CHARACTERIZATION OF THE PROTEIN PHOSPHATASE 2A REGULATORY SUBUNIT PR70

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Dedicated To My Wife

For her constant love and support

CHARACTERIZATION OF THE PROTEIN PHOSPHATASE 2A REGULATORY SUBUNIT PR70

by

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Protein phosphatase 2A (PP2A) is a phosphoserine/threonine phosphatase that controls the phosphorylation of numerous proteins in eukaryotic cells. PP2A consists of a core dimer composed of a scaffolding subunit (A-subunit) and a catalytic subunit (C-subunit) that interacts with a variety of regulatory subunits. There are four families of regulatory subunits: R2, R3, R4, and R5. The diversity of regulatory subunits gives rise to multiple PP2A holoenzymes and accounts for the ability of PP2A to regulate diverse cellular processes. Relatively little is known about the molecular basis for the interaction of the regulatory subunits with the core dimer and substrates. A more thorough understanding of

these interactions would provide insights into how the regulatory subunits target PP2A to different cellular processes.

The R3 regulatory subunit termed PR70 was identified in a yeast two hybrid screen with the DNA replication protein Cdc6 as bait. PR70 interacts with the PP2A core dimer and Cdc6 *in vivo* and *in vitro*. Biochemical approaches were used to identify regions and residues within PR70 that are important for mediating protein-protein interactions with the PP2A core dimer and Cdc6. PR70 contains two conserved calcium binding EF hand motifs and binds calcium *in vitro*. Calcium enhances the binding of PR70 to the A-subunit but not to Cdc6. Although calcium did not enhance the binding of PR70 to Cdc6, it did result in an increase in the amount of PP2A associated with Cdc6. Both calcium binding and enhanced interactions with the A-subunit require functional EF hand motifs. A conserved motif within the conserved R3 family domain was identified that is sufficient for the interaction of PR70 with PP2A. The C-terminal region was shown to be important for the interaction of PR70 with Cdc6, but not with the A-subunit. This result suggested that different portions of PR70 are important for mediating interactions with PP2A and Cdc6. Finally, PR70 is phosphorylated in intact cells at threonine 76 and serine 543. A functional analysis indicated that mutation of these sites does not affect the ability of PR70 to interact with PP2A, suggesting phosphorylation plays some other role in regulating PR70.

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List of Abbreviations

APC	adenomatous polyposis coli
APC/C	anaphase promoting complex/cyclosome
A-subunit	scaffolding subunit of protein phosphatase 2A
ASBD	A-subunit binding domain
ATP	adenosine 5'-triphosphate
BCA	bicinchoninic Acid
BLAST	basic local alignment search tool
BSA	bovine serum albumin
CAK	cdk activating kinase
CDK	cyclin dependent kinase
Cdc6	cell division cycle mutant 6
cDNA	complimentary deoxyribonucleic acid
CG-NAP	centrosome and Golgi localized PKN-associated protein
CL-A	calyculin A
C-subunit	catalytic subunit of protein phosphatase 2A
C-terminal	carboxy terminal
DMEM	Dulbecco's Modified Eagle Medium
DTT	dithiothreitol
EDTA	ethylenediamine tetraacedtic acid
EF	EF hand
EGTA	ehtyleneglycol bis-aminoethylether

eIF2α	eukaryotic translation initiation factor 2α
Erk	extracellular regulated kinase
EST	expressed sequence tag
GST	glutathione-S-transferase
HA	hemagglutinin
HEAT	Huntington/elongation/A subunit/Tor
HEPE	4-(2-hydroxyethyl)-1- piperazineethanesulfornic acid
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranoside
K_D	equilibrium dissociation constant
KSR	kinase suppressor of ras
M	mitosis
MAPK	mitogen activated protein kinase
MBP	myelin binding protein
MCM	mini-chromosomal maintenance complex
MEK	mitogen/extracellular kinase
MEKK	MEK kinase
N-terminal	amino terminal
OA	okadaic acid
ORC	origin recognition complex
PCR	polymerase chain reaction
PKA	protein kinase A

PKC	protein kinase C
PKN	protein kinase N
PKR	double-stranded RNA dependent protein kinase
PP2A	protein phosphatase 2A
pre-RC	pre-replication complex
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	short interfering ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction

Chapter 1

Introduction

Reversible protein phosphorylation is one of the major mechanisms that regulates cellular processes in eukaryotes. Protein phosphorylation is controlled by the coordinated actions of protein kinases and phosphatases. Signaling pathways use this modification for transducing extracellular signals that regulate cellular homeostasis and growth. Phosphorylation occurs on serine, threonine, and/or tyrosine residues on target proteins, and is responsible for conformational and functional changes to these targets. Phosphatases responsible for dephosphorylation belong to three distinct categories of phosphatases: tyrosine phosphatases, serine/threonine phosphatases, and dual specificity phosphatases. There are eight classes of serine/threonine phosphatases present in eukaryotes (protein serine/threonine phosphatases 1, 2A, 2B/calcineurin, 4, 5, 6, and 7) that contain a conserved phosphatase domain. Compared to the role of kinases in signaling, the regulation of cellular processes by protein phosphatases is poorly understood.

1.1 Protein Phosphatase 2A

Protein phosphatase 2A (PP2A) is ubiquitously expressed and comprises 0.3-1% of all cellular protein (Ruediger et al., 1991). PP2A accounts for a significant proportion of the phosphatase activity in cells, and regulates diverse cellular processes, including development, metabolism, proliferation, transformation, transcription, translation, apoptosis, and stress

response (Janssens and Goris, 2001). The importance of PP2A in cellular processes is further supported by genetic studies which show that deletion of PP2A in yeast (Kinoshita et al., 1991), Drosophila (Orgad et al., 1990), and mice (Gotz et al., 1998) is lethal.

PP2A was once thought of as a single broad specificity enzyme, but it is actually many different enzymes composed of a core dimer that interacts with a variety of regulatory subunits (Figure 1). The core dimer consists of a 36 kDa catalytic subunit (C-subunit) and a 65 kDa scaffolding subunit (A-subunit). There are two isoforms of the C-subunit (α and β) that share 97% identity in their primary amino acid sequence (Arino et al., 1988). The catalytic subunit has remained highly conserved during evolution, as 80% identity between yeast and man is observed (Janssens and Goris, 2001). The A-subunit also has two isoforms (α and β), which share 80% identity (Hemmings et al., 1990). The Aα subunit is composed of 15 non-identical HEAT (Huntington/elongation/A subunit/Tor) repeats consisting of 39 amino acids. Each HEAT repeat is composed of two alpha-helices connected by an intra-repeat loop, and adjacent repeats are connected by inter-repeat loops (Groves et al., 1999). Early work mapped the binding specificity of the C-subunit and regulatory subunits to the A-subunit. The regulatory subunits bind to the intra-repeat loops of repeats 1-10 and the C-subunit binds to repeats 11-15 of the A-subunit (Ruediger et al., 1994). Mutational analysis of the A-subunit identified residues that are important for interacting with all regulatory subunits and others that are specific for binding individual regulatory subunit families (Ruediger et al., 1999).

The interaction of regulatory subunits with the core dimer is critical for PP2A function. The regulatory subunits specifically target PP2A to substrates, signaling

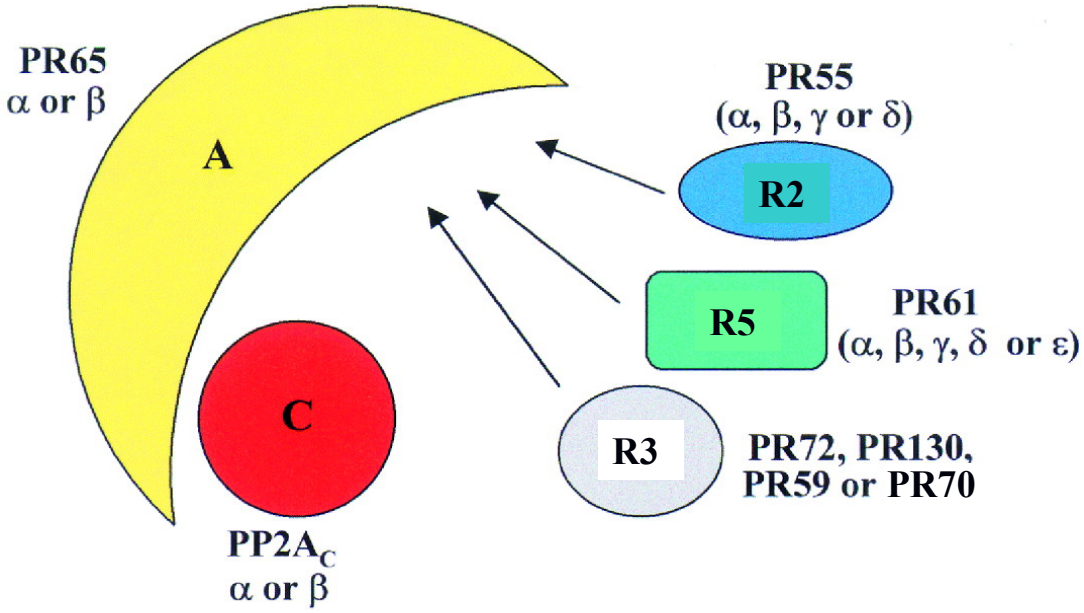

Figure 1.1 Structure of PP2A Holoenzymes. The catalytic subunit (C) is constitutively bound to the scaffolding subunit (A) to form the core dimer of PP2A. The core dimer can interact with a variety of regulatory subunits (R) to generate a diversity of forms. The regulatory subunits specifically target the enzyme to specific substrates and subcellular locations. Derived from figure from Janssens et al, 2001.

complexes, and subcellular localizations. There are four classes of regulatory subunits termed R2, R3, R4, and R5 using nomenclature derived from their official human gene symbols (Silverstein et al., 2003). The diversity of regulatory subunits gives rise to multiple PP2A holoenzymes and accounts for the ability of PP2A to regulate diverse cellular processes. The presence of regulatory subunits affects the kinetics of dephosphorylation (Price and Mumby, 2000). This supports the idea that the regulatory subunits recruit substrates to PP2A. Genetic analysis in *Saccharomyces cerevisiae* (Zhao et al., 1997), insect cells (Silverstein et al., 2002), and mammalian cells (Adams et al., 2005) also support the hypothesis that specific biological functions of PP2A are mediated by distinct regulatory subunits.

Relatively little is known about the molecular basis for the interaction of the regulatory subunits with the core dimer and substrates. The regulatory subunits exhibit mutually exclusive binding to the A-subunit (Ruediger et al., 1992; Ruediger et al., 1994), but surprisingly, there is little sequence conservation between the individual regulatory subunit families. A loosely conserved A-subunit binding domain (ASBD) was identified in the regulatory subunit families (Li and Virshup, 2002). This putative domain suggests that the regulatory subunits may contain a conserved structure responsible for interaction with the PP2A core dimer. A recent study identified a cluster of conserved charged residues in the R2 family which are important for interaction with the core dimer (Strack et al., 2002). However, specific motifs or amino acid residues that mediate interaction of these regulatory subunits with substrates have not been identified. A better understanding of the basis for the

interaction with the PP2A core dimer and substrates would provide new insights into how the regulatory subunits target PP2A to different cellular processes.

The R2 subunits are the best characterized family of regulatory subunits of PP2A and consists of 4 members (α, β, γ, and δ). The R2α subunit targets PP2A to the Ras-Raf-Mek-Erk pathway, and regulates signaling at multiple steps of this pathway. Knockdown of the R2 subunit in Drosophila S2 cells by RNAi (Silverstein et al., 2002) or displacement of R2α from the core dimer by the SV40 small tumor antigen (small-t) (Sontag et al., 1993) activates the mitogen activated protein kinase (MAPK), Erk. Once thought to primarly be a negative regulator of the Ras-Raf-Mek-Erk pathway, it is now known that PP2A also positively regulates the pathway. The R2α associates with Kinase Suppressor of Ras (KSR) and Raf-1 and promotes dephosphorylation of inhibitory phosphorylation sites on these substrates (Ory et al., 2003). The R2α and R2β subunits also target PP2A to microtubules via a heat labile anchoring activity present in microtubule-associated and microtubule-interacting proteins (Price et al., 1999). The R2α and R2β subunits also target PP2A to the microtubule associated protein, tau (Sontag et al., 1996). Over-expression of tau with SV40 small-t results in hyperphosphorylation of tau on multiple sites, dissociation of tau from microtubules, and destabilization of microtubules.

The R3 subunit family consists of 3 members (PR72, PR59, and PR70). The gene encoding PR72 produces two alternatively spliced transcripts encoding proteins of 72 and 130 kDa (Hendrix et al., 1993). PR72 was recently shown to target PP2A to the Wnt signaling cascade (Creyghton et al., 2005a) by interacting with the human Naked cuticle protein. Over-expression of PR72 results in repression of the classical Wnt signaling cascade

and the presence of PR72 is required for the inhibitory effect of Naked cuticle on Wnt signaling. The members of this family also have been implicated in targeting PP2A to proteins involved in cell cycle regulation. The splice variant PR130 targets PP2A to the scaffolding protein centrosome and Golgi localized PKN-associated protein (CG-NAP) (Takahashi et al., 1999). CG-NAP anchors a signaling complex to the centrosome and golgi apparatus in a cell cycle dependent manner. PR59 was discovered in a yeast two hybrid screen with the retinoblastoma-related protein p107 as bait (Voorhoeve et al., 1999). Over-expression of PR59 results in dephosphorylation of p107 and cell cycle arrest in G_1 phase. PR70 was identified in a yeast two hybrid screen with the DNA replication protein Cdc6 as bait (Yan et al., 2000). The interaction of PR70 with Cdc6 is discussed in more detail in the next section.

The R5 subunit family consists of 5 members (α, β, γ, δ, and ε). R5 subunits target PP2A to the Wnt signaling pathway. R5α interacts with the adenomatous polyposis coli (APC) proteinin a yeast two hybrid system (Seeling et al., 1999a). APC forms a complex with axin and glycogen synthase kinase 3β (GSK-3β) that mediates the regulation of β-catenin. R5β and γ interact with axin which further supports the role of PP2A in regulating Wnt signaling (Yamamoto et al., 2001). Over-expression of R5α reduced the level of β-catenin protein and inhibited the expression of β-catenin target genes (Seeling et al., 1999b). PP2A also plays a positive role in Wnt signaling. The R5ε subunit is required upstream of β-catenin and regulates disheveled which a positive regulator of the pathway (Yang et al., 2003). The R5 subunits also target PP2A to Cyclins G1 and G2. R5α and β interact with cyclin G1 and cyclin G1 recruits PP2A heterotrimers containing R5α to the p53 regulator

Mdm2 (Okamoto et al., 1996)Okamoto et al., 2002). This interaction results in dephosphorylation of threonine 216 of Mdm2 and is thought to regulate both Mdm2 and p53 activity. Rα and β also interact with cyclin G2 (Bennin et al., 2002). Over-expression of cyclin G2 resulted in aberrant nuclei and a G_1/S phase cell cycle arrest. The forced cell cycle arrest by over-expression of G2 is dependent on its ability to interact with R5 subunits. Surprisingly, cyclin G2 was found only in complex with R5α and β and the catalytic subunit, but not the scaffolding subunit.

1.2 DNA Replication and Cdc6

Precise coordination of DNA replication is required to ensure that daughter cells inherit an intact complement of genetic material. A two step process regulates the initiation of DNA replication. First, assembly of pre-replication complexes (pre-RCs) on replication origins is initiated either as the cell exits mitosis or during early G1 phase (Figure 1.2). Cdc6 and Cdt1 are recruited to the origins by the origin recognition complex (ORC), and once bound; Cdc6 and Cdt1 recruit the putative DNA helicase mini-chromosomal maintenance complex (MCM) to complete the formation of the pre-RC. The formation of the pre-RCs at the origin licenses chromatin to be competent for replication. In the second step, initiation of replication occurs by activation of the S-phase cyclin dependent kinases (CDKs) and the Cdc7/Dbf4 kinase. Additional replication factors, including Cdc45, Sld3, RPA, and DNA polymerase α are recruited to the site of replication and convert the pre-RCs to replisomes (Bell and Dutta, 2002). Once the replisome is assembled, synthesis of new DNA can begin. Once replication is initiated, formation of new pre-RCs is inhibited until the end of M phase.

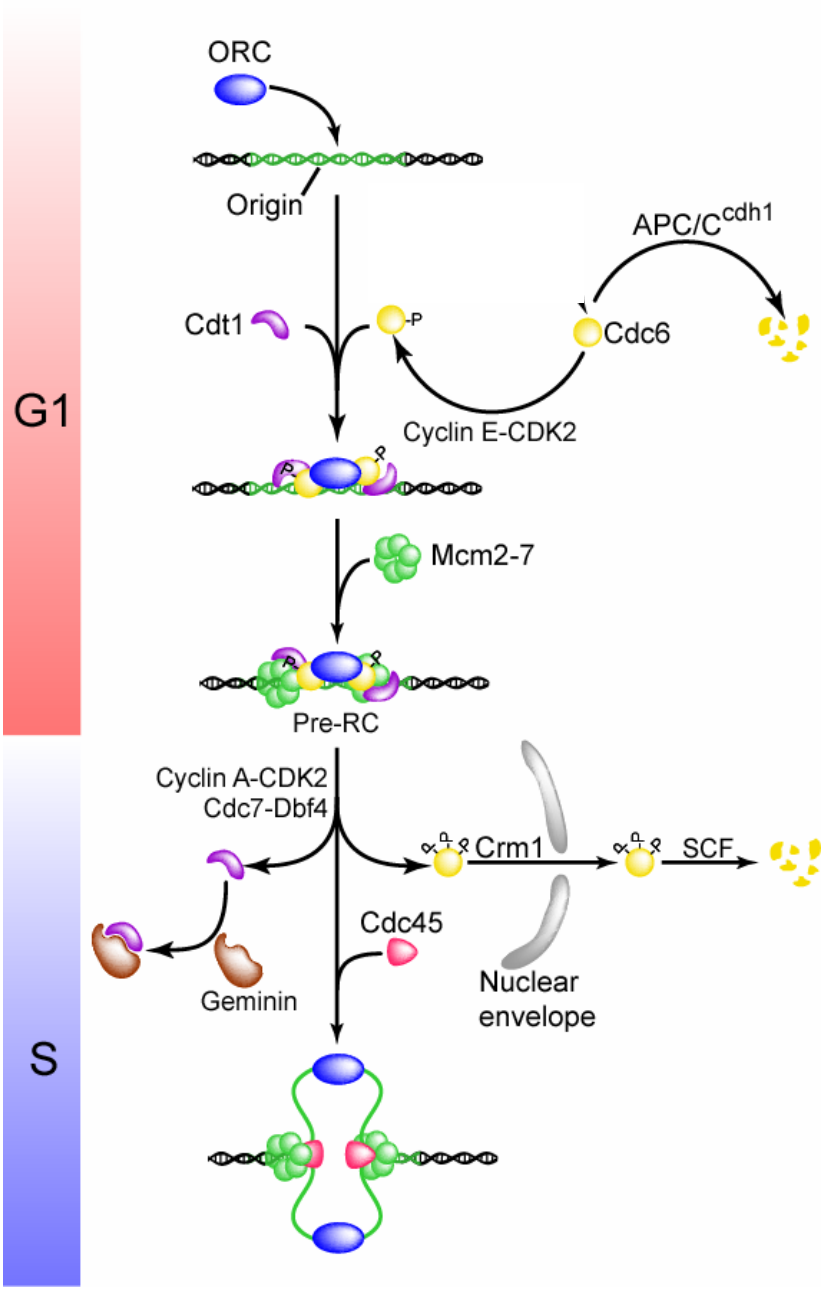

Figure 1.2 Model of DNA Replication. Details of the model are described in the text.

Thus, pre-RCs only assemble on origins during a short window in the cell cycle between the end of mitosis and a point in late G1 phase. This temporal regulation of pre-RC assembly is a key regulatory process that ensures origins are fired only once per cell cycle.

The assembly of pre-RCs in late M/early G1 phase occurs during a period of high proteolysis and low Cdk activity (Ang and Harper, 2004; Diffley, 2004). The high proteolytic activity during G1 correlates with activation of the E3 ubiquitin ligase, anaphase promoting complex (APC). Two activation subunits, Cdc20 and Cdh1, confer substrate specificity for APC. During late M/G1 phase, the Cdh1 subunit is active. Cdh1 binds to substrates at their N-terminus and requires an RxxL destruction box (D-box) and a KEN box motif for binding (Harper et al., 2002). APC^{Cdh1} promotes pre-RC formation by mediating destruction of the pre-RC formation inhibitors geminin and cyclin A. Geminin inhibits pre-RC assembly by binding to Cdt1 and blocking recruitment of the MCM complex. Cyclin A-Cdk2 phosphorylates Cdh1 and blocks its ability to bind to APC. Emi1, an APC^{Cdh1} inhibitor, expression increases at the end of the G1 phase. This results in APC^{Cdh1} inhibition, and subsequently, cyclin A-cdk2 activation, geminin accumulation, and entry into S phase. The activity of cyclin A and geminin inhibit pre-RC assembly until late mitosis (Blow and Dutta, 2005).

Cdks are central to the regulation of DNA replication. They are essential for triggering the initiation of DNA replication and play a direct role in preventing assembly of pre-RCs once initiation of DNA replication has started. Cdk-mediated phosphorylation regulates Cdc6 function. Cdc6 is phosphorylated *in vitro* by cyclin A-cdk2 and cyclin E-cdk2 at three N-terminal sites, S54, S74, and S106 (Herbig et al., 2000; Jiang et al., 1999).

Cdks regulate the nuclear localization of ectopically expressed Cdc6, which is nuclear in G1 phase but translocated to the cytoplasm in S and G2 (Alexandrow and Hamlin, 2004; Coverley et al., 2000; Mendez and Stillman, 2000). The translocation of Cdc6 requires the cdk phosphorylation sites. Cyclin A-cdk2 can also target Cdc6 for proteolysis (Coverley et al., 2000). The proteolysis only affects soluble forms of Cdc6, as chromatin bound Cdc6 persists through S and G2 phases (Coverley et al., 2000; Mendez and Stillman, 2000; Alexandrow and Hamlin, 2004). This suggests free nuclear Cdc6 is targeted for proteolysis to prevent pre-RC formation after the onset of S phase.

In quiescent and early G1 cells, Cdc6 protein levels are low. Low Cdc6 protein levels are maintained by ubiquitin-mediated degradation via APC^{Cdh1} (Petersen et al., 2000). Cdc6 contains D-box and KEN-box motifs, which are necessary for ubiquitination and degradation (Petersen et al., 2000) . A recent report demonstrated that phosphorylation of Cdc6 by cyclin E-cdk2 stabilizes Cdc6 in G1 phase (Mailand and Diffley, 2005). Cdc6 is stabilized by phosphorylation of the Cdk sites, based on observations that mutation of the phosphorylation sites to alanine destabilizes whereas mutation to phospho-mimicking aspartate stabilizes Cdc6. The association of APC^{Cdh1} with Cdc6 is blocked when the phosphorylation sites are mutated to aspartate. The association is lost because phosphorylation blocks binding to the D-box and KEN-box motif which are next to the phosphorylation sites. Cdk-mediated stabilization allows Cdc6 to accumulate before geminin and cyclin A and allows a period of time when Cdc6 can induce pre-RC assembly. A similar mechanism is involved in inhibition of DNA replication following ionizing radiation. Activation of p53 by DNA damage results in enhanced destruction of Cdc6 by APC^{Cdh1}. The p52-mediated proteolysis of Cdc6 is

mediated by expression of the cdk inhibitor p21 and inhibition of cdk-mediated phosphorylation at S54 of Cdc6.

The regulation of Cdc6 function and protein stability by phosphorylation is an important mechanism in the control of DNA replication. Since phosphorylation is controlled by competing kinases and phosphatases, the role of phosphatases in DNA replication and Cdc6 regulation is important to understand. Currently, very little is known about the role of phosphatases in DNA replication. As described above, the PP2A regulatory subunit PR70 was identified in a yeast two hybrid screen using Cdc6 as bait (Yan et al., 2000). PR70 binds specifically to the N-terminus of Cdc6, which is the region that contains the three cdk phosphorylation sites. Forced expression of PR70 causes a G1 arrest, which suggests Cdc6 function and DNA replication may be regulated through targeting of PP2A by PR70.

