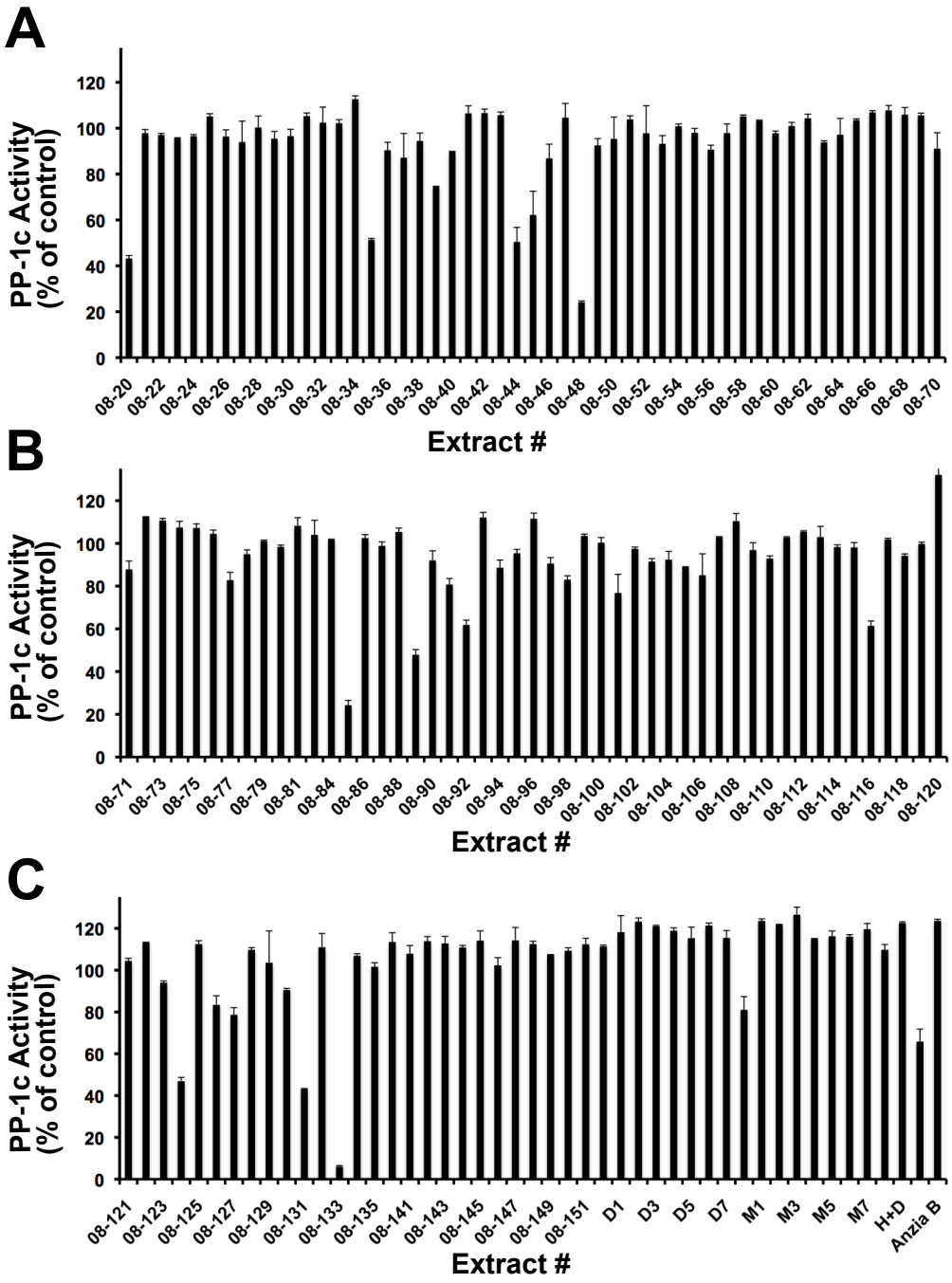


Figure 5.10: **Identification of Marine Crude Extracts that Inhibit Protein Phosphatase-1c Activity Towards pNPP.** Marine crude extracts were tested for their ability to inhibit PP-1cα using the 60 μl pNPP PP-1c activity assay. Extracts were incubated with PP-1cα for 10 min at 37 °C prior to the addition of *p*NPP (5 mM final concentration). Reactions were then incubated for an additional hr at 37 °C, after which the absorbance of each well was measured at 405 nm. The activity of PP-1c in the presence of each marine crude extract is shown as a percentage of a control sample that contained PP-1cα and 4 μl of MeOH.

for their ability to inhibit PP-1c activity towards pNPP (Figure 5.10) and only marine crude extracts that inhibited PP-1c less than 10% were tested using the solid-phase disruption assay.

As an initial "proof of principle" experiment, the ability of the ASPP2 RVXF peptide (RVKFNPLALLLDSS, residues 921 – 934) to disrupt the binding of PP-1c to ASPP2 and iASPP within the solid-phase disruption assay was tested. Results show that pre-incubation of PP-1c with the ASPP2 RVXF peptide decreased the total amount of PP-1c bound to iASPP or ASPP by 27 and 34%, respectively (Figure 5.11).

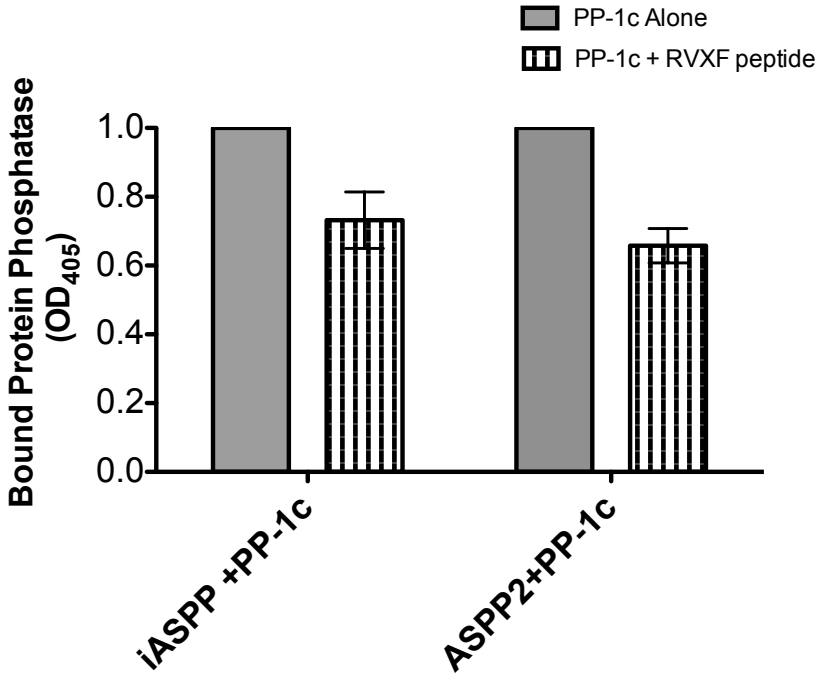


Figure 5.11: **The ASPP2 RVXF Peptide Disrupts the Ability of PP-1c to Bind to iASPP and ASPP2**. iASPP and ASPP2 was immobilized onto nickel (Ni) coated plates (200 nM per well) and incubated overnight at 4 °C. Ni plates were then incubated with 50 μl of 0.8 μM PP-1cα per well, in the absence or presence of 0.5 mM of the ASPP2 RVXF peptide, for an additional hour at 4 °C. After sufficient washing, the amount of bound PP-1c protein was determined by the addition of *p*NPP substrate (10 mM) and the absorbance at 405 nm was measured after incubation at room temperature for 60 min. Non-specific binding to the Ni plate was determined by measuring the absorbance of wells incubated with PP-1cα alone.

Next, the ability of the Papua New Guinea marine crude extracts to disrupt

the PP-1c•iASPP complex was tested using the solid-phase disruption assay. The results of the solid-phase disruption assays are shown in Figures 5.12 and 5.13. Since the ASPP2 RVXF peptide disrupted the binding of PP-1c to iASPP by about 27%, marine crude extracts that disrupted the binding of PP-1c to iASPP more than 25% were selected for further purification. Out of 124 marine crude extracts tested, 11 extracts decreased the ability of PP-1c to bind to iASPP greater than 25% (Table 5.2).

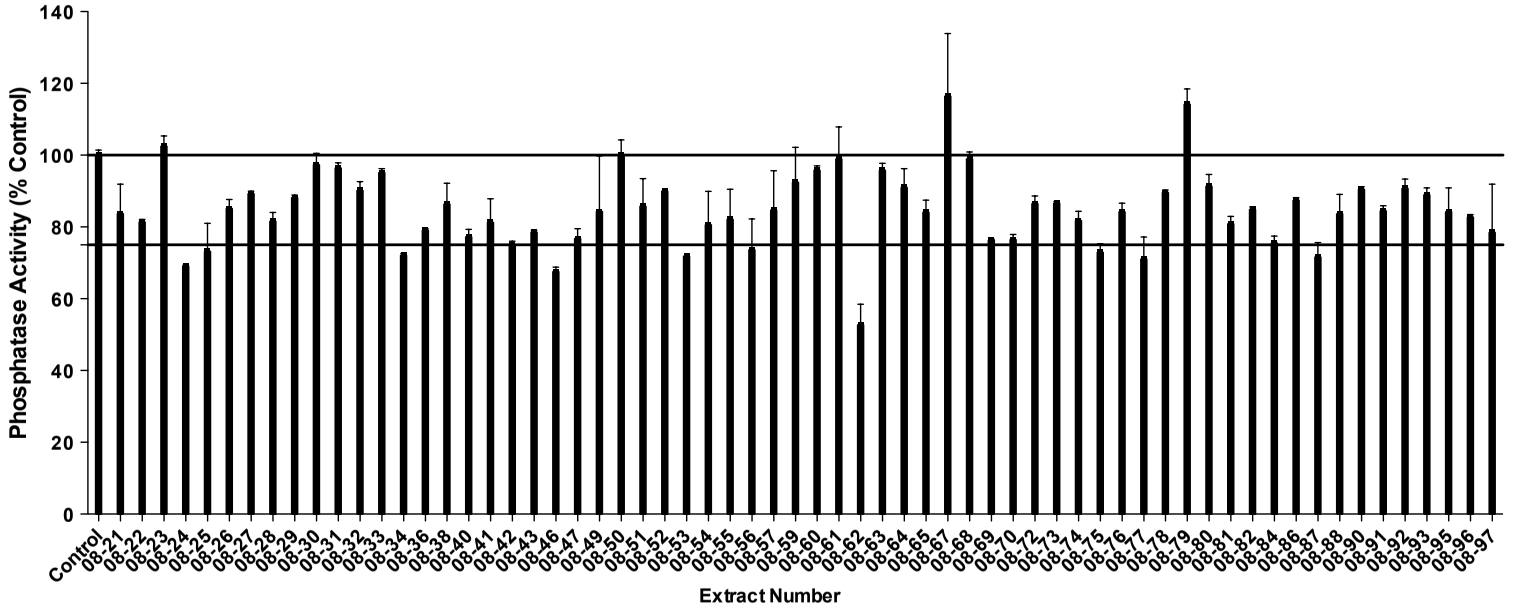


Figure 5.12: **Identification of Natural Extracts that Disrupt the PP-1c•iASPP Complex via the Solid-phase Disruption Assay (Extracts 08-21 to 08-87)**. iASPP$_{608-828}$ was immobilized on 96-well Ni NTA coated plates (Pierce Scientific), 20 pmol, overnight at 4 °C. Wells were washed and incubated for 1 hour with approximately 0.8 μM PP-1cα +/- marine natural product extract (0.16 μg of total extract per well). After sufficient washing, 100 μl of 10 mM pNPP (in dilution buffer) was added and absorbance at 405 nm was measured after 1 hour at room temperature. Activity of PP-1cα in the presence of extracts is shown as a percentage of the control sample that contained PP-1cα and 2 μl of MeOH. Crude extracts are listed as labelled by the Ray Andersen laboratory.

