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Protein-protein interaction between multi-functional protein CAD and protein phosphastase 1 (PP1)

Meenal Mhaskar

Protein-Protein interaction between the multi-functional protein CAD and protein phosphatase 1

by

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in

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April 15, 2014

Ypsilanti, MI

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By

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ABSTRACT

Uncontrolled cell proliferation, a hallmark of cancer, is associated with activation of CAD, a multifunctional protein that catalyzes the first three steps in pyrimidine biosynthesis. The cell cycle dependent regulation of pyrimidine biosynthesis is a consequence of sequential phosphorylation of CAD Thr-456 and Ser-1406 by the MAP kinase and PKA cascades, respectively. The mechanism that controls the timing of these events is not well understood. Our hypothesis is that timing of the activation and nucleocytoplasmic dynamics of CAD is controlled by signaling complexes with kinases and phosphatases. Interestingly, a consensus sequence for PP1 targeting proteins is located immediately adjacent to Thr-456. Peptides were synthesized corresponding to residues 444-460 of CAD that encompasses both the PP1 consensus sequence and Thr-456 as well as two mutant peptides in which Thr-456 was replaced with Ala or Asp. The wild type peptide and Ala mutant were able to bind to PP1 while the Asp mutant which, mimics phosphorylated CAD, does not.

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CHAPTER 1: INTRODUCTION

The Cell: The Fundamental Unit of Life

A cell is the structural and functional unit of all living organisms. It is the fundamental unit in biology similar to an atom which occupies the center position in the physical science. A cell carries out all the vital functions such as transport of nutrients, respiration, and reproduction. Living organisms are divided as unicellular or multicellular. Multi-cellular organisms (Figure. 1) show increasing complexity by allowing the differentiation of numerous cellular lineages with specialized functions within the organism. Prokaryotes are usually single-celled organisms that lack a cell nucleus, the most common examples of which are *Escherichia coli* and cyanobacteria, the blue green algae. The cells of eukaryotes have a well-defined nucleus surrounded by a nuclear membrane. Examples of eukaryotes include all plants and animal cells including unicellular fungi (yeast, mold) and protozoans. The cell organelles have specialized functions which are essential for growth and survival. The mitochondria metabolize energy; lipids and glycoproteins are synthesized in the endoplasmic reticulum; lysosomes, present only in animal cells, degrade worn out and dead cellular constituents; and chloroplasts, found in only plant cells, carry out photosynthesis. The nucleus of the cell is the administrative headquarter because it encloses the cell's genome, i.e., the DNA (deoxyribonucleic acid) comprised of two helical strands that are coiled around a common axis forming the double helix packaged within the chromosomes. Living beings depend on genes, as they specify all proteins and functional RNA chains. DNA and RNA are molecular precursors which store information and transmit this information from one generation to the next which is the fundamental condition of life.

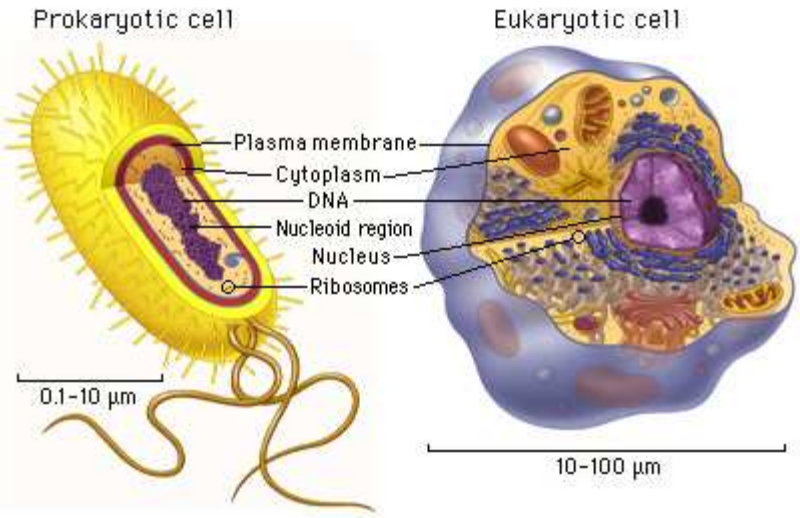


Figure 1. Prokaryotic and Eukaryotic cells[1]

The Eukaryotic Cell Cycle

A cell goes through many functional stages during its life cycle (Figure 2). It undergoes cell division in need to replace worn out or dead cells. Whether the cell will divide or not is a highly regulated process. For example, when a parent stem cell in the bone marrow undergoes cell division to form two daughter cells, the daughter cells exit the cell cycle and are differentiated to form specialized blood cells that might undergo apoptosis at a later time. When a particular cell divides, new cells are produced. After cell division, the two daughter cells contain an identical set of the parental cell's genetic material. Cell division is preceded by a replication program that is largely executed by

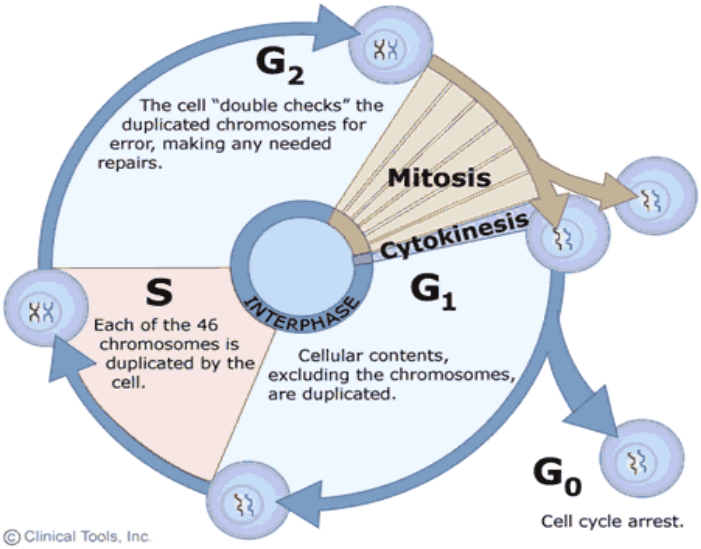


Figure 2. Schematic representation of the eukaryotic cell-cycle[2]

specific proteins. The cell cycle is a tightly programed and regulated mechanism in which the initiation of specific stages occurs only after the successful completion of early ones and the progress is tightly controlled by specific check points. Most eukaryotic cells operate according to internal clocks via a highly regulated mechanism and proceed through a sequence of cell- cycle phases during which DNA is duplicated during the synthesis (S) phase and the copies are then distributed to opposite ends of the parent cell during the mitotic (M) phase. Transition from one phase to the other is highly regulated at various check points. The four phases in a eukaryotic cell cycle are G1, S, G2 and M. Interphase comprises G1, S, and G2 phases. DNA is synthesized during the S phase and other macromolecules are synthesized throughout the interphase. Whether a cell can grow and divide is a highly controlled process at the cellular level.

Cancer is the second leading killer disease in the U.S and in a number of European countries.[3] In cancer, cell division is no longer restrained and cells continue to divide and grow uncontrollably, forming malignant tumors, possibly invading nearby parts of the body. In order to fully understand how a normal cell becomes cancerous, extensive research efforts are devoted to intensely study the mechanism of cell division. A normal cell converting into a cancerous one is a complex multi-step process involving various factors such as genetic instability and increased DNA synthesis during the S-phase of the cell cycle that requires an increased demand for pyrimidine and purine biosynthesis.
To understand how a normal cell might turn cancerous requires substantial analysis of the proteome in both the normal and cancerous cells. Cancer can be considered as proteomic disease since proteins have specific functions in cells and tissues and many are

implicated to play critical roles in tumor genesis and influence the conversion of normal cells into malignant ones. Currently, vast amounts of information are continuously generated for proteomes of different types of cancer. Comparison of patterns of protein expression in different types of cancers is anticipated to result in individualized therapy in the long run. Proteomic techniques are largely used to investigate how signaling pathways are altered in a way that may allow a normal cell to turn cancerous in the hopes of identifying and ultimately developing new drugs that will improve our understanding of how to target various signaling pathways to combat cancer.