1.3 Goal of the project

Understanding how regulatory subunits interact with the PP2A core dimer and substrates is critical for understanding how PP2A regulates many diverse biological processes. The goal of the project was to identify the molecular basis for PR70 interactions with the PP2A core dimer and Cdc6 and how these interactions regulate PR70 function. Insights into PR70 regulation may elucidate the role of PR70 PP2A-mediated regulation of Cdc6 function and DNA replication.

Specific aims:

One: Characterize PR70 Expression and interactions of PR70 with PP2A and Cdc6.

Two: Determine how calcium binding affects the function of PR70.

Three: Identify specific regions and residues of PR70 mediating interactions with the PP2A core dimer and Cdc6.

Four: Determine if phosphorylation of PR70 affects the function of PR70.

Chapter 2

Characterization of Full Length Human PR70

2.1 Introduction

The R3 regulatory subunit termed PR48 was originally identified in a yeast two hybrid screen using Cdc6 as bait (Yan et al., 2000). The cDNA clone identified an open reading frame encoding a protein of 48kDa. Although the open reading frame extended further in the 5' direction, neither BLAST searches of the human EST database nor PCR could identify a longer cDNA with an initiation codon. Subsequently, a homologous PR48 cDNA with an N-terminal extension that encodes a protein of 70 kDa was identified from *Xenopus* (Stevens et al., 2003). Examination of the human EST database identified several ESTs that overlapped with the N-terminal extension of the *Xenopus* cDNA. Comparison of the human ESTs and the *Xenopus* sequence showed extensive homology. Theoretical translation of the human EST sequences could generate a protein of similar length as the *Xenopus* protein, which were named PR70.

Since the EST data suggested that human PR48 was a partial cDNA clone, it was important to identify and characterize the full length transcript. The results presented in this chapter show that this R3 subunit is actually encoded by a longer cDNA with an N-terminal extension, which in agreement with the previous report will be called PR70. The expression of PR70 mRNA and protein, as well as assays to detect interaction with PP2A and Cdc6 are

described in this chapter. The results indicate that the full length PR70 is ubiquitously expressed and interacts with both PP2A and Cdc6 *in vivo*.

2.2 Materials and Methods

2.2.1 Human EST Database Search and Cloning of Full Length PR70

A BLAST search for human ESTs containing the PR70 start codon and overlapping 5' sequence (nucleotides 1-790) (GenBank Accession NM_013239) was performed using the MegaBLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/). Alignments of the PR70 cDNA sequence and the identified human ESTs were performed using the AlignX program of the Vector NTI suite (Invitrogen). PR70 cDNA was assembled using the PR48 cDNA and the IMAGE Human Clone ID 5728169 (GenBank Accession Number BM544432) (Invitrogen). The sequence of BM54432 was verified by automated sequencing and contained the hypothetical translational start codon of full length PR70 and 3'sequences overlapping with the PR48 cDNA. The full length PR70 cDNA was constructed using an internal NcoI restriction site. A PCR fragment that contained the translational start codon and the 5' end of the cDNA was generated using the BM54432 cDNA as template with the following primers: 5'-CGGGATCCATGCCGCCCGGCAAAGT-3' (sense strand) and 5'-GCGCCTTGATCCGGC-3' (anti-sense strand). Following PCR amplification, the product was digested with the restriction enzymes BamHI and NcoI. The 3' portion of the PR48 cDNA was excised with the restriction enzymes NcoI and HindIII. The two fragments were ligated with T4 ligase (New England BioLabs) and subcloned into pCMV-Tag2B (Stratagene), which had been digested with BamHI and HindIII. The resulting construct

encodes a full length PR70 cDNA fused to an N-terminal FLAG epitope tag. The sequence was verified by automated sequencing.

2.2.2 Cell Culture and Expression of FLAG-tagged PR70 and PR70 Mutants

COS-7, HeLa, HEK293, MCF-7, MDA-MB-231, and HT-29 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) in an atmosphere of 5% CO_2. For transient expression of FLAG-PR70, COS-7 cells were seeded into 10cm dishes for transfection the next day at 90-95% confluence ($1.5x10^6$ cells/dish). Cells were tranfected with 7μg DNA and 14μg Lipofectamine 2000 Reagent using the manufacturer protocol (Invitrogen) and incubated for 24 hours before harvesting.

2.2.3 Total RNA Isolation and RT-PCR

COS-7, HeLa, HEK293, MCF-7, MDA-MB-231, and HT-29 cells were plated at 70% confluence in 35-mm dishes the day before harvest. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and both cDNA synthesis and PCR was performed using the SUPERSCRIPT One-step RT-PCR with PLATINMUM *Taq* kit as described by the manufacturer (Invitrogen). The primers used to amplify PR70 were 5'-ATGGATGACATGGGCCTG-3' (sense strand) and 5'-TCACAGCGGCTCCAGGT-3' (anti-sense strand). The primers used to amplify Cdc6 were 5'-ATGCCTCAAACCCGATCC-3' (sense strand) and 5'-TTAAGGCAATCCAGTAGCTA-3' (anti-sense strand). Five microliters (1/10) of the reaction was resolved on a 1% agarose gel.

2.2.4 siRNA Treatment of HeLa cells

HeLa cells were seeded in 35mm dishes for transfection the next day when they were typically 50 to 60% confluent. Small interfering RNA (siRNA) transfections were performed

using Oligofectamine as instructed by the manufacturer (Invitrogen). The sequence of the siRNAs were as follows: PR48 #1, 5'-CUACCUGGUGCAGGAGGACTT-3' (sense strand) and 5'-GUCCUCCUGCACCAGGUAGTT-3' (anti-sense strand); PR48 #2, 5'-GGAGGCGUCCGAGUUCCACTT-3' (sense strand) and 5'-GUGGAACUCGGACGCCUCCTT-3' (anti-sense strand); PR48 #3, 5'-CACGCACCCGGGGCUGUCGTT-3' (sense strand) and 5'-CGACAGCCCCGGGUGCGUGTT-3' (anti-sense strand); PR48 #4, 5'CGUCUUCUUCGACACCUUCTT-3' (sense strand) and 5'-GAAGGUGUCGAAGAAGACGTT-3' (anti-sense strand); and MEKK2, 5'-AGCCGCCUUGGGCUGAAUUTT-3' (sense strand) and 5'-AAUUCAGCCCAAGGCGGCUTT-3' (anti-sense strand). The siRNA oligonucleotides were chemically synthesized at an in-house facility at UT Southwestern. The annealing and transfection of the siRNAs were performed as previously described [Elbashir et al., 2001]. After 48 h, cells were lysed with IP lysis buffer (recipe below), centrifuged to remove cellular debris, and protein concentrations determined using Bicinchoninic Acid (BCA) assay (Pierce). Equal amounts of protein were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose membranes. The membranes were probed with anti-PR70 antiserum and anti-MEKK2 antibodies (Santa Cruz). The blots were developed using the enhanced chemiluminescence detection system (Amersham Biosciences).

2.2.5 Immunoprecipitations and Western Blotting

All immunoprecipitations followed the protocol described previously (Yan et al., 2000). Briefly, the media was aspirated and the cells were washed with cold PBS. The cells were incubated on ice for 20 minutes in IP lysis buffer containing 20mM Tris-HCl (pH 7.5), 0.2% Nonidet P-40, 20% glycerol, 200mM NaCl, 1mM EDTA, and protease inhibitor cocktail (Roche). Lysates were centrifuged at 14,000 x g for 10 min, and protein complexes were immunoprecipitated from the supernatant.

Immunoprecipitations of endogenous PR70 and Cdc6 were performed from exponentially ($1.2x10^6$ cells/10cm dish) growing HeLa cells. PR70 was immunoprecipitated using a rabbit polyclonal antiserum against PR70 that was generated against the peptide CDLYEYACGDEDLEPL conjugated to keyhole limpet haemocyanin (KLH). Cdc6 was immunoprecipitated using a rabbit polyclonal antibody generated against a full length Cdc6 GST fusion protein previously described (Yan et al., 1998). As a negative control, immunoprecipitations were performed using pre-immune serum from the rabbits immunized against PR70 or Cdc6. Anti-PR70, anti-Cdc6, and preimmune sera were a kind gift of Zhen Yan (Duke University). For immunoprecipitation of endogenous proteins, 10 microliters of antiserum and 40 microliters of protein A-sepharose (Sigma-Aldrich) were added to 300 microliters supernatant and incubated for 2 hours at 4°C followed by 3 washes with lysis buffer. The immunoprecipitates were solubilized in 60 microliters of SDS-PAGE loading buffer, 30 microliters (50%) were resolved on an 10% SDS-PAGE gel, and transferred to nitrocellulose membranes. The membranes were then probed with anti-PP2A C-subunit monoclonal antibody (1F6), anti-PP2A A-subunit antiserum (C-20, Santa Cruz

Biotechnology), anti-cdc6 monoclonal antibody (clone DCS-180, Upstate), or anti-PR70 antiserum. Blots were developed using the enhanced chemiluminescence detection system (Amersham Biosciences).

Transiently expressed FLAG-PR70 was immunoprecipitated using 10 micrograms anti-FLAG polyclonal antibody (Sigma Aldrich) and 40 microliters of protein A-sepharose (Sigma-Aldrich) for 2 hours at 4°C followed by 3 washes with lysis buffer. The immunoprecipitates were solubilized in 60 microliters of 2x SDS-PAGE loading buffer, 30 microliters (50%) was resolved on a 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane. The membrane was then probed with anti-FLAG monoclonal antibody (M2, Stratagene), anti-PP2A C-subunit (1F6), and anti-PP2A A-subunit (C-20, Santa Cruz Biotechnology). Blots were developed using the enhanced chemiluminescence detection system (Amersham Biosciences).

2.3 Results

2.3.1 Identification of Human ESTs Encoding Full Length PR70

As stated above, the original human PR48 cDNA was likely a truncation of a longer transcript. To further text this possibility, the putative N-terminal portion of the PR70 nucleotide sequence (nucleotides 1-790), reported by Stevens et al was used to perform a BLAST search for human ESTs using the MegaBLAST tool of NCBI. The search identified the same EST (GenBank Accession AL555389) previously reported and twenty three additional humans ESTs. Alignment with the PR70 cDNA sequence identified seven ESTs (GenBank Accession Numbers AL555389, BM544432, BI82834, BX353646, BM476655, BX355787, and BI555823) that contained the proposed PR70 initiation codon and additional

3' sequence (Figure 2.1). The 3' ends of three of the human ESTs (AL555389, BX355787, and BI555823) overlapped with PR48 cDNA (Figure 2.1). The results confirmed the previous report and show that PR48 is encoded by a longer cDNA. In accordance with the previously established nomenclature, the protein encoded by the full-length cDNA will be termed PR70.

2.3.2 Subcloning and Expression of Full Length PR70

The BLAST results established that previous work had been carried out with a truncation of PR70. It was therefore necessary to prepare a full length PR70 cDNA. A BLAST search with PR70 nucleotides 1-790 of the Invitrogen clone collections using CloneRanger (http://clones.invitrogen.com/cloneranger.php) was used to identify available PR70 cDNAs. This search identified twenty clones, including AL555389 and IMAGE ESTs clones BM54432 and BI828434 previously identified in searches of the NCBI EST database. All three clones were purchased from Invitrogen. Automated sequencing verified the cDNA sequence of clones BM54432 and BI82834, but sequencing of AL555389 was unsuccessful. Surprisingly, sequencing of BM54432 and BI82834 suggested that they may contain the full length PR70 cDNA. Subsequent PCR analysis showed that a product of similar size as the full length PR70 cDNA could be amplified from both cDNAs (Data not shown). However, the complete sequence of BM54432 and BI82834 could not be verified. Consequently, a full length PR70 cDNA was generated using an internal NcoI restriction site present in the region of these ESTs that overlapped the original PR48 cDNA. The sequence of the region of human EST BM54432 that spans the translational start codon and the internal NcoI site was verified by automated sequencing. The start codon and sequence to the NcoI site was

amplified using PCR primers that introduced a BamHI site, adjacent the start codon, and the NcoI site. The C-terminal portion of the cDNA was excised from a PR48 cDNA using NcoI and HindIII. Following gel purification, the two cDNA fragments were ligated and cloned into the pCMV-Tag2B expression vector (Stratagene) to produce an N-terminal FLAG tagged protein. The sequence of the full length PR70 cDNA was verified by automated sequencing. To verify proper construction of the cDNA, COS-7 cells were transiently transfected with FLAG-PR70 cDNAs and expression of FLAG-PR70 tested. FLAG-tagged PR70 was expressed in COS-7 cells (six different isolates from the subcloning are shown) and migrates at the predicted molecular mass of 73kDa (Figure 2.2, Lanes 3-8).

2.3.3 Expressed FLAG-PR70 Incorporates into PP2A Complexes

Previously, it was shown that PR48 interacted with the PP2A A-subunit in a yeast two hybrid assay and that active PP2A holoenzymes containing PR48 could be reconstituted (Yan et al., 2000). To test the ability of full length PR70 to associate with PP2A, FLAG-tagged PR70 was expressed in COS-7 cells and immunoprecipitated. Immunoprecipitation of FLAG-PR70 resulted in co-immunoprecipitation of the A- and C-subunits of PP2A, illustrating that epitope tagged PR70 interacts with endogenous PP2A subunits in intact cells. (Figure 2.3, Lanes 3 and 6). Expressed FLAG-PR70 also interacted with endogenous PP2A subunits in COS-7, HeLa, and NIH-3T3 cells (Figure 2.3, Lane 3, Figure 5.2, Lane 4, and Figure 5.2, Lane 2, respectively). Data in Chapter 3 show that full length PR70 also interacts with Cdc6 *in vitro* (Figure 3.3, Lanes 2 and 7, respectively). Numerous attempts to co-immunoprecipitate endogenous Cdc6 or over-expressed HA-tagged Cdc6 with FLAG-PR70 produced mixed results. Recent data has provided a possible explanation for this and will be

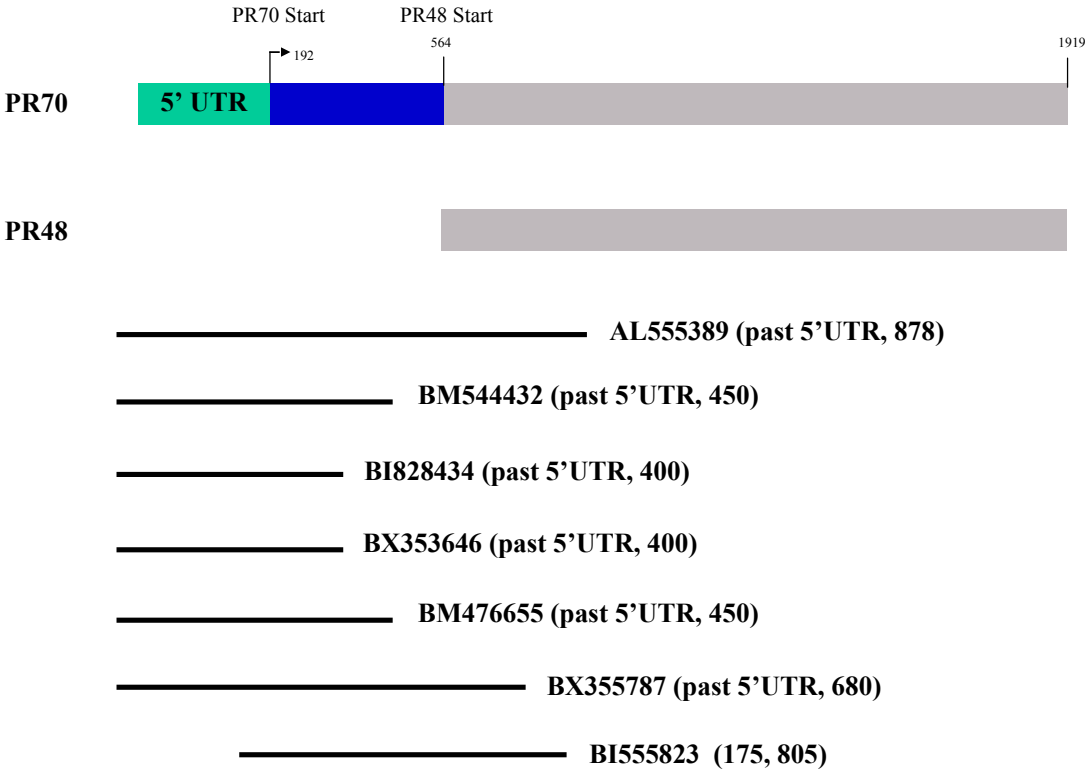

Figure 2.1 Screening of the human ESTs Database Identified ESTs Overlapping with PR70 N-terminal Sequence. Alignment of the 7 human ESTs identified in a BLAST search, which contain the hypothetical start of PR70 and 5' sequence. The BLAST search was conducted using the first 790 nucleotides of the PR70 cDNA and identified 24 total ESTs inthe BLAST search. The human ESTs AL555389, BX355787, and BI555823 were the only ESTs identified that contained the hypothetical start of PR70 and PR48.

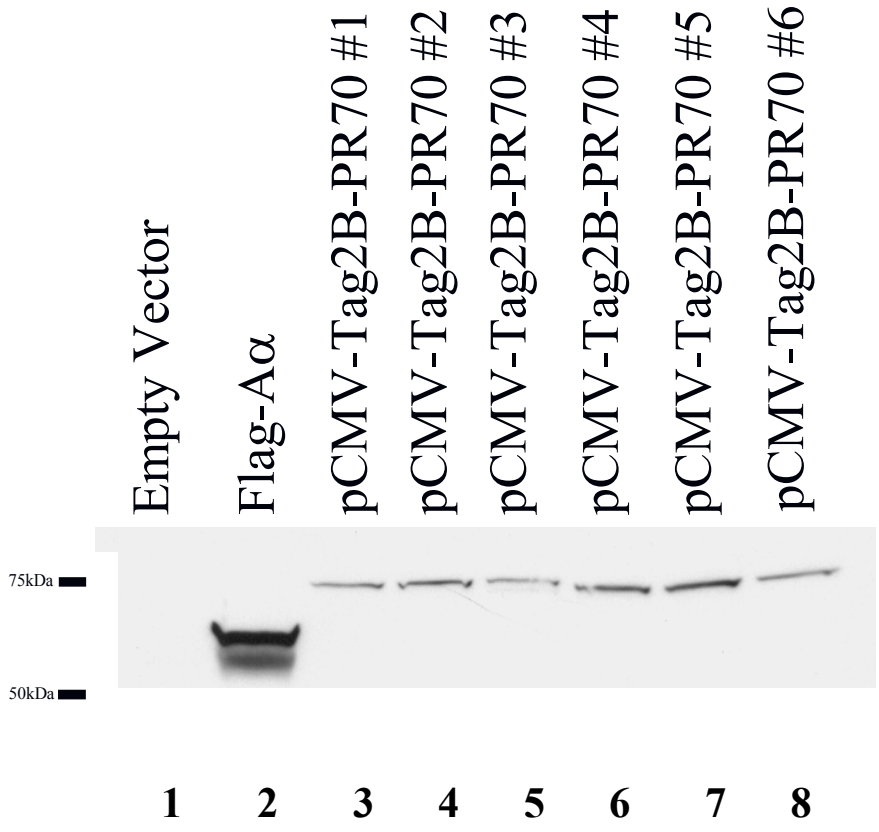

Figure 2.2 Expression of FLAG-PR70 in COS-7 Cells. Expression of six different N-terminal FLAG epitope tagged PR70 clones created in the subcloning were transiently over-expressed in COS-7 cells. Following 24 hour incubation, whole cell lysates were resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was immunoblotted with anti-FLAG antibodies to detect FLAG-PR70 protein expression. FLAG-PP2A A-subunit served as a control for transfection and blotting.

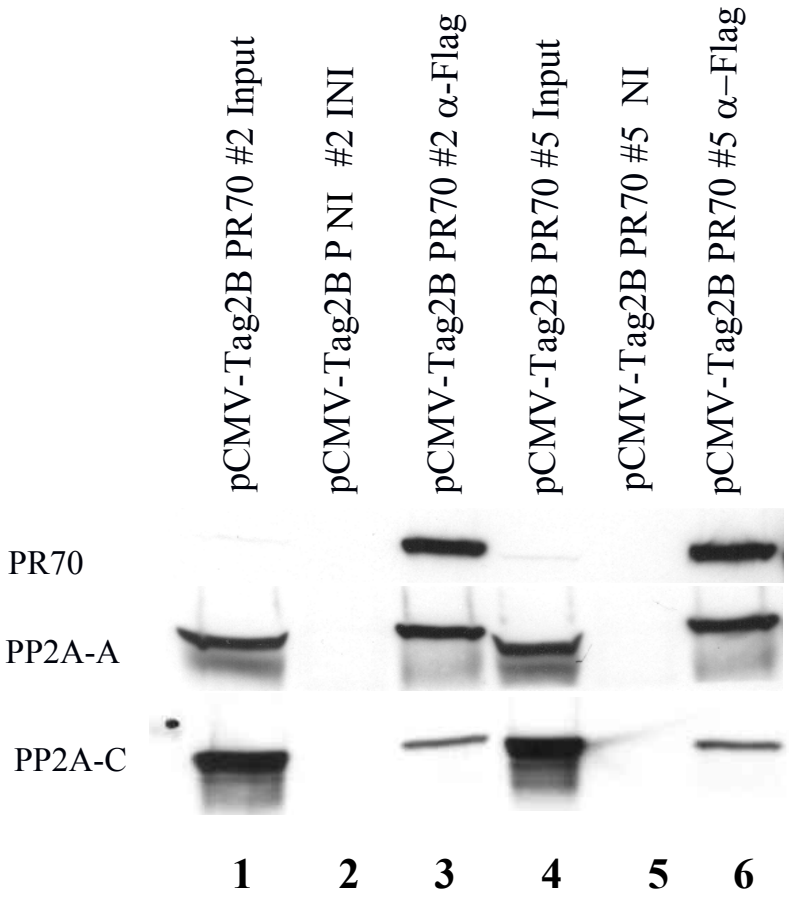

Figure 2.3 Over-expressed PR70 Interacts with the PP2A Core Dimer *In Vivo*. Two sequenced verified FLAG-PR70 cDNAs were transiently transfected in COS-7 cells. Following 24 hour incubation, the cells were lysed and the FLAG-PR70 was immunoprecipitated using anti-FLAG antibody. The immunoprecipitations were resolved on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blotted with anti-FLAG, anti-PP2A A-subunit, and anti-PP2A C-subunit antibodies.

discussed in Chapter 6. The data shows that FLAG epitope tagged full length PR70 interacts with the PP2A core dimer and Cdc6 in a manner similar to the truncated PR48 protein.

2.3.4 Expression of Endogenous PR70

The members of the R3 family of regulatory subunits have varying expression profiles. The two splice variants of PR72 (PR72 and PR130) are predominantly expressed in heart and skeletal muscle. PR72 expression is not observed in any other human tissues, but PR130 has low expression in brain, placenta, lung, liver, kidney and pancreas (Hendrix et al., 1993). Surprisingly, the related R3 subunit, PR59, is expressed in heart, but not skeletal muscle (Voorhoeve et al., 1999). Similar to PR130, PR59 has low expression in all other tissue. In order to determine the pattern of expression, PR70 mRNA expression in different mammalian cell lines was assessed using a one step RT-PCR assay. Total RNA was isolated from HeLa (human cervix), HEK293 (human kidney), MCF-7 (human breast), H1299 (human lung), MDA-MB-231 (human breast), HT-29 (human colon), and COS-7 (monkey kidney) cells and RT-PCR was performed using primers specific for amplification of PR70 sequence. As a positive control, the expression of Cdc6 was also assessed. PR70 mRNA expression was detected in the human cell lines, and at lower levels in the monkey cell line (Figure 2.4A). Expression of PR70 protein was also tested by Western blotting whole cell lysates from the same cell lines. A rabbit polyclonal antibody to PR70 was developed by our collaborator, Zhen Yan (Duke University). The antibody was generated by immunization of rabbits with the peptide CDLYEYACGDEDLEPL conjugated to KLH which corresponds to the C-terminus of the PR70 amino acid sequence. PR70 protein was detected in all the cell types (Figure 2.4B). MCF-7 cells had the highest expression, followed

by HEK-293 cells (Figure 2.4B, Lanes 3 and 2, respectively). Low expression of PR70 was observed in the other cell lines tested. Two prominent bands were recognized by the PR70 antibody, one that migrates at a molecular weight of 70kDa and another at 48kDa (Figure 2.4B). To determine which band corresponded to the PR70 protein, knockdown of the protein with siRNAs directed at different sequences within the PR70 mRNA were performed in HeLa cells. Knockdown of MEKK2 was used a positive control. Transfection with siRNAs #1, #2, and #3 (Figure 2.5, Lanes 2, 3, and 4) knocked down the protein band at 70kDa but not the 48kDa band compared to mock treated (Figure 2.5, Lane 1) or MEKK2 siRNAs (Figure 2.5, Lane 6). These data show that PR70 is expressed in the mammalian cell lines tested, and indicate that the endogenous protein has an apparent molecular weight consistent with the full length PR70 cDNA.

