

Structural and biochemical insight into the inositol polyphosphate  
4-phosphatase family of proteins

by

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A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Immunobiology

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2004

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**For the Major Program**

This work is dedicated to my parents Donald F. Shearn Sr. and Catherine R. Shearn and the

7<sup>th</sup> west floor of University Hospital (Denver, Colorado)

“Live each day to it’s fullest for the gift of life is truly precious”

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## ABSTRACT

Inositol polyphosphate 4-phosphatases (IP4Ps) are lipid phosphatases that remove the 4-position phosphate from the lipid second messenger phosphatidylinositol 3,4-bis-phosphate converting it to phosphatidylinositol 3-phosphate. There are 2 families of IP4P's that are 37% homologous to each other denoted type I and type II. Previously, several different isoforms of both type I and type II IP4P had been identified. These include the  $\beta$ -spliceoforms of both type I and II that contained a hydrophobic tail instead of a hydrophilic tail and a novel deletion spliceoform found in brain tissue that is approximately 1kDa smaller than type I $\alpha$  called type I $\alpha_2$ . I have identified a novel splice variant of type I IP4P. IP4P type I $\alpha_3$  is an 110kDa splice variant of type I IP4P's that contains an additional 40 amino acids creating a PEST region. The presence of the additional PEST region could play a role in proteolytic regulation of type I IP4P. This novel spliceoform is predominantly located in T-cells and in other hematopoietic cells. IP4P I $\alpha_3$  could have specialized roles in immune related functions. IP4P are lipid phosphatases, therefore they must be able to get to membranes to have access to their substrate. In this dissertation we identify a lipid-binding C2 domain located on the N-terminal end of both type I and type II IP4Ps. These C2 domains also bind calcium but in the presence of calcium lipid binding is decreased. In addition, we identify a short spliceoform of type II IP4P which we denote Stubby. This spliceoform lacks the phosphatase domain and consists primarily of a C2 domain. The C2 domain of Stubby is not complete, by sequence homology only  $\beta$ -strands 1-6 and all three calcium-binding loops are present. Comparing the lipid binding properties of Stubby, type I IP4P C2 domain and the type II IP4P C2 domain revealed different substrate specificity. In a lipid overlay, the IP4P type I C2 domain and



Stubby prefer to bind phosphatidylinositol 3,5-bisphosphate whereas the type II C2 domain prefers phosphatidylinositol 3,4,5-tris-phosphate. This suggests that different forms of IP4Ps are localized to different places in the cell.

## CHAPTER 1. GENERAL INTRODUCTION

### Introduction

Since the discovery that calcium regulation in cells is partially controlled by cleavage of phosphatidylinositol 4,5-bisphosphate by the enzyme Phospholipase C, phosphatidylinositol signaling has come to the forefront of cell mediated signal transduction. The list of proteins that bind or modify phosphatidylinositols has rapidly grown and these proteins are now known to be involved in virtually every aspect of the cell from cell growth to production of oxidative intermediates during innate immune responses. Some examples of proteins involved in phosphatidylinositol signaling include the protein kinase AKT, synaptotagmins and p47<sup>phox</sup> all of which bind phosphatidylinositols. Phosphatidylinositol lipid modifying proteins include phosphatidylinositol 3-kinases and phosphatidylinositol lipid phosphatases.

The primary focus of this dissertation is the study of novel lipid binding C2 domain found in inositol polyphosphate 4-phosphatases (IP4P). This research provides insight on the mechanism of membrane recruitment for IP4P, an important characteristic of IP4P that until now has not been characterized. In addition, this research describes two new spliceoforms in the IP4P family, type I $\alpha_3$  and Stubby. Although the precise function of these proteins is not clear, the identification of these spliceoforms enhances the diversity of the IP4P family found in humans.

## Dissertation Organization

The first chapter of the dissertation will provide details of the current body of knowledge regarding mechanisms of the production of phosphatidylinositol lipids, their modifications and their ability to recruit proteins to the membrane.

Chapter 2, which has been published in *Biochemical and Biophysical Research Communications*, is the identification of a novel spliceoform of type I IP4P that is primarily found in hematopoietic cells. An examination of the genomic sequence of type I IP4P is also reported.

Chapter 3, to be submitted to the journal *Biochemistry*, involves the identification of a novel C2 domain found in type I IP4P. This C2 domain has the currently unique feature of having a lipid binding mechanism that is inhibited by increasing amounts of calcium. In addition, residues involved in lipid and calcium binding are investigated.