In several types of cancer cells, key differences exist in the expression and/or malfunction of certain proteins that regulate signaling cascades involved in, for example, metabolism, DNA repair and cell-cycle regulation. Growth factors such as EGF (epidermal growth factor) and PDGF (platelet derived growth factor) activate the mitogen activated protein kinase (MAPK) cascade which is required for the proliferation of certain cells and differentiation of others. However, uncontrolled activation of this pathway has been implicated in cancer development.[4] When one of the proteins in the pathway is mutated, it can be constitutively stuck in the "on" or "off" position, which is a prerequisite for the development of many cancers. Overexpression and malfunction of different proteins, protein kinases and phosphatases and other components of the MAPK/ERK pathway are believed to result in several types of cancer. A considerable number of drugs have been developed that target particular components in this pathway. Many naturally occurring toxins and pathogens can lead to mutations in particular protein kinases and phosphatases that can result in a number of disorders due to altering the phosphorylation states of intra-cellular targets of many signaling cascades including the MAPK cascade.

Overview: Phosphorylation

Phosphorylation is a ubiquitous cellular mechanism. In the early part of the 19th century, the existence of phosphorylation was considered a biological method of merely providing phosphorus as a nutrient and nothing more.[5] All this changed in the late 1930s when Carl and Getty Cori found that glycogen phosphorylase could be isolated in two forms as phosphorylase b and phosphorylase a.[4] A year later, Fischer and Krebs as well as Wosilait and Sutherland found that the interconversion of phosphorylase b to phosphorylase a involved a phosphorylation/dephosphorylation mechanism.[4] This idea was later shown to be true and has now become an integral example of cell biology and signaling. It has been estimated that 30% of the proteins encoded by the human genome contain covalently bound phosphate, and abnormal phosphorylation is now considered to be a root cause of diseases such as cancer and other proliferative diseases.[4] The extent of phosphorylation of a protein is the result of a dynamic equilibrium between the activity of protein kinases and protein phosphatases.

The addition of the phosphate group is catalyzed by enzymes known as kinases and removal of phosphate groups is catalyzed by enzymes known as phosphatases (Figure 3).

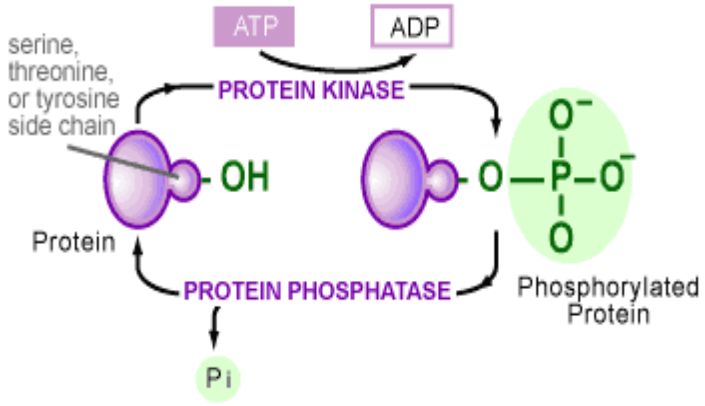


Figure 3. Schematic representation of phosphorylation/dephosphorylation by protein kinases and protein phosphatases [6]

Phosphorylation predominantly involves formation of a phospho-ester of serine, threonine and tyrosine –OH side chains and sometimes phosphoramidate bonds of histidine –NH side chains by the transfer of the γ- phosphoryl group of ATP (or sometimes GTP) to the –OH group or –NH group in the side chains of amino acid residues (Figure 3).

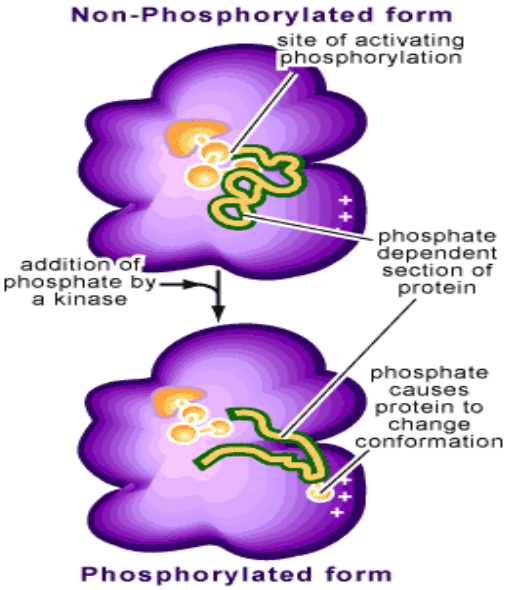


Figure 4. Schematic representation of how phosphorylation can cause conformational changes in a protein [6]

How Can Phosphorylation Control Enzyme Activity?

Protein phosphorylation is one type of post-translational modification. Since phosphate groups are negatively charged, phosphorylation of proteins might alter its overall charge which can then alter the conformation of the protein and ultimately its functional activity and/or protein interacting partners (Figure 4). A change in the phosphorylation state of a given protein can be due to change in the activities of either protein kinase(s) or protein phosphatase(s). The activity of both protein kinases and protein phosphatases is tightly regulated to allow the timely optimal functioning of the protein. The activity of some protein kinases and protein phosphatases depends on the concentration of second messengers such as cAMP, cGMP, Ca^{2+} or diacylglycerol.[4] Other protein kinases or phosphatases are membrane associated receptors (e.g., insulin receptor) and are activated upon binding of their corresponding ligands.[4] Signals from, for example,

EGF and PDGF can activate protein kinases and phosphatases extracellularly. All signal transduction pathways are regulated on some level by phosphorylation making phosphorylation relevant to most, if not all areas of cell signaling. Upon reception of a signal, these phosphorylation cascades continue to function until protein phosphatases are activated and down-regulate the cascade. A typical outcome of phosphorylation in response to a signal is activation of certain kinases and phosphatases which may then alter the accessibility of binding sites on proteins for their binding and interacting partners, altering their function.

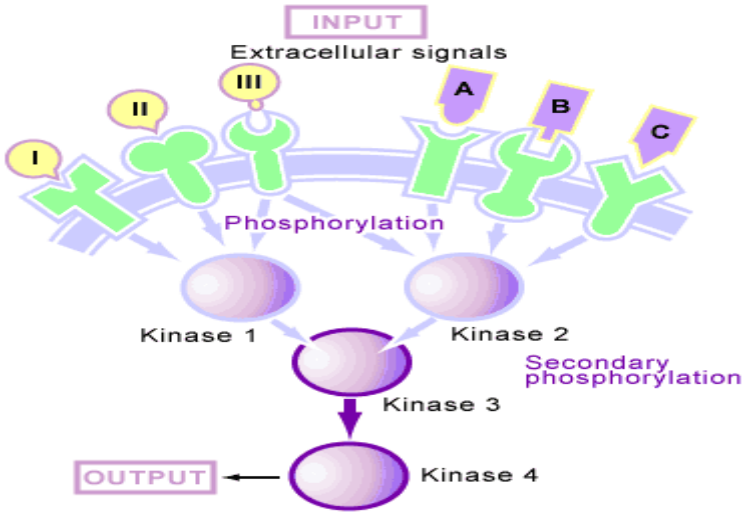


Figure 5. Schematic representation of Phosphorylation Cascade [6]

Phosphorylation (Figure 5) is a molecular switch that directly turns on or off the functions of proteins. Besides controlling enzyme activity and regulation, other functions include regulating and controlling intracellular dynamics and interactions with other proteins or peptides, or their possible degradation by proteases. With such mechanisms, the cell is able to regulate a diverse set of processes including cellular movement, reproduction and metabolism. The mammalian cell constantly receives signals from its surroundings to

which it has to respond appropriately. Growth factors lead to growth of a cell, its differentiation or proliferation. Defects in these tightly regulated and controlled processes can cause cancer and chronic inflammatory diseases.[4]

Protein kinases and protein phosphatases are classified into three classes depending on whether they act either on serine/threonine residues or on tyrosine residue or on all three residues. A further classification is based on the structural relationship and enzymatic properties of the catalytic subunits, subcellular localization, substrate specificity and/or the sensitivity to specific activators and inhibitors.[7]

Protein Phosphatase 1:

Extensive research has been carried out in understanding the role of protein serine/threonine phosphatases in control mechanisms in eukaryotes that regulates processes as diverse as metabolic pathways, hormonal response and cellular signaling, gene transcription, translation, cell division, learning and memory.[8] The human genome encodes ~ 500 kinases of which two thirds are serine/threonine kinases; thus, diversity of kinases has kept pace with increasing complexity of organisms more so than protein phosphatases. There are four distinct families of protein phosphatases which comprise ~ 150 members, of which only 40 are serine/threonine phosphatases.[7] The PTP family is composed of protein tyrosine phosphatases and the dual-specificity protein phosphatases which dephosphorylate phosphoserine and phosphothreonine as well as tyrosine residues. Members of the PPM and FCP1 families are metal dependent protein serine /threonine phosphatases. The PPM family includes the Mg^{2+} dependent PP2C enzymes while the structurally unrelated FCP family has a single member, FCP1. All other phosphoserine/phosphothreonine specific protein phosphatases belong to the PPP family

which is further divided into sub-families PP1, PP2A (including PP4 and PP6), PP2B and PP5. Protein phosphatase 1 (PP1) in particular is ubiquitously distributed and regulates a broad range of cellular functions including glycogen metabolism, cell cycle progression and muscle relaxation.[9] PP1 has evolved to contain an efficient catalytic subunit but lacks substrate specificity. The catalytic subunit of PP1 (PP1c) is highly conserved. PP1 is 37kDa major serine/threonine phosphatase that regulates a variety of cellular functions. PP1 consists of a catalytic subunit and regulatory subunits that determine the subcellular localization of PP1 and/or regulate its function. The PP1 inhibitory subunits are themselves regulated by phosphorylation.