2.3.5 Endogenous PR70 Interacts with Endogenous PP2A and Cdc6

While the human R3 family members have been shown to interact with the PP2A core dimer in expression studies (Voorhoeve et al., 1999; Janssens et al., 2003), no data exists demonstrating that endogenous human R3 family members are components of PP2A holoenzymes. PR72 was identified in a PP2A holoenzyme preparation purified from rabbit skeletal muscle (Hendrix et al, 1993). However, the endogenous human PR72-containing heterotrimer has not been isolated. To test for PR70 in endogenous human PP2A heterotrimers, the polyclonal antibody against PR70 was used to immunoprecipitate PR70 from HeLa cells. Immunoblotting showed that the anti-PR70 antibodies co-precipitated PP2A A- and C-subunits (Figure 2.6A, Lane 4). Despite numerous attempts, Cdc6 could not be detected in PR70 immunoprecipitates. In contrast, immunoprecipitation of endogenous

Cdc6 from HeLa cells co-precipitated PR70 andthe A- and C-subunits of PP2A (Figure 2.6B, Lane 4). Together with the FLAG-PR70 immunoprecipitation data, these results showed that PR70 interacts with the PP2A core dimer and establishes its role as a PP2A regulatory subunit. They also show that a quaternary complex consisting of the PP2A core dimer, PR70, and Cdc6 can be isolated from HeLa cells.

2.4 Discussion

RT-PCR-based expression analysis shows that PR70 mRNA levels are fairly consistent among several human cell lines with COS-7 cells having lower expression [Figure 2.4A]. PR70 mRNA is also expressed in human heart (Data not shown). A similar analysis was performed with PR72 and PR130 [Hendrix et al, 1993]. PR72 mRNA expression was not observed in any of the same human cell lines, but was detected at high levels in heart and skeletal muscle. These observations suggest that PR72 carries out a function unique to muscle. PR130 expression was observed in most human tissues (no expression in liver and pancreas), but expression varied in the human cell lines. PR130 mRNA was detected in HeLa and MCF-7 cells, but not MDA-MB-231 cells. PR59 expression was observed in all tissues tested, except muscle, suggesting that PR59 and PR72 do not have redundant functions. Expression of human PR70 appears to be ubiquitous, which is comparable to the expression of Xenopus PR70 (Janssens et al., 2003; Stevens et al., 2003). The expression profile of PR70 is not surprising, since it is implicated in regulating the DNA replication factor Cdc6.

The present study demonstrates that epitope-tagged PR70 incorporates into PP2A heterotrimers (Figure 2.3). This is not surprising since the N-terminal truncated protein

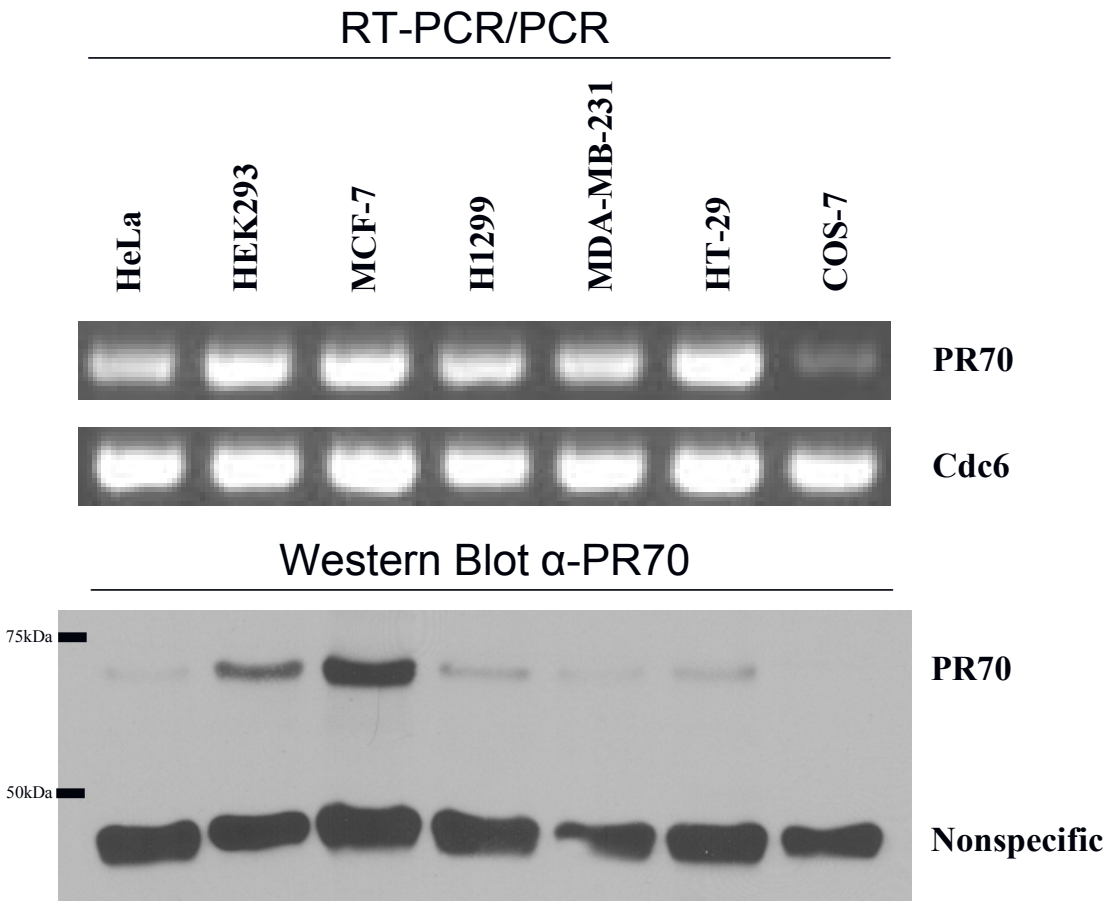

Figure 2.4 Analysis of PR70 mRNA and Protein Expression In Different Mammalian Cell Lines. (A) Total RNA was isolated from mammalian cell lines and subjected to RT-PCR/PCR with primers specific for the PR70 transcript. Primers specific for Cdc6 were used as a control. The PCR product was analyzed on a 1% agarose gel. (B) Whole cell lysates from mammalian cell lines were blotted for endogenous PR70 using rabbit polyclonal antiserum against PR70. Two bands were present, one at 70kDa which represents endogenous PR70, and a non-specific band at 48kDa.

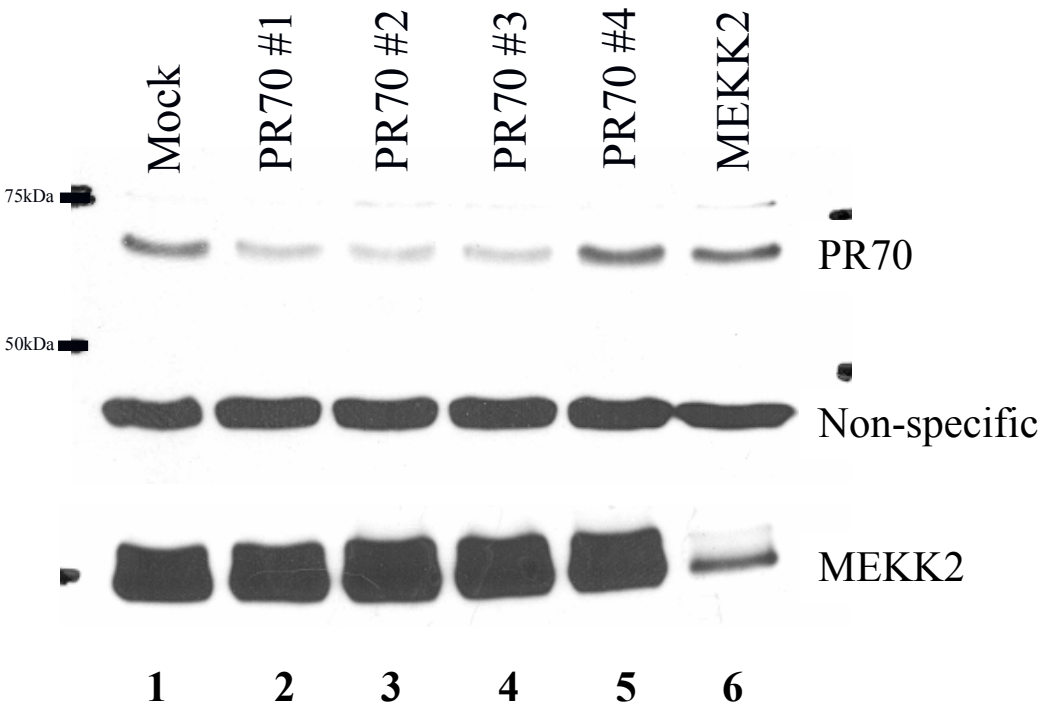

Figure 2.5 siRNA Treatments Knockdown PR70 Protein Levels. HeLa cells were treated with siRNAs directed at different sequences of PR70 for 48 hours. Following the incubation, the samples were resolved on a SDS-PAGE gel and transferred to a nitrocellose membrane. The membrane was blotted with anti-PR70 antibodies. MEKK2 siRNAs were used as a positive control for siRNA treatment.

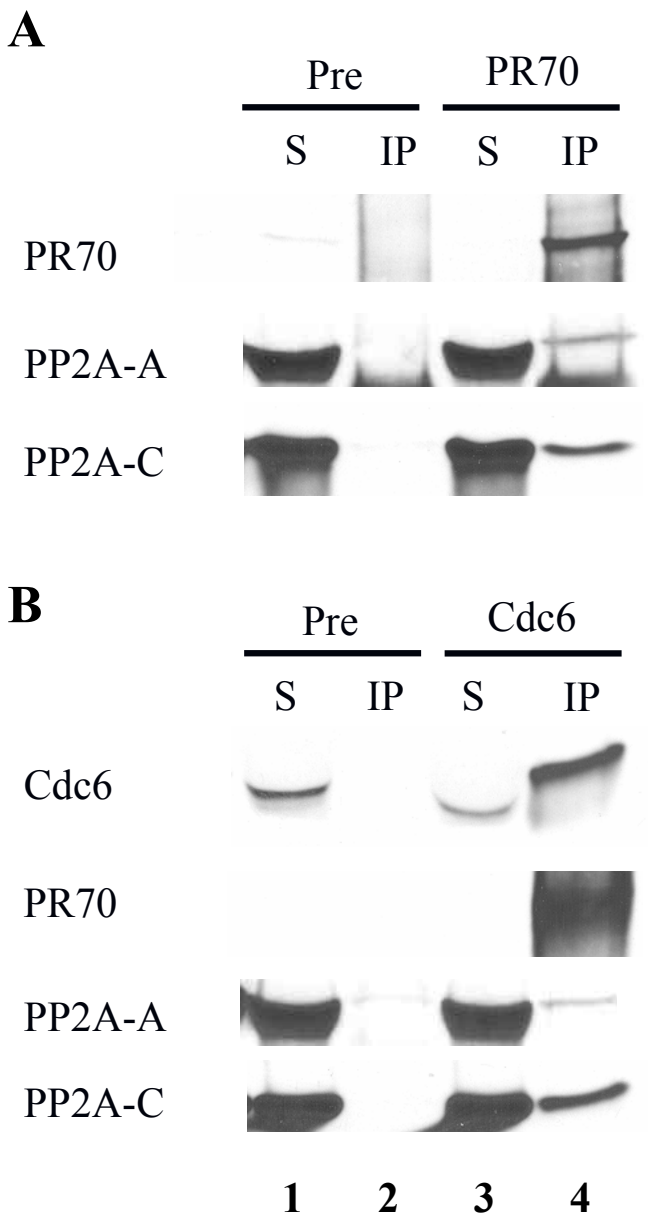

Figure 2.6 Endogenous PR70 Interacts with the PP2A Core Dimer and Cdc6 *in vivo*. (A) Endogenous PR70 was immunoprecipitated from exponentially growing HeLa cells using an antibody specific for PR70. The immunoprecipitations were resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blotted with anti-PR70, anti-A-subunit, and anti-C-subunit antidbodies (B) Endogenous Cdc6 was immunoprecipitated from exponentially growing HeLa cells using an antibody specific for Cdc6. Membrane was blotted with anti-Cdc6 antibody and the antibodies described above.

(PR48) was previously shown to interact with PP2A. This result further validates that PR70 is a bona fide regulatory subunit of PP2A. This is the first study to illustrate that an endogenous human R3 family member interacts with PP2A. Endogenous Cdc6 was not detected in PR70 immunoprecipitates, but PR70 was detected in Cdc6 immunoprepitates. This inconsistency suggests that PR70 interacts with other substrates, and only a proportion of PR70 interacts with Cdc6. This is the first report to illustrate immunoprecipitation of an endogenous quaternary protein complex consisting of the PP2A core dimer, a regulatory subunit, and a putative substrate (Cdc6). Recently, a quaternary complex consisting of human PP2A, PR72, and the Naked protein was reported (Creyghton et al., 2005b). However, this relied on over-expression of epitope-tagged proteins, and no evidence for an endogenous complex was reported. This data illustrates that PR70 forms a complex with Cdc6 and recruits the PP2A core dimer *in vivo*.

Chapter 3

Effects of Calcium on PR70 Function

3.1 Introduction

Calcium is an intracellular second messenger and changes in intracellular calcium concentration control diverse biological processes including muscle contraction, fertilization, apoptosis, gene transcription, and cell division (Berridge et al., 1998). Many of these effects are modulated by calcium binding proteins. The largest class of calcium binding proteins is the EF hand group, which shares a common calcium binding motif. The classical EF hand motif is a helix-loop-helix that contains twelve amino acids, six of which participate in metal coordination (Lewit-Bentley and Rety, 2000). There are two classes of EF hand calcium-binding proteins, calcium buffers and calcium sensors. The calcium buffers bind calcium with lower K_D than the sensors and calcium binding does not cause a conformation change. Binding of calcium by the EF hands in the calcium sensor proteins occurs with a higher K_D and alters the conformation of the motif to expose an adjacent hydrophobic protein-binding surface. As a result, these proteins are able to interact with and regulate secondary effector proteins in a calcium-dependent manner.

The known members of the R3 family of PP2A regulatory subunits all contain two EF hand sequences. A recent report showed that PR72 binds calcium directly and that binding was mediated by the EF hand motifs (Janssens et al., 2003). Mutation of one of the EF hands resulted in a loss of binding to the A subunit of PP2A and altered subcellular localization of

PR72. Although calcium caused an apparent conformational change in PR72, addition of calcium did not affect the phosphatase activity of PR72 containing heterotrimers *in vitro*.

Analysis of the PR70 amino acid sequence identified two EF hand motifs, which lie within a domain conserved within the R3 family. An important question was whether PR70 binds calcium and whether calcium binding affects PR70 function. Results described in this chapter show that PR70 binds to calcium *in vitro* and this binding is weakened when the EF hands are mutated. Addition of calcium enhances the interaction of PR70 with the A subunit of PP2A but not Cdc6. The calcium enhanced binding to the A-subunit is mediated by the second EF hand (EF2). Furthermore, addition of calcium results in recruitment of the PP2A core dimer to the Cdc6-PR70 protein complex. Finally, the addition of calcium to *in vitro* phosphatase assays results in enhanced dephosphorylation of Cdc6 by the PP2A heterotrimers containing a truncated form of PR70.

3.2 Materials and Methods

3.2.1 Mutagenesis of EF hand Motifs

Point mutations were introduced into each EF hand using a PCR-based site directed mutagenesis method (mutated residues underlined). For the introduction of the point mutations, PCR was performed with pCMV-Tag2B containing the full length PR70 cDNA as template with the following primers: **EF1 (x,y)** 5'-CAAGTTCTGGGAGCTGGCCACGGCCCACGACCTGCTCATCG-3' (sense strand) and 5'-CGATGAGCAGGTCGTGGGCCGTGGCCAGCTCCCAGAACTTG-3' (anti-sense strand), **EF1 (-z)** 5'- TTGTGCCGCGCCAGGTTGTCCGCGTCGATGAGC GCTCATCGACGCGGACAACCTGGCGCGGCACAA-3' (sense strand) and 5'- -3' (anti-

sense strand), **EF2 (x,y)** 5'-TGGTTCCGCTGCATGGCCCTGGCCGGGGACGGCGCCCTG-3' (sense strand) and 5'-CAGGGCGCCGTCCCCGGCCAGGGCCATGCAGCGGAACCA-3' (anti-sense strand), and **EF2 (-z)**, 5'-GCGCCCTGTCCATGTTCCAGCTCGAGTACTTCTAC-3' (sense strand) and 5'-GTAGAAGTACTCGAGCTGGAACATGGACAGGGCGC-3' (anti-sense strand). Mutations in both EF hands were introduced using the EF1 mutant cDNAs as template for PCR with primers for introduction of EF2 point mutants. The EF1 sequence was changed from ^{166}**D**T**D**HDLLADADD^{177} to ^{166}**A**T**A**HDLLADADD^{177} for the EF1 (x,y) mutant and ^{166}DTDHDLLADAD**N**^{177} for the EF1(-z) mutant. The EF2 sequence was changed from ^{240}**D**L**D**GDCALSMFE^{251} to ^{240}**A**L**A**GDCALSMFE^{251} for the EF2 (x,y) mutant and ^{240}DLDGDCALSMF**N**^{251} for the EF2 (-z) mutant. The double mutants are a combination of the above mutants (Figure 3.1). The mutations were verified by automated sequencing.

3.2.2 Subcloning and Purification of GST-PR70 and EF hand Mutants

PR70 cDNA was cloned in pGEX-4T-1 (Amersham Biosciences) using the PR70 cDNA sequence described in 2.2.1 and the PR70 EF-hand mutants created in 3.2.1. PCR was performed with pCMV-Tag2B containing the full length PR70 or EF hand Mutant cDNA as template with the following primers: 5'-CGGGATCCATGCCGCCCGGCAAAGT-3 (sense strand) and 5'-ATTTGCGGCCGCTCACAGCGGCTCCAGGTC-3' (anti-sense strand). Following PCR amplification, the product was digested with BamHI and NotI and ligated into pGEX 4T-1 which had been cut with the same restriction enzymes. The resulting construct encodes a full length PR70 or PR70 with EF hand mutations fused to an N-terminal glutathione-S-transferase (GST) epitope tag.

For expression and purification of GST-PR70 and EF hand mutants, 100mL culture of *Escherichia coli (E. coli)* transformed with the cDNAs constructed above were induced for 150 minutes at 37°C with 400μM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich). The cells were pelleted and washed once with PBS. Cells were resuspended in 4mL buffer C (10 mM Tris-Cl, pH 7.4, 1 mM DTT, and protease inhibitor cocktail) and lysed by the addition of lysozyme (2 mg/mL), 130μL of 5 M NaCl, and 180μL 25% Triton X-100 at 4°C for 30 minutes with shaking. The lysate was sonicated, and centrifuged at 30,000 x g for 30 minutes at 4°C to remove cellular debris. The lysate was mixed with 400μL glutathione-agarose (Sigma-Aldrich) overnight at 4°C. The resin was recovered by centrifugation and washed four times with buffer B (20 mM HEPES-KOH, pH 7.6, 100 mM KCl, 1 mM DTT, 1 mM EDTA, and 20% glycerol). The glutathione agarose beads were resuspended in 400μL SDS-PAGE loading buffer, and eluted proteins resolved on a 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue stain. The eluted samples were stored at -80°C until used.

3.2.3 Calcium overlay assay

In vitro $^{45}Ca^{2+}$ overlay assays followed a protocol described previously (Maruyama et al., 1984). Briefly, purified GST-fusion proteins were resolved on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then washed three times in IMK buffer (10mM imidazole-HCl, pH 6.8, 5mM $MgCl_2$, and 60mM KCl) for one hour at room temperature. The membrane was then incubated in IMK buffer containing 5μCi/mL [$^{45}Ca^{2+}$] for 10 minutes. After incubation, the membrane was washed three times in 30% ethanol for five minutes followed by a twelve hour exposure to X-ray film.

3.2.4 Transient Expression, Immunoprecipitations, and Western Blotting of FLAG PR70 and EF Hand Mutants

Protocols were followed as described in 2.2.2 and 2.2.5.

3.2.5 Purification of GST-Cdc6 and GST-A Fusion Proteins

GST-Cdc6 fusion proteins were prepared as previously described with modifications (Herbig et al., 2000). Briefly, 1 liter of Sf9 cells ($2x10^6$ cells/mL) were infected with recombinant GST-Cdc6 baculoviruses (a gift of Dr. Ellen Fanning) at an Sf9 culture:baculovirus ratio of 1:20 (vol/vol) for 60 hours. The cells were pelleted and washed once with PBS. Cells were lysed on ice in 40mL of buffer A (100mM Tris-HCl, pH 7.4, 100mM NaCl, 5mM KCl, 0.5mM $MgCl_2$, 0.5% Nonidet P-40, 1mM DTT, 10mM NaF, 1mM EGTA, 2mM EDTA and a protease inhibitor tablet) using a Dounce homogenizer. Lysates were centrifuged at 30,000 x g for 30 minutes at 4°C to remove cellular debris and the lysate was mixed with 2mL glutathione-agarose (Sigma-Aldrich) for 2 hours at 4°C. The resin was recovered by centrifugation and washed twice with PBS, once with PBS containing 1.5M NaCl, and once with PBS containing 1.5mM NaCl and 0.1% (vol/vol) Nonident P-40, and then reequilibrated in PBS. The GST-Cdc6 fusion protein immobilized on glutathione agarose beads was resuspended in buffer B (20mM HEPES-KOH, pH 7.6, 100mM KCl, 1mM DTT, 1mM EDTA, and 50% glycerol) and stored at -80°C.

GST-A fusion protein was prepared as previously described with modifications (Sontag et al., 1993). 1L cultures of *E. coli* transformed with the cDNAs encoding GST-A were induced for 2 hours at 30°C with 400μM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich). The cells were pelleted and washed once with PBS. Cells were

resuspended in 40mL buffer C (10mM Tris-Cl, pH 7.4, 1mM DTT, and protease inhibitor cocktail) and lysed by the addition of lysozyme (2mg/mL), 1.3mL of 5M NaCl, and 1.8mL 25% Triton X-100. The lysate was sonicated, and centrifuged at 30,000 x g for 30 minutes at 4°C to remove cellular debris. The lysate was mixed with 2mL glutathione-agarose (Sigma-Aldrich) for 2 hours at 4°C. The resin was recovered by centrifugation and washed four times with buffer B with 20% glycerol. The GST-A fusion protein immobilized on glutathione agarose beads was resuspended in buffer B and stored at -80°C until use.

Purified GST protein was a gift of M.H. Cobb lab.

3.2.6 GST- pulldown Assays

GST, GST-A, and GST-Cdc6 immobilized on glutathione agarose beads were used to conduct GST-pulldown experiments. FLAG-PR70 or FLAG-PR70 mutants were over-expressed in transfected COS-7 cells (2.2.2) and cells were lysed as described previously (2.2.5). GST-pulldowns were conducted by incubating the cleared COS-7 lysate with either GST, GST-A or GST-Cdc6. The GST-pulldowns were conducted in lysis buffer (N), in lysis buffer containing 10mM EDTA (E) or lysis buffer containng 10mM $CaCl_2$ (Ca). The samples were incubated for one hour at room temperature with agitation. The calpain inhibitor calpeptin (50μM) (Calbiochem) was added to some experiments. Following incubation, the sample was washed three times with IP Lysis buffer supplemented with EGTA, $CaCl_2$, or $CaCl_2$ and calpeptin and the beads were collected by centrifugation. GST-pulldowns were conducted with increasing amounts of calcium to determine the concentration needed to enhance binding of PR70 to the A-subunit. Free calcium concentrations were calculated using Webmaxc Standard

(http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm). After washing, the pull-downs were solubilized in 60 microliters of 2x SDS-PAGE loading buffer, 30 microliters (50%) were resolved on an 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was probed with anti-FLAG (M2, Stratagene), PP2A C-subunit (1F6), anti-PP2A A-subunit (C-20, Santa Cruz Biotechnology), and anti-GST (Santa Cruz). Blots were developed using the enhanced chemiluminescence detection system (Amersham Biosciences).