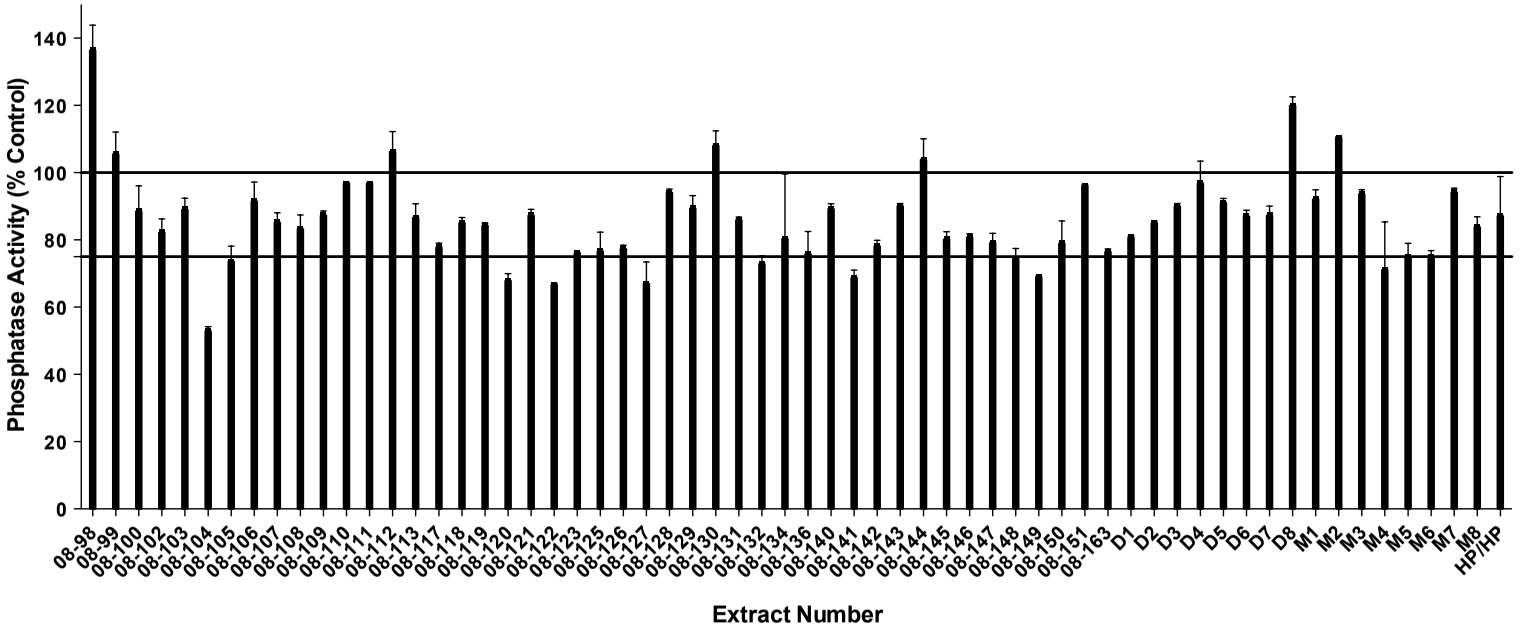


Figure 5.13: **Identification of Natural Extracts that Disrupt the PP-1c•iASPP Complex via the Solid-phase Disruption Assay (Extracts 08-98 to HP/HP)**. iASPP$_{608-828}$ was immobilized on 96-well Ni NTA coated plates (Pierce Scientific), 20 pmol, overnight at 4°C. Wells were washed and incubated for 1 hour with approximately 0.8 μM PP-1cα +/- marine natural product extract (0.16 μg of total extract per well). After sufficient washing, 100 μl of 10 mM pNPP (in dilution buffer) was added and absorbance at 405 nm was measured after 1 hour at room temperature. Activity of PP-1cα in the presence of extracts is shown as a percentage of the control sample that contained PP-1cα and 2 μl of MeOH. Crude extracts are listed as labelled by the Ray Andersen laboratory.

Table 5.2: **Summary of Marine Crude Extracts that Disrupt the Formation of the PP-1c•iASPP Complex**. Table lists marine crude extracts that were identified to disrupt the PP-1c•iASPP complex using the solid-phase disruption assay.

Extract No.	% PP-1c Inhibition
08-24	31
08-34	28
08-46	32
08-53	28
08-62	47
08-104	47
08-120	32
08-122	33
08-127	33
08-141	31
08-149	31

5.2.6 Identification of Marine Crude Extracts that Disrupt the PP-1c•iASPP Complex Using the Microcystin-Sepharose Disruption Binding Assay

Marine crude extracts that inhibited the activity of PP-1c towards pNPP could not be tested within the solid-phase disruption assay, since inhibition of PP-1c activity would not allow for the proper measurement of the amount of PP-1c in each sample. The 16 marine crude extracts that inhibited PP-1c pNPP activity (Table 5.1) and the 11 marine crude extracts that disrupt the formation of the PP-1c•iASPP complex within the solid-phase assay (Table 5.2) were further investigated using the MC-Sepharose disruption assay.

MC-Sepharose disruption experiments were carried out in duplicate and a representative experiment is shown in Figure 5.14. Results show that only three of the marine crude extracts tested disrupted the PP-1c•iASPP complex :08-35, 08-39, and 08-133.

While marine crude extracts 08-62 and 08-141 clearly disrupted the formation of the PP-1c•iASPP complex (decreased the amount of PP-1c bound by 47 and 31%, respectively), 08-62 and 08-141 had no effect on the binding of PP-1c to iASPP when tested with the MC-Sepharose disruption assay. A flowchart of the experimental

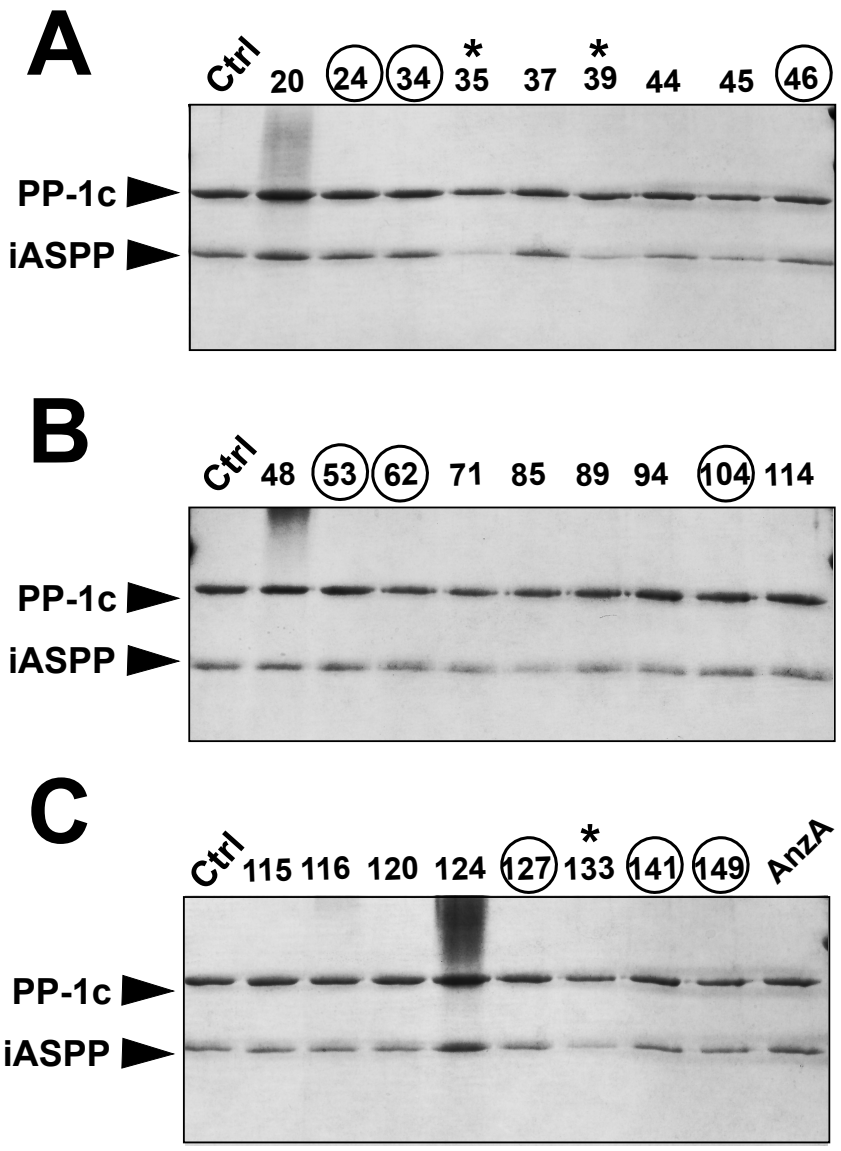


Figure 5.14: **Identification of Marine Crude Extracts that Disrupt the iASPP•PP-1c Complex Using a Microcystin-Sepharose Disruption Assay**. PP-1cα was incubated with 20 μl of MC-Sepharose for approximately 1 hour at end over at 4 °C. After washing with Buffer A containing 0.15 M NaCl, resin was incubated for an additional hour with $iASPP_{608-828}$ in the absence (control containing 10 μl MeOH) or presence of a crude marine extract (2 μl of 10 mg/ml extract per sample). Extracts were washed with Buffer E containing 0.15 M NaCl and 0.1% Tween-20 and subsequently eluted with SDS-PAGE loading buffer and boiled at 100 °C for 5 min. Disruption assay was carried out in duplicate and a representative gel is shown. Samples that are circled indicate they were first identified within the solid-phase disruption assay. Samples highlighted with an asterisk were selected for further study based on the results of these experiments.

design and a summary of the marine crude extracts identified with each assay is shown in Figure 5.2.6.