The PP1 catalytic subunit (PP1c) can form complexes with greater than 50 regulatory subunits. The regulatory subunits that interact with PP1c control the specificity and diversity of PP1 function.[7] Most forms of regulation are achieved through the regulatory subunits, although a small number of PP1c molecules may be inhibited by phosphorylation during the cell cycle. Interaction of targeting subunits with PP1c modifies its substrate specificity, most often by an allosteric mechanism.

A plethora of PP1 subunits have been identified and more than 50 regulatory subunits have been discovered in higher eukaryotes.[7] In eukaryotes multiple genes encode PP1c isoforms.[7] The four isoforms of mammalian PP1c are designated PP1α, PP1β (also known as PP1δ) , PP1γ1 and PP1γ2, the latter two arising from alternative splicing.[7] PP1α variant PP1α2 is similar to the PP1α isoform that is predicted from the human genome sequence (Ensembl, ENSP00000176139) indicating that alternative splicing gives rise to two α alpha isoforms.[7] Mammalian PP1c isoforms possess distinct tissue distribution and subcellular localizations as shown in Figure 6.

PP1 Holoenzymes

Cellular functions of protein phosphatase 1 are defined by the association of PP1 catalytic subunit with endogenous protein inhibitors and regulatory subunits. The PP1

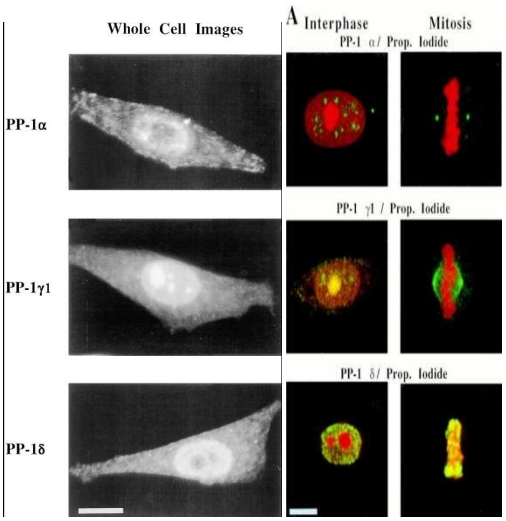


Figure 6. Subcellular localization of PP1c isoforms. PP-1 isoforms are differentially localized both during interphase and mitosis in HeLa cells. (*A*) Images for PP-1 α, γ1, and δ (*green*) merged with respective images of propidium iodide counterstain (*red*) are shown for cells at interphase and at mitotic metaphase.[10]

catalytic subunit forms complexes with more than 50 regulatory subunits in a mutually exclusive manner of which a few are shown in Figure 7 and Table 1.

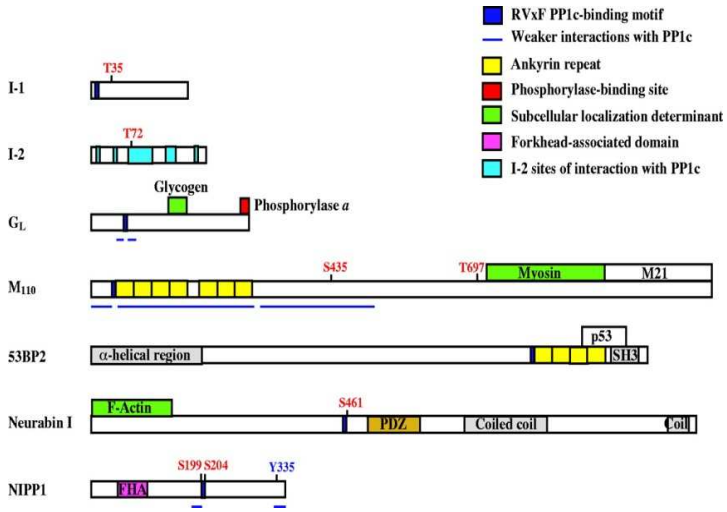


Figure 7. Schematic representation of mammalian PP1 binding subunits.[7]

Table 1. PP1 regulatory subunits [8]

The major region of PP1c that interacts with regulatory subunits consists of 1) a hydrophobic groove to which a RVXF motif binds, and 2) a neighboring negatively charged region. Many PP1 regulators consist of a consensus RVXF sequence that docks within the hydrophobic groove on the surface of the PP1 catalytic subunit. Site-directed mutagenesis studies have narrowed down the consensus sequence for this motif as [RK]-X_{0-1}[VI]-{P}-[FW] where X is any residue except proline.[11] While there are other binding sites on many of the regulatory subunits for PP1, mutations of the RVXF sequence hinders PP1 binding.[12] Crystallization of a small region of a GM peptide with PP1c identified the RVXF motif as the main sequence that mediates the interaction with a hydrophobic groove on the surface of PP1c, that is distinct from the active site, that binds to the substrate and the phosphorylated residues of inhibitor proteins. The hydrophobic RVXF-binding groove is located near the C-terminus of PP1c and includes residues Ile169, Leu243, Phe257, Leu289-Cys291 and Phe293, which interact mainly with valine and phenylalanine residues in the GM peptide containing the RVXF motif.[12] PP1C contains a negatively charged region that accommodates basic residues preceding the RVXF motif.

The RVXF motif, found in about 100 PP1c-binding proteins, binds to a hydrophobic surface groove located on a surface behind the PP1c active site. The RVXF binding channel is a hydrophobic groove opposite the catalytic site and is formed by top and rear edges of two central beta sheets. Binding via the RVXF motif does modify the conformation of PP1 but does not significantly affect the catalytic activity. The available data suggests that the RVXF motif serves as an anchor for the initial binding of the regulatory units thereby promoting binding to secondary sites that often bind with lower affinity but affect the overall activity and substrate specificity of PP1.[12]

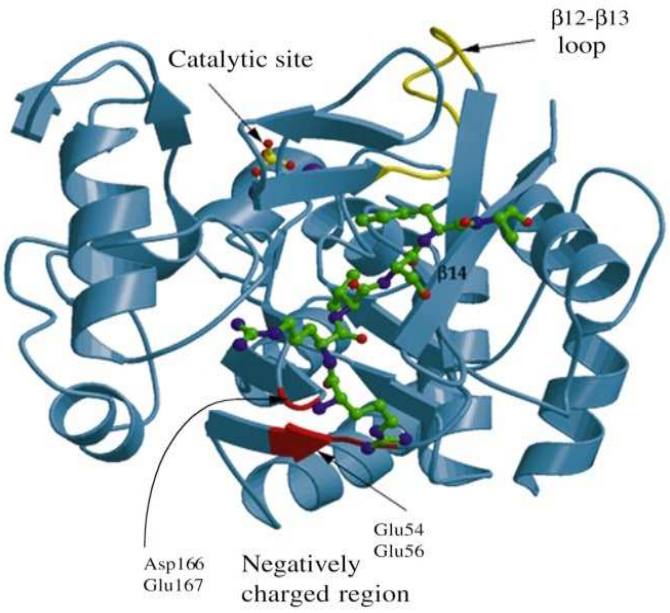


Figure 8. Structure of the PP1c–RVXF-containing- microcystin peptide complex. PP1c is shown as a ribbon structure. The β12-β13 loop that covalently binds to microcystin is indicated in the middle.[7]

The β-12 and β-13 loop form a flexible binding site of PP1 through which toxins, including microcystin, and inhibitors such as Inhibitor-1, DARP-32 and Inhibitor-2 bind with PP1.[13] The loop partially guards the entrance to the active site and undergoes conformational changes upon binding to partners. The β-12 and β-13 loop contains Cys273 that covalently binds to microcystin.