3.2.7 His_6-CyclinA-GST-Cdk2 Purification and Activation

GST-Cdk2 was expressed in bacteria and purified following the protocol outlined in 3.2.5 (Purification of GST-A fusion proteins). CyclinA-Cdk2 is activated by cdk activating kinase (CAK). Sf9 cells contain endogenous CAK, so purification of active cdk2/cylcinA was performed by incubating purified GST-cdk2 with Sf9 lysates containing expressed His_6-cyclin A. Sf9 cells were infected with recombinant His_6-cyclin A baculoviruses at an Sf9 culture:baculovirus ratio of 1:20 (vol:vol) for 72 hours. The cells were pelleted and washed once with 20mM HEPES, pH 7.0 and 135mM NaCl. The Sf9 pellet was lysed on ice in Sf9 Lysis Buffer (75mM HEPES, pH 7.4, 75mM NaCl, 2.5mM $MgCl_2$, 0.1mM EDTA, 0.1% Triton X-100, 20mM β-glycerolphosphate, 250μM Na_3VO_4, 1mM DTT, 1mM PMSF, and protease inhibitor cocktail) with a Dounce homogenizer. The lysate was incubated 30 minutes on ice and cleared by centrifugation at 14,000 x g for 30 minutes. For the isolation of the purified active cyclin A-cdk2 complex, 5μg of GST-cdk2, immobilized on glutathione agarose, was incubated with 1mL cleared Sf9 lysate containing His_6-cyclinA supplemented with 1mM ATP. The mixture was incubated at room

temperature with mixing for one hour. This incubation allowed Cdk2 and Cyclin A to interact and CAK to active the cyclin A-cdk2 complex. Following incubation, the beads were collected by centrifugation and washed four times with beads washing buffer (20mM HEPES, pH 7.4, 50mM NaCl, 2.5mM $MgCl_2$, 0.1mM EDTA, 0.1% Triton-X 100, 0.1mM Na_3VO_4, and 10mM β-glycerolphosphate). Following the last wash, the activated GST-cdk2-cyclin A, immobilized on glutathione agarose, was resuspended in kinase buffer without phosphatase inhibitors (50mM HEPES, pH 7.4, 10mM $MgCl_2$, 1mM DTT). Phosphatase inhibitors were not included because they could affect the subsequent dephosphorylation assays. Activity of the purified cdk2/cyclinA was checked by an *in vitro* kinase assay with histone H1 (Calbiochem) following the protocol as described below (3.2.8).

3.2.8 GST-Cdc6 Phosphorylation

To phosphorylate purified GST-Cdc6, 1μg Cdc6 immobolized on glutathione agarose was mixed with 8μL of the cyclin A-cdk2 complex in kinase buffer supplemented with 50μM ATP and 10μCi [γ-^{32}P]-ATP. The sample was incubated for one hour at 30ºC. Following the incubation, the beads were collected by centrifugation and washed four times in wash buffer containing 20mM HEPES, pH 7.4 and 10mM EDTA. The wash buffer contained EDTA to chelate the excess magnesium to stop the kinase reaction. To verify phosphorylation of GST-cdc6, one half of the reaction was solubilized in SDS-PAGE loading buffer and resolved on a 10% SDS-PAGE gel. The gel was fixed and stained with Coomassie Brilliant Blue stain to visualize proteins. The gel was dried and exposed to X-ray film to assess the degree of Cdc6 phosphorylation. Large scale phosphorylation of GST-

Cdc6 was performed for subsequent use in PP2A dephosphorylation assays. For the large scale phosphorylation, 100µg of Cdc6 was phosphorylated as described above using conditions scaled to fit the reaction conditions. Following the washes, the phosphorylated GST-Cdc6 was resuspended in phosphatase buffer (50mM Tris, pH 7.6, 0.7mg/mL BSA, 50mM NaCl, 0.4mM EDTA) supplemented with 20% glycerol, aliquoted, and stored at -80°C until used.

3.2.9 GST-Cdc6 Dephosphorylation Assays

Dephosphorylation assays of ^{32}P-labeled GST-Cdc6 with different PP2A oligomers were performed. PP2A heterotrimers (including AC-PR48, AC-Bα, and AC-B56γ3) and the PP2A core dimer were reconstituted in Sf9 cells and purified by members of the Mumby lab as described previously (Yan et al., 2000). For dephosphorylation assays, 10µg of phosphorylated Cdc6 was incubated with 5µg of each PP2A oligomer in phosphatase buffer at 30°C for 30 minutes. AC-PR48 was incubated alone or in the presence of 10mM EGTA or 10mM $CaCl_2$. Following the incubation, the beads were pelleted and the supernatant was removed. The reaction was stopped by addition of SDS-PAGE loading buffer and resolved on a 10% SDS-PAGE gel. The gel was fixed and stained with Coomassie Brilliant Blue stain. The gel was dried and exposed to X-ray film to assess the degree of dephosphorylation. The ImageQuant software of PhosphorImager (Molecular Dynamics) was used to scan the Coomassie stained Cdc6 band to normalize for apparent protein concentration. The protein bands were then excised (including a comparable sized band with no protein as background control) from the dried gel and radioactivity measured by liquid scintillation counting (Beckman) to quantitate the ^{32}P content of GST-Cdc6.

Phosphorylated GST-Cdc6 without phosphatase treatment was used at the standard (100%) phosphorylation level of Cdc6. ^{32}P remaining was calculated by first dividing the CPM of Cdc6 without phosphatase treatment with its relative protein level. This calculated value was set at 100% phosphorylation. The CPM/relative protein level of each sample was determined similarly. The % ^{32}P remaining for each sample was calculated by dividing its calculated value by the calculated value of Cdc6 without phosphatase treatment value.

3.3 Results

3.3.1 PR70 Contains Two Conserved EF hand Calcium Binding Motifs and Binds Calcium in vitro

Analysis of the amino acid sequence of PR70 identified two conserved EF hand calcium binding motifs. These motifs are conserved throughout the R3 family of regulatory subunits (Figure 3.1A). To test whether PR70 is a calcium binding protein and if calcium affects function, two sets of point mutants were constructed with inactivating substitutions of amino acids directly involved in calcium binding (Strynadka and James, 1989). Mutations included alanine substitutions at both the X and Y coordinates and a conservative change at the –Z coordinate (Figure 3.1B). *In vitro* $^{45}Ca^{2+}$ overlay assays were performed with GST-PR70 and the EF hand mutants. PR70 bound calcium (Figure 3.2, Lane 2). Mutation of EF1 resulted in reduced binding of calcium compared to wild-type PR70 (Figure 3.2, Lanes 3 and 4), whereas mutation of EF2 and double mutation of EF1 and EF2 disrupted the ability of PR70 to interact with with calcium (Figure 3.2, Lanes 6-9). The negative control, GST-A, did not bind to calcium (Figure 3.2, Lane 1). The data demonstrate PR70 binds calcium *in vitro* and this binding requires functional EF hand motifs.

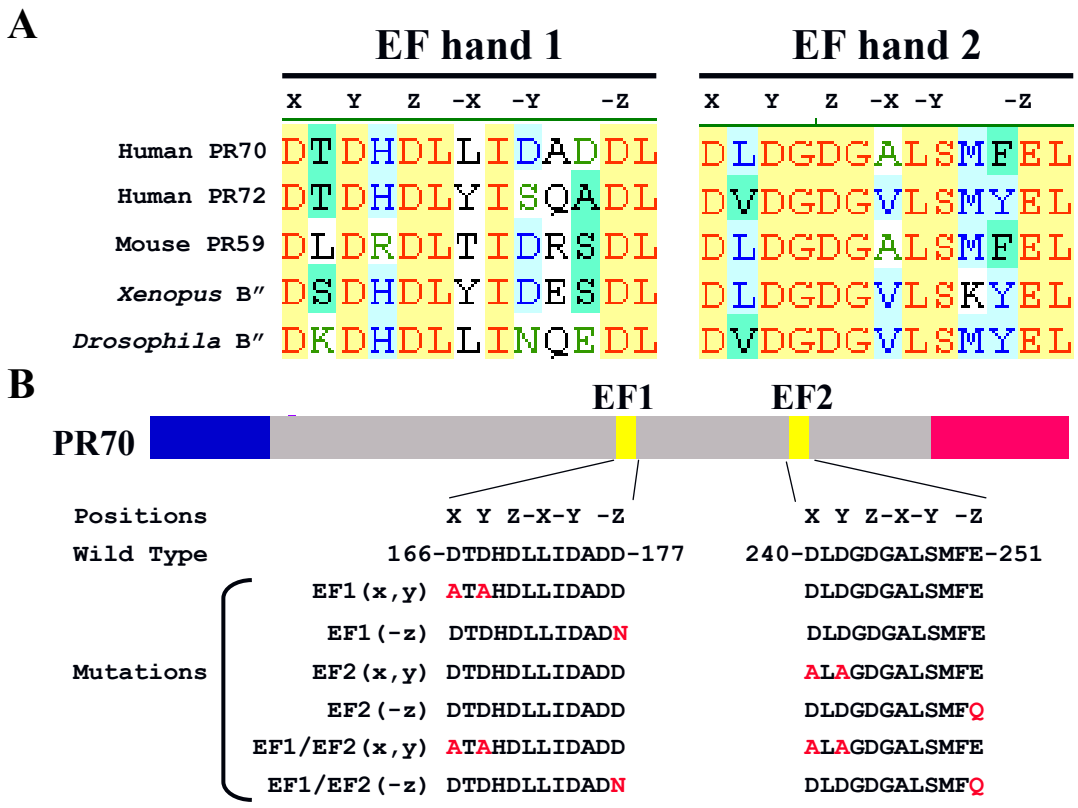

Figure 3.1 Two Putative EF Hand Motifs Are Conserved In the R3 Regulatory Subunit Family of PP2A. (A) Alignment of EF hand Motifs of eukaryotic R3 subunits. Identical residues present in every family member are shaded in yellow. Conservedresidues are shaded in teal and blue. Coordinates of the EF hand motif are marked above the alignment. (B) Mutations of EF1 and EF2 created. Mutated residues are in red.

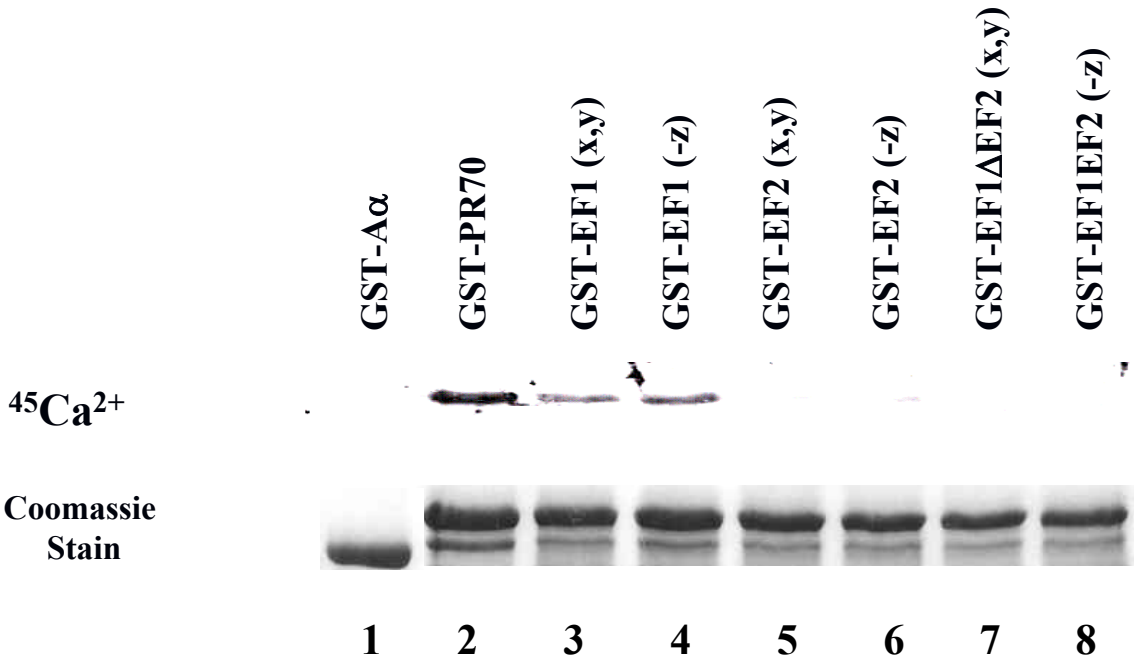

Figure 3.2 PR70 Binds to Calcium in a Gel Overlay Assay and Calcium Binding is Mediated by Functional EF Hand Motifs. PR70 and EF hand mutant proteins were resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was washed and then incubated with 5μCi/mL [$^{45}Ca^{2+}$] for minutes followed by three washes. The nitrocellulose was dried and calcium binding was visualized by autoradiography.

3.3.2 Mutation of EF2 Results in Weakened Interaction of PR70 with PP2A

To determine if mutations to the EF hands affect the ability of PR70 to interact with PP2A, FLAG-tagged versions of each mutant were transfected into COS-7 cells. FLAG-PR70 and EF hand mutants were immunoprecipitated and incorporation into PP2A heterotrimers was assessed by blotting FLAG immoprecipitates for PP2A A- and C-subunits. Wild-type PR70 and EF1 mutants interacted with endogenous PP2A (Figure 3.3, Lane #2, 3, and 4). Mutation of EF2 or both EF1 and EF2 resulted in a significant loss of interaction with PP2A (Figure 3.3, Lane 5, 6, 7, and 8). A longer exposure of the blot shows a weak interaction of the A- and C-subunits with EF2 mutants and EF1 EF2 double mutants (Figure 3.3 OE, Lane 5, 6, 7, and 8). These results show that mutation of EF2 affects binding of PR70 to the PP2A core dimer.

3.3.3 Calcium Enhances the Binding of PR70 to GST-A and Recruits A- and C-Subunits of PP2A to GST-Cdc6

The effects of calcium on the interaction of PR70 with PP2A and Cdc6 were determined using GST pull-downs assays. FLAG-tagged PR70 was expressed in COS-7 cells and cells were lysed in lysis buffer containing EDTA. The lysates were incubated with GST-A or GST-Cdc6 and bound proteins detected by immunoblotting. PR70 interacted with GST-A and GST-Cdc6 in these cell free conditions (Figure 3.4, Lanes 2 and 7 respectively). Compared to lysates alone or lysates containing EGTA, the addition of calcium enhanced the binding of PR70 to GST-A but not to GST-Cdc6 (Figure 3.4, Lanes 4 and 9 respectively) There was a small amount of cleavage of FLAG-PR70 present in the calcium lane, which was blocked by the addition of the calpain inhibitor calpeptin (Figure 3.4, Lane 4 OE).

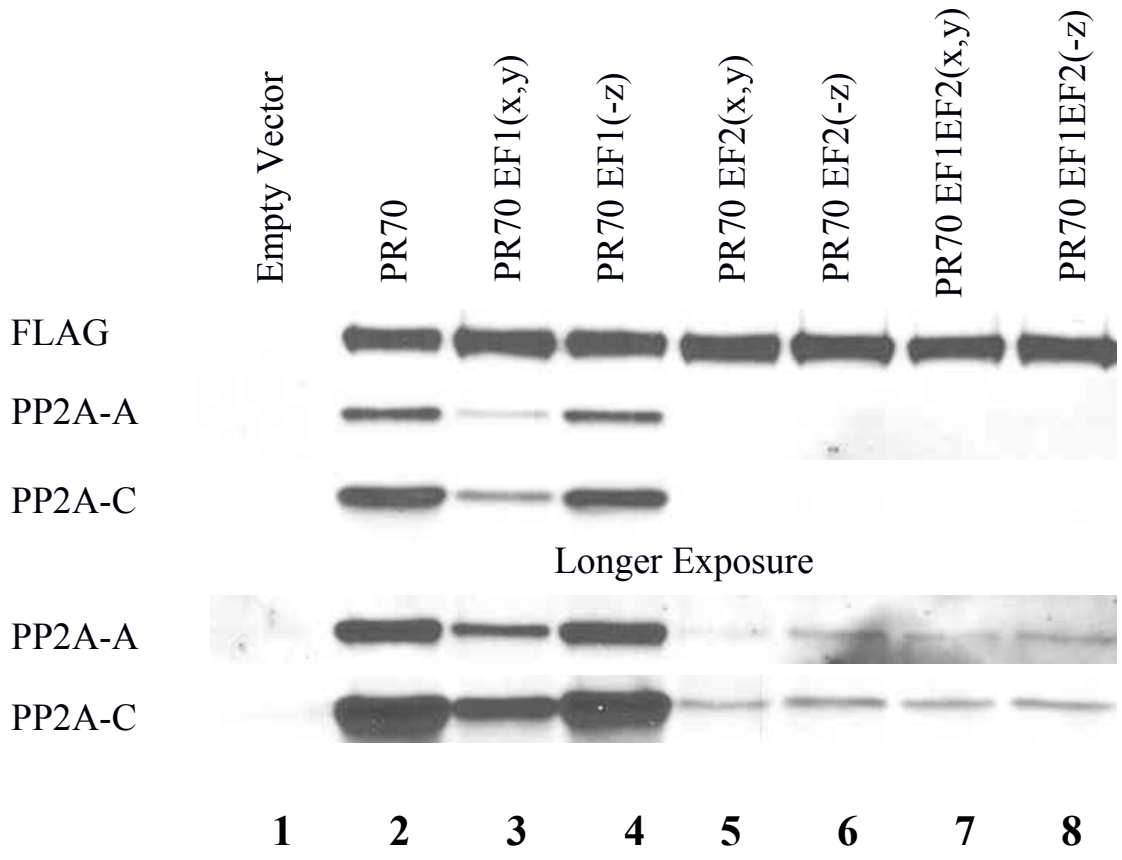

Figure 3.3 Mutation to EF2 Affects Interaction of PR70 with PP2A Core Dimer. COS-7 cells were transiently transfected with cDNA for FLAG epitope tagged PR70 and EF hand mutants. Following 24 hour incubation, the cells were lysed and the FLAG-PR70 and EF hand mutants were immunoprecipitated using anti-FLAG antibody. The immunoprecipitations were resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blotted with anti-FLAG, anti-PP2A A-subunit, and anti-PP2A C-subunit antibodies.

Although calcium did not enhance the binding of PR70 to GST-Cdc6, it did result in an increase in the amount of PP2A A- and C-subunit associated with GST-Cdc6 (Figure 3.4, Lane 9).

A large excess of free calcium (10mM) was used in the initial PR70 pull-down experiments with GST-A and GST-Cdc6. In order to test whether physiological levels of calcium had similar effects, calcium titrations were performed. GST-pulldown assays were performed with increasing amounts of calcium and binding of FLAG-PR70 to GST-A was monitored. There is a small amount of PR70 binding to GST-A even in the presence of very low calculated free calcium concentrations (1nM) (Figure 3.5). An increase in the binding of FLAG-PR70 to GST-A was observed at calculated free calcium of 30nM calcium and the effect appeared to saturate at 100μM free calcium (Figure 3.5). The data shows that calcium enhances the binding of PR70 to GST-A, even at physiological calcium concentrations. Futhermore, addition of calcium does not enhance the interaction of PR70 with Cdc6, but recruits PP2A core dimer to the PR70-Cdc6 complex.

3.3.4 Mutation of EF2 Affects the Calcium Enhanced Binding of PR70 to GST-A and Recruitment of PP2A A- and C-Subunits to Cdc6

To test the function of the EF hands in the calcium dependent interaction of PR70 with the AC core dimer, GST pull-downs were repeated with the EF hand mutants containing conservative changes at the –Z position. Compared to assays in the presence of EGTA, the addition of calcium resulted in enhanced binding of PR70 and the EF1 mutant to GST-A, but not to GST-Cdc6 (Figure 3.6A and B, Lanes 3 and 5, respectively). However, the addition of calcium increased the association of the PP2A core dimer with GST-Cdc6 (Figure 3.6B,

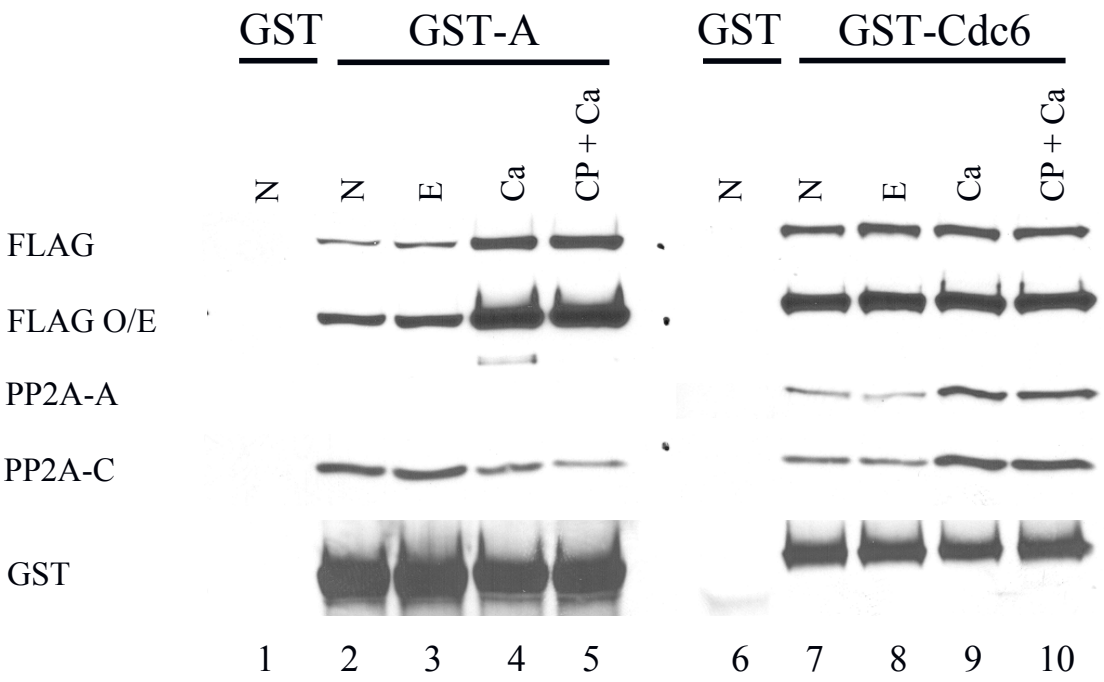

Figure 3.4 Calcium Enhances Binding of PR70 to the A-subunit of PP2A but not to Cdc6. FLAG-PR70 was transiently expressed in COS-7 cells for 24 hours and the cells were lysed. GST-pulldowns were performed using immobilized GST-A and GST-Cdc6 with lysates alone, or in the presence of 10mM EGTA or 10mM $CaCl_2$. 50μM calpeptin was added to block proteolysis of FLAG-PR70 in the presence of calcium. The proteins bound to GST-A and GST-Cdc6 were resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blotted with anti-FLAG, anti-PP2A A-subunit, anti-PP2A C-subunit,and anti-GST antibodies. (N=Lysis buffer, E=10mM EGTA, Ca=10mM $CaCl_2$, CP=Calpeptin)

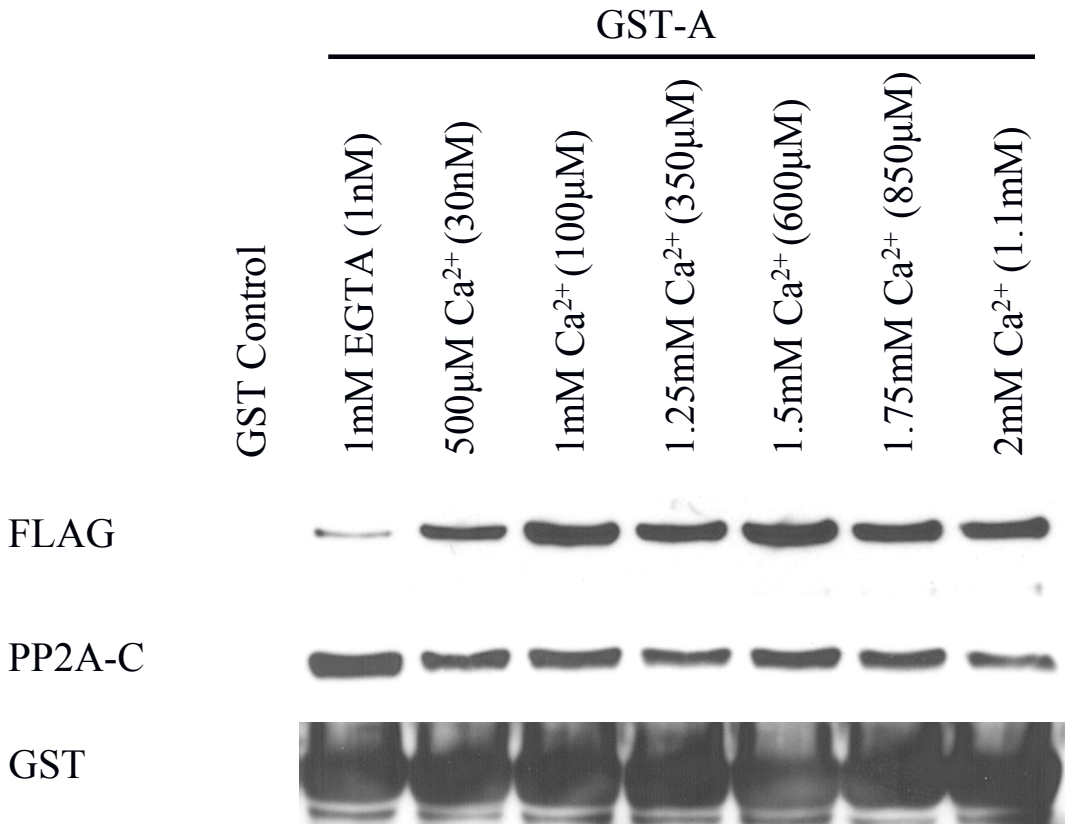

Figure 3.5 Calcium Titration for Calcium Enhanced Binding of PR70 to GST-A. FLAG-PR70 was transiently expressed in COS-7 cells for 24 hours and the cells were lysed. GST-pulldowns were performed using immobilized GST-A and lysates containing increasing concentrations of free calcium (shown as the calculate values). The proteins bound to GST-A were resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blotted with anti-FLAG, anti-PP2A A-subunit, anti-PP2A C-subunit, and anti-GST antibodies.