5.2.7 Assay Guided Purification of 08-35 and 08-39 Marine Crude Extracts

The marine crude extracts 08-35 and 08-39 were further fractionated by solvent partitioning into aqueous (Aq) and ethyl acetate (EtOAc) fractions by David Williams from the Andersen lab (University of British Columbia, Vancouver), as described in detail in Section 5.2.4. Unfortunately, the 08-133 marine crude extract could not be investigated further because there insufficient material for further purification.

The Aq and EtOAc fractions of the 08-35 and 08-39 marine crude extracts were tested for their ability to disrupt both the PP-1c•iASPP and PP-1c•ASPP2 complexes using the MC-Sepharose PP-1c disruption assay. Experiments were carried out in duplicate and a representative experiment is shown in Figure 5.16. Results showed that the 08-35 Aq and the 08-39 EtOAc fractions almost completely disrupted the ability of PP-1c to bind to iASPP. In contrast, the 08-35 EtOAc and the 08-39 Aq fractions had little effect the binding of PP-1c to either iASPP or ASPP2.

The Aq and EtOH fractions for 08-35 and 08-39 were also tested for their ability to inhibit PP-1c pNPP activity. The results showed that both 08-35 Aq and 08-39 EtOAc fractions inhibited the activity of PP-1c towards pNPP (Figure 5.17). In contrast, the 08-35 EtOAc and 08-39 aqueous fractions did not have any effect on the activity of PP-1c.

The 08-35 Aq and 08-39 EtOAc fractions were further purified by solvent portioning and Sephadex LH20 chromatography by Dr. David Williams from the Andersen laboratory, yielding six fractions for 08-35 (Aq-Aq, BuOH, BuOH-A, BuOH-B, BuOH-C, and BuOH-C) and four fractions for 08-39 (EtOH-A, EtOH-B, EtOH-C, and EtOH-D). These fractions were then tested for their ability to inhibit PP-1c pNPP activity and for their ability to disrupt the PP-1c•iASPP complex. The results from these experiments are shown in Figure 5.18. Results showed that the 08-35 BuOH and 08-35 BuOH-C LH20 fractions marginally inhibited the activity of PP-1c (Figure 5.18, panel A). However, both of these fractions also reduced the ability of PP-1c to bind to iASPP (Figure 5.18, panel B).

The 08-39 EtOH-A and EtOH-B fractions marginally inhibited the activity of PP-1c towards pNPP (Figure 5.18, panel A). The effect of the 08-39 EtOH-C fraction

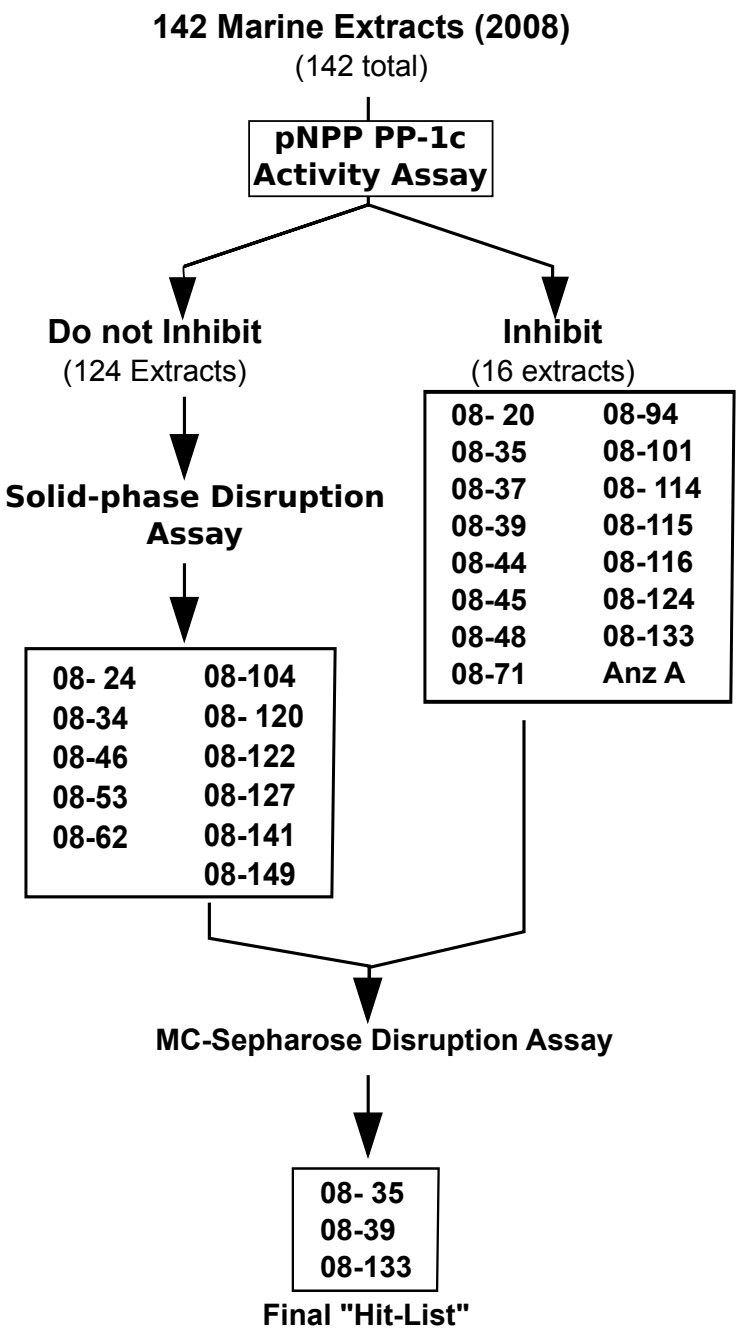


Figure 5.15: **Summary of the Experimental Design for the Identification of Marine Crude Extracts that Disrupt the PP-1c•iASPP Complex**. Out of 142 samples, 18 exhibited moderate inhibition of PP-1c pNPP activity (greater than 10% inhibition). The extracts that did not inhibit PP-1c were tested for their ability to disrupt the PP-1c•iASPP complex using the solid-phase disruption assay and 11 samples were identified to disrupt the ability of PP-1c to bind to iASPP. These extracts, in addition to the extracts that inhibited PP-1c, were tested for their ability to disrupt the PP-1c•iASPP complex using MC-Sepharose binding experiments. The results from these experiments resulted in a final "hit list" of 3 marine extracts: 08-35, 08-39, and 08-133.

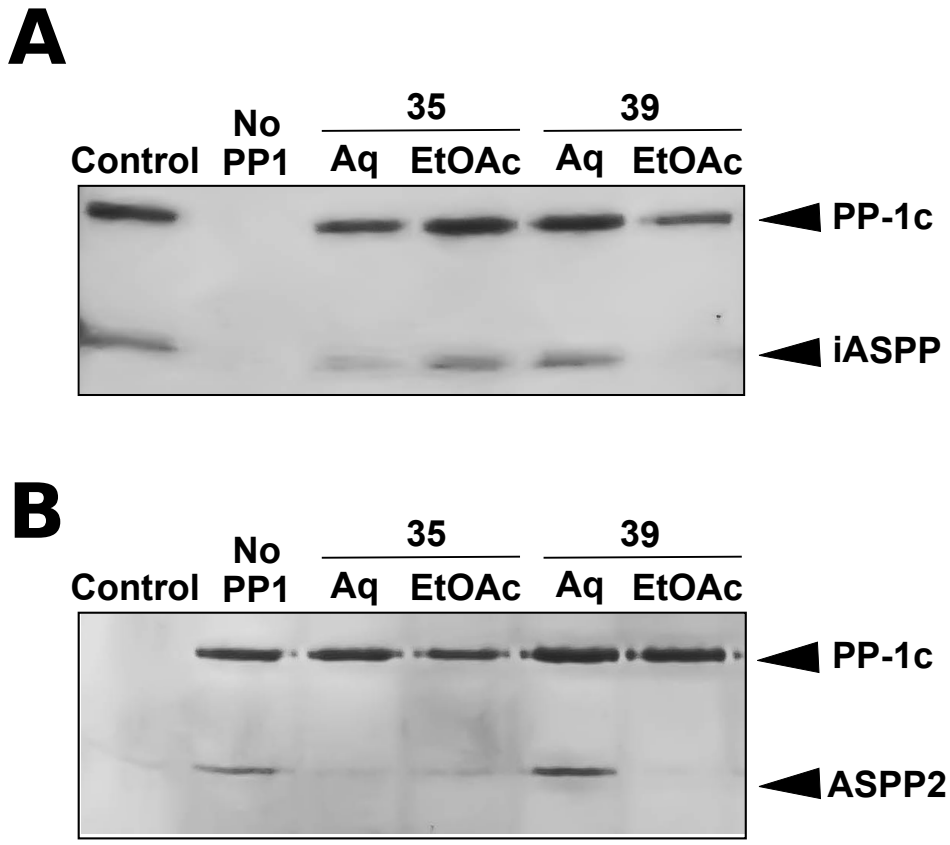


Figure 5.16: **Identification of 08-35 and 08-39 Aqueous and Ethyl Acetate Fractions that Disrupt the iASPP•PP-1c and ASPP2•PP-1c Complexes (Microcystin-Sepharose Round 2)**. PP-1cα was incubated with 20 μl of MC-Sepharose for approximately 1 hour at end over at 4 °C. After washing with Buffer A containing 0.15 M NaCl, resin was incubated for an additional hour with iASPP$_{608-828}$ in the absence (control containing 10 μl MeOH) or presence of a 08-35 or 08-39 aqueous (Aq) or ethyl acetate (EtOAc) fraction (2 μl of 10 mg/ml extract per sample). Resin was washed with Buffer E containing 0.15 M NaCl and 0.1% Tween-20 and bound protein was subsequently eluted with SDS-PAGE loading buffer and boiled at 100 °C for 5 minutes. Disruption assay was carried out in duplicate and a representative gel is shown in *Panel A*. In addition, the same 08-35 and 08-39 Aq and EtOAc fractions were tested for their ability to disrupt the binding of PP-1cα to ASPP2$_{905-1128}$ (*Panel B*).