Inhibitor-2

The function of PP1 is largely determined by its associated regulatory subunits that can direct the enzyme to various subcellular compartments to be in close proximity to its substrates. Inhibitor-2 (I-2) was the first protein phosphatase inhibitor identified and known to form a stable high affinity complex with PP1 with nanomolar affinity (*Kd*=2 M).[14] Studies have shown that I-2 mediated inhibition of PP1 regulates cardiac contractility.[16] PP1-I2 complex also plays a role in other processes such as cell division.[17]

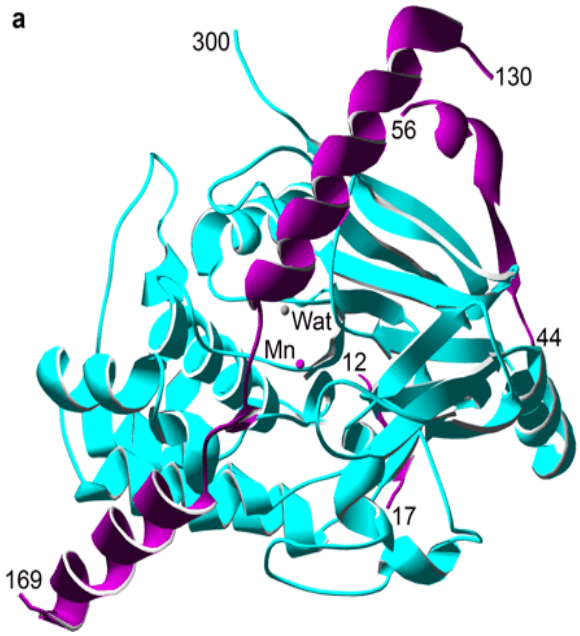


Figure 9. Ribbon structure PP1c-I2 complex. [14]

I-2 associates with the PP1 catalytic subunit to form a complex that is subsequently regulated by the phosphorylation of I-2. I-2 inhibits the PP1 free catalytic subunit and controls the state of activation of PP1. The bound I-2 causes conformational changes which are reversed upon phosphorylation of a conserved Thr (Thr-Pro) site on I-2.[15] The inactive complex can be reactivated through phosphorylation of I-2 at Thr72 by glycogen synthase kinase-3 or the extracellular-regulated kinase 2 (ERK2).[15]

Different domains of I-2 are involved in inhibition, inactivation, and reactivation of PP1. The first 35 residues on the N-terminus and more specifically the motif, 10–13 (IKGI) of I-2, play a role in inhibition.[15] This motif of I-2 binds to a negatively charged region close to the RVXF-binding groove. This region is similar to that known to bind basic residues upstream of the RVXF motif in many PP1 targeting subunits. The region encompassing Thr72 is involved in inactivation of PP1, whereas the COOH-terminal region of I-2 is required for reactivation of the inactive complex.[15]

Multiple regulatory subunits as well as inhibitor proteins besides I-2 compete for a binding site on PP1c. It is proposed that PP1c binds only one protein at a time either to a regulatory subunit or inhibitor proteins such as I-2.[18]

CAD

Over the past decades, extensive research has been carried out to better understand the mechanisms by which signals are transmitted from cell surface receptors to intracellular targets. Protein phosphorylation is a post-translational modification of proteins whereby a serine, a threonine or a tyrosine residue is phosphorylated by a protein kinase by the addition of a covalently bound phosphate group. Phosphorylation of proteins is one of the most common modes of regulation of protein function and activity. Growth signals typically initiate receptor-mediated signal transduction cascades which involve reversible protein phosphorylation that facilitates changes in, for example, transcriptional, translational and cell-cycle regulatory events.

Among the large number of proteins regulated by phosphorylation, several are key enzymes in basic metabolic pathways including *de novo* nucleotide biosynthesis, for example CAD, the key large mammalian multifunctional protein that catalyzes the first three steps in the *de novo* pyrimidine biosynthetic pathway.[19] Proliferating cells require increased amounts of pyrimidine nucleotides for the synthesis of DNA, RNA, phospholipids, glycogen and other biological molecules involved in various processes. CAD consists of six copies of a 243 kDa polypeptide comprised of 2225 amino acid residues.[19] The tri-functional polypeptide is organized into discrete functional domains that carry glutamine-dependent carbamoyl phosphate synthetase (CPSase), aspartate transcarbamoylase and dihydroorotase (DHOase) activities.[19]

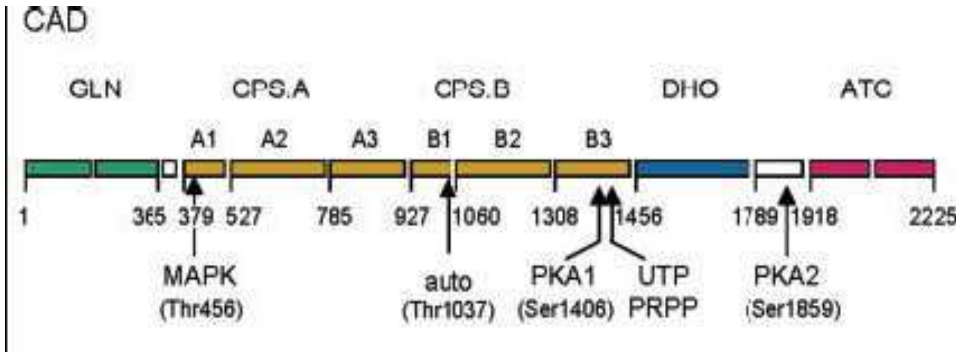


Figure10. The domain structure of the multifunctional protein CAD that catalyzes the first three steps in *de novo* pyrimidine biosynthesis[19]

CAD is regulated by a number of different mechanisms including allosteric and transcriptional regulation, phosphorylation and selective degradation. The activity of the protein invariably increases in tumor cells to meet the increased demand for pyrimidines during proliferation. The CAD complex is activated by MAP kinase (Erk1/2) just prior to the S phase of the cell cycle, when DNA synthesis occurs. MAP kinase is an enzyme that phosphorylates proteins involved in cell growth and division.[20] Growth signals initiate a signaling cascade that activates MAP kinase and leads to the phosphorylation of CAD on Thr-456 in the A1 subdomain (Figure 10). Upon completion of DNA synthesis, the cells emerge from S phase, CAD is dephosphorylated and a second kinase, protein kinase A (PKA), phosphorylates CAD at Ser-1046 in the B3 subdomain leading to deactivation and a decreased flux through the *de novo* pyrimidine biosynthetic pathway (Figure 11).[20]

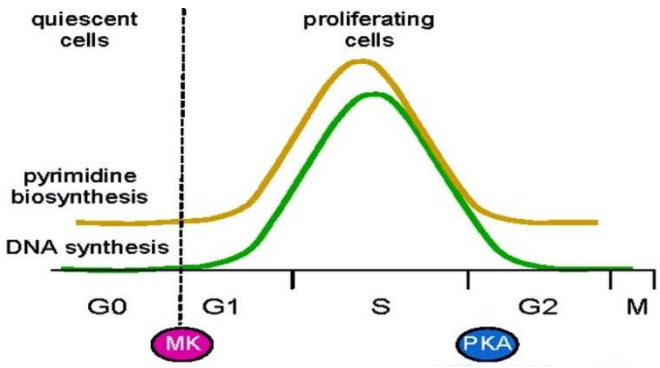


Figure 11. Schematic representation of cell cycle regulation of *de novo* pyrimidine biosynthesis [19]

The timing of the up and down regulation of CAD must be precise but the control mechanisms that regulate the processes are not yet fully understood. Studies have shown that phosphorylation of CAD by PKA down regulates the pathway.[21] It has also been shown that MAP kinase and PKA form stable complexes with CAD suggesting that mutual antagonism is the result of steric effect by the bound kinases.[22] The cytoplasmic level of CAD is higher in resting cells and is primarily unphosphorylated.[23] Cell fractionation and fluorescence microscopy studies (Figure 12) showed that Thr (P)-456 CAD was primarily localized within the nucleus.[23]