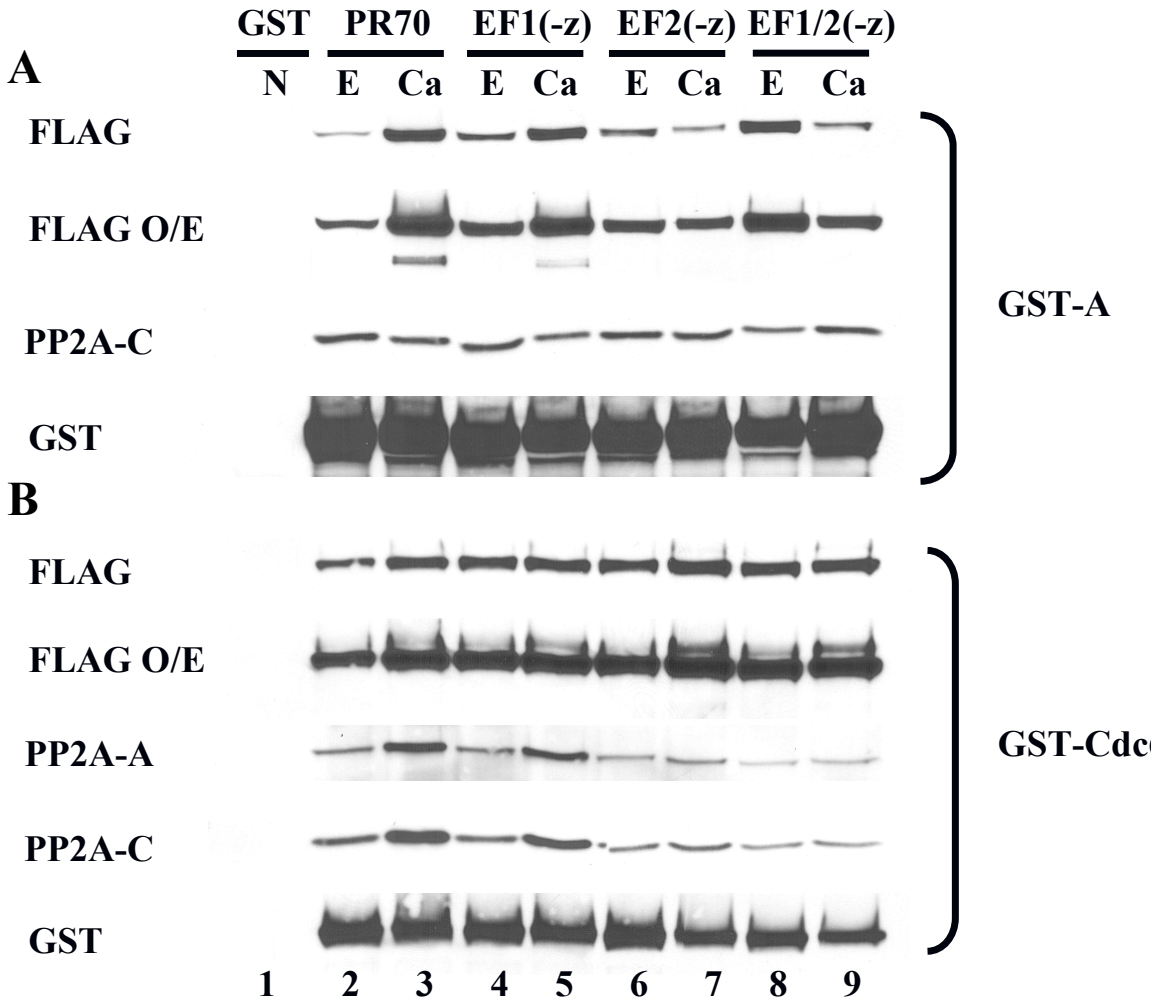

Figure 3.6 Mutation to EF2 Affects the Calcium Enhanced Binding of PR70 to GST-A and Recruitment of PP2A A- and C-Subunits to Cdc6. FLAG-PR70 and EF hand mutants were transiently expressed in COS-7 cells for 24 hours and the cells were lysed. GST-pulldowns were performed using immobilized GST-A and GST-Cdc6 with lysates alone, or in the presence of 10mM EGTA or 10mM $CaCl_2$. The proteins bound to GST-A and GST-Cdc6 were resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blotted with anti-FLAG, anti-PP2A A-subunit, and anti-PP2A C-subunit, and anti-GST antibodies.

Lanes 3 and 5, respectively). Mutation of EF2 or double mutation of EF1 and EF2 resulted in a loss of the calcium-enhanced binding of PR70 to GST-A (Figure 3.6A, Lanes 7 and 9). Consistent with the loss of calcium enhanced binding to GST-A, there is a loss in the calcium-dependent recruitment of the PP2A core to GST-Cdc6. This data along with the previous data illustrate that calcium binding enhances the interaction of PR70 with PP2A and recruits the PP2A core dimer to the PR70-Cdc6 complex.

3.3.5 Calcium Stimulates Dephosphorylation of Cdc6 by the AC-PR48 Heterotrimer

Since calcium enhances the interaction of PR70 with the PP2A core dimer and the association of the PP2A core dimer with Cdc6, a key question was whether addition of calcium stimulated dephosphorylation of Cdc6 by the AC-PR70 heterotrimer. To address this question, *in vitro* phosphatase assays were performed using recombinant GST-Cdc6 protein purified from Sf9 cells, which had been labeled *in vitro* with Cyclin A-Cdk2 and [γ-^{32}P]ATP. ^{32}P-labeled Cdc6 was incubated with different purified PP2A oligomers. These experiments were done prior to construction of a full-length PR70 cDNA and utilized the truncated PR48 form of the protein. Since PR48 interacts with PP2A and Cdc6 similarly to full length PR70, the results are likely to be similar to those with full length PR70. The dephosphorylation assays with AC-PR48 were performed in the presence of 10mM calcium or 10mM EGTA. Each PP2A oligomer had a basal level of phosphatase activity towards ^{32}P-labeled Cdc6 (Figure 3.7). In the absence of calcium, the holoenzyme containing PR48 had similar activity towards ^{32}P-labeled Cdc6 as the other PP2A oligomers (Figure 3.7). In the presence of free calcium, dephosphorylation of Cdc6 by AC-PR48 was significantly enhanced (Figure 3.7). The addition of EGTA had no effect on AC-PR48 activity. These

results suggest that calcium stimulates the phosphatase activity of the AC-PR48 holoenzyme toward Cdc6.

3.4 Discussion

PR70 contains two conserved EF hand motifs that are shared with the other members of the R3 family. Like PR72, PR70 binds calcium *in vitro*. Mutation of EF1 reduced calcium binding, whereas mutation of EF2 completely abolished calcium binding in a gel overlay assay. The results show that EF2 mutants did not bind to calcium, even though they had intact EF1 motifs. Many calcium binding proteins contain tandem EF hands. The pairing of adjacent EF hands results in cooperativity in calcium binding. It is likely that EF2 mutants do not bind calcium because calcium binding to EF2 stimulates the ability of EF1 to bind calcium. When combined with the data on PR72, these results argue that, all members of the R3 family will have enhanced interaction with the AC core dimer in the presence of calcium.

Mutations of EF1 did not affect the ability of PR70 to incorporate into PP2A heterocomplexes. In contrast, mutation of EF2 resulted in weakened binding to the PP2A core dimer. This result was probably not due to a change in the overall structure of the protein, because (a) EF2 mutants were able to interact with GST-A *in vitro* (b) EF2 mutants were able to interact with GST-Cdc6 *in vitro* and (c) similarmutations in the PR72 protein did not change the protein structure as measured by intrinsic tryptophan fluorescence (Janssens et al., 2003). This suggests that the effects seen with EF2 are due to a loss in calcium binding and not structural changes of the protein.

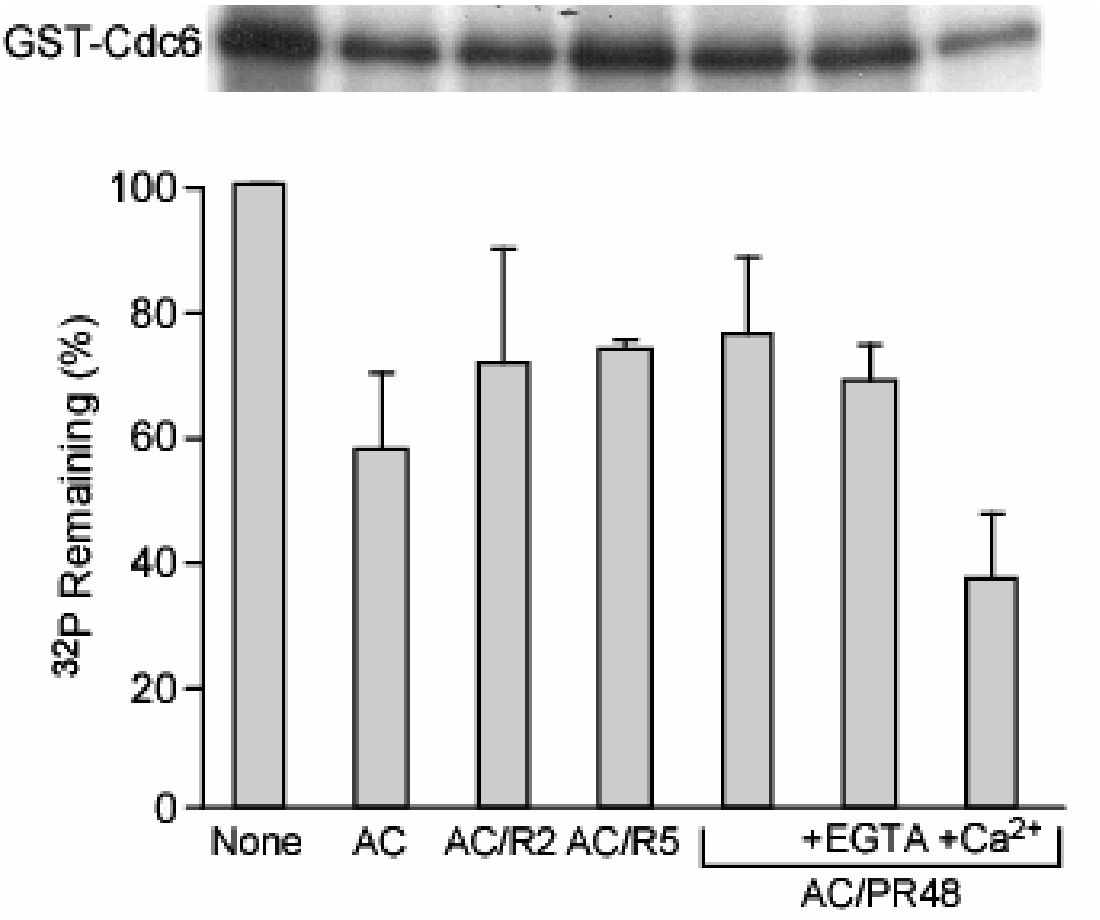

Figure 3.7 Ca^{2+} Stimulates Dephosphorylation of Cdc6 by the AC-PR48 Heterotrimer. (A) Recombinant (Sf9 cells) GST-Cdc6 was phosphorylated in vitro with cyclin A/CDK2 and [^{32}P]-ATP. Phosphorylated Cdc6 was incubated with different purified PP2A oligomers for 30 minutes. AC-PR48 was incubated alone or with 10mM calcium or 10mM EGTA. The level of Cdc6 phosphorylation was determined by SDS-PAGE and autoradiography. (B) Results from four independent Cdc6 dephosphorylation assays. [^{32}P] remaining was determined by scintillation counting and protein concentrations were normalized.

The R3 family members are probably in the 'calcium sensor' class of the EF hand proteins. In most calcium sensors, binding of calcium induces a large conformational change that exposes a hydrophobic patch. Exposure of the patches leads to enhanced interactions with substrates and effector proteins and is the basis for calcium-dependent regulation by these proteins. The enhanced binding of PR70 to the A-subunit in the presence of calcium may be due to a conformational change in PR70, which exposes a hydrophobic patch. There is a calcium-induced conformational change in PR72 (Janssens et al., 2003). It seems likely that calcium-binding causes a similar conformational change in PR70.

Neither the addition of calcium, nor mutation of the EF hands affected the binding of PR70 to GST-Cdc6. However, calcium caused a significant increase in the amount of PP2A A- and C-subunits associated with Cdc6. This recruitment of PP2A core dimer to GST-Cdc6 is dependent on a functional EF2. In the presence of free calcium, dephosphorylation of Cdc6 by AC-PR48 was significantly enhanced. These results suggest that PR70 binds to Cdc6 irrespective of calcium, but upon calcium binding, affinity for the PP2A core dimer and Cdc6 dephosphorylation are enhanced. The finding that calcium recruits the PP2A core dimer to the PR70-Cdc6 protein complex may represent a novel calcium signaling pathway that can affect DNA replication.

Addition of calcium resulted in proteolytic cleavage of FLAG-PR70, even in the presence of a general protease inhibitor tablet. This proteolysis was blocked by the addition of the calpain inhibitor, calpeptin. mCalpain has previously been reported to interact with PR72, and mCalpain cleaves PR72 (personal communication with Christine van Hoof) Our data show that mCalpain cleaves at the C-terminus of PR70, because the N-terminal FLAG

epitope is still intact. EF2 mutants and EF1 EF2 double mutants are not cleaved by calpain (Figure 3.5, Lanes 7 and 9, respectively), but wild-type and EF1 are (Figure 3.5, Lanes 3 and 5, respectively), which suggests that calpain recognizes a site on PR70 exposed when it is in complex with the PP2A core dimer or when calcium is bound. This also suggests that calpain cleavage of PR70 requires binding of calcium to EF2. The calpain cleavage product was still able to interact with PR70, but not with Cdc6. Data in Chapter 4 show that deletion of the C-terminus of PR70 results in weakened binding to Cdc6 *in vitro*. Based on these observations, a model can be proposed in which, an influx of calcium causes calcium binding to PR70, recruitment of the PP2A core dimer to PR70-Cdc6 protein complexes, and enhanced dephosphorylation of Cdc6. This calcium flux would also activate the calcium regulated protease mCalpain, which would cleave at the C-terminus of PR70 and block the interaction of PP2A with Cdc6. This would lead to phosphorylation of Cdc6 and stabilization of the protein by cyclin E-cdk2 and allow formation of pre-RCs and DNA replication.

Chapter 4

The Molecular Basis for PR70 Interactions with PP2A and Cdc6

4.1 Introduction

Interaction of the regulatory subunits with the core dimer is critical for PP2A function. However, relatively little is known about the molecular basis for these interactions. The regulatory subunits bind to the core dimer through contacts with both the A- and the C-subunits. The A-subunit serves as a scaffold for assembly of the heterotrimer and consists of 15 nonidentical HEAT repeats (Groves et al., 1999). Early work mapped the binding sites for the C-subunit and regulatory subunits in the A-subunit. The regulatory subunits bind to repeats 1-10 and the C-subunit binds to repeats 11-15 of the A-subunit (Ruediger et al., 1994). The regulatory subunits bind to the core dimer in a mutually exclusive manner such only a single regulatory subunit is present in the holoenzyme. Mutational analysis of the A-subunit identified residues that are important for interacting with all of the regulatory subunits and others specific for interaction with individual regulatory subunit families (Ruediger et al., 1999). In contrast to the A-subunit, relatively little is known about the residues in the regulatory subunits important for mediating the interaction to the PP2A core dimer. The PP2A regulatory subunits have little amino acid conservation between the different families. A loosely conserved A-subunit binding domain (ASBD) was identified in multiple regulatory subunit families (Li and Virshup, 2002). This report suggested that the regulatory subunits contain a conserved structure responsible for

interacting with the PP2A core dimer, but no residues involved in mediating these interactions were identified. The R2 regulatory subunits contain WD domains, which have been proposed to be a conserved motif responsible for interaction with the PP2A core dimer (Griswold-Prenner et al., 1998). A recent study identified a cluster of conserved charged residues of the R2 family member Bγ which are important for interaction with the PP2A core dimer (Strack et al., 2002). This is the first report to identify specific residues within a regulatory subunit important for interaction with the A-subunit. Even less is known about regions or residues that are important for the interactions of regulatory subunits with PP2A substrates.

Comparison of the amino acid sequences of members of the R3 regulatory subunit family identified a highly conserved R3 domain. This conserved domain is flanked by regions unique to individual family members. In order to understand how PR70 functions, it is important to identify the specific region(s) and residue(s) responsible for mediating interaction of PR70 with the PP2A core dimer and Cdc6. A series of PR70 truncation mutants were constructed to define region(s) of PR70 important for interaction with PP2A and Cdc6. Truncation analysis of PR70 identified a region in the N-terminus of the conserved R3 domain important for mediating interaction with PP2A. Sequence analysis of this region identified a hydrophobic patch important for the interaction of PR70 with the core dimer. The PR70-unique region at the C-terminus was shown to be important in mediating the interaction between PR70 and Cdc6.

4.2 Materials and Methods

4.2.1 Generation of Truncation Mutations and Site-directed Mutagenesis

PR70 truncation mutants were generated by PCR amplification using the PR70 cDNA as template. ΔN1 (aa 125-575) corresponds to PR48 described previously (Yan 2000). ΔN2, ΔN3, and ΔC1 were generated using the following primers: ΔN2 (aa 136-575) 5'-CGGGATCCGCCACCATGGATGACATG-3', ΔN3 (aa 162-575) 5'-CGGGATCCAGGACTCCGTCAACGTG-3', and ΔC1 (1-441) 5'-CCCAAGCTTCATCTGGCAGAGGCAGTC-3'.

Point mutations were introduced in the FYFP motif of PR70 using a PCR-based site directed mutagenesis method (mutated residues underlined). For the introduction of the point mutations, PCR was performed with pCMV-Tag2B containing the full length PR70 cDNA as template with the following primers: **AYFP**, 5'-GCCAAAGCATTCCGACCGCCTACTTCCCCAGAGGACG-'3 (sense strand) and 5'-CGTCCTCTGGGGAAGTAGGCGGTCGGAATGCTTTGGC-3' (anti-sense strand), **FAFP**, 5'-CCAAAGCATTCCGACCTTCGCCTTCCCCAGAGGACGCC-3' (sense strand) and 5'-GGCGTCCTCTGGGGAAGGCGAAGGTCGGAATGCTTTGG-3' (anti-sense strand), **FYAP**, 5'-GCATTCCGACCTTCTACGCCCCCAGAGGACGCCCGC-3' (sense strand) and 5'-GCTTTCGTCCTCTGGGGGCGTAGAAGGTCGGAATGC-3' (anti-sense strand). To make the AYAP mutant, the AYFP cDNA was used as a template and PCR mutagenesis was done with the following primers **AYAP**, 5'-CATTCCGACCGCCTACGCCCCCAGAGGACGCCCG-3' (sense strand) and 5'-CGGGCGTCCTCTGGGGGCGTAGGCGGTCGGAATG-3' (anti-sense strand). To make

the AAAP mutant, the AYAP cDNA was used as a template and PCR mutagenesis was done with the following primers **AAAP**, 5'-GCATTCCGACCGCCGCCGCCCCCAGAGGACG-3' (sense strand) and 5'-CGTCCTCTGGGGGCGGCGGCGGTCGGAATGC-3' (anti-sense strand). The mutations were verified by automated sequencing.

4.2.2 Transient Expression and Immunoprecipitations of FLAG-PR70 and Mutants and Western Blotting

Protocols were followed as described in 2.2.2 and 2.2.5.

4.2.3 GST-Pulldowns Assays

Protocols were followed as described in 3.2.6

4.3 Results

4.3.1 Two N-terminal truncation mutants fail to interact with PP2A

Analysis of the amino acid sequence of the R3 regulatory subunit family identified a conserved region in the central domain of each family member and unique sequences at the N- and C-termini (Figure 4.1A). A series of PR70 truncation mutants were constructed to define which region(s) within the R3 domain of PR70 are important for interaction with PP2A (Figure 4.1B). N-terminal FLAG epitope tagged truncation mutants of PR70 were transiently expressed in COS-7 cells for and immunoprecipitated with anti-FLAG antibody. To assay the ability of the truncation mutants to incorporate into PP2A heterotrimers in intact cells, immunoprecipitates were blotted for endogenous PP2A A- and C-subunits. ΔN1 (previously known as PR48, aa 125-575), which deletes the N-terminal PR70 unique sequence, but contains the entire conserved R3 domain, interacts with PP2A similarly to full length PR70 (Figure 4.2, Lanes 2 and 3, respectively). Deletion of the C-terminal PR70

unique sequence (ΔN1) also had no effect on the ability of PR70 to interact with PP2A (Figure 4.2, Lane 6). Two deletions into the N-terminus of the conserved R3/PR72 domain, ΔN2 (aa 125-575) and ΔN3 (aa 136-575), resulted in proteins that failed to interact with PP2A *in vivo* (Figure 4.2, Lanes 4 and 5, respectively). The data shows that the R3 domain is sufficient to interact with the PP2A core dimer.