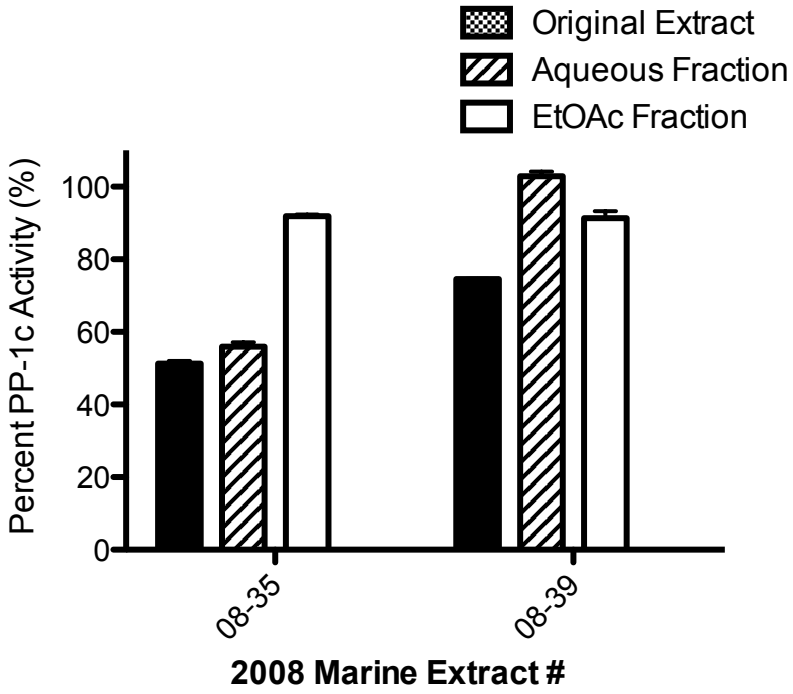


Figure 5.17: **Inhibition of PP-1c by 08-35 and 08-39 Aqueous and EtOAc Fractions**. The original marine crude extract or the Aqueous and EtOAc extract fractions were tested for PP-1c inhibition using the pNPP activity assay using 2 μl of a 10 mg/ml extract. Extracts were incubated with PP-1cα for 10 min prior to the addition of pNPP (5 mM final concentration). Reactions were then incubated for an additional hour at 37 °C, after which the absorbance for each reaction was measured at 405 nm. The percent PP-1c activity is shown as a percentage of a control sample that contained PP-1c and MeOH.

on activity of PP-1c was unable to be tested because the extract precipitated out of solution when added to pNPP assay buffer (buffer L). However, no precipitation was seen when the extract was diluted into MC-Sepharose binding buffer (buffer E), so the 08-39 EtOAc-C fraction could still be tested for its ability to disrupt the PP-1c•iASPP complex. Significantly, both 08-39 EtOH-C and EtOH-D subtractions almost completely abolished the ability of PP-1c to bind to PP-1c (Figure 5.18, panel B).

5.2.8 Identification of Sokotrasterol Sulphate as the Active Compound in 08-39 EtOH-C Fraction

The 08-35 BuOH-C fraction was analyzed by NMR by David Williams from the Anderson laboratory at the University of British Columbia (Vancouver, BC) and the fraction was identified as a sulphated sterol compound, sokotrasterol sulphate (Figure 5.19). Sokotrasterol sulphate was first isolated in 1983 from the *Halichondriidae* marine sponge isolated from Sokotra Island in the Arabian Sea [189]. While it is similar to other marine sterol compounds, such as halistanol sulphate [83], sokotrasterol sulphate is unique in that it contains three additional methyl groups, two at C26 and one at C25.

Sokotrasterol sulphate is structurally similar to cholesterol; therefore, it was of interest to test whether cholesterol also inhibits the enzymatic activity of PP-1c or disrupts the ability of PP-1c to bind to the iASPP protein. PP-1cα activity was measured using the pNPP PP-1c activity assay in the presence of increasing amounts of sokotrasterol sulphate or cholesterol (ranging from 0.1 nM to 1 μM). The effect of sokotrasterol sulphate on the activity of PP-1c towards pNPP at higher concentrations were unable to be tested due to the precipitation of sokotrasterol sulphate at concentrations over 1 mM. Results from these pNPP experiments show that while sokotrasterol sulphate inhibits the activity of PP-1c with an IC_{50} of approximately 500 nM, cholesterol has no effect on the (Figure 5.20).

Results show that cholesterol,at concentrations ranging from 100 – 150 nM, did not to disrupt the formation of the PP-1c•iASPP complex within the MC-Sepharose disruption assay (Figure 5.21). In contrast, sokotrasterol sulphate drastically impaired the ability of PP-1c to bind to iASPP, even at low concentrations (10 – 50 nM).

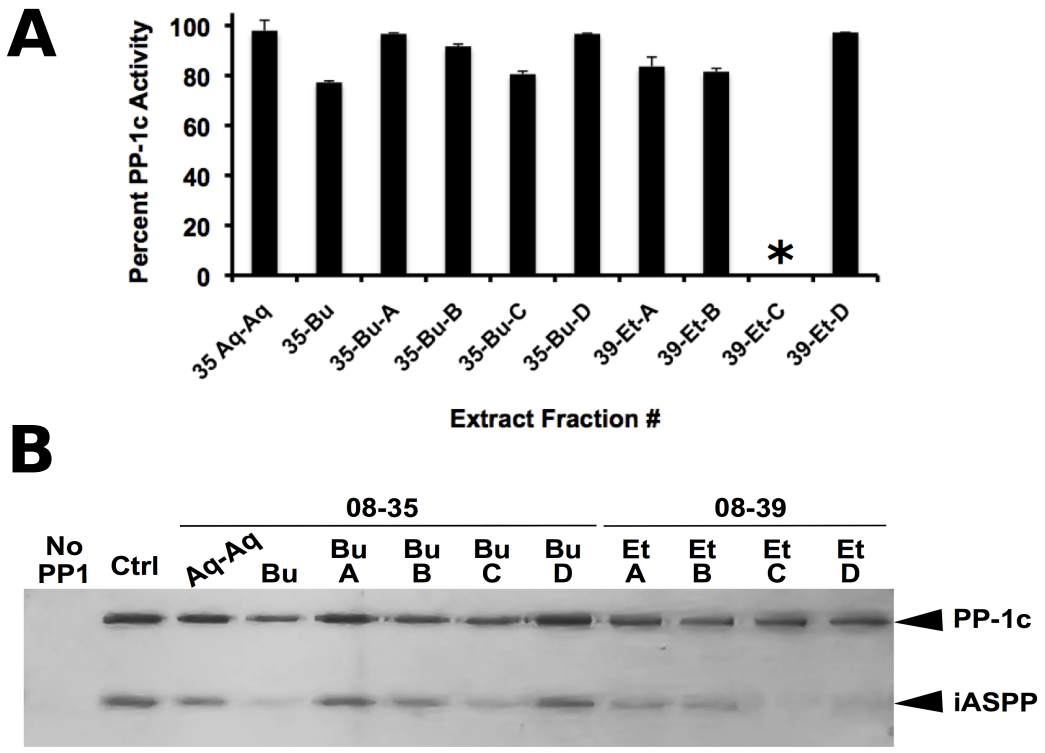


Figure 5.18: **Identification of 08-35 and 08-39 Fractions That Disrupt the iASPP•PP-1c Complex (Microcystin-Sepharose Round 3)**. Aq and EtOAc fractions of 08-35 and 08-39 were further analyzed for their ability to inhibit the activity of PP-1cα towards pNPP (*panel A*) and to disrupt the formation of the PP-1c•iASPP complex (*panel B*). PP-1cα was incubated with 20 μl of MC-Sepharose for approximately 1 hour at end over at 4 °C. After washing with Buffer A containing 0.15 M NaCl, resin was incubated for an additional hour with iASPP$_{608-828}$ in the absence (control containing 10 μl MeOH) or presence of a crude marine extract (2 μl of 10 mg/ml extract per sample). Extracts were washed with Buffer E containing 0.15 M NaCl and 0.1% Tween-20 and subsequently eluted with SDS-PAGE loading buffer and boiled at 100 °C for 5 minutes. Disruption assay was carried out in duplicate and a representative gel is shown in *panel B*

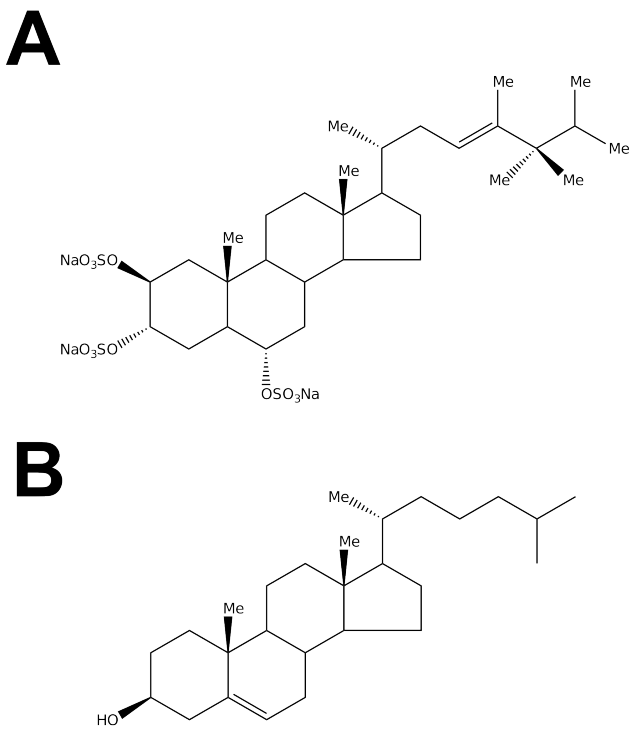


Figure 5.19: **The Chemical Structure of Sokotrasterol Sulphate and Cholesterol**. The figure depicts the chemical structure of sokotrasterol sulphate (*panel A*) and cholesterol (*panel B*). Sokotrasterol sulphate is a proangiogenic steroid produced by marine sponges. Structurally similar to cholesterol, sokotrasterol sulphate contains a classic steroid ring structure comprised of four fused rings. The structure of sokotrasterol sulphate is unique among other steroid compounds, such as cholesterol, as it contains three additional methyl groups within its sterol side chain.