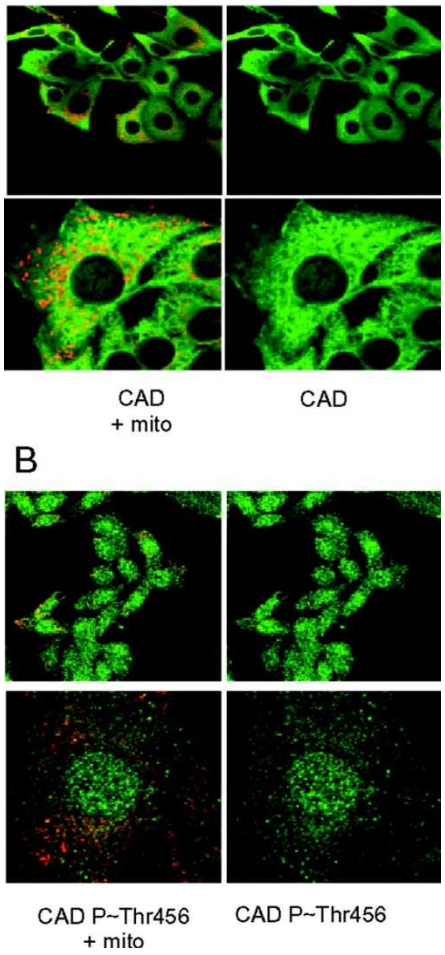


Figure 12. Confocal microscopic images of intracellular localization of CAD. Dephosphorylated is cytosolic whereas phosphorylated CAD P-Thr456 is nuclear[22]

The rate of the *de novo* pyrimidine biosynthetic pathway was found to be up-regulated in MCF7 breast cancer cells. The activity was 4.4 fold higher than the normal MCF10A breast cells.[23] The growth dependent changes in the CAD phosphorylation state and the associated changes in the rate of pyrimidine biosynthesis are similar in normal breast cells MCF10A and BHK21 cells but quite different in the highly malignant MCF7 cells.[23] In MCF10A cells, the rate of pyrimidine biosynthesis is low and CAD is dephosphorylated prior to S phase.[23] As the cultured cells enter the S phase, MAPK is activated and CAD-Thr456 is phosphorylated and pyrimidine biosynthesis is up-regulated. As the cells become confluent, the activity of MAP kinase decreases and CAD-Thr456 is dephosphorylated. Subsequently, CAD Ser1406 is phosphorylated and pyrimidine biosynthesis is down regulated. In MCF7 cells, CAD-Thr456 is persistently phosphorylated while Ser1406 is dephosphorylated leading to a constant rate in pyrimidine biosynthesis.[23] The activity of the pathway was high in newly passaged cells and increased only slightly as the culture became confluent. It has been shown that there was no down regulation of the pathway at higher cell densities.[23] Thus, the cell cycle- dependent regulation of pyrimidine biosynthesis is tightly regulated by reciprocal antagonism of CAD phosphorylation by MAP kinase and PKA.[23]

HYPOTHESIS

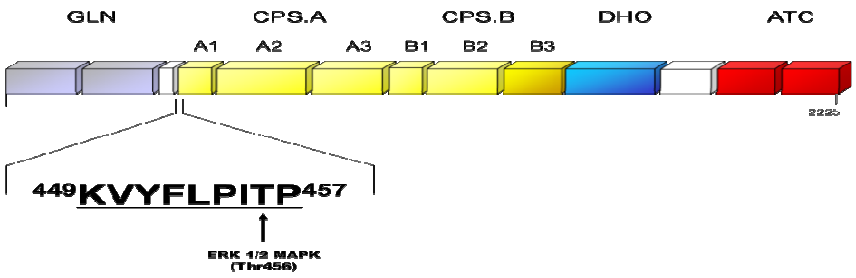
Why CAD and PP1?

This thesis project focused on the interaction between CAD and PP1. PP1 is known to bind to many different targeting proteins. The sequence recognized by PP1 as the binding site of a large number of targeting proteins is highly conserved, Lysine (or Arginine)-Valine-X-Phenylalanine, RVXF or KVXF, where X is any amino acid except Proline as Proline causes steric effects.[11] There is only one such sequence in the CAD complex located immediately adjacent to the MAP kinase site, Thr-456 (Figure 13).

Our working hypothesis is that phosphorylation of Thr-456 blocks the binding of PP1 and thus prevents the premature dephosphorylation and down regulation of the pathway. At the end of S phase, the phosphorylation of CAD by PKA alters the conformation of the protein allowing dephosphorylation of Thr-456 by PP1.

The objectives of this project are to determine 1) the binding or docking site on CAD for PP1 and 2) whether the binding of PP1 depends on whether or not Thr-456 is phosphorylated.

Accomplishing these two objectives is significant as it provides insight into the timing of the phosphorylation/dephosphorylation events of CAD and the resulting outcome on pyrimidine biosynthesis.



Consensus sequence: KVYF

Figure 13. Schematic representation of CAD, residues 1-2225, comprising the putative PP1 binding sequence, KVXF

CHAPTER 2: EXPERIMENTAL METHODS

Several different methods were employed to address the objectives of the project.

Immunoprecipitation

Immunoprecipitation (Figure 14) was used to establish that a complex forms between CAD and PP1. This experiment was carried out by my lab colleagues, Kyle Poulsen and David McDiarmid in Dr. Evans' Lab at Eastern Michigan University.

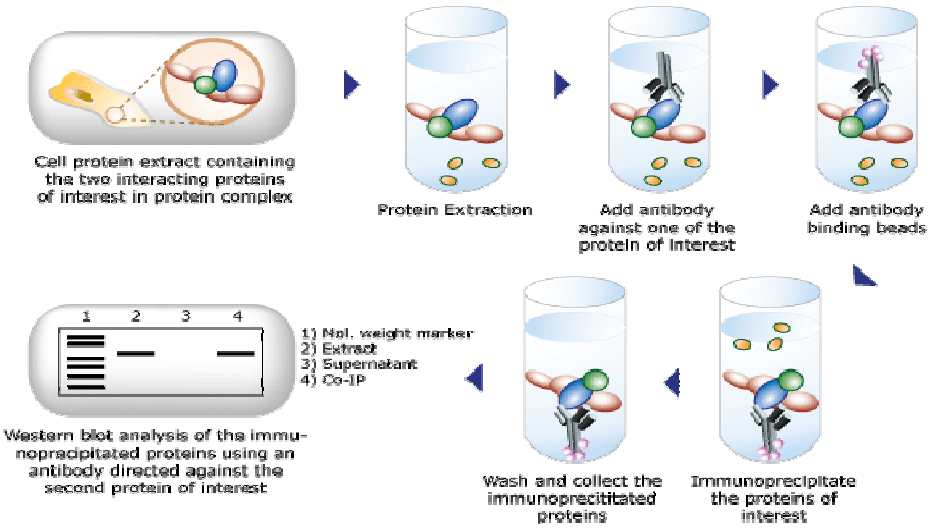


Figure 14. Schematic representation of immunoprecipitation [24]

In this method, one of the proteins, for example, CAD is reacted with CAD-specific antibodies and then incubated with beads that have high affinity for immunoglobins. The protein bound to the bead is then reacted with a second antibody, for example, directed against PP1, to determine whether it forms a complex with the CAD that was previously bound to the beads. The immunoprecipitated proteins are analyzed by Western blotting.

The Microcystin pull down assay was performed by Frederic Sigoillot at Wayne State University. Microcystins are hepatotoxic cyclic peptides produced by cyanobacteria (e.g. *Microcystis aeruginosa*). Microcystins strongly inhibit protein phosphatases type 1 (PP1).

Pull down assays are *in-vitro* assays used to confirm the existence of protein-protein interactions previously predicted by other research techniques such as immunoprecipitation (IP). The working principle is similar to that of immunoprecipitation with the only difference being that a tag is used, such as a histidine-tag, instead of an antibody.

Peptide Synthesis

In order to determine the location of the PP1 binding site on CAD, a 17 amino acid residue peptide (residues 444-460) was synthesized in collaboration with Dr. Debbie Heyl-Clegg which corresponded to the naturally occurring sequence in CAD.

Wild-Type peptide

444Gln-Gly-Leu-Ala-Asp-**Lys-Val-Tyr-Phe**-Leu-Pro-Lle-**Thr**-Pro-His-Tyr-Val460

Putative PP1 binding site MAP kinase site

This peptide was used in competition experiments to determine whether it could bind to PP1 and block the binding and effect of Inhibitor-2 (I-2) on PP1 catalytic activity. No sequence analogous to the consensus RVXF motif is found in I-2. However, the fact that DARPP-32 peptide containing a KIQF is able to antagonize PP1 inhibition by I-2 suggests that I-2 contains a similar sequence. Inhibition of PP1 was measured by assaying the hydrolysis of *p*-nitrophenyl phosphate which produces a colored product that can be measured using a spectrophotometer.