Chapter 4, to be submitted to the *Biochemical Journal*, involves the identification of a novel alternatively spliced form of type II IP4P which we denote Stubby. This spliceoform is interesting due to the fact that it does not contain the phosphatase domain. In fact it consists almost exclusively the N-terminal C2 domain of type II IP4P. Lipid and calcium binding properties of both Stubby and the type II IP4P C2 domain are characterized.

Chapter 5 will present general conclusions from this work and where this work can be taken to learn more about mechanisms of regulating the IP4P family of proteins.

## Literature Review

### *Introduction to phosphatidylinositol lipid signaling*

Although phosphatidylinositols (PtdIns) were discovered over 45 years ago, it has only been in the last 15 to 20 years that research has shed significant light on their function within the cell. Phosphatidylinositols are derived from a D-*myo*-inositol that is attached to diacylglycerol by a phosphodiester linkage at the 1' position (1,2). They are unique molecules that can be phosphorylated at the 3',4',5' hydroxyl groups. This makes them very versatile in that the addition or subtraction of phosphate groups can lead to the presentation of an entirely different motif. This ability leads to signaling specificity being conferred by head group configuration.

In the last 15 years, phosphatidylinositols (PtdIns) have been shown to be necessary for membrane recruitment of many proteins and it is this primary characteristic that makes them extremely important regulators of cellular processes (3,4). Protein domains such as PH, PX and C2 domains can bind polyphosphorylated phosphatidylinositols providing membrane recruitment (5-7). Some examples of proteins containing these domains include the PtdIns(3,4)P<sub>2</sub> binding PX domain found in p47<sup>phox</sup> (a protein involved NADPH oxidase activation), the PH domain of the protein kinase PDK1 that binds PtdIns(3,4,5) P<sub>3</sub> and the C2 domain of Rhabphilin3A, which binds PtdIns(4,5)P<sub>2</sub> and is involved in synaptic vesicle trafficking (7-11). Future research in the field of phosphatidylinositol metabolism should lead to important insight on cellular function.

### *Phosphoinositide kinases*

Phosphoinositide kinases are lipid kinases that phosphorylate phosphatidylinositol on the 3' hydroxyl, the 4' hydroxyl or the 5' hydroxyl on the d-*myo*-inositol ring of

phosphatidylinositol. These reactions are performed by phosphatidylinositide 3-kinases (PtdIns 3-kinases), phosphatidylinositol phosphate 4-kinases (PIP 4-kinases), and phosphatidylinositol 4-phosphate 5-kinases (1,2,12,13). Since the substrate and the products of IP4P are both involved in phosphatidylinositol 3-kinase signaling, Phosphatidylinositide 3-kinases will primarily be discussed. Other phosphatidylinositol lipid kinases are reviewed elsewhere (13,14).

### *Phosphatidylinositide 3-kinases*

There are three classes of PtdIns 3-kinases that are involved in many cellular functions (2,15). Class I PtdIns 3-kinases consists of an 110 kDa catalytic subunit (p110 $\alpha$ , $\beta$ , $\delta$ , $\gamma$ ) and a regulatory subunit (p85 $\alpha$ , $\beta$ , p55 $\gamma$  and p101) (1,15). Class I PtdIns 3-kinases phosphorylate the 3' hydroxyl of PtdIns, PtdIns(4)P, and PtdIns(4,5)P<sub>2</sub> producing the lipid second messengers PtdIns(3)P, and PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>. These kinases signal downstream of receptor tyrosine kinases and heterotrimeric G protein receptors. Activation is via Src-homology 2 (SH2) domains present on the regulatory subunit to phosphorylated tyrosines and by binding of the small G-protein Ras or in the case of class Ib by the  $\beta\gamma$  subunits of large heterotrimeric G-proteins (16). All class I PtdIns 3-kinases also contain C2 domains on their catalytic subunit (15,17).

Class II PtdIns 3-kinases are relatively unknown. They possess a lipid binding C2 domain C-terminus that binds lipids by a calcium independent mechanism (18,19). It should also be noted that deletion of the C2 domain increased lipid activity of PtdIns 3-Kinase C2 $\beta$  but mutation of the PtdIns 3-Kinase C2 $\alpha$  does not affect its localization (16,17). In addition, they contain a PX domain (20). C2 and PX domains will be discussed in more detail later. Class II PtdIns 3-kinases are thought to be involved in insulin dependent skeletal

muscle pathways, production of phosphatidylinositols in the nucleus (21). Moreover and in *Drosophila melanogaster*, the Class II homolog acts antagonistically to the EGF receptor pathway in wing patterning (22).