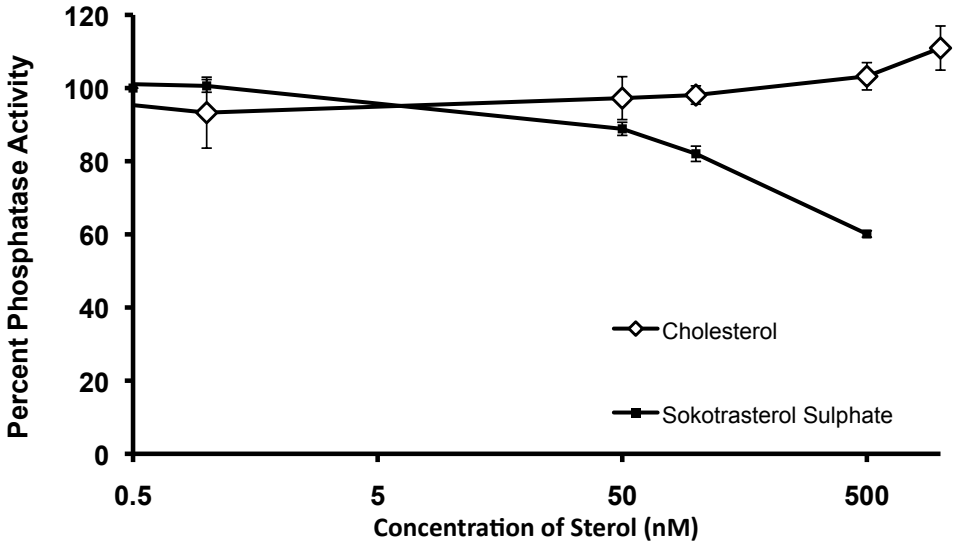


Figure 5.20: **Cholesterol Does Not Inhibit the Activity of Protein Phosphatase-1c Towards pNPP**. The activity of PP-1cα was tested using the pNPP PP-1c activity assay in the presence of either sokotrasterol sulphate or cholesterol, concentrations ranging from 0.1 nM to 1 μM. Sokotrasterol sulphate is a very weak PP-1c inhibitor, inhibiting the activity of PP-1c in the high nM range. Cholesterol has no affect on the activity of PP-1c towards pNPP.

5.2.9 Marine Extract 08-39 Contains Novel Compounds Related to the Tetronic Acid Hippospongin

The 08-39 EtOAc-C fraction was further analyzed by Dave Williams (from the Andersen lab at the University of British Columbia, Canada) using HPLC and NMR spectroscopy. NMR spectra of the 08-39 EtOAc-C-1 fraction revealed that the extract was likely a mixture of two related compounds. Subsequent HPLC and NMR analysis of 08-39 EtOAc-C-1 fraction after treatment with diazomethane (CH_2N_2) revealed that this crude extract likely contains two novel compounds (labelled as 08-39 EtOAc-C-1+CH_2N_2-1 and 08-39 EtOAc-C-1+CH_2N_2-2), that are related to hippospongin (Figure 5.22). The hippospongin structure contains a furan ring and a tetranoic acid on opposite ends of the structure. It was first isolated from the sponge *Hippospongia* and has induces antispasmodic activity in the ileum of guinea pig [139].

Tetronic acids are often difficult to chromatograph and the 08-39 EtOAc-C-1 fraction is still in the process of being purified. Several other sesterterpenes purified from the marine crude extract 76253 (compounds are not formally named but are

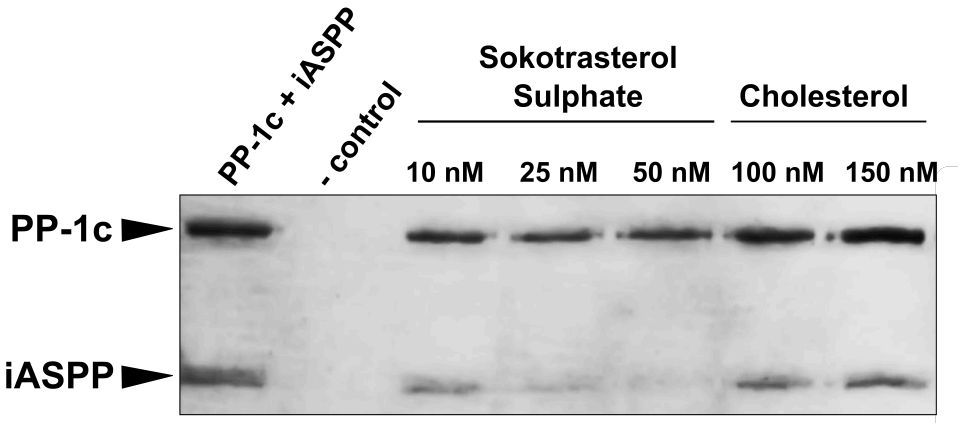


Figure 5.21: **Cholesterol Does not Disrupt the Formation of the PP-1c•iASPP Complex**. PP-1cα was incubated with 20 μl of MC-Sepharose for approximately 1 hour at end over at 4 °C. After washing with Buffer E containing 0.15 M NaCl, resin was incubated for an additional hour with iASPP$_{608-828}$ in the absence (control containing 10 μl MeOH) or presence of sokotrasterol sulphate or cholesterol. Resin was washed with Buffer E containing 0.15 M NaCl and 0.1% Tween-20 and subsequently eluted with SDS-PAGE loading buffer and boiled at 100 °C for 5 min. The disruption assay was carried out in duplicate and a representative gel is shown.

referred to as 76253 EtOAc C-1, C-2, and C-3) have a chemical structure similar to the 08-39 EtOAc+Ch_2N_2-1 compound (Figure 5.22). The 76253 EtOAc C-1, C-2, and C-3 compounds were further tested for their ability to disrupt the formation of the PP-1c•iASPP complex, using the MC-Sepharose disruption assay. The disruption assay was repeated twice and a representative experiment is shown in Figure 5.23. The results from this experiment show that similar to the 08-39 active fractions, 76253 EtOAc-C1 and C-3 compounds clearly disrupt the ability of PP-1c to bind to iASPP.

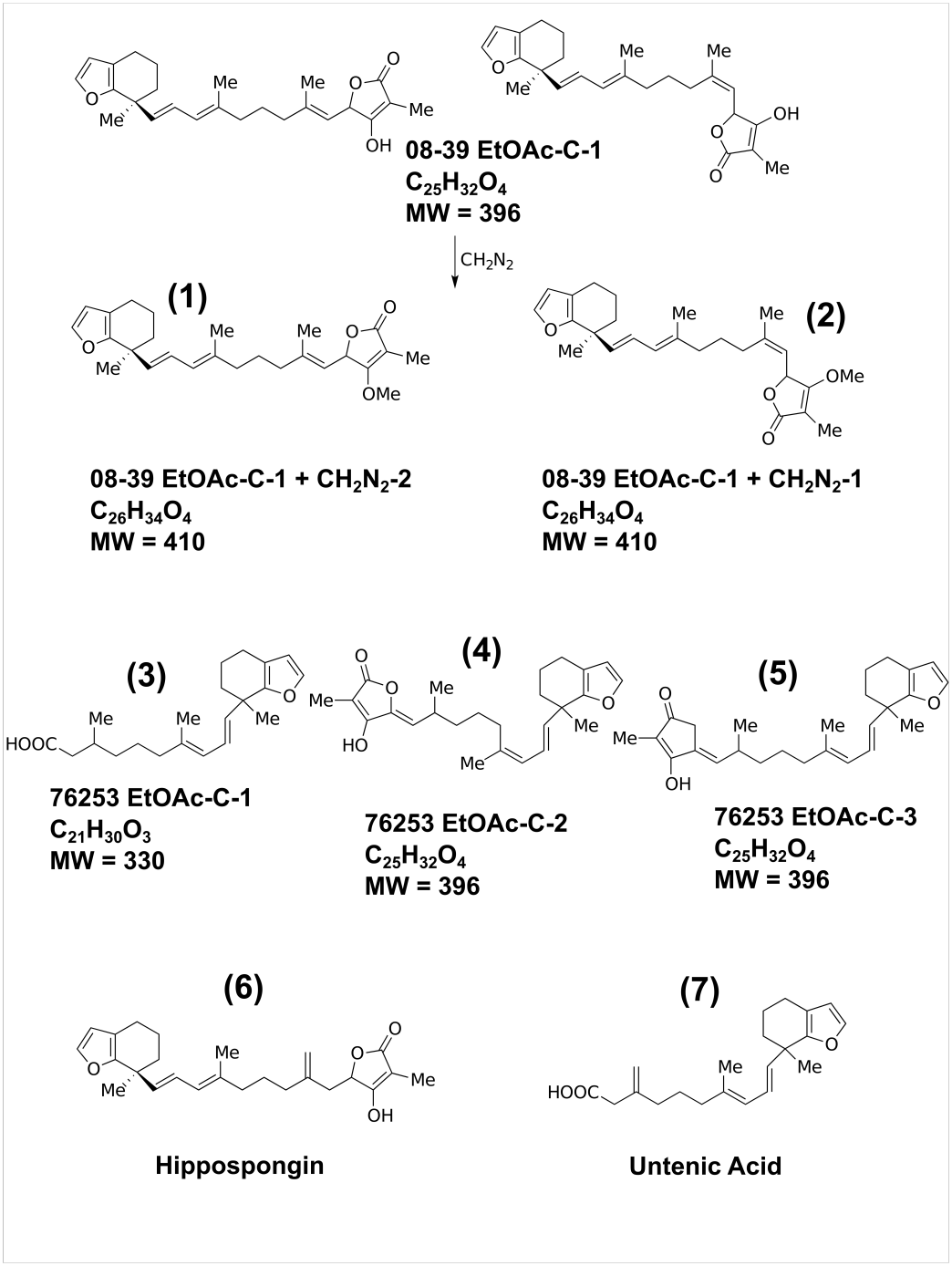


Figure 5.22: **The Chemical Structures of Hippospongin and Related Identified Sesterterpenes**. The 08-39 EtOAc-C-1 fraction was shown to contain two novel bioactive compounds, after treatment with diazomethane (labelled 1 and 2). These compounds are closely related to hippospongin (6) and untenic acid (7). Due to the fact that it was difficult to separate the structurally similar compounds from 08-39 EtOAc-C-1, similar compounds 76253 EtOAcC-1, C-2, and C-3 (labelled 3 – 5) were further tested for their ability to disrupt the PP-1c•iASPP complex.

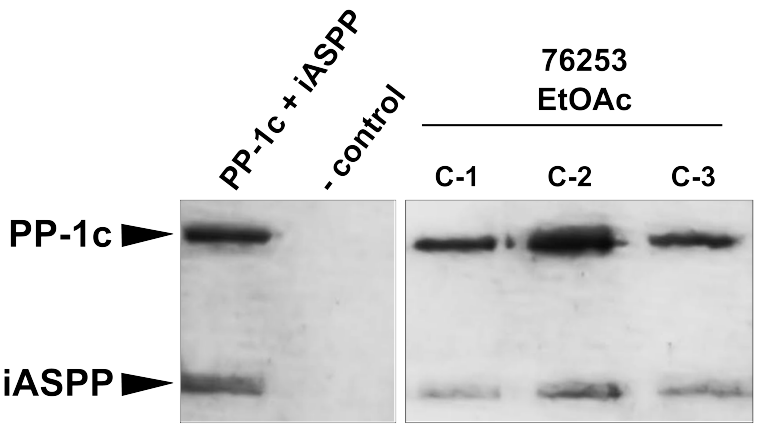


Figure 5.23: **Hippospongin Related Compounds 76253 EtOAc-C-1, C-2, and C-3 Partially Disrupt the Formation of the PP-1c•iASPP Complex**. PP-1cα was incubated with 20 μl of MC-Sepharose for approximately 1 hour at end over at 4 °C. After washing with Buffer E containing 0.15 M NaCl, resin was incubated for an additional hour with iASPP$_{608-828}$ in the absence (control containing 10 μl MeOH) or presence of either 76253 EtOAc-C-1, C-2, or C-3 (100 μM final concentration). Resin was washed with Buffer E containing 0.15 M NaCl and 0.1% Tween-20 and subsequently eluted with SDS-PAGE loading buffer and boiled at 100 °C for 5 minutes. The disruption assay was carried out in duplicate and a representative gel is shown.