Our hypothesis is that if the peptide binds to PP1, then it should result in blocking or reducing PP1 inhibition by I-2. Moreover, to determine whether the phosphorylation state of Thr456 influences the interaction of CAD with PP1, two additional peptides were synthesized. In one, Thr456 was replaced with Alanine (Ala) as it cannot be phosphorylated.[27] The second peptide was synthesized replacing Thr-456 with Aspartate (Asp). Aspartate has been reported in several studies to mimic a phosphorylated residue both *in vitro* and *in vivo* as when a protein is

phosphorylated, a negative charge is added to the amino acid side chain that mimics that of aspartate.[28]

Mutant peptide1

444Gln-Gly-Leu-Ala-Asp-**Lys-Val-Tyr-Phe**-Leu-Pro-Lle-**Ala**-Pro-His-Tyr-Val460

Mutant peptide 2

444Gln-Gly-Leu-Ala-Asp-**Lys-Val-Tyr-Phe**-Leu-Pro-Lle-**Asp**-Pro-His-Tyr-Val460

Solid Phase Peptide Synthesis

Solid phase synthesis is the most commonly used methodology to synthesize peptides. The fundamental premise of this method is that amino acids are coupled in the desired sequence followed by removal of reversible protecting groups while the C-terminal of the first amino acid is anchored to an insoluble support, a resin. Double coupling was carried out in the chain assembly in order to achieve better yields.

Solid phase peptide synthesis (Figure 15) involves repetition of two steps, coupling and deprotection of amino acids assisted by various reagents. Wash steps remove impurities several times during each cycle. Synthesis starts with the C-terminal amino acid of the sequence. The C-terminal amino acid is attached to a cross-linked polystyrene resin via an acid labile bond with a linker molecule. The resin is placed in the reaction vessel and subsequently deprotected. The desired amino acids are added one at time always at the C-terminus of the amino acid at the end of the growing chain. The N-terminal α- amine group of the added amino acid is protected during coupling and then deprotected awaiting the next amino acid to be added to it. Following this pattern, the peptide is synthesized with amino acids added in order and then the N-terminus can be capped with acetic anhydride to form an acetylated amine.

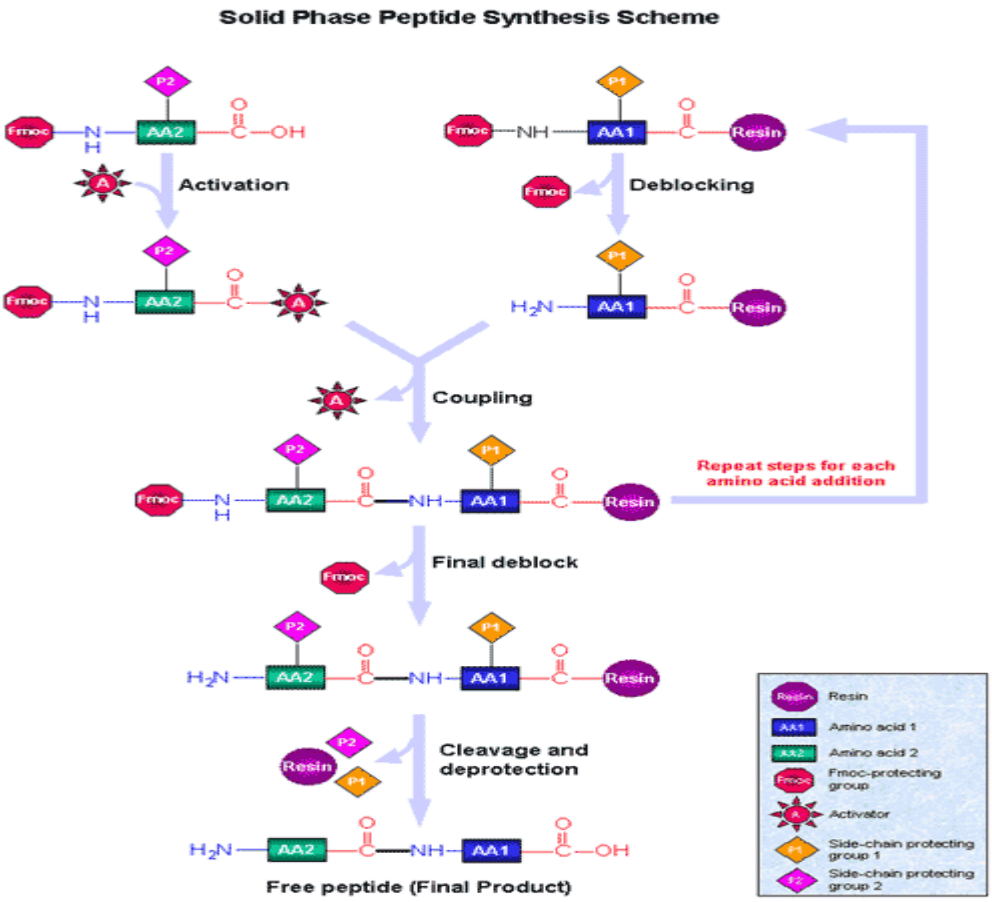


Figure 15. Schematic representation of solid phase peptide synthesis [25]

Three steps are repeated each time as shown in Figure 15:

1) Deprotection of the N-terminal amino acid of the peptide carried out by 20% piperidine in DMF to remove the Fmoc (fluorenylmethyloxycarbonyl) group from the α-amine.
2) Activation and coupling of the next amino acid using HBTU (O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate)
3) Deprotection of the N-terminus of the new amino acid and repetition until the desired peptide is assembled on the resin. A biotin tag was further added at the N-terminus of the three peptides.

The Rainin PS3 synthesizer monitors the deprotection and coupling steps. At the end of the synthesis, the peptide is cleaved from the resin and the side chain protection groups are then removed from the peptide. This is achieved by using a mixture of 90% trifluoroacetic acid (TFA) and scavengers (phenol, water, anisole) that result in solubilization of the peptide. The peptide is then precipitated in cold diethyl ether, re-dissolved in water lyophilized and purified by RP-HPLC.

Crude peptides were purified to homogeneity by preparative reversed-phase high performance liquid chromatography (RP-HPLC) on a Waters instrument with a Phenomenex Jupiter C18 column (2.2 x 25.0 cm, 10mL/min). A linear gradient of 105 acetonitrile (0.1% TFA)/water (0.1%TFA) to 50% acetonitrile (0.1% TFA)/water (0.1%TFA) was employed, followed by lyophilization. Peptide purity was assessed by analytical RP-HPLC. Peaks were monitored at 214, 230, 254 and 280 nm. The peptides were >97% pure as analyzed by peak integration. Electrospray mass spectrometry confirmed the appropriate molecular weights.

Protein Phosphatase Assay:

p-Nitrophenyl Phosphate (PNPP) is a non-specific, chromogenic substrate used to assay (Figure 16) most phosphatases such as alkaline phosphatases, acid phosphatases, protein tyrosine phosphatases and serine/threonine phosphatases. The PNPP phosphatase activity is a spectrophotometric assay based on the ability of phosphatases to catalyze the hydrolysis of PNPP to *p*-nitrophenol, a chromogenic product with absorbance at 405 nm. The reaction product, *p*-nitrophenol, which is soluble under alkaline conditions, has an intense yellow color.