4.3.2 A Hydrophobic Patch in the N-terminus of the R3 Domain is Involved in Interaction with PP2A

The data described in 4.3.1 shows that amino acids 125-136 at the N-terminus of the conserved R3 domain are important for the association of PR70 with the A- and C-subunits. Detailed examination of this region identified a region that is highly conserved within the R3 family members that contains a hydrophobic patch consisting of amino acids FYFP (Figure 4.3A). FxFP motifs have been shown to be important in mediating protein-protein interactions between MAPK ERK and substrates (Jacobs et al., 1999). The sequence is also similar to sequence motifs (FxFG, GLFG, and PSFG) that are important for mediating the interaction between the nucleoporins and importins (Bayliss et al., 2000). In order to test for a possible role of the FxFP motif in the interaction of PR70 with the A- and C-subunits, these residues were altered by site-directed mutagenesis. The FYFP motif was changed to alanines, including double and triple mutations (Figure 4.3B), and the ability of the mutants to interact with PP2A was tested. Mutation at any of the sites within the FYFP motif resulted in a loss of interaction with PP2A (Figure 4.4, Lanes 4-8). A longer exposure of the blot showed there is a small amount of A- and C-subunit that interacted with each of the FYFP mutants (Data not shown). These results show that mutation to the FYFP motif affects

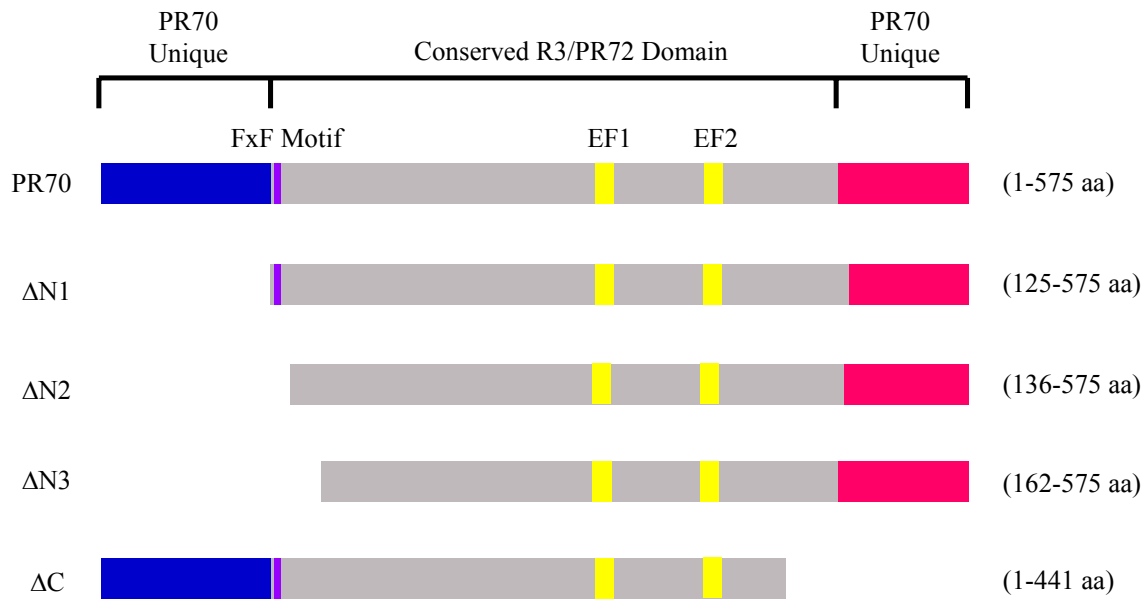

Figure 4.1 Schematic of PR70 Regions and Deletion Mutants Created. Alignment of the R3/PR72 family identified a conserved region in the central portion of the protein, and unique sequences at the N- and C-termini of each family member. Deletions mutations were constructed and tested for their ability to associate with endogenous PP2A core dimer.

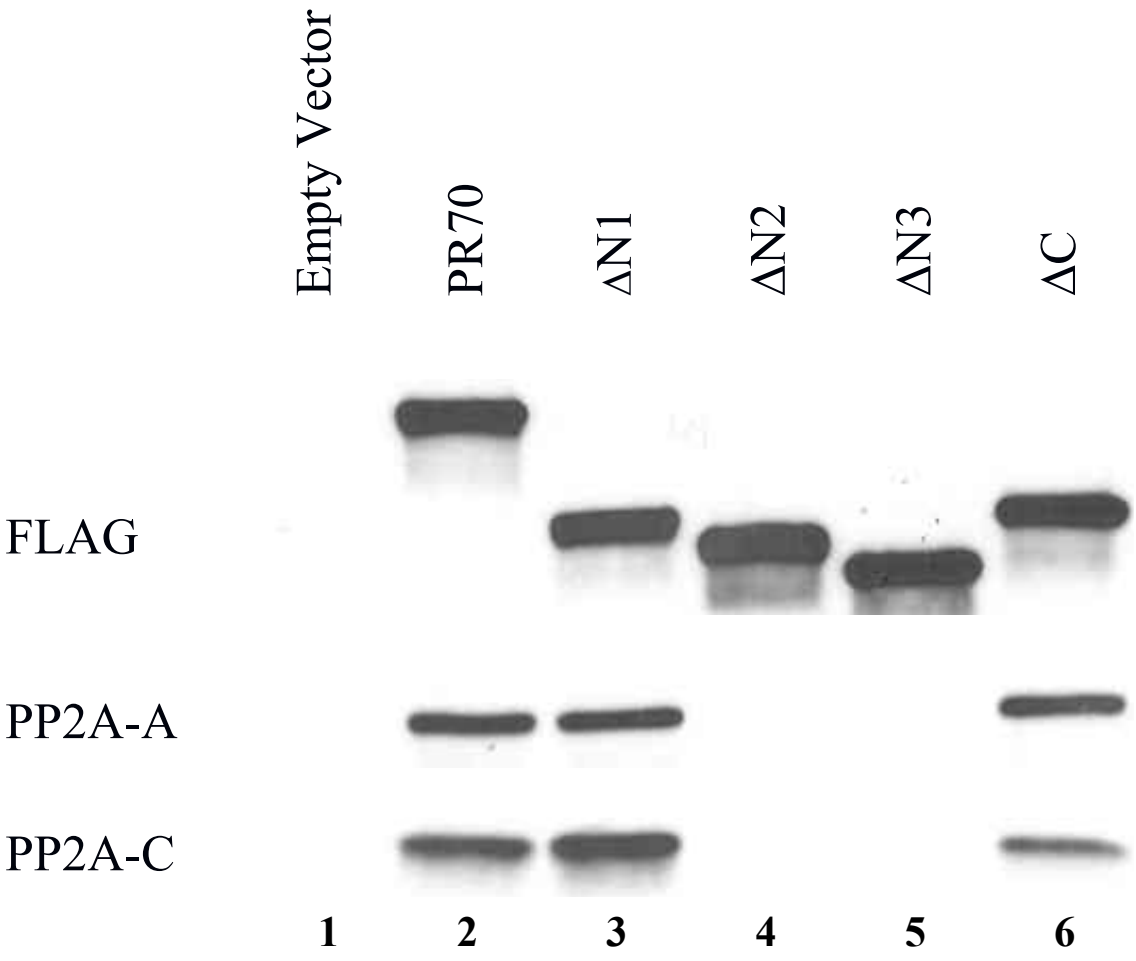

Figure 4.2 Mapping of PP2A Association Domains of PR70 By Deletion Mutagenesis. COS-7 cells were transiently transfected with cDNA for FLAG epitope tagged PR70 and various truncation mutants. Following 24 hour incubation, the cells were lysed and the FLAG-PR70 and FYFP mutants were immunoprecipitated using anti-FLAG antibody. The immunoprecipitations were resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blotted with anti-FLAG, anti-PP2A A-subunit, and anti-PP2A C-subunit antibodies.

binding of PR70 to the PP2A core dimer. When the FYFP mutants were assayed in GST-pulldown experiments, they all bound to GST-A (Figure 4.5). This suggests that the FYFP is not absolutely essential for interaction of PR70 with the A-subunit.

Although no FYFP motif is found in the R2 or R5 subunit families, sequence alignments identified related FDY and FDF conserved motifs in each family (Figure 4.6). Similar to the R3 family members, these conserved sequences are located in the N-terminus of the protein. To test if the FDF motif might play a role in the interaction of these subunits, both phenylalanines of the R5 family member R5γ3 were mutated to alanine. Mutation of the FxF residues in R5γ3 did not affect binding to PP2A (Figure 4.7). This data suggests interaction with the PP2A core dimer by a hydrophobic patch is only present in the R3 regulatory subunit family.

4.3.3 Different Regions of PR70 Mediate Interactions with PP2A and Cdc6

In previous experiments, the addition of calcium resulted in a small amount of cleavage of PR70 (Figure 3.3, Lane 4). This cleavage occurs at the C-terminus, because the N-terminal FLAG epitope is still intact. In those experiments, the cleaved product interacted with PR70, but not with Cdc6 (Figure 3.3, Lanes 4 and 9, respectively). This observation led to the hypothesis that the C-terminus of PR70 mediates interaction with Cdc6. To test this, GST pulldowns were performed with full length PR70, the ΔN3 and ΔC mutants in the absence and presence of calcium. As described previously, full length PR70 and ΔC interacted with GST-A (Figure 4.8A, Lane 2 and 8, respectively), and ΔN3 did not (Figure 4.8A, Lane 5). Furthermore, there was an enhanced interaction of PR70 and ΔC with GST-A

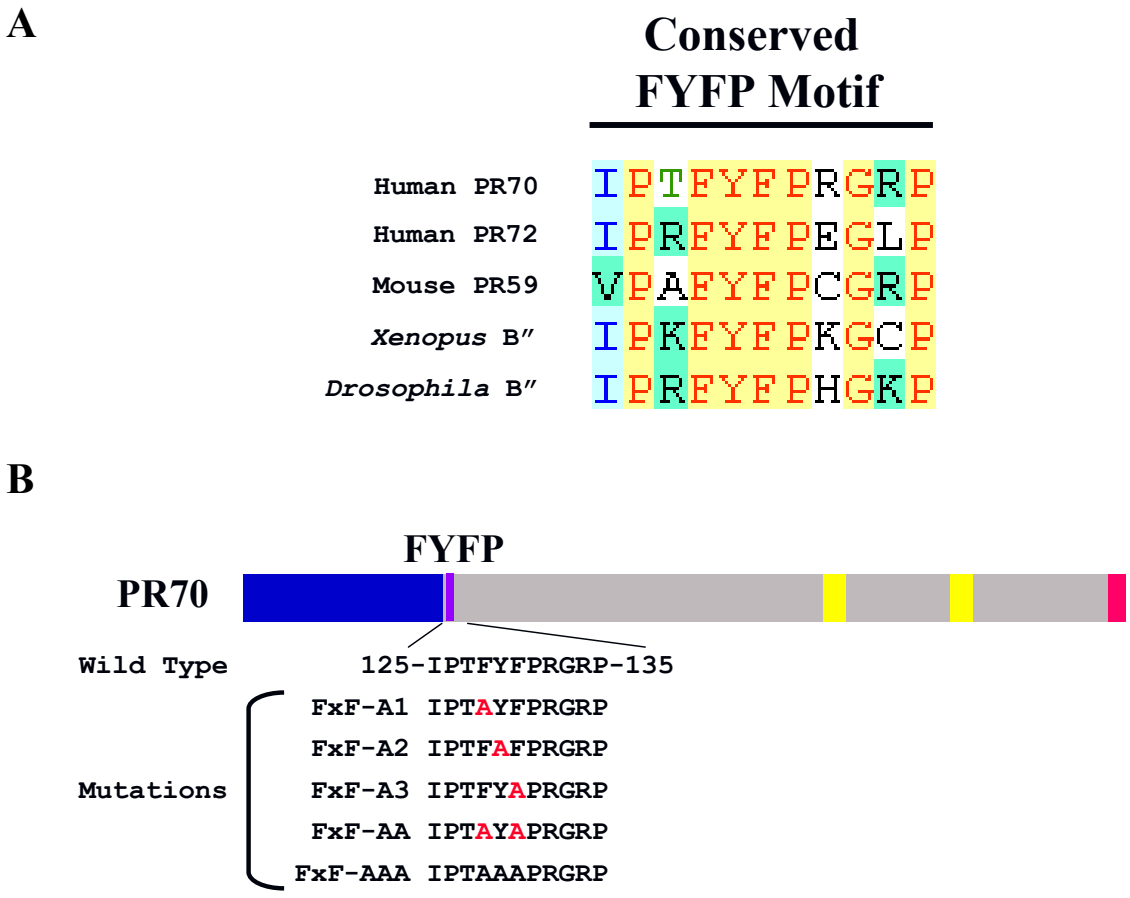

Figure 4.3 FYFP Motif is Conserved in the R3 Regulatory Subunit Family of PP2A.
(A) Alignment of FYFP motif of eukaryotic R3 subunits. Identical residues present in every family member is shaded yellow. Conserved residues are shaded in teal and blue.
(B) Mutations of FYFP motif created. Mutated residues are in red.

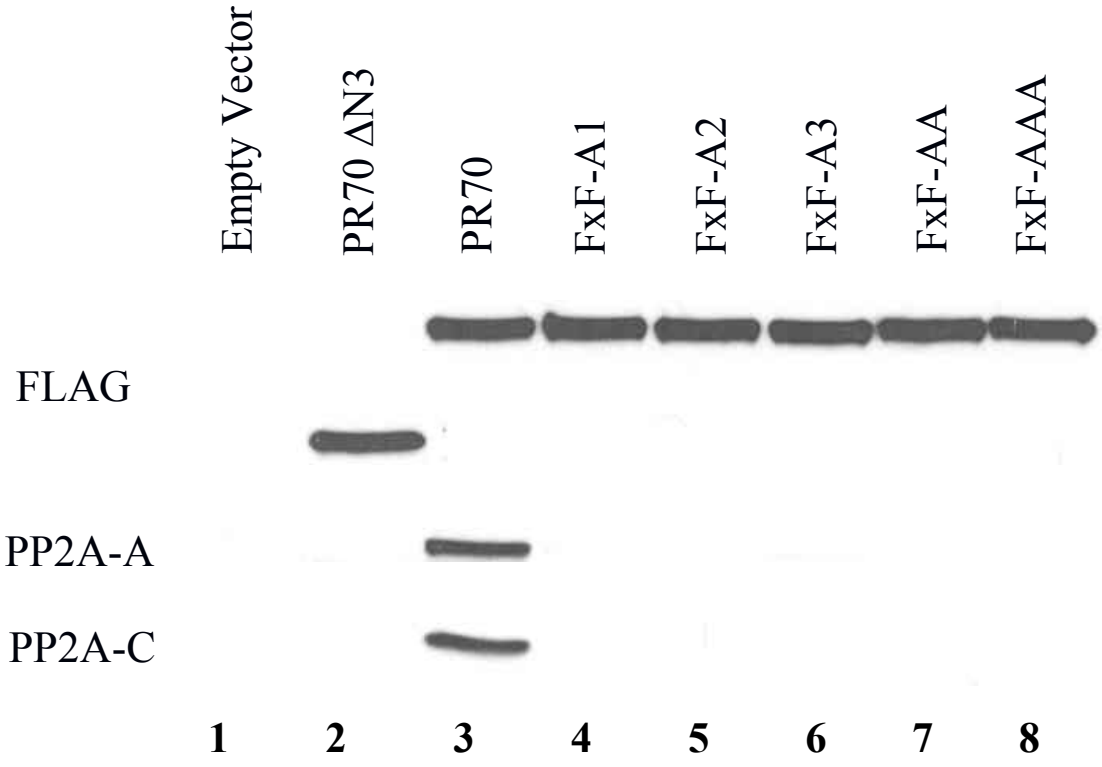

Figure 4.4 Mutation to the FYFP Motif Affects Interaction with PP2A Core Dimer. COS-7 cells were transiently transfected with cDNA for FLAG epitope tagged PR70 and FYFP mutants. Following 24 hour incubation, the cells were lysed and the FLAG-PR70 and FYFP mutants were immunoprecipitated using anti-FLAG antibody. The immunoprecipitations were resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blotted with anti-FLAG, anti-PP2A A-subunit, and anti-PP2A C-subunit antibodies.

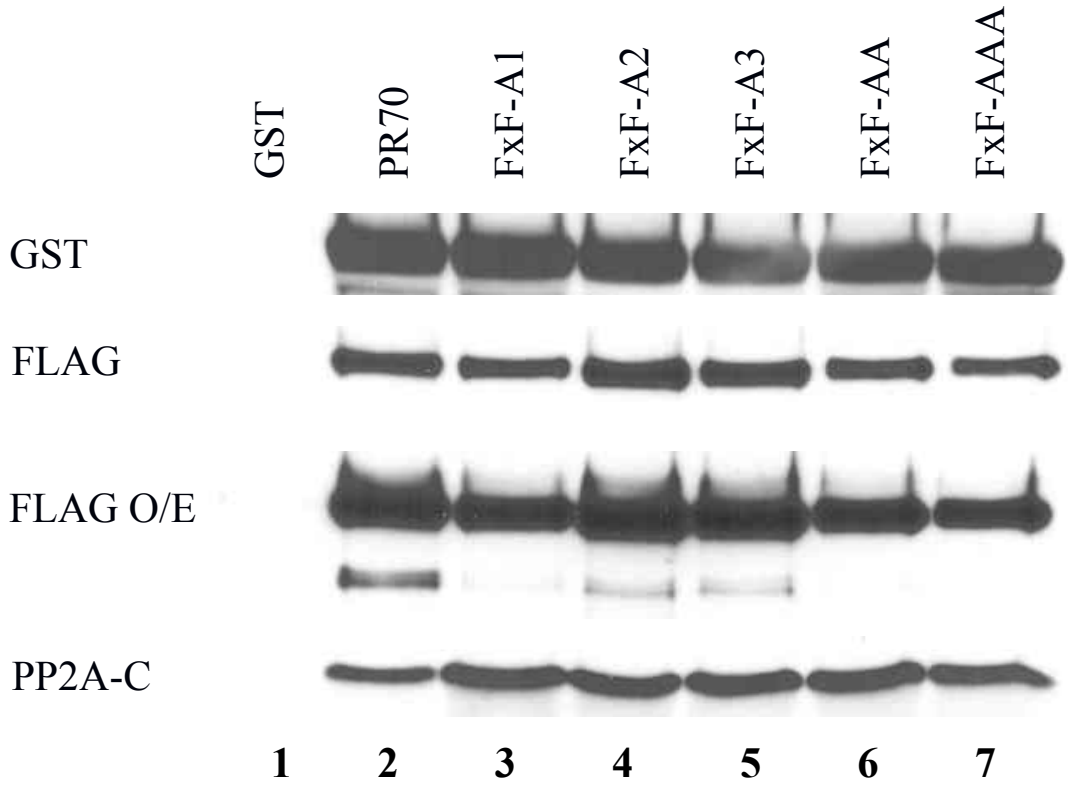

Figure 4.5 FYFP Mutants Bind to GST-A *in vitro*. FLAG-PR70 and FYFP mutants were transiently expressed in COS-7 cells for 24 hours and the cells were lysed. GST-pulldowns were performed using immobilized GST-A with lysates in the presence of 10mM $CaCl_2$. The proteins bound to GST-A were resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blotted with anti-FLAG, anti-PP2A A-subunit, anti-PP2A C-subunit, and anti-GST antibodies.

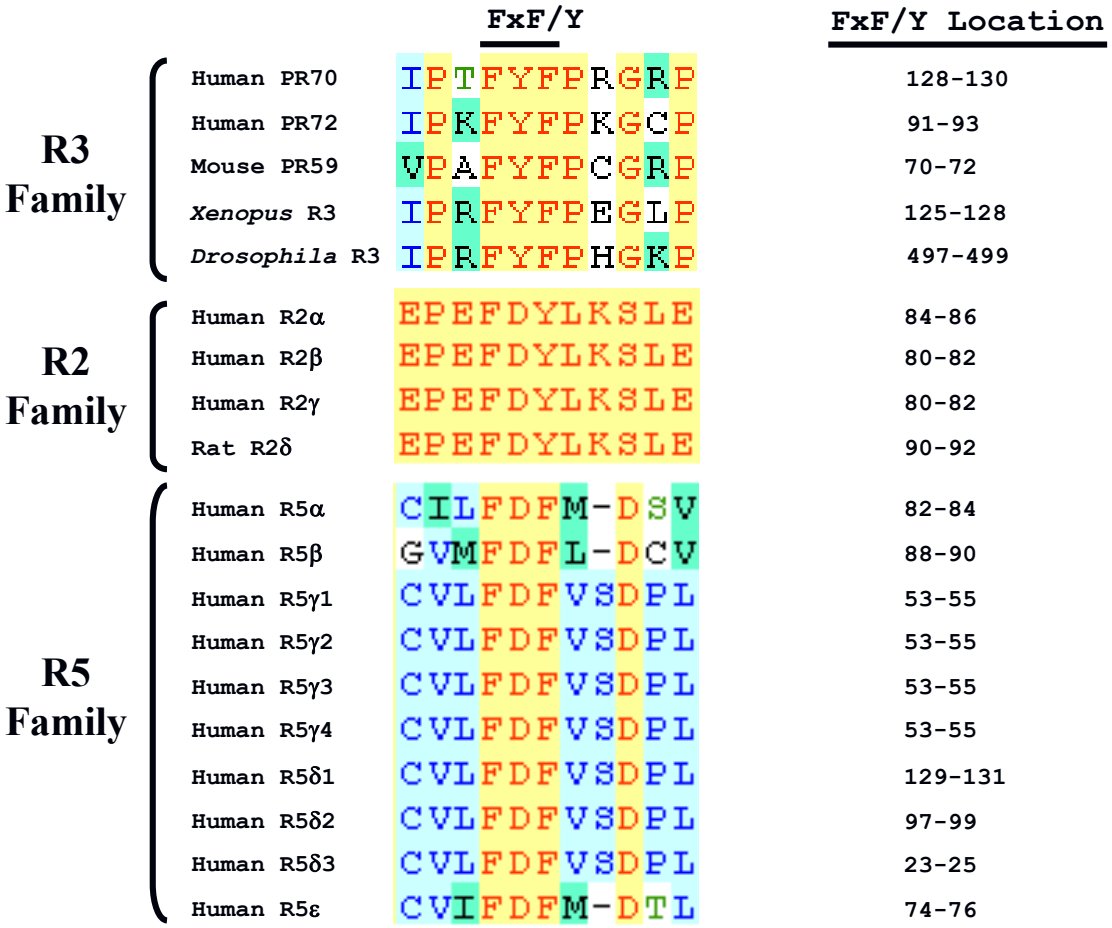

Figure 4.6 FxF/Y Sequence is Conserved in the Regulatory Subunit Families. Alignments of the regulatory subunit families of PP2A. Identical residues present in every family member is shaded yellow. Conserved residues are shaded in teal and blue.

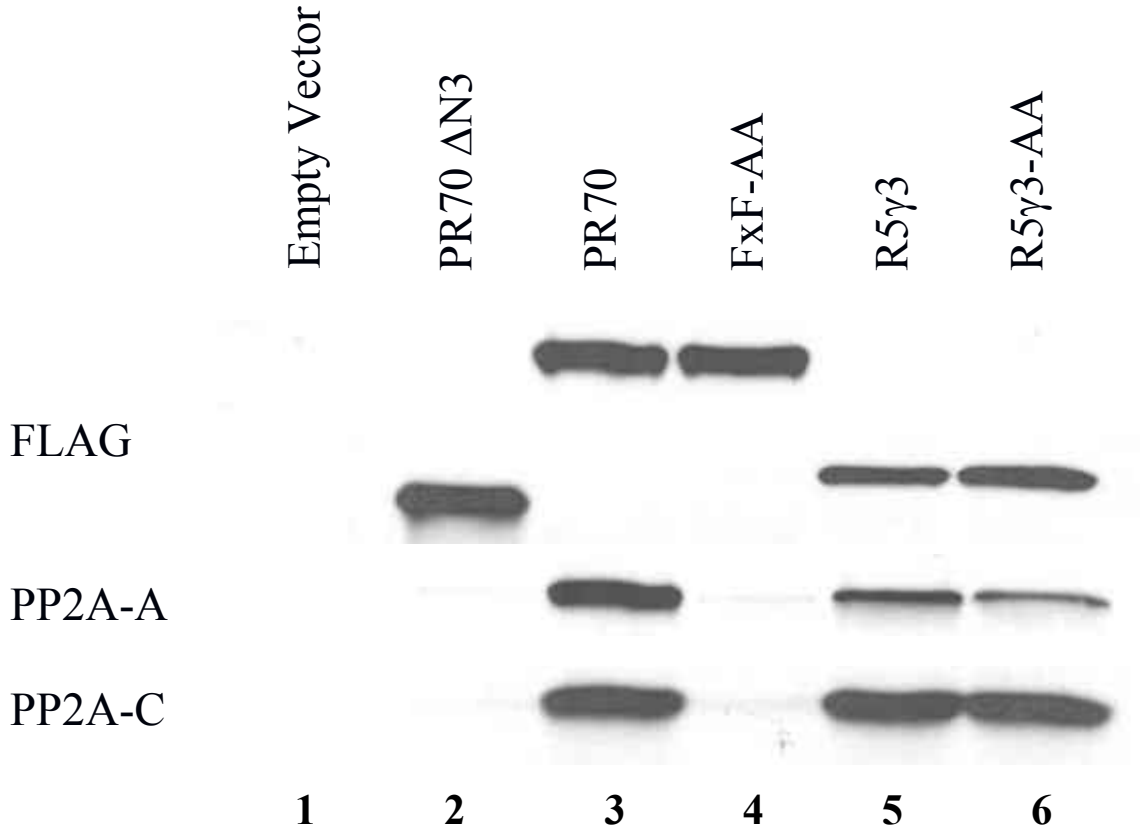

Figure 4.7 Mutation of the FxF Sequence in R5γ3 Does Not Affects Interaction with PP2A Core Dimer. COS-7 cells were transiently transfected with cDNA for FLAG epitope tagged PR70, FxF-AA, R5γ3, R5γ3-AA. Following 24 hour incubation, the cells were lysed and the FLAG-tagged proteins were immunoprecipitated using anti-FLAG antibody. The immunoprecipitations were resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blotted with Abs for anti-FLAG, anti-PP2A A-subunit, and anti-PP2A C-subunit.

in the presence of calcium (Figure 4.8A, Lanes 3 and 9, respectively). Both full length PR70 and ΔN3 interacted with GST-Cdc6 (Figure 4.8B, Lanes 2 and 5, respectively), but only full length PR70 was able to recruit the PP2A A- and C-subunits in the presence of calcium (Figure 4.8B, Lane 3). Compared to full length PR70 and ΔN3, the ΔC mutant bound weakly to GST-Cdc6 and calcium-induced recruitment of PP2A core dimer was lost (Figure 4.8B, Lanes 8 and 9). A small amount of A- and C-subunits still bound to GST-Cdc6 with the ΔC mutant. This is due to endogenous PR70 recruiting the core dimer to Cdc6, as GST-pulldown with naïve lysate still show interaction of A- and C-subunits to Cdc6 (Data not shown). This data suggest that calcium-induced recruitment of the PP2A core dimer to Cdc6 is dependent on the ability of PR70 to interact with the A- and C-subunits. This data illustrates that different portions of PR70 are important for mediating protein-protein interactions with PP2A and Cdc6.