5.2.10 Discussion

The iASPP protein is over expressed in multiple forms of cancer. We hypothesize that the PP-1c•iASPP complex is an ideal and suitable target for the development of protein-protein disrupting compounds that could potentially be used cancer therapy. In Chapters 2 and 3 of this thesis, several interaction "hot spots" were identified within the PP-1c•ASPP protein complex that are essential for complex formation. In particular, the interaction of the C-terminal tail of PP-1c and the ankyrin-repeat and SH3 domains of the ASPP proteins appears to be unique among PP-1c regulatory protein interactions. Surface plasmon resonance experiments revealed that the C-terminal tail of PP-1c binds with higher affinity to iASPP versus ASPP2 (Chapter 3). In addition, chemical shift mapping data from Jonathan Pan (from the laboratory of Dr. Brian Sykes at the University of Alberta) and Inja Byeon (from the laboratory of Dr. Angela Gronenborn at the University of Pittsburgh) demonstrates that a PP-1c C-terminal peptide binds to the ankyrin repeat and SH3 domains of both iASPP and ASPP2; however, there are subtle differences in the binding of the PP-1c C-terminus to each ASPP protein (discussed in Chapter 6, see Figure 6.1).

The identification of a novel protein-protein disruption compound that targeted specifically targeted the interaction between the C-terminal tail of PP-1 and the iASPP ankyrin repeat and SH3 domains would be ideal, since it would not impair the ability of PP-1c to bind to other PP-1c regulatory proteins. However, it may be impossible to avoid also disrupting the interaction of PP-1c with ASPP1 or ASPP2. Considering that ASPP1 and ASPP2 are often down regulated in many forms of cancer, this may be less of a concern when designing a novel PP-1c•iASPP disrupting compound.

In this study, over 150 marine crude extracts were screened for the ability to disrupt the PP-1c•iASPP complex. In order to screen a large number of marine crude extract samples, two new protein-protein disruptions assays were developed and optimized: the MC-Sepharose PP-1c disruption assay and the His-tagged solid-phase disruption assay. From these initial screens, we identified 2 marine crude extracts (08-35 and 08-39), which disrupted the formation of the PP-1c•iASPP complex, 08-35 and 08-39. In collaboration with Dr. David Williams from the Andersen laboratory, we carried out disruption assay guided purification of 08-35 and

08-39. Three marine natural products were identified to clearly disrupt the binding of PP-1c to iASPP - sokotrasterol sulphate and two hippospongin-like compounds 76253 EtOAc-C-1 and 76253 EtOAc-C-3.

Further investigation is required to assess the effects of Sokotrasterol sulphate and Hippospongin compounds on the activity of PP-1c both *in vitro* and *in vivo*. In cells, sokotrasterol sulphate has been previously shown to be proangiogenic and induce the expression of several proteins involved in angiogenesis, including Tie-1 and α_v integrin [215, 129]. Additional experiments are required to investigate whether sokotrasterol sulphate or the hippospongin-related compounds can disrupt the binding of PP-1c with other regulatory proteins, besides the ASPP proteins.

Chapter 6

General Discussion and Conclusion

6.1 iASPP Binds to PP-1c Via Multiple Sites of Interaction

In this thesis, I have characterized the binding of protein phosphatase-1c to the ASPP proteins and demonstrated that all three ASPP proteins bind to PP-1c via multiple sites of interaction. Whilst ASPP1 and ASPP2 both contain classical RVXF PP-1c binding motifs, the equivalent motif in iASPP is RARL (residues 622–625). Deletion or mutation of the iASPP RARL motif partially disrupted the ability of iASPP to bind to PP-1c, demonstrating that iASPP binds to PP-1c in part via its non-classical RARL motif. Despite the fact that iASPP lacks a true RVXF motif, surface plasmon resonance (SPR) experiments showed that full-length PP-1cα binds preferentially to iASPP versus ASPP2, with dissociation constants (K_D) of 26 nM and 123 nM, respectively.

In addition to the RVXF motif, we have also identified two additional PP-1c regions that are important for binding to the ASPP proteins: 1) a positively charged region of PP-1c, comprising residues Lys-260 and Arg-261 and 2) the C-terminal tail of PP-1c (discussed further in Section 6.2). Previous studies carried out by our laboratory, showed that residues Lys-260 and Arg-260 of PP-1c may also be important for binding to other proteins including the cdk2•cyclin A complex and phosphorylase *a*. Mutation of PP-1cγ Arg-261 to Ser resulted in the reduced the ability of cdk2•cyclin A to phosphorylate the C-terminus of PP-1c by approximately 50%. Therefore, the interaction between PP-1c and the ASPP proteins may potentially block the subsequent phosphorylation of PP-1c by cdk2•cyclin A complexes. In addition, mutation of PP-1cγ Lys-260 to Asp increased the specific activity of PP-1cγ towards phosphorylase *a* approximately two fold, suggesting that the positively charged region of PP-1c is also important for binding to phosphorylase *a* [Andrea Fong, unpublished data]. This may explain why PP-1c has a decreased ability to dephosphorylate phosphorylase *a* when bound to either ASPP2 or iASPP (Chapter 2, Figure 2.5).

6.2 The Importance of the Protein Phosphatase-1c C-terminal Tail for Binding to ASPP Proteins

Analysis of the PP-1c•ASPP2 complex model suggested that the PP-1c C-terminus, was also important for binding to the ASPP proteins and could interact directly with

ankyrin-repeat and SH3 domains of the ASPP proteins. Significantly, all three PP-1c isoforms (α, β, and γ) contain a putative type-2 SH3 binding motif (PxxPxR). A previously characterized cdk2•cyclinA phosphorylation site is located directly within this motif (Thr-320 and Thr-311 in PP-1cα and PP-1cγ, respectively). Deletion or mutation of the PP-1c C-terminus disrupted the ability of PP-1c to bind to all three ASPP proteins. Furthermore, SPR experiments showed that a PP-1c C-terminal peptide, comprising the C-terminal tail of PP-1c (residues 301–330), binds with high affinity to both iASPP and ASPP2, with an apparent K_D of 53 nM and 136 nM respectively. Together, these experiments demonstrate that the C-terminus of PP-1c is critical for binding to the ASPP proteins.

Structural studies are currently being carried out to further understand how the C-terminal tail of PP-1c interacts with the ASPP proteins. Jonathan Pan, a graduate student from laboratory of Dr. Brian Sykes (University of Alberta) and Inja Byeon, a graduate student from the laboratory of Dr. Angela Gronenborn (University of Pittsburgh) are currently generating NMR structures of the C-terminal ankyrin-repeat and SH3 domains of iASPP and ASPP2 bound to a peptide derived from the C-terminal tail of PP-1cα (residues 301–330). Initial chemical shift mapping data, using labelled C-terminal constructs of iASPP or ASPP2 and unlabelled PP-1c peptide, reveal that while the binding of the PP-1c C-terminus to iASPP and ASPP2 is similar, there may be subtle differences in the binding (Figure 6.1). Specifically, the PP-1c peptide appears to bind tightly to a hydrophobic groove formed between the ankyrin-repeat and SH3 domains of iASPP. However, the PP-1c C-terminus binds only weakly to the equivalent groove in ASPP2. This may explain the differences in the binding affinities of the PP-1c C-terminus to iASPP and ASPP2 observed in our surface plasmon resonance experiments. It may also explain why PP-1c is unable to interact directly with the SH3 domain from Src, since the majority of SH3 domains (including Src) do not have an adjacent ankryin repeat domain and therefore do not contain a similar groove.

Sequence differences between the C-termini of PP-1c isoforms α, β, γ may explain why all three ASPP proteins bind preferentially to the α isoform of PP-1c, over β and γ. Firstly, differences in the total length of the PP-1c C-terminal tails may impact the optimal binding of the PP-1c PxxPxR SH3 binding motif with the SH3 domain of each ASPP protein. The overall length of the C-terminal tail differs among the three PP-1c isoforms, with PP-1cα being the longest, at 30 residues long

and PP-1cγ being the shortest, consisting of only 23 residues (Figure 3.1). Secondly, differences in the amino acid sequence surrounding the PxxPxR motif of PP-1cα, β, and γ may also impact binding to the ASPP proteins. For example, both PP-1cα and PP-1cβ contain a short hydrophobic domain located N-terminal to their PxxPxR SH3 binding motifs (PP-1cα residues 305–316, GKYGQFSGLNPGG) that is absent in the PP-1cγ isoform (Chapter 3, Table 3.1). This hydrophobic region of PP-1cα/β may facilitate interactions with the hydrophobic groove formed between the ankyrin repeat and SH3 domains of the ASPP proteins (Figure 6.1). The lack of a hydrophobic domain within the PP-1cγ C-terminus may explain why PP-1cγ has a reduced ability to bind to all three ASPP proteins, particularly iASPP (Chapter 2, Figure 2.10).

6.2.1 Alternative Roles for PP-1c in ASPP Mediated Pathways

All three ASPP proteins had been shown to interact with multiple protein binding partners (Chapter 1, Table 1.4). The results from this thesis demonstrate that while iASPP targets PP-1c to specifically dephosphorylate p53, ASPP2 does not. This begs the question whether ASPP1 and ASPP2 can target PP-1c to other proteins within eukaryotic cells and regulate other PP-1c mediated site-specific dephosphorylation reactions. For example, ASPP1 has been shown to block the phosphorylation and subsequent inhibition of YAP1, a pro-survival co-transcriptional activator (described in detail in Chapter 1, Section 1.5.2) [13]. This could be from either directly blocking Lats2-mediated phosphorylation or alternatively, enhancing the dephosphorylation of YAP1 by a specific Ser/Thr protein phosphatase such as PP-1c. To date, the specific phosphatase that dephosphorylates YAP1 has not been identified [288]. It would be interesting to test whether ASPP1 can bind to both YAP1 and PP-1c simultaneously and facilitate the dephosphorylation of YAP1 by PP-1c. This could be tested using *in vitro* methods described in this thesis, such as gel filtration chromatography and an *in vitro* YAP1 dephosphorylation assay.