Class III PtdIns 3-kinases are found in organisms ranging from yeast (Vps34p) to humans (p150) (1). They are involved in regulating vesicular transport processes such as in phagocytosis (23). Class III PtdIns 3-kinase has a preferred substrate of PtdIns and is thought to be the primary producer of PtdIns(3)P in cells (1).

#### *Phosphoinositide phosphatases*

The removal of phosphate groups from the inositol headgroup to create new second messengers is regulated by phosphoinositide polyphosphate phosphatases. These are a large family of lipid phosphatases including 3-phosphatases such as PTEN and Myotubularins, 4-phosphatases and 5-phosphatases such as SHIP (24,25). The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) was first identified as a putative tyrosine phosphatase that is mutated in human prostate, breast and brain tumors. PTEN removes the 3-position phosphate from PtdIns(3,4,5)P<sub>3</sub> converting it to PtdIns(4,5)P<sub>2</sub>. Mutations in PTEN give rise to Cowden syndrome, which has the characteristic of increased breast and thyroid tumors (24,26,27). PTEN is a member of the protein tyrosine phosphatase (PTP) superfamily of enzymes. The primary characteristic of PTP proteins is the presence of a conserved active site motif (CX<sub>5</sub>R) or in PTEN CKAGKGR. The presence of Lys residues at positions C<sup>+1</sup> and C<sup>+4</sup> makes PTEN different from other PTP family members (28). This allows for the catalysis of the primary substrate of PTEN PtdIns(3,4,5)P<sub>3</sub>. PtdIns(3,4,5)P<sub>3</sub> is produced by PtdIns 3-kinases and as stated previously is involved in the regulation of

numerous cellular activities including cell growth, differentiation and modulation of immune functions (1).

PTEN<sup>-/-</sup> mice die before birth with defects in development and regions of increased cellular proliferation (26,29). PTEN also is expressed highly in Purkinje cells and antisense oligonucleotides that suppressed PTEN levels lead to death of neurons (29). An examination of cells that have mutations in PTEN contain elevated protein kinase B (AKT) activity leading to increased cellular survival (30-32). Other proteins that are regulated by PTEN include p70S6 kinase and Tek homology kinases such as ITK (33,34). AKT, p70S6 kinase and ITK are all downstream effectors of PtdIns 3-kinase signaling and either are activated by proteins that can interact with phosphoinositides or interact with phosphoinositides themselves. Thus, PTEN is a negative regulator of PtdIns 3-kinase signaling.

Myotubularins are also phosphoinositide 3-phosphatases, but they have different lipid specificity when compared to PTEN. They will convert PtdIns(3)P and PtdIns(3,5)P<sub>2</sub> to PtdIns and PtdIns(5)P respectively, by hydrolyzing the 3-position phosphate (25). Myotubularins are a very large family containing 13 members, eight of these members have been shown to be active (25). The active site of Myotubularins contains a conserved CX<sub>5</sub>R motif, but instead of the Lys residues found in catalytic site of PTEN they contain Asp residues at C<sup>+1</sup> and C<sup>+4</sup> and a Trp residue at C<sup>+3</sup> (35). It is thought that these amino acids provide lipid specificity towards their substrates.

Myotubularins have been implicated in two different genetic diseases. First, is a mutation in MTM1 that will lead to a X-linked myotubular myopathy, a disease characterized by muscle weakness in newborns (36,37). A role for MTM1 could be in the regulation of myotube formation. The overexpression of inactive MTM1 (D278A) leads to a reduced

accumulation of PtdIns(5)P suggesting that its primary substrate is PtdIns(3,5)P<sub>2</sub> (38).

PtdIns(5)P has been implicated in osmotic stress responses in mammals and plants (39,40).

Also, stimulation of EGF recruits MTM1 to late endosomes. Overexpression of WT MTM1 leads to abrogation of the transition from late endosomes to lysosomes (41).