4.4 Discussion

Alignment of the amino acid sequences of the R3 family identified a highly conserved R3 domain. The high degree of similarity (82.2% conserved and 66.3% identical) suggested that this region is important for mediating the interaction of R3 regulatory subunits with the A-subunit of PP2A. This conserved domain also contains the EF hand motifs. Deletion of the unique PR70 sequences in the N- and C-terminus did not affect the ability of PR70 to incorporate into PP2A heterotrimers (Figure 4.2, Lanes 2 and 6, respectively). Further deletion of the N-terminal portion of the R3 domain (aa 1-135) results in complete loss of interaction with PP2A in intact cells (Figure 4.2, Lane5) and *in vitro* (Figure 4.8A, Lane 5). This loss of binding is probably not due to misfolding, because ΔN3 is still able

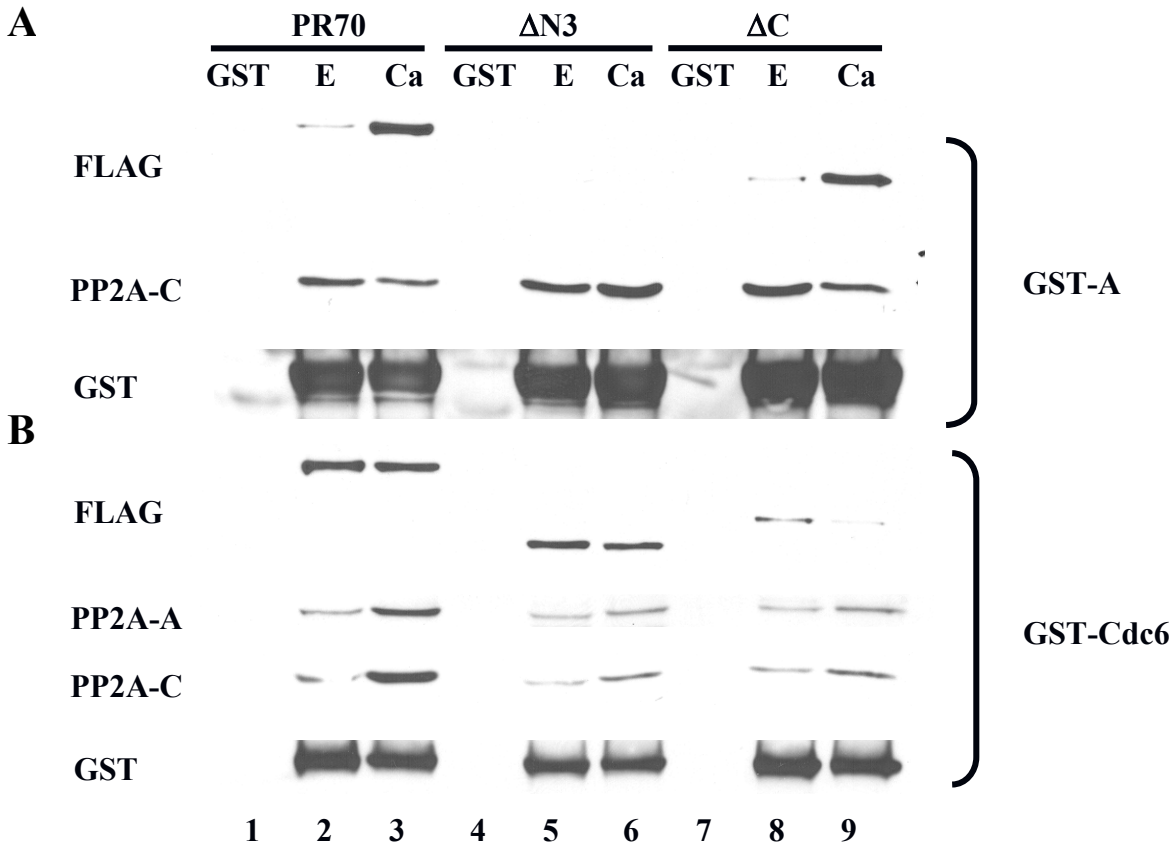

Figure 4.8 The C-terminus of PR70 Mediates Interaction with Cdc6. FLAG-PR70 and truncation mutants were transiently expressed in COS-7 cells for 24 hours and the cells were lysed. GST-pulldowns were performed using immobilized GST-A and GST-Cdc6 with lysates in the presence of 10mM EGTA or 10mM $CaCl_2$. The proteins bound to GST-A and GST-Cdc6 were resolved on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blotted with anti-FLAG, anti-PP2A A-subunit, anti-PP2A C-subunit, and anti-GST antibodies.

to interact with GST-Cdc6 (Figure 4.8B, Lane 5). Although the mutant was not constructed, it is assumed that a truncation mutant which consists solely of the R3 domain would interact with PP2A. These results suggest that the R3 domain is necessary and sufficient for interaction of PR70 with PP2A.

A stretch of 11 amino acids (125-136) important for incorporation of PR70 into PP2A heterotrimers *in vivo* was identified. Sequence alignment of this region identified a conserved FYFP motif. The FxFP sequence is an evolutionary conserved docking site that mediates ERK binding to substrates in multiple protein families (Jacobs et al., 1999). Mutation of the FxF motif in a number of Erk substrates shows this motif is sufficient but not absolutely necessary for Erk binding. Hydrogen exchange mass spectrometry experiments with Erk2 and a peptide containing the Elk1 FxF motif revealed a hydrophobic pocket in Erk2 for FxF binding (Lee et al., 2004). The FYF hydrophobic patch is also similar to the FxFG, GLFG, and FG sequences of nucleoporins that mediate interactions with importin-β (Bayliss et al., 2000). Importins transport cargo in and out of the nucleus in a process hypothesized to occur by interaction with FxFG motifs of the nucleoporins. This is of interest, because importin-β, like the A-subunit of PP2A, is a HEAT repeat protein. The crystal structure of a complex formed between importin-β and five tandem FxFG repeats from the nucleoporin Nsp1p has been described (Bayliss et al., 2000). The nucleoporin FxFG motif binds to a hydrophobic pocket at a primary site between the A helices of HEAT repeats 5 and 6 and to a secondary site between HEAT repeats 6 and 7 of importin-β. Mutation of the PR70 FYFP motif resulted in a weakened interaction with PP2A *in vivo* (Figure 4.4). Similar to EF2 mutants, the FYFP mutants were still able to interact with GST-A *in vitro*

(Figure 4.5). These results suggest a model for the interaction between R3 family members and the A-subunit in which an exposed FYFP motif contributes to the interaction of PR70 with the HEAT repeats of the A-subunit. This interaction could be mediated in two different ways. The crystal structure of PP2A A-subunit reveals exposed hydrophobic surfaces in the region of the protein that interacts with regulatory subunits (Figure 4.9A). The FYFP motif may form a hydrophobic interaction with one or more of these hydrophobic surfaces. Another possibility is that the phenylalanines in the FYFP motif could complex with the A-subunit between the A-helices of HEAT repeats similar to importin-β and nucleoporins (Figure 4.9B).

Data in chapters 2 and 3 show a number of point and truncation mutants that have weakened binding to PP2A in intact cells. Surprisingly, each of these mutants is able to interact with GST-A *in vitro* and in most cases with similar apparent affinity as wild-type PR70. A potential explanation for this phenotype is that an excess of GST-A in the pull-downs pushes the equilibrium toward interaction between the A-subunit and PR70. Thus, interaction would be detected even if the affinity of mutant regulatory subunits for the A-subunit is low compared to wild-type. As noted earlier, the regulatory subunits bind to the core dimer through contacts with both the A-subunit and the C-subunit. Another explanation for weakened binding *in vivo* but binding *in vitro*, is that interaction with PP2A *in vivo* is dependent on interaction with A- and C-subunits. So, the PR70 mutants may not be defective in interaction with the A-subunit, but with the C-subunit. If this was true, the regulatory subunit would still be able to interact with the A-subunit *in vitro*. The data suggests that there are multiple sites of interaction of PR70 with PP2A, in which

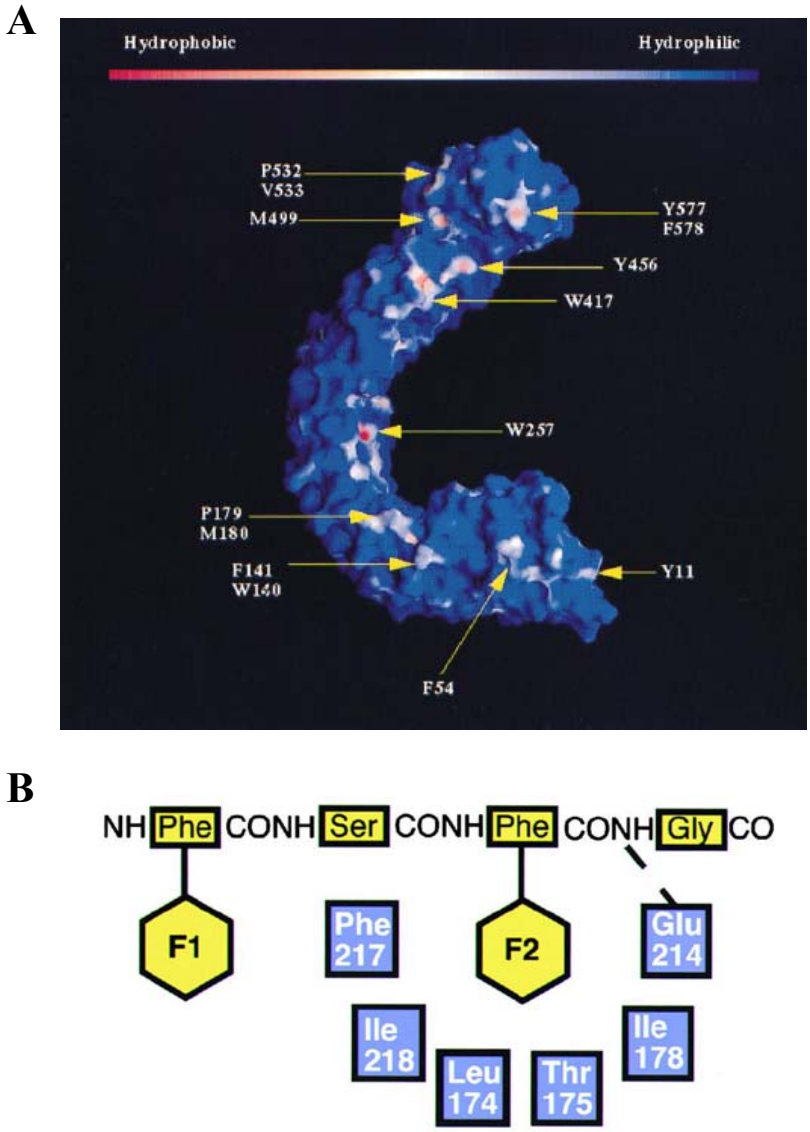

Figure 4.9 Model of FYFP Mediated Binding to PP2A. (A) Hydrophobic surfaces of the A-Subunit of PP2A. Hydrophobic surfaces are colored in red and white, with hydrophilic surfaces in blue. Figure from Groves et al., 1999. (B) Schematic of the importin-βresidues that interact with the FxFG core at the primary site. Figure from Bayliss et al., 2000.

none are absolutely necessary, but in conjunction, mediate a tight interaction with the AC core dimer.

If the conserved R3 domain is responsible for the interaction with the core dimer, than an important question is if the PR70-unique sequences at the N- and C-termini are important for mediating interaction with substrates or effectors. The N- and C-termini are poorly conserved among the R3 family members (13% conserved and 7% identity for the C-terminus of PR70 compared to PR72). GST-Cdc6 pulldown experiments with the ΔN3 and ΔC mutants show that the C-terminus is important for the interaction of PR70 with Cdc6. It cannot be ruled out that portions of the of the conserved R3 domain are also needed, because the ΔC mutant has a portion of this domain deleted. In summary, the data presented in this chapter suggests that different regions of PR70 are important for mediating interactions with PP2A and Cdc6.

Chapter 5

Phosphorylation of PR70

5.1 Introduction

The regulation of PP2A can occur through several mechanisms including association with regulatory subunits, differential subcelluar localization, and post-translational modification. The C-subunit of PP2A can be phosphorylated *in vitro* by the tyrosine kinases $p60^{v\text{-}src}$, $p56^{tck}$, epidermal growth factor receptor, and insulin receptor (Chen et al., 1992). The phosphorylation occurs on Tyr^{307} and has been suggested to cause inactivation of the enzyme. The addition of growth factors and cellular transformation can stimulate transient Tyr307 phosphorylation, implying that PP2A is down regulated in response to stimulatory events (Chen et al., 1994).

Very little is known about post-translational modification of the regulatory subunits of PP2A.. The R5 regulatory subunits B56α, B56β, B56γ3, B56δ, and B56ε are phospho proteins in intact cells (McCright et al., 1996). B56δ is phosphorylated by Protein Kinase A (PKA) *in vitro*. Phosphorylation of B56δ does not affect interaction with the PP2A dimer, but does stimulate activity toward two general phosphatase substrates (Usui et al., 1998). B56α is phosphorylated by double-stranded RNA-dependent protein kinase (PKR) (Xu and Williams, 2000). Similar to B56δ, phosphorylation of B56α does not affect interaction with the PP2A dimer, but does. stimulate activity toward Protein Kinase C (PKC) phosphorylated myelin binding protein (MBP) and PKR phosphorylated eukaryotic

translation initiation factor 2α (eIF2α) *in vitro*. These reports suggest that phosphorylation of regulatory subunits can affect the phosphatase activity of PP2A.

Important questions are whether PR70 is phosphorylated, which residues are phosphorylated, and whether phosphorylation affects interaction with the PP2A core dimer. The results described in this section show that PR70 is phosphorylated in intact cells and that phosphorylation does not affect interaction with the PP2A core dimer.

Materials and Methods

5.2.1 Cell Culture and Expression of FLAG-tagged PR70 and Phosphorylation site Mutants

NIH-3T3 cells were maintained in DMEM containing 10% FCS in an atmosphere of 5% CO_2. For transient expression of FLAG-PR70, NIH-3T3 cells were seeded into 10cm dishes for transfection the next day at ~60% confluence ($1.0x10^6$ cells/dish). Cells were transfected 10μg DNA, 60μL PLUS reagent, and 20μL Lipofectamine as instructed by the manufacturer (Invitrogen) and allowed to incubate for 48 hours.

HeLa cells were maintained as described in 2.2.2. For transient transfection of FLAG-PR70, HeLa cells were seeded into 10cm dishes for transfection the next day at ~80% confluence ($1.2x10^6$ cells/dish). Cells were transfected 7μg DNA, 42μL PLUS reagent, and 14μL Lipofectamine as instructed by the manufacturer (Invitrogen) and allowed to incubate for 24 hours.

COS-7 cells were maintained and transfected as described in 2.2.2.

5.2.2 In vivo ^{32}P labeling of FLAG-PR70

Following the tranfection, the cells were washed with 5mL phosphate

free DMEM and 4mL of phosphate free DMEM was added to each plate. The cells were then metabolically labeled with 1mCi/mL [^{32}P]-H_3PO_4 (MP Biomedicals) in phosphate free DMEM for four hours. For calyculin A (CL-A) treatment, 15 minutes before the end of the four hour labeling, CL-A was added at a concentration of 100nM. Following the incubation, the media was aspirated and the cells were washed 3 times with PBS. The cells were lysed on ice for 20 minutes in IP lysis buffer. The lysates were centrifuged at 14,000 x g for 10 minutes, and FLAG-PR70 was immunoprecipitated as described in 2.2.5. The immunoprecipitates were solubilized in 80µL SDS-PAGE sample buffer and 40µL (50%) was resolved on 10% SDS-PAGE gel. The gel was fixed and stained with Coomassie Brilliant Blue stain to visualize the protein. The gel was dried and exposed to X-ray film overnight to asses the degree of ^{32}P labeling.

To verify the immunoprecipitation, 20µL (25%) of the sample was resolved on an SDS-10% polyacrylamide gel, and transferred to a nitrocellulose membrane. The membrane was then probed with anti-FLAG (M2, Stratagene) and anti-PP2A C-subunit (1F6). Blots were developed using the enhanced chemiluminescence detection system (Amersham Biosciences). The nitrocellulose membrane was also exposed to X-ray film overnight to assess ^{32}P labeling.

5.2.3 Large Scale Immunoprecipitation of FLAG-PR70 and Identification of Phosphorylation Sites

The transient transfection protocol outlined above (5.2.3) was scaled up four fold for over-expression in NIH-3T3 cells. Following the transfection, the media was aspirated and the cells were washed twice with ice cold PBS. The cells were lysed for 20 minutes in IP

Lysis buffer on ice. The lysates were centrifuged at 30,000rpm for 30 minutes at 4ºC to remove cellular debris. The supernatant was then passed through a 0.45μM filter and a small aliquot was saved for immunoblotting. FLAG-PR70 and was immunoprecipitated using 150μL packed bead volume of FLAG M2 Agarose (Sigma Aldrich) for 2 hours at 4ºC followed by 3 washes with lysis buffer. The immunoprecipitated protein complexes were then eluted from the M2 agarose by 3 elutions with 150μL of 0.1mg/mL 3X FLAG peptide in 1x TBS. A small aliquot of the elution was saved for immunoblotting. The eluate was then concentrated using a Millipore micro concentrator (YM-10, Millipore) and a small aliquot was saved for immunoblotting. The rest of the eluate was solubilized in 4x SDS-PAGE sample buffer (amount of sample buffer was determined by the volume of eluate for each sample). The entire eluate was resolved on an 10% SDS-PAGE Criterion gel (Bio-Rad). The gel was stained with Coomassie Colloidal Blue Stain to visualize the proteins. The protein bands corresponding to FLAG-PR70 was excised and given to Dr. Hongjun Shu in the Protein Lab Chemistry of the Alliance for Cellular Signaling for nano-electrospray ionization mass spectrometry analysis.

To verify immunoprecipitation of FLAG-PR70, each the samples were resolved on an SDS-10% polyacrylamide gel, and transferred to a nitrocellulose membrane. The membrane was probed with anti-FLAG (M2, Stratagene) and anti-PP2A C-subunit (1F6). Blots were developed using the enhanced chemiluminescence detection system (Amersham Biosciences).

5.2.4 Mutagenesis of Phosphorylation Sites

Point mutations to either non-phosphorylatable alanine or phospho-mimicking aspartic acid were introduced in each phosphorylation site using a PCR-based site directed mutagenesis method (mutated residues underlined). For the introduction of the point mutations, PCR was performed with pCMV-Tag2B containing the full length PR70 cDNA as template with the following primers: **T76A** 5-GAACCCCCGGGAGCCCCCGGGCCGG-3' (sense primer) and 5'-CCGGCCCGGGGGCTCCCGGGGGTTC-3' (anti-sense primer), **T76D** 5-GAACCCCCGGGAGACCCCGGGCCGG-3' (sense primer) and 5'-CCGGCCCGGGGTCTCCCGGGGGTTC-3' (anti-sense primer), **S543A** 5'-GAGTGCGCTGCGCGCCCCGCTGGCCCAG-3' (sense primer) and 5'-CTGGGCCAGCGGGGCGCGCAGCGCACTC-3' (anti-sense primer), **S543D** 5'-GAGTGCGCTGCGCGACCCGCTGGCCCAG-3' (sense primer) and 5'-CTGGGCCAGCGGGTCGCGCAGCGCACTC-3' (anti-sense primer). For the double mutants, the mutations were introduced as follows: **T76AS543A** T76A was used as a template, and the S543A primer for introduction of the mutation, **T76AS543D** T76A cDNA template, S543D primer for introduction of the mutation, **T76DS54D** T76D cDNA template, S543D primer for introduction of the mutation, and **T76DS543A** T76D cDNA template, S543A primer for introduction of the mutation. The mutations were verified by automated sequencing.

5.2.5 Expression, Immunoprecipitation, and Western Blotting of FLAG-PR70 and Phosphorylation Site Mutants

Protocols were followed as described in 2.2.2 and 2.2.5.

5.3 Results

5.3.1 PR70 is Phosphorylated in Intact Cells

An FxFP amino acid motif has been shown to mediate MAPK ERK docking to substrates. The identification of the FxFP motif in PR70 raised the possibility that PR70 is phosphorylated. The PR70 amino acid sequence was analyzed using the Scansite algorithm (http://scansite.mit.edu/) to identify potential phosphorylation sites within PR70. The analysis identified a putative phosphorylation site for ERK1/2 upstream of the FxFP motif. To determine if PR70 is phosphorylated in intact cells, FLAG-PR70 was transiently transfected in NIH-3T3 cells. After 48 hours, the cells were metabolically labeled with [^{32}P]-H_3PO_4 for 4 hours and FLAG-tagged PR70 was immunoprecipitated. Autoradiography showed that PR70 was phosphorylated (Figure 5.1, Lane6). Addition of the phosphatase inhibitor calyculin A (CL-A) resulted in greater ^{32}P-labeling, suggesting the phosphorylation is sensitive to a PP2A-like phosphatase (Figure 5.1, Lane 8). PR70 was also phosphorylated in NIH-3T3, COS-7, and HeLa cells, illustrating that PR70 is phosphorylated in multiple cell lines (Figure 5.2) These data show that expressed PR70 can be phosphorylated in intact cells.

5.3.2 Identification of Phosphorylation Sties

Since PR70 can be phosphorylate in intact cells, the next step was to identify the phosphorylation sites. FLAG-PR70 was expressed in four 10cm dishes of NIH-3T3 cells and immunoprecipitated with anti-FLAG antibody. FLAG-PR70 was eluted, concentrated, and resolved on an SDS-PAGE gel. A band corresponding to PR70 was observed (Figure 5.3).