In addition, the ASPP proteins themselves are also phosphorylated on multiple residues throughout the cell cycle [57, 226]. Phosphoproteomic studies have identified 17 Ser/Thr phosphorylation sites on iASPP (Table 6.1) [57, 226]. Many of the known iASPP phosphorylation sites are not found in either ASPP1 or ASPP2, suggesting that the ASPP proteins are differentially regulated via phosphorylation/dephosphorylation reactions. Reversible phosphorylation of iASPP may affect

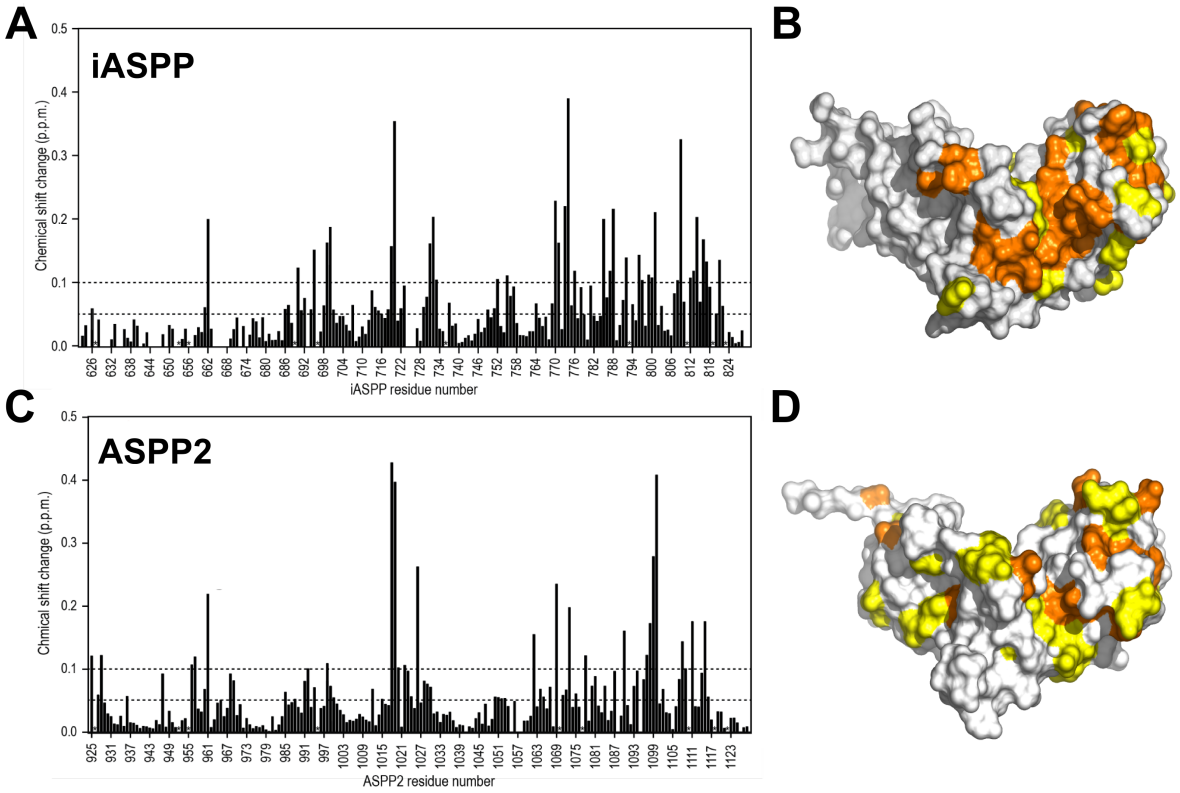


Figure 6.1: **NMR Chemical Shift Mapping Between the iASPP, ASPP2 and the C-terminus of PP-1cα.** ^{15}N-labelled ASPP2 (residues 925–1128) or iASPP (residues 622-828) was titrated with unlabelled PP-1c C-terminal peptide (PP-1cα residues 301-330). *Panels A* and *C* depict bar diagrams showing the chemical shift changes at a PP-1c peptide:ASPP ratio of 1:1 molar. The chemical shift changes were transposed onto the structures of the C-terminal ankyrin repeat and SH3 domains of ASPP2 and iASPP and are shown in *Panels B* and *D* [90, 235]. The ASPP2 and iASPP residues showing chemical shift changes between 0.5 and 1.0 parts per million (ppm) are labelled yellow and changes above 1.0 ppm are labelled orange. Data for figure provided by Inja Byeon from the Gronenborn laboratory.

the subcellular localization of iASPP and/or its ability to bind to different interacting protein partners. For instance, Ser-102 is a known phosphorylation site of iASPP (Table 6.1) and is located directly within a putative mode–I 14-3-3 binding site (RSX(pS/pT)XP, iASPP residues 99–104, RSE(pS)AP) [300, 124]. 14-3-3 proteins are dimeric proteins that bind to pSer/pThr recognition sequences on target proteins and in some cases can alter the subcellular localization of a protein by sequestering it in one cellular compartment and preventing further translocation of the target phosphoprotein. Studies have demonstrated that full-length iASPP is primarily cytoplasmic, whereas the shorter iASPP splice variant (comprising residues 421–828) that does not contain the putative 14-3-3 binding site and is located for the most part in the nucleus [252]. Therefore, the association between iASPP and 14-3-3 proteins may partially explain the differences in the observed subcellular localizations of full-length iASPP and the shorter iASPP splice variant. It is tempting to speculate that the formation of a dimeric PP-1c•iASPP complex may also facilitate the site-specific dephosphorylation of iASPP on one or more phosphorylated Ser/Thr residues, including pSer-102. If PP-1c can specifically dephosphorylate iASPP on residue pSer-102, this may potentially prevent the association of iASPP with 14-3-3 proteins and induce the translocation of iASPP into the nucleus where it can bind to both PP-1c and p53 to facilitate site-specific dephosphorylation of p53.

ASPP1 also contains a unique N-terminal phosphorylation site that affects its subcellular localization. Upon oncogenic stress, the Lats2 kinase phosphorylates the ASPP1 N-terminus on Ser-383, promoting the translocation of ASPP1 into the nucleus and its association with chromatin [13]. While ASPP1 contains a classical Lats1 kinase consensus site, HXRXXS (HGRSKS, ASPP1 residues 378–383) [311, 13], this sequence is not conserved in either ASPP2 or iASPP. It is predicted that the subsequent dephosphorylation of ASPP1 pSer-383 would prevent ASPP1 nuclear translocation; however, the Ser/Thr protein phosphatase that carries out this dephosphorylation has not been identified. It is tempting to further speculate that the formation of a dimeric PP-1c•ASPP1 complex may facilitate dephosphorylation of ASPP1 on one or more phospho-Ser residues, specifically pSer-383. This question could be tested by developing an *in vitro* ASPP1 dephosphorylation assay, whereby ASPP1 is first phosphorylated by Lats2 and subsequently incubated with PP-1c.

Table 6.1: Identified Phosphorylation Sites Within iASPP. PKA, protein kinase A; CDK1, cyclin dependent kinase 1; CK1, casein kinase; ATM, ataxia-telangiectasia mutated kinase; ATR, ataxia telangiectasia and Rad3 related kinase

Residue Number	**Sequence**	**Also Present in ASPP1/ASPP2?**	**Identified Kinase**
Ser-102	RSE**S**SAPT	no/no	unknown
Ser-109	TLHPY**S**PLSP	yes/yes	MAPK
Ser-119	PKGRP**S**SPR	yes/no	CDK1
Ser-120	PKGRPS**S**PR	yes/no	CDK1
Thr-123	PR**T**PLYQPD	no/no	MAPK
Ser-134	AYG**S**LDRAT	no/no	unknown
Ser-158	GRAP**S**PRP	no/no	CDK1/MAPK
Ser-183	RAG**S**PRG	yes/yes	CDK1/MAPK
Ser-187	PRG**S**PLA	yes/yes	MAPK
Ser-203	RGP**S**PRPP	no/no	CDK1/MAPK
Ser-225	LLG**S**GGSA	no/no	unknown
Ser-316	YKV**S**PLAS	no/no	CDK/MAPK
Ser-332	YRR**S**LGS	no/no	PKA
Ser-426	RPLKRRG**S**MEQAPAV	yes/yes	unknown
Ser-567	PEPEL**S**PITEGSE	no/no	MAPK
Ser-594	PAP**S**QSSPPE	no/no	ATM
Ser-597	PAPSQS**S**PPE	no/no	CK

6.2.2 Small Marine Compounds Disrupt the Ability of PP-1c to Bind to the iASPP Protein

The results from this thesis have demonstrated that the PP-1c•iASPP complex is a potentially valuable molecular target for the development of novel small molecule drug therapies that may be applicable for the treatment of cancer. The marine environment produces a multitude of chemically diverse bioactive compounds for chemical defense and these same compounds are also invaluable molecular leads for drug design. Chapter 5 of this thesis describes the development of two novel protein-protein disruption assays to screen over 150 marine extract leads from the waters of Papua New Guinea. Using these assays, three compounds were identified that disrupted the formation of a PP-1c•iASPP complex: sokotrasterol sulphate and two previously unidentified hippospongin-related compounds.

Further investigation of the effects of sokotrasterol sulphate and the hippospongin related compounds is necessary to evaluate the potential for these compounds to be used as a cancer drug therapy. Firstly, the specificity of these compounds for disrupting the PP-1c•iASPP complex will need to be tested. Preliminary experiments demonstrate that sokotrasterol sulphate also disrupts the ability of PP-1c to bind to ASPP2 (Figure 5.16). Disruption of the binding of PP-1c to ASPP2 would be less of a concern, due to the fact that iASPP is over expressed in many forms of cancer, while the expression of ASPP1/2 is often down-regulated. However, extensive screening is required to determine whether these compounds disrupt the ability of PP-1c to bind to other regulatory proteins such as inhibitor-2, MYPT1, and Repo-Man. This will be carried out using the *in vitro* MC-Sepharose assay as described in Chapter 5. Secondly, the *in vivo* effects of sokotrasterol sulphate and the hippospongin-related compounds will also be evaluated. The growth inhibitory and anticancer effects of all three compounds are currently being tested within the National Cancer Institutes NCI-60 cancer cell line panel. The NCI-60 cell line comprises 60 different cell lines from nine different tumour types, including leukaemia, melanoma, lung, breast, and prostate cancers [248]. Typically, the effect of a compound on the growth of each cell line is determined using a colourimetric sulforhodamine B (SRB) assay, which is used to measure the total protein content (which can be extrapolated to the relative cell density) of each cell line sample after exposure to a test compound.