Phosphatase

p-Nitrophenyl phosphate ⟶ p-Nitrophenol + phosphate

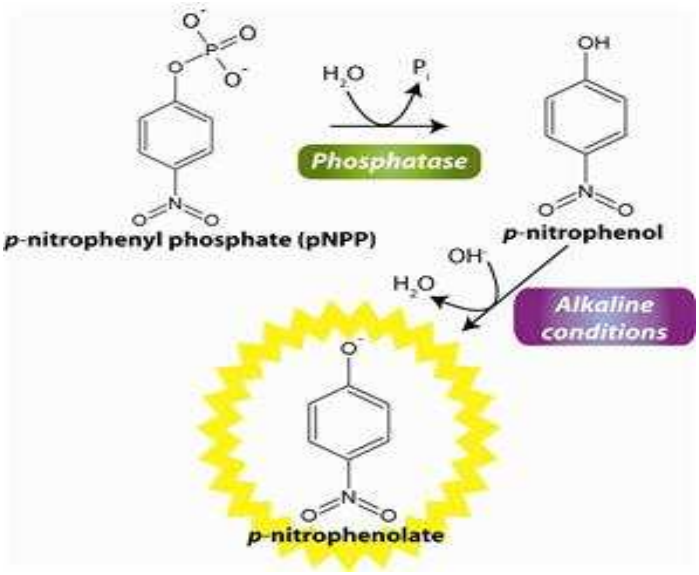


Figure16. The biochemical principle of the alkaline phosphatase assay. [26]

The 1ml of the biotinylated peptides (concentration 1mg/ml, 567 µM) was dissolved in 50 µl DMSO prior to use. The total reaction mixture (50 µl) consisting of 5 µl 10X PP1 reaction buffer, 5 µl of 10X $MnCl_2$, 800 units (0.8 µl) I-2 and 1.25 units (0.5 µl) PP1 was incubated prior to addition of the substrate PNPP (5 µl of 500mM PNPP, final concentration of 50mM and the reaction was quenched by addition of 1 ml of 1N NaOH or 1 ml of 0.5M EDTA. The amount of product, *p*-nitrophenol, was determined by measuring the absorbance at 405 nm. One unit of protein phosphatase activity is defined as the amount of enzyme that hydrolyzes 1 nanomole of PNPP in one minute at 30°C in a total reaction volume of 50 µl under standard reaction conditions.

CHAPTER 3: RESULTS AND DISCUSSION

CAD co-immunoprecipitates with Protein Phosphatase 1

Results obtained from western blot analysis after immunoprecipitation indicated that CAD co-immunoprecipitates with PP1 as shown in Figure 17. The bands in lane 2 and lane 4 indicate the presence of CAD in the supernatant of BHK cells. Immunoprecipitation of PP1 using PP1-specific antibodies contained CAD indicating that CAD forms a complex with PP1 (lane 3 and lane 5).

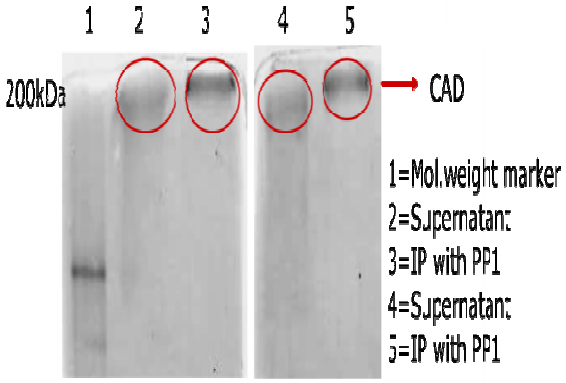


Figure 17. Co-immunoprecipitation of CAD with PP1

Pull Down Assay

Microcystin binds with PP1 and acts as its inhibitor. Microcystin pull down assays further confirmed that CAD and PP1 form a complex. Microcystin agarose beads were used in the pull down assays. No PP1 was pulled down with the control protein G agarose beads. PP1 pulled down with the microcystin beads also revealed the presence of CAD indicating formation of a

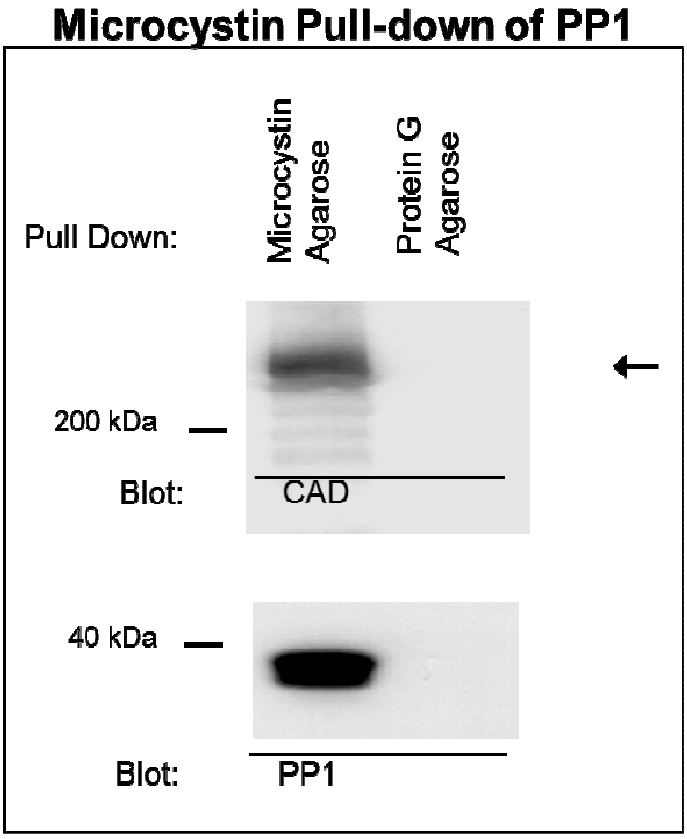


Figure 18. Western Blot of the Microcystin pull-down assay

complex.

Protein Phosphatase Assay

As mentioned earlier, the PP1 binding site (PP1c) can bind either to a regulatory subunit or to inhibitor proteins such as I-2[18]. This can be demonstrated by measuring

spectrophometrically the activity of PP1 with I-2 in presence of the WT peptide and two mutant peptides at 405nm.

Also as discussed earlier, our hypothesis is that if the peptide binds to PP1, it should block the inhibition of PP1 by its endogenous inhibitor, Inhibitor-2 (I-2). The WT peptide that corresponds to the naturally occurring sequence in CAD (Wild-Type peptide: 444Gln-Gly-Leu-Ala-Asp-**Lys-Val-Tyr-Phe**-Leu-Pro-Lle-**Thr**-Pro-His-Tyr-Val460) was used for this assay.

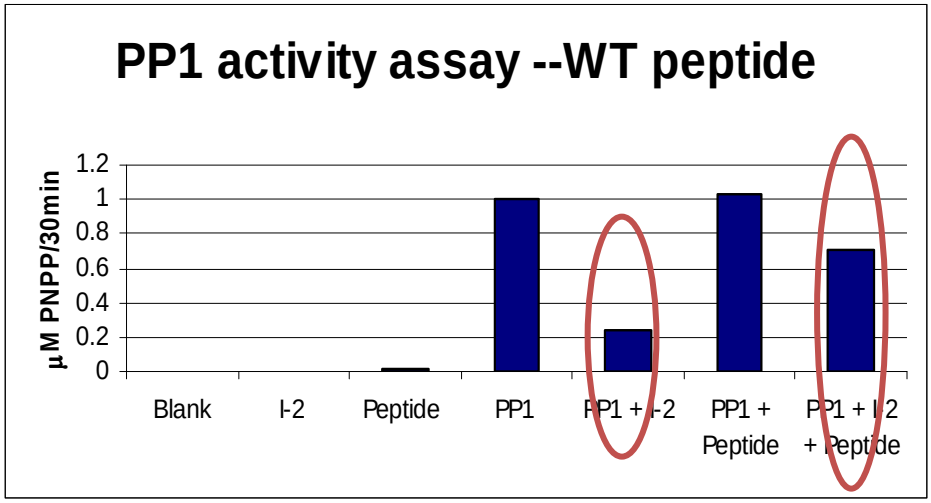


Figure 19. PP1 activity with I-2 in the presence of the WT CAD peptide

As shown in Figure 19, PP1 by itself shows significant activity when it reacts with the substrate PNPP and also shows similar activity in presence of only the WT peptide indicating that the WT peptide binds to PP1 but does not inhibit its activity.

In the presence of only its endogenous inhibitor I-2, PP1 binds with I-2, I-2 inhibits the activity of PP1 represented in the bar graph above encircled in red. However, a significant amount of activity is recovered in presence of the WT peptide. These results suggest that I-2 is not able to inhibit PP1 significantly indicating that the peptide maybe competing for the same binding site on PPI.

In the second assay, a mutant peptide in which Thr-456 is replaced by Alanine (Ala) (Mutant peptide: 444Gln-Gly-Leu-Ala-Asp-**Lys-Val-Tyr-Phe**-Leu-Pro-Lle-**Ala**-Pro-His-Tyr-Val460) was used instead of the WT peptide. This peptide mimics non-phosphorylated CAD because Alanine lacks the side chain that can be phosphorylated. The bar graph below (Figure 20) indicates similar activity of PP1 with its substrate, PNPP, as in the previous assay (Figure 19) and the presence of the Ala mutant peptide does not significantly affect the activity of PP1.