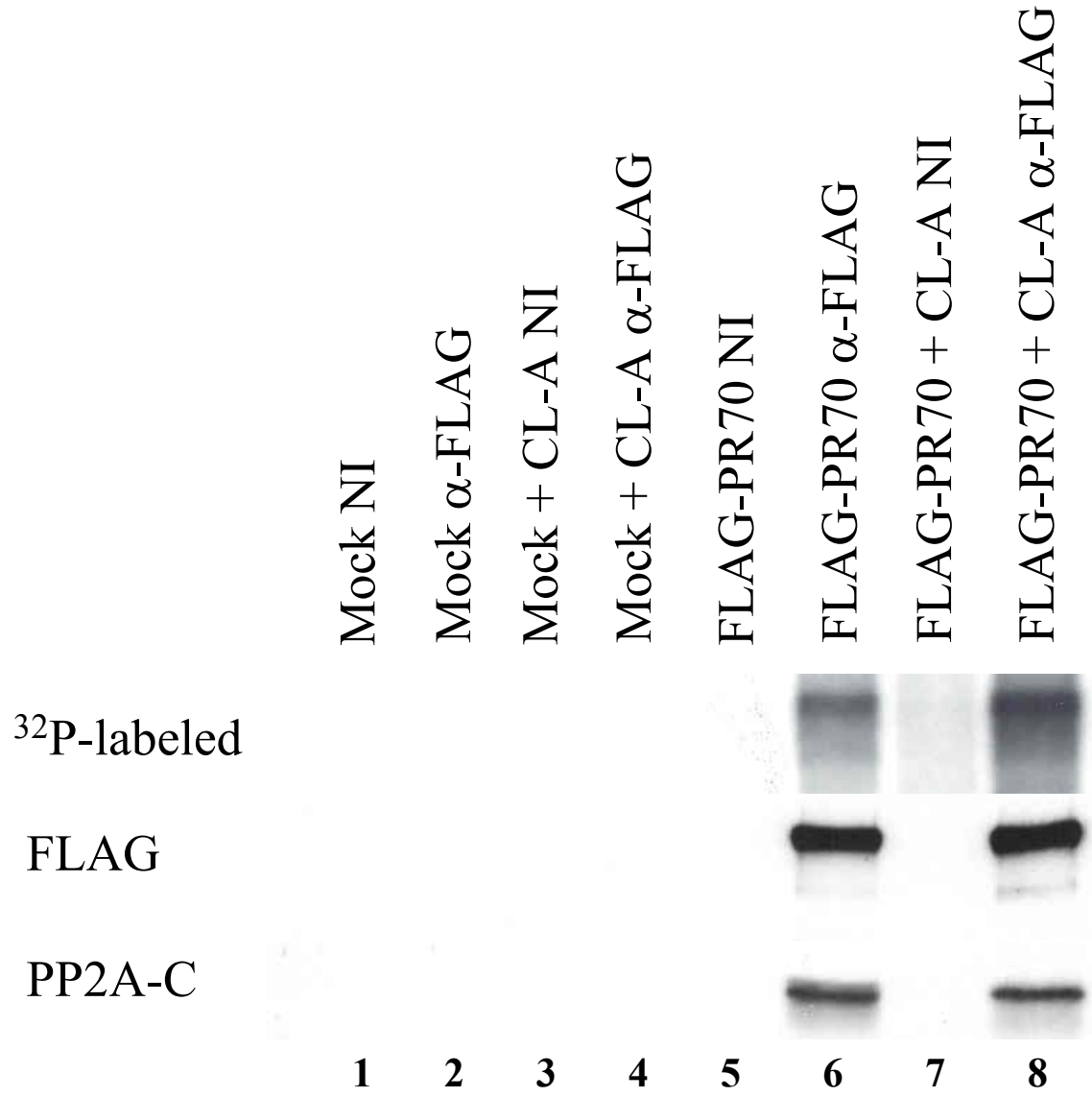

Figure 5.1 PR70 is Phosphorylated in Intact Cells. NIH-3T3 cells were transiently transfected with cDNA for FLAG epitope tagged PR70. Following 24 hour incubation, the cells were metabolically labeled with 1mCi/mL [^{32}P]-H_3PO_4 for 4 hours. The cells were lysed and the FLAG-PR70 was immunoprecipitated using anti-FLAG antibody. The immunoprecipitations were resolved on a SDS-PAGE gel, dried, and exposed to X-ray film overnight. Also, a gel was transferred to a nitrocellulose membrane. The membrane was blotted with anti-FLAG and anti-PP2A C-subunit antibodies.

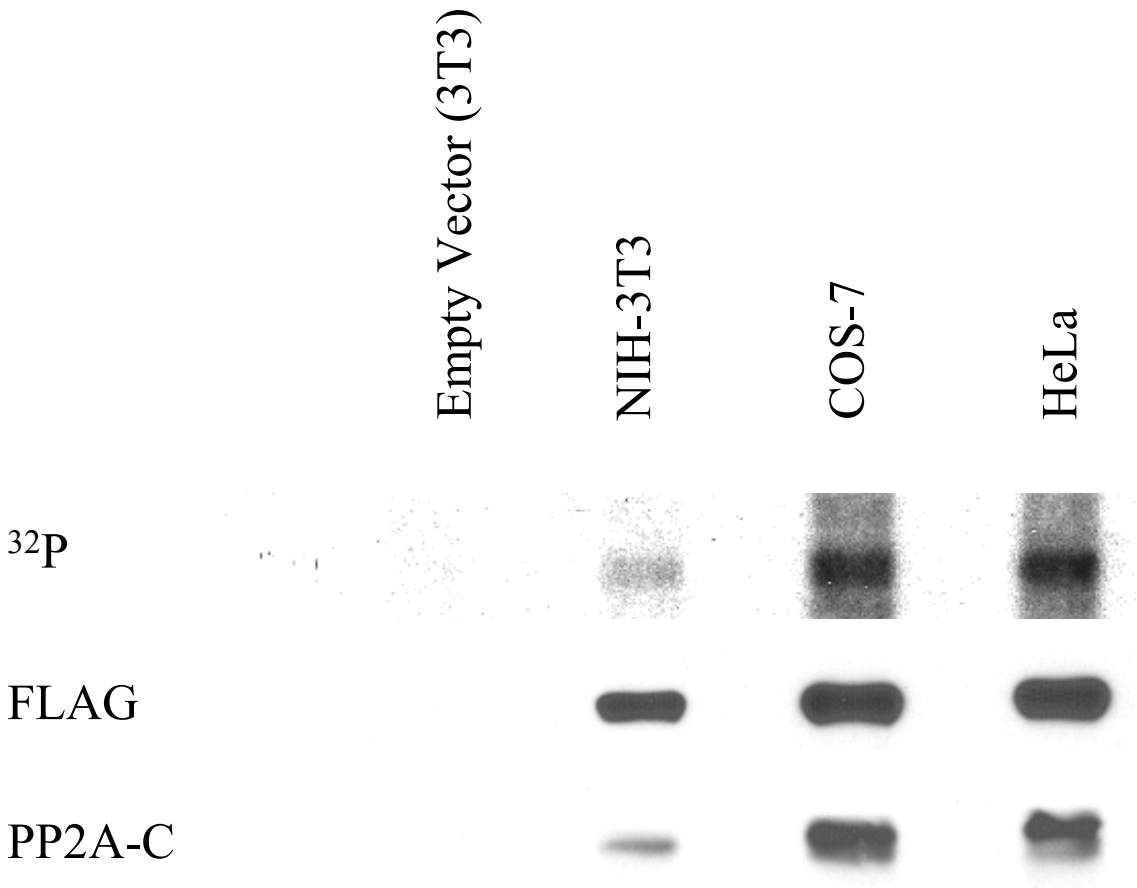

Figure 5.2 PR70 is Phosphorylated in Different Mammalian Cell Lines *in vivo*. NIH-3T3, COS-7 and HeLa cells were transiently transfected with cDNA for FLAG epitope tagged PR70. Following 24 hour incubation, the cells were metabolically labeled with 1mCi/mL [^{32}P]-H_3PO_4 for 4 hours. The cells were lysed and the FLAG-PR70 was immunoprecipitated using anti-FLAG antibody. The immunoprecipitations were resolved on a SDS-PAGE gel, dried, and exposed to X-ray film overnight. Also, a gel was transferred to a nitrocellulose membrane. The membrane was blotted with anti-FLAG and anti-PP2A C-subunit antibodies.

Treatment the cells with CL-A produced what appeared to be a single band of PR70 compared to the doublet in the untreated sample, suggesting that a greater portion of PR70 was phosphorylated in the presence of CL-A (Figure 5.3). A band corresponding to the A-subunit was also observed in immunoprecipitations from PR-70 transfected cells, but not in cells transfected with the empty vector (Figure 5.3). The bands corresponding to PR70 were excised and sent for mass spectrometry analysis to identify phosphorylation sites. Two phosphopeptides were identified. The phosphopeptides identified were PSGLEPPGTPGPGPALPLGAASSPR (aa 68-92) and LSALRSPLAQR (538-548) (Figure 5.4A). The analysis could not identify the exact amino acid phosphorylated in the peptides. But, the analysis did eliminate each of the serines and threonines in the first peptide except the threonine at position 76 and eliminated the only other serine in the second peptide except serine at position 543. Due to too little protein in the sample, no phosphopeptides were identified from the CL-A treated sample. The data illustrates that PR70 is phosphorylated in intact cells and the probable phosphorylation sites are threonine 76 (T76) and serine 543 (S543).

5.3.3 Mutation of the Phosphorylation Sites Does Not Affect the Interaction of PR70 With PP2A

In order to test for a possible role of the phosphorylation sites in the interaction of PR70 with the A- and C-subunits, these sites were altered by site-directed mutagenesis. Two different sets of phosphorylation site mutants were constructed. Threonine 76 and Serine 543 were mutated to alanine, to ablate the phosphorylation site, or to aspartic acid, to mimic phosphorylation (Figure 5.4B). To determine if mutations of the putative

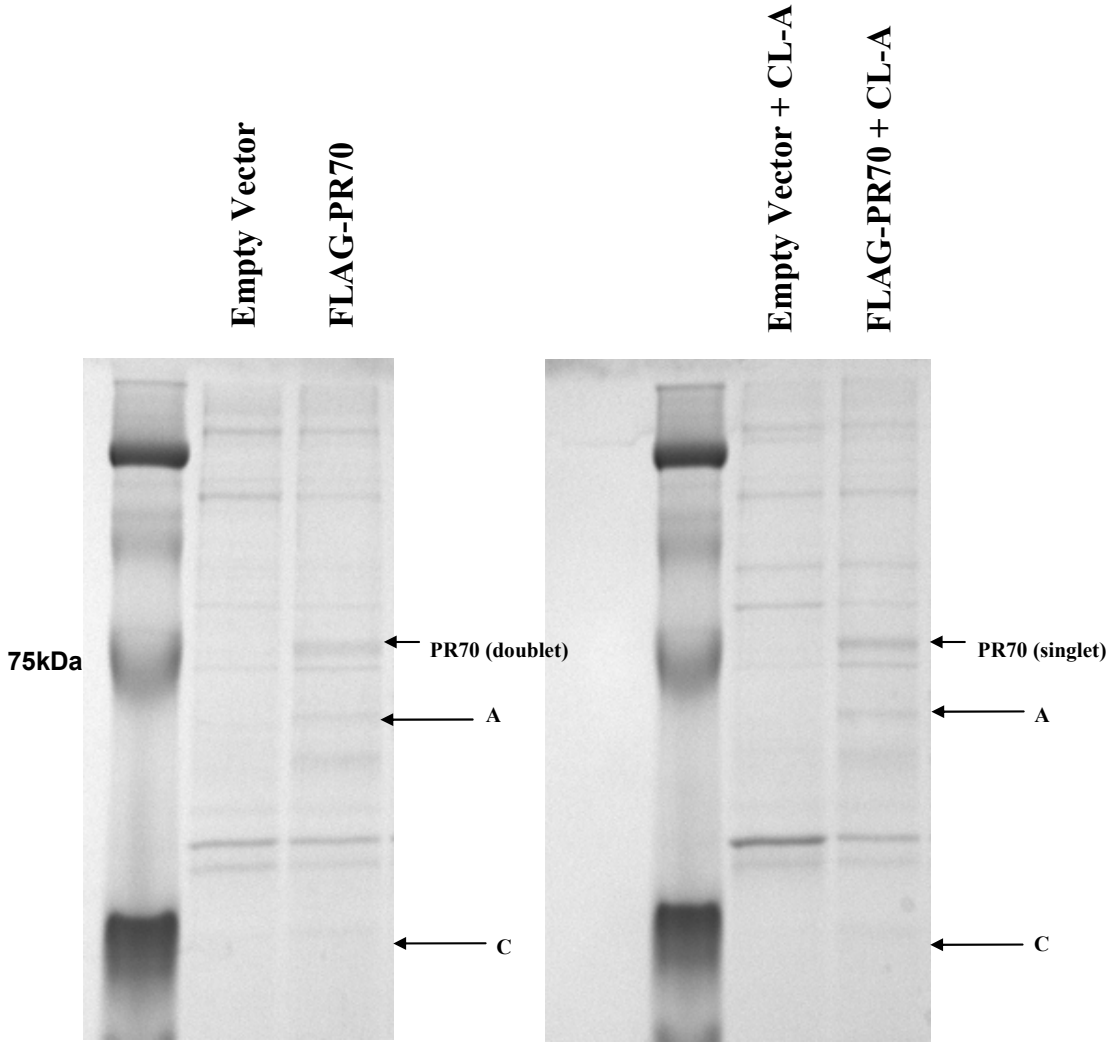

Figure 5.3 Large Scale Immunoprecipitation of PR70. NIH-3T3 cells were transiently transfected with cDNA for FLAG epitope tagged PR70. Following 24 hour incubation, the cells were lysed and the FLAG-PR70 was immunoprecipitated using anti-FLAG M2 Agarose. The immunoprecipitations were eluted with 3X FLAG-peptide, concentrated, and resolved on a SDS-PAGE gel. The gel was stained with Colloidal Coomassie Blue stain and the protein band corresponding to FLAG-PR70 was excised and given to the Protein Lab at the Alliance for Cellular Signaling for nano-electrospray ionization mass spectrometry analysis to identify phospho-peptides.

A

Flag-PR70: Two phosphopeptides detected:

PSGLEPPGTPGPGPALPLGAASSPR(1phospho)
LSALRSPLAQR(1phospho)

Flag-PR70 + CL-A: No phosphopeptide detected

B

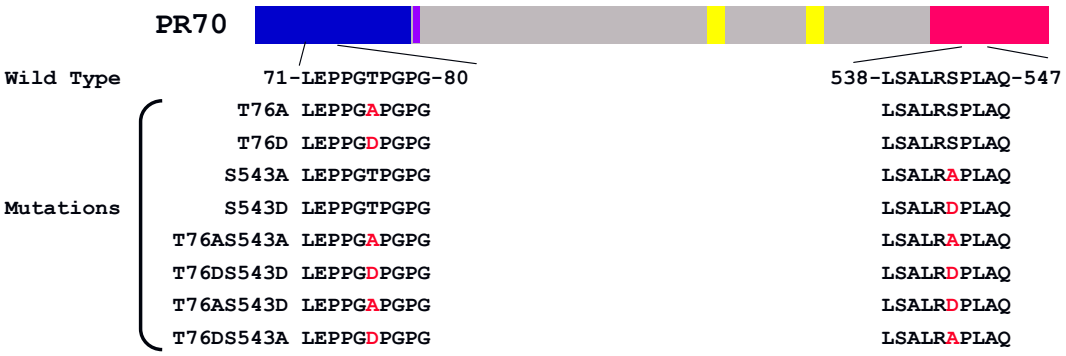

Figure 5.4 Identification of Two Phospho-peptides of PR70. (A) Phospho-peptides of PR70 identified by nano-electrospray ionization mass spectrometry analysis. (B) Mutations of phospho-sites created. Mutated residues are in red.

phosphorylation sites affected the ability of PR70 to interact with PP2A, FLAG-tagged versions of each mutant were expressed in COS-7cells. FLAG-PR70 and the phosphorylation site mutant proteins were immunoprecipitated and incorporation into PP2A heterotrimers was assessed by blotting for endogenous A- and C-subunits. Each phosphorylation site mutant interacted with endogenous PP2A (Figure 3.3). These data show that mutation of the phosphorylation sites, by either ablating the phosphorylation site or producing a phosphorylation mimic, had no apparent affect on binding of PR70 to the PP2A core dimer.

5.4 Discussion

Scansite analysis predicted a putative ERK1/2 phosphorylation site upstream of the FxFP motif in PR70. Incorporation of ^{32}P into expressed PR70 shows that the protein can be phosphorylated in intact cells. Mass spectometry analysis of FLAG-PR70 identified two phosphopeptides. Although the exact phosphorylation sites were not identified, elimination of serine and threonine residues, suggested that the two phosphorylation sites are T76 and S543. T76 corresponds to the phosphorylation site identified by Scansite search to be a potential substrate for Erk1/2. There is no consensus motif at S543 that clearly identifies a candidate kinase responsible for phosphorylating this site. Since S543 is followed by a proline residue, it is probable it is phosphorylated by a Cdk, MAPK or GSK-3β. An initial *in vitro* kinase assay with immunoprecipitated Erk1/2 did not show PR70 phosphorylation. [^{32}P]-labeling experiments with the alanine mutants show reduced labeling of the T76AS543A mutant compared to wild-type. The T76AS543A mutation did not completely ablate the [^{32}P]-labeling, so it is possible that there are other phosphorylation sites in the

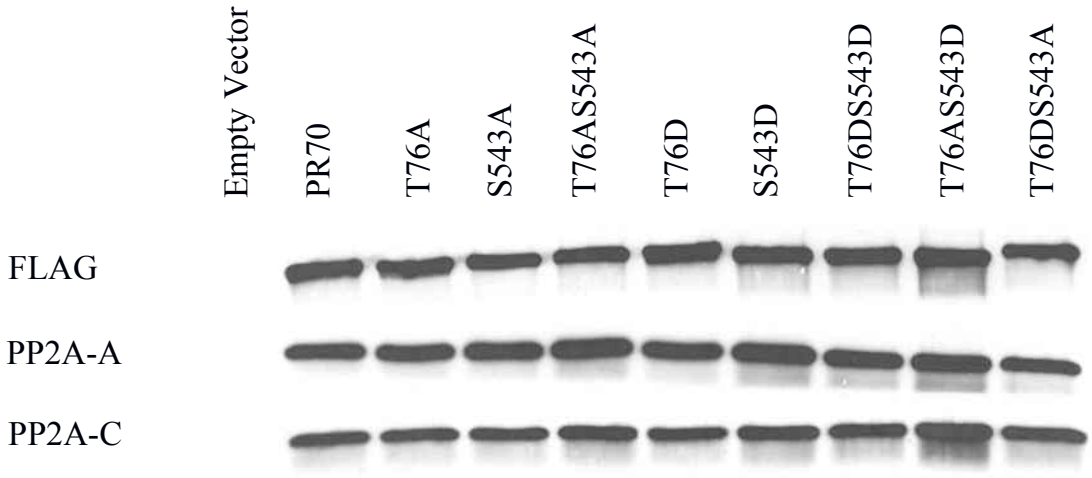

Figure 5.5 Mutation to the Phosphorylation Sites Does Not Affect Interaction with PP2A Core Dimer *in vivo*. COS-7 cells were transiently transfected with cDNA for FLAG epitope tagged PR70 and phospho-site mutants. Following 24 hour incubation, the cells were lysed and the FLAG-PR70 and phospho-site mutants were immunoprecipitated using anti-FLAG antibody. The immunoprecipitations were resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blotted with anti-FLAG, anti-PP2A A-subunit, and anti-PP2A C-subunit antibodies.

protein (Data not shown). This is the first report showing that a R3 subunit can be phosphorylated in intact cells.

Two PP2A regulatory subunits have been shown to be phosphorylated in intact cells, both belonging to the R5 family, B56α and B56δ. Phosphorylation of either subunit did not cause dissociation from the PP2A core dimer, but did stimulate activity *in vitro*. Since phosphorylation of B56α and B56δ results in activation, a model is that phosphorylation positively affects interactions of regulatory subunits with substrates and/or effectors. This model may also fit with PR70, since phosphorylation occurs in the unique PR70 sequence of the N- and C-termini, which may direct interaction with substrates. This model suggests phosphorylation of S543 may affect the interaction of PR70 with Cdc6.

B56α and B56δ are dephosphorylated by PP2A. Auto-dephosphorylation of regulatory subunits may be an important mechanism by which PP2A controls its own activity. PR70 phosphorylation may be regulated by PP2A, because treatment of cells with CL-A resulted in an increase in [^{32}P]-labeling. This model suggests dephosphorylation of PR70 by PP2A may regulate the interaction of PR70 with Cdc6.

Chapter 6

Future Directions

6.1 Interaction of PR70 with A-subunit and Cdc6.

The addition of calcium to GST-pulldown experiments causes enhanced binding of PR70 to the A-subunit *in vitro*. For most calcium sensors, binding of calcium induces a large conformational change that exposes a hydrophobic patch. Exposure of the patches leads to enhanced interactions with substrates and effector proteins and is the basis for calcium-dependent regulation by these proteins. The enhanced binding of PR70 to the A-subunit in the presence of calcium may be due to a calcium-induced conformational change that exposes a hydrophobic patch. A calcium-induced conformational change in PR72 has been reported (Janssens 2003). Calcium-binding may cause a similar conformational change in PR70. It is therefore important to determine if PR70 undergoes a similar calcium-induced change and whether this exposes residues that enhance the interaction with the A-subunit.

Intrinsic tryptophan fluorescence was used to assess whether calcium binding to PR72 triggers a conformation change. The experiment was performed using a fragment of PR72 comprising both EF hand motifs (aa 262-449) which only contains three tryptophans, and two of them are located between the two EF hands. The exposed hydrophobic patch involved in interaction with effectors is usually located between the two EF hand motifs in calcium sensors. Therefore, this region within PR70 may be important for interaction with

the A-subunit. The hydrophobic residues mediating calcium-induced interaction of PR70 with the A-subunit should be identified. The corresponding PR70 fragment (aa 300-487), including the same three tryptophan residues, should be constructed. Intrinsic tryptophan fluorescence will be performed, and a calcium-dependent conformational change in PR70 should be tested. As a control, the double EF hand mutant will be used. The potential hydrophobic residues between EF hands, including FVW (aa 377-379) and YWF (aa 395-387) to mediate the interaction with the A-subunit could be tested by mutating to alanine. The ability of these mutants to interact with the core dimer should be assessed along with assessing whether calcium binding elicits a conformational change.

The residues mediating the interaction of PR70 with Cdc6 should also be identified. GST-Cdc6 pulldown experiments with the ΔN3 and ΔC mutants show that the C-terminus is important for the interaction of PR70 with Cdc6, but did not rule a role for portions of the conserved R3 domain. To determine the residues of PR70 responsible for the interaction with Cdc6, a set of C-terminal deletion mutants could be constructed. Similar to the deletion analysis of PR70 with the A-subunit, this may provide insight about important residues necessary for binding to Cdc6.

6.2 Regulation of Cdc6 by PR70-PP2A

As mentioned in Chapter 2, numerous attempts to co-immunoprecipitate endogenous Cdc6 or over-expressed HA-tagged Cdc6 with FLAG-PR70 produced mixed results. Recently, a paper was published showing that phosphorylation of Cdc6 by cyclin E-cdk2 stabilizes the protein by preventing its proteolytic destruction via the anaphase promoting complex (APC) (Mailand et al., 2005). This may explain why

immunoprecipitation experiments have failed with co-expressed PR70 and Cdc6. Dephosphorylation of Cdc6, mediated by the targeting of PR70-containing PP2A, may target Cdc6 for destruction. To support this hypothesis, preliminary data show that co-expression of PR70 and Cdc6 results in destabilization of Cdc6. Furthermore, destabilization of Cdc6 by PR70 does not occur when the truncation ΔN3 is expressed with Cdc6. This is probably due to the inability of ΔN3 to interact with the core dimer and suggests that destabilization of Cdc6 by PR70 is due to recruitment of the PP2A core dimer and dephosphorylation of Cdc6. These results need to be verified. It is also important to determine if PR70-induced degradation of Cdc6 is mediated by dephosphorylation. Mutation of the three cdk phosphorylation sites to aspartate has been shown to stabilize the protein. If PR70-mediated degradation is dependent on dephosphorylation at these sites, the aspartate mutants should be resistant to PR70. Finally, it should be determined if PR70-mediated degradation of Cdc6 can be blocked by increased Cdk activity. PR70 could be co-expressed with cyclin A or cdk2 and the stability of Cdc6 assessed. It is expected that co-expression of cyclin A and cdk2 will dampen the effect of PR70.

6.3 Phosphorylation of PR70 and Regulation of Function

The results show PR70 is phosphorylated in intact cells, but mutation of the phosphorylation sites by either ablating the phosphorylation site or producing a phosphorylation mimic of PR70 has no affect on binding to the PP2A core dimer. Identifying the PR70 kinase may provide insights into the reason PR70 is phosphorylated. The T76 phosphorylation site was identified in the Scansite search to be a potential substrate for Erk1/2. *In vitro* kinase assays with immunoprecipitated Erk should be performed to

determine if PR70 is an Erk substrate *in vitro*. Also, there is data suggesting the FxFP motif directs binding of p38 substrates, so the kinase assay should also be performed with p38. There is no consensus motif at S543 that identifies what type of proline-directed kinase might be responsible for phosphorylating this site. Purified cyclin-cdk complexes or other proline-directed available kinases could be used in *in vitro* kinase assays to determine whether PR70 is phosphorylated by a specific kinase. If these assays fail, a yeast two hybrid screen could be performed with the unique N- and C-termini of PR70, which contain the phosphorylation sites, as bait to identify the potential kinase responsible for phosphorylating PR70.

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