6.3 Regulation of p53 Signaling by PP-1c and the ASPP Proteins: An Updated Model

In normal functioning cells, p53 is kept at low levels mainly due to the binding of mdm2 (mouse double minute 2), an E3 ubquitin ligase that carries out the poly-ubiquitination of p53 and targets p53 for proteosomal degradation in the cytoplasm. p53 is also regulated via multiple post-translational modifications (described in detail in Chapter 1, Section 1.4.2) that integrate signals from multiple cellular pathways to tightly control p53 activity. In response to various cellular stresses such as DNA damage, hypoxia, and oncogene activation, p53 expression and activity is increased via multiple phosphorylation within its N-terminal transactivation domains (TAD1 and TAD2). N-terminal phosphorylation disrupts the binding of mdm2 and promotes the association of other stabilizing proteins such as the acetyltransferase p300. Once activated, p53 functions within the nucleus as a transcription factor promoting the expression of target genes involved in cell cycle arrest and apoptosis.

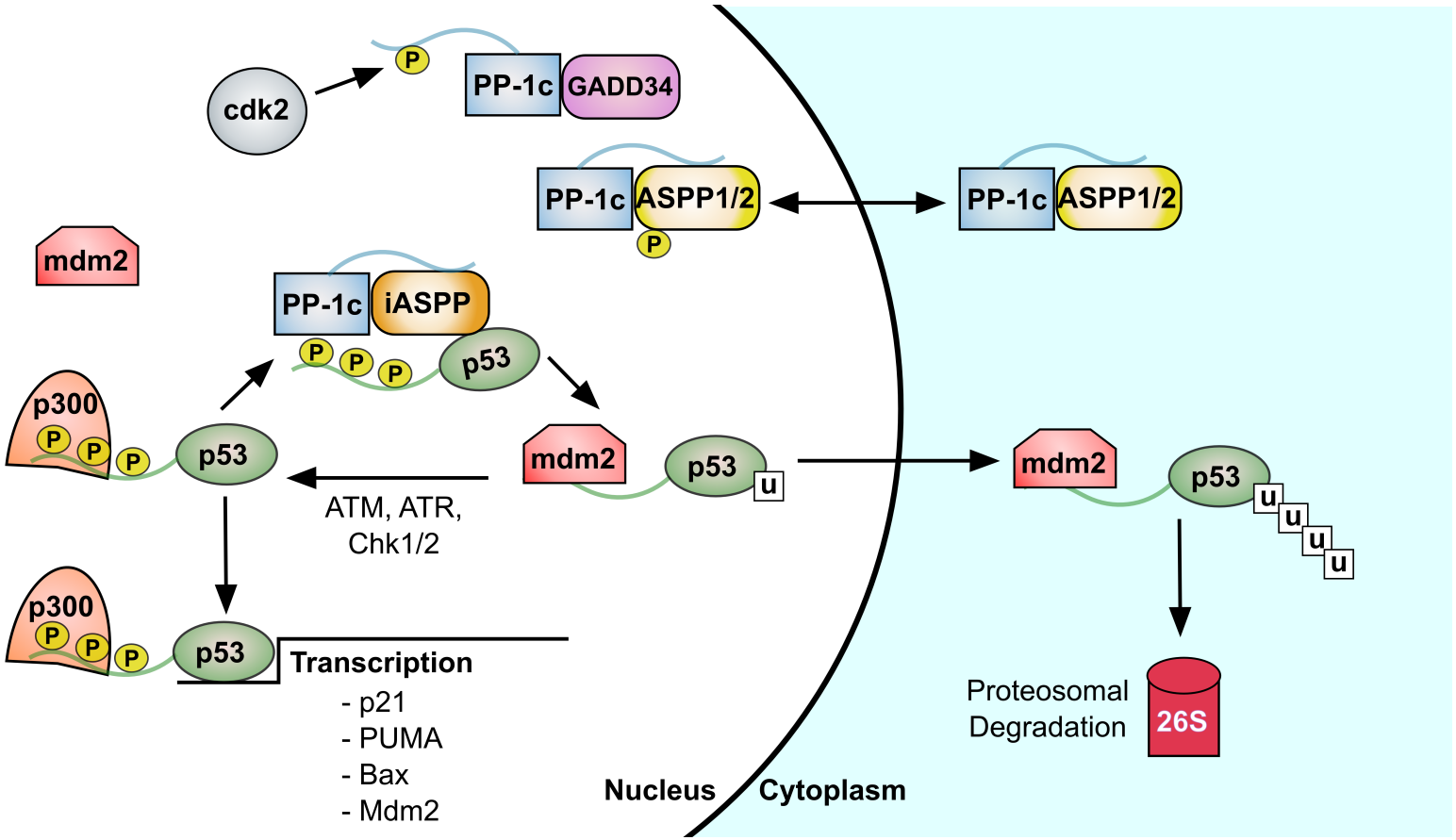
Once a particular cellular stress has been dealt with, p53 signalling must be turned off in order to regain normal cell functions. Protein phosphatases, such as PP-1c and PP-2A negatively regulate p53 signalling by reversing this modification, resulting in the decreased expression and activity of p53. Prior to this study, PP-1c was previously shown to carry out the dephosphorylation of pSer-15 and pSer-37 residues within the p53 TAD1 domain [96, 161]. PP-1c and p53 do not bind directly to one another [161]; however, to date no PP-1c regulatory protein that targets PP-1c directly to p53 has been identified.

Based on the results described in this thesis, I propose a model for how p53 is regulated in part by PP-1c and the ASPP proteins (Figure 6.2). Formation of the trimeric PP-1c•iASPP•p53 complex may facilitate site-specific dephosphorylation of p53 by PP-1c on pSer-15, counteracting the actions of stress-induced Ser/Thr protein kinases such as ATM and ATR. Dephosphorylation within the transactivation domains of p53 is predicted to promote the association of p53 with mdm2, which leads to the cytoplasmic proteosomal degradation of p53, preventing p53 from inducing the expression of target genes involved in cell cycle arrest or apoptosis. On the other hand, ASPP1 and ASPP2 can not interact with both PP-1c and p53 simultaneously; therefore, the formation of the PP-1c•ASPP1/2 complexes directly inhibit PP-1c mediated p53 dephosphorylation. The interaction between PP-1c and

ASPP1/2 may also regulate the subcellular localization of ASPP1/2 and promote the translocation of ASPP1/2 from the nucleus to the cytoplasm, where ASPP1/2 may target PP-1c to dephosphorylate other cellular protein substrates.

In normal dividing cells, the C-terminus of PP-1c is phosphorylated during mitosis, which causes the auto-inhibition of PP-1c and contributes to a high level of mitotic phosphoproteins that allow cells to proceed through mitosis [226]. When phosphorylated, PP-1c cannot interact with iASPP; therefore, PP-1c would not be able to dephosphorylate p53. However, it is predicted that pre-formation of the heterodimeric PP-1c•ASPP complex would prevent the subsequent phosphorylation of the PP-1c C-terminus by cdk2•cyclin A complexes. This would allow sub-pools of PP-1c to remain active during mitosis and inhibit p53 signalling via the dephosphorylation of key residues within the p53 N-terminal transactivation domains. When PP-1c is associated with other regulatory proteins that do not bind directly to the PP-1c C-terminal tail, the C-terminal tail would remain exposed and be accessible for phosphorylation by cdk2•cyclin A complexes. Therefore, within these heterodimeric PP-1c complexes the activity of PP-1c would be inhibited during mitosis.

Based on this model, relative expression levels of the ASPP proteins appear to be critical for determining whether the p53 signalling pathway may be activated in response to a cellular stress. Indeed, iASPP is over expressed in many cancer types while the expression of ASPP1 and ASPP2 is often decreased. Increased iASPP expression would sequester PP-1c within the PP-1c•iASPP•p53 trimeric complex, resulting in the constitutive hypophosphorylation of p53 and the inability of wild-type p53 to be activated in response to cellular stress. Based on the results described in this thesis, the PP-1c•iASPP complex is a potentially valuable drug target for cancer therapies. Disruption of the PP-1c•iASPP complex by small molecular protein-protein disrupting compounds would prevent the formation of the PP-1c•iASPP•p53 trimeric complex and promote the increased expression and activation of p53 via hyperphosphorylation of the p53 N-terminal transactivation domain.


cdk2
P
PP-1c
GADD34
PP-1c
ASPP1/2
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PP-1c
ASPP1/2
mdm2
PP-1c
iASPP
P
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P
p53
p300
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p53
mdm2
p53
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ATM, ATR,
Chk1/2
mdm2
p53
u
u
u
u
p300
P
P
P
p53
Transcription
- p21
- PUMA
- Bax
- Mdm2
Proteosomal
Degradation
26S
Nucleus
Cytoplasm

Figure 6.2

Figure 6.2: **An Updated Model of the Regulation of p53 by Dephosphorylation**. In normal functioning cells, p53 is kept at low levels due to the actions of mdm2, an E3 ubiquitin ligase that promotes the poly-ubiquination of the p53 C-terminus. Ubiquitination of p53 leads to its translocation from the nucleus to the cytoplasm and targets p53 for proteosomal degradation. In the event of cellular stress, such as DNA damage, several Ser/Thr protein kinases are activated that phosphorylate the N-terminal transactivation domain of p53. This leads to the stabilization and activation of p53, as phosphorylation disrupts the binding of mdm2 and promotes the association of proteins that stabilize p53. Upon activation of p53, p53 binds to specific DNA promoters for gene targets involved primarily in cell cycle arrest or apoptosis. In order to turn off p53 signalling after a stress has been dealt with, p53 is dephosphorylated by several Ser/Thr protein phosphatases including protein phosphatase-1c (PP-1c). The formation of a trimeric PP-1c•iASPP•p53 complex facilities the site specific dephosphorylation of p53 on Ser-15. ASPP1 and ASPP2 do not bind trimerically to PP-1c and p53; therefore, when ASPP1/2 are bound to PP-1c they likely function to inhibit PP-1c mediated p53 dephosphorylation. iASPP has been shown to interact directly with the C-terminal tail of PP-1c. Within the PP-1c•iASPP•p53 complex, PP-1c may be protected from cdk2•cyclin A phosphorylation and thus remain active throughout the cell cycle. When PP-1c is bound to other regulatory proteins that do not directly interact with the C-terminal tail, cdk2 can phosphorylate the PP-1c C-terminus, thus inhibiting the enzymatic activity of p53. Additionally, PP-1c may also regulate ASPP activity by dephosphorylating the ASPP proteins themselves. Dephosphorylation of ASPP1 N-terminal residue 383 may directly affect the subcellular localization of ASPP1 and promote a cytoplasmic distribution.

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