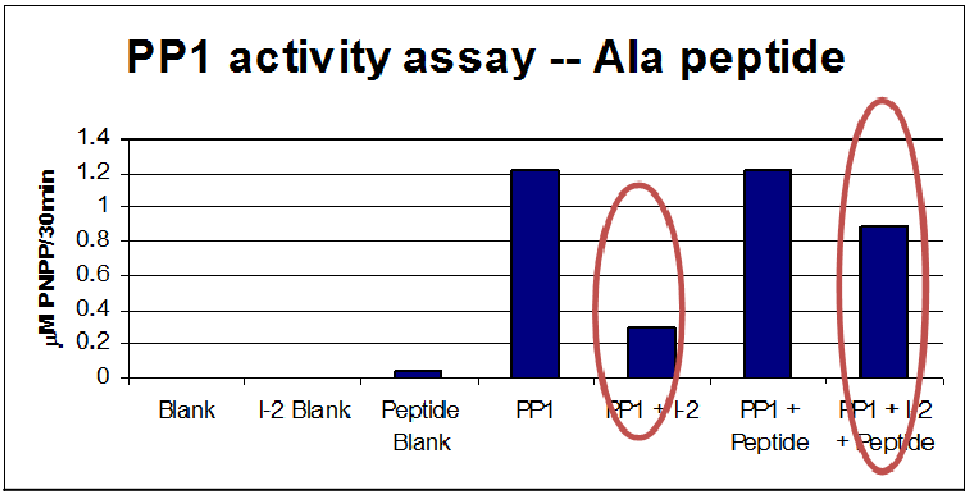


Figure 20. PP1activity with I2 in the presence of the CAD (T456A) peptide

The activity of PP1 was inhibited as expected in the presence of I-2 but in the presence of the mutant peptide CAD (T456A), the ability of I-2 to inhibit PP1 is disrupted as indicated by the bar graph encircled in red (Figure 20). The result of this assay indicates that the binding of the WT CAD peptide and the Thr-456 Ala mutant peptide have the same affinity for PP1 and is able to block the inhibition by I-2.

The result of the third assay was significant in terms of the second goal of the project which is to determine if binding of the CAD peptides to PP1 depends on whether or not Thr-456 is phosphorylated. In this assay, the third mutant peptide was used in which Thr-456 was replaced by Aspartate (Asp) which mimics phosphorylated CAD (Mutant peptide: 444Gln-Gly-Leu-Ala-Asp-**Lys-Val-Tyr-Phe**-Leu-Pro-Lle-**Asp**-Pro-His-Tyr-Val460). As indicated by the bar graph, the CADT456D peptide was largely ineffective against I-2. PP1 was inhibited by Inhibitor-2 (I-2) clearly indicating that the interaction of Inhibitor-2 and PP1 is not disrupted in presence of the Asp mutant peptide (Figure 21). Figure 22 summarizes the effect of the three peptides.

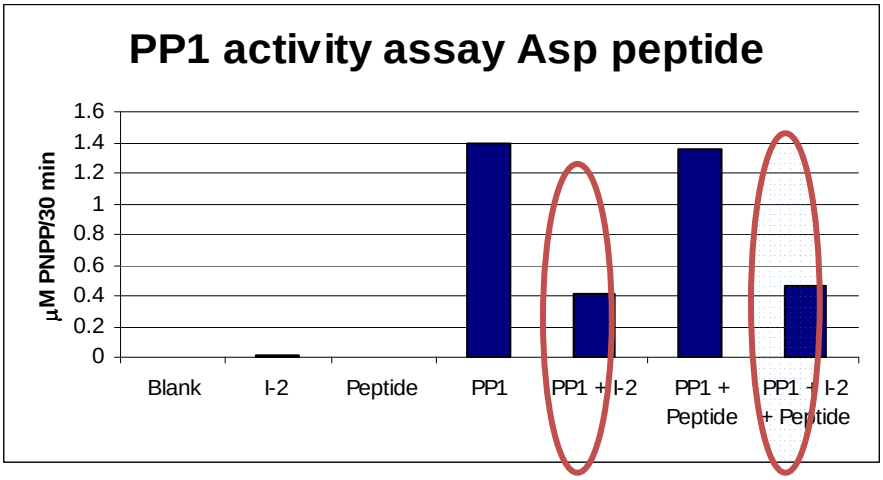


Figure 21. PP1activity with I2 in the presence of the CAD (T456D) peptide

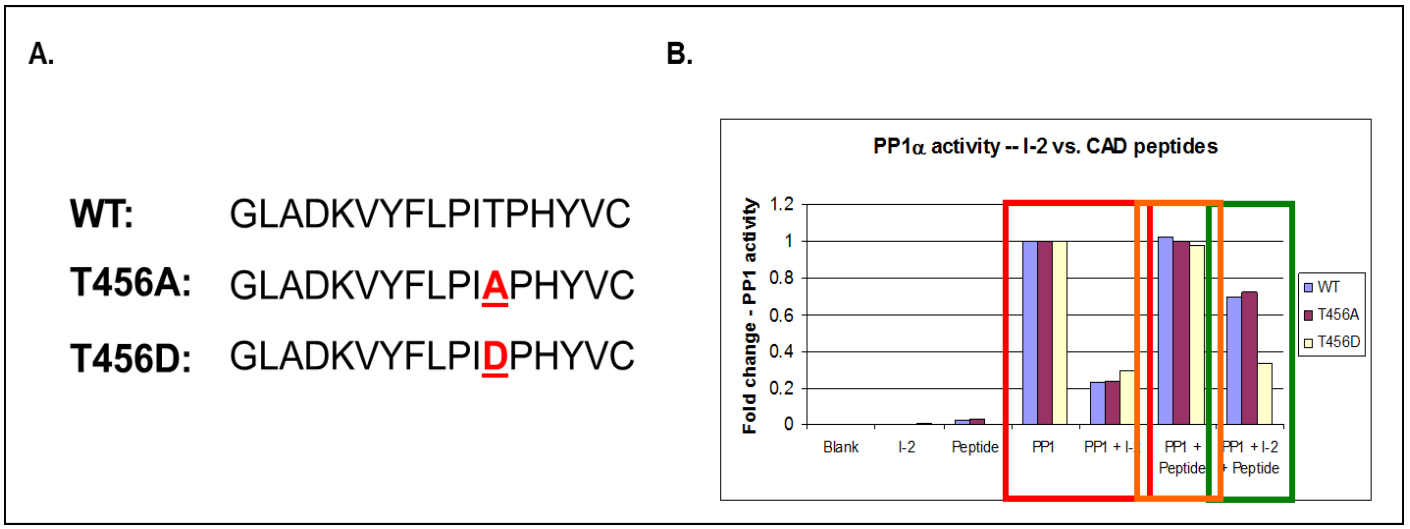


Figure 22. PP1α activity-I2 vs. CAD peptide

An unanticipated result was that when the full length purified CAD protein was incubated with PP1, the phosphatase activity increased more than 2-fold. The significance of this result is not yet clear but it may be necessary mechanism to prevent premature phosphorylation of the PKA phosphorylation site on CAD.

CHAPTER 4: CONCLUSION

Results of the phosphatase assays provide evidence that the peptides, synthesized based on the putative PP1 binding sequence in CAD, have the ability to affect the activity of inhibitor-2 (I-2) on PP1 as summarized in Figure 22.

The observation that the effect of the mutant CAD peptide, T456A, reduces the ability of I-2 to inhibit PP1, and this peptide mimicked unphosphorylated wild type sequence, provides support for the hypothesis that binding of the peptide to PP1 depends on unphosphorylated Thr456 via the putative binding sequence, KVXF, of CAD.

The PP1 binding site on CAD is adjacent to the MAP kinase phosphorylation site. The mutant peptide, Thr456D, which mimics phosphorylated CAD does not appear to hinder the inhibitory effect of I-2, supporting the hypothesis that binding to PP1 depends on whether CAD has been phosphorylated by MAP kinase on Thr456.

This project paves the way for future studies of the complex regulatory mechanisms of CAD. The next step would be to investigate whether PKA binding alters the interactions of CAD with PP1 and the phosphorylation state of Thr-456.

While the up- and down-regulation of the *de novo* pyrimidine pathway has been well-established, the understanding of the mechanism by which PP1 can affect CAD activity is the unique contribution of this project. This project narrows down the region of CAD that interacts with PP1 and suggests the possibility for the formation of a regulated complex. This project begins to fill the gap in our understanding of how PP1 regulates CAD and ultimately the *de novo* pyrimidine biosynthetic pathway and may serve in the design of drugs that could interrupt the activation of this pathway in rapidly proliferating cells.

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