SHIP (SH2 domain-containing Inositol 5-Phosphatase) contains two primary members (SHIP1 and SHIP2) and several alternatively spliced isoforms. SHIP family members convert PtdIns(3,4,5)P<sub>3</sub> to PtdIns(3,4)P<sub>2</sub>. SHIP1 is expressed primarily in hematopoietic cells but can also be detected in testis (41-43). To date SHIP1 has been linked to numerous processes involved in the immune system. SHIP1 is a negative regulator of myeloid cell development, mast cell activation and B-cell activation (42). It also localizes to the phagosomal cup during phagocytosis, which will be discussed later (44). Activation of SHIP occurs via phosphorylation of the SH2 domain and membrane recruitment. During B-cell activation and mast cell degranulation SHIP1 is recruited to the FcγRIIB and FcεRI receptors respectively (45). In neutrophils SHIP is activated downstream of Lyn kinase after stimulation of the oxidative burst suggesting an additional function besides initiation of the burst. This has been shown to lead to increased apoptosis in neutrophils (46). A knockout mouse of SHIP1 has been created. These mice die from myeloproliferative disease consistent with a function of regulating the development and proliferation of lymphocytes and other immune cells (42).

#### *Inositol polyphosphate 4-phosphatases*

Inositol polyphosphate 4-phosphatases (IP4P) are a family of enzymes that catalyze the hydrolysis of the PtdIns 3-kinase lipid second messenger PtdIns(3,4)P<sub>2</sub> to PtdIns(3)P and have been implicated in the regulation of phosphatidylinositol 3-kinase lipid second



messenger signaling (47). In humans, two different genes encode IP4Ps, type I 4-phosphatases are located on chromosome 2q11.2 and type II 4-phosphatases are located on chromosome 4q28.1-q31.1 (48). An examination of the amino acid sequences of type I and type II IP4P shows that they are 37% homologous to each other (24). Type I and type II IP4P's are alternatively spliced (49). Type I is spliced in two places, the first site of splicing is located on the C-terminal tail and leads to the production of either a hydrophilic tail denoted type I $\alpha$  or a hydrophobic tail denoted type I $\beta$  (49). The second site of alternative splicing affects the central region of the protein and leads to a short 104-kDa form (I $\alpha_2$ ) or a longer 105-kDa form (I $\alpha$ )(50). To date no activity has been identified for the I $\beta$  isoforms of both type I and type II IP4P (49). Both type I and type II IP4Ps are magnesium independent and IP4P family members contain the conserved CX<sub>5</sub>R PTP active site motif (CKSA/GKDRT). Using Northern blotting type I IP4P has been shown to be located in brain>heart =skeletal muscle>spleen>kidneys (50). The current function of type II IP4P is currently unknown. It has a molecular weight of 105 kDa and has been identified by Northern blot to be expressed primarily in skeletal muscle>heart>>>brain=placenta=pancreas>liver> kidney and lung tissue (49). Using lysates of platelets, rat brain, heart, skeletal muscle and spleen >95% of IP4P activity can be immunoprecipitated using an antibody that recognizes the C-terminus of type I IP4P (50,51). This suggests that the relative abundance of type II IP4P in these tissues is very low.

Very little is known about the functional regulation of type I IP4P in cells. In platelets, type I IP4P is proteolytically cleaved by calpain proteases after thrombin or calcium ionophore stimulation (52). Using EDTA or the calpain inhibitor calpeptin blocks IP4P degradation (52). In addition, in platelets the p85 subunit of PtdIns 3-kinase has been shown

to interact with type I IP4P in co-immunoprecipitation experiments (47). Also in a GATA I transcription factor knockout mouse, type I IP4P transcription was found to be decreased (53). In a GATA<sup>-/-</sup> megakaryocyte cell line addition of type I IP4P via retroviral transfection resulted in decreased lifespan. IP4P also could play a role in cell proliferation, using NIH 3T3 cells there is a 2.5 fold decrease in proliferation in IP4P overexpressing cells when compared to control cells (53). Recently an IP4P<sup>-/-</sup> mouse has been created; this mouse dies approximately 21 days after birth. The overall phenotype is failure to thrive with abnormal neuronal development, seizures and defects in hematopoietic development (54). Type I IP4P has also been implicated in regulation of the NADPH oxidative burst in neutrophils. Using an *in vitro* assay and superoxide production two proteins were identified that could promote the formation of superoxide. These proteins were the inositol polyphosphate 5-phosphatase SHIP1 and IP4P (55).

In summary, the addition or removal of phosphate groups from polyphosphatidylinositols by PtdIns kinases and phosphatases allows for the production of completely new signaling mediators. These signals are then propagated by the ability of proteins to associate with membranes in a lipid specific mechanism. IP4P is a lipid phosphatase involved in the production of PtdIns(3)P from PtdIns(3,4)P<sub>2</sub>. This enzymatic action terminates signaling downstream of the lipid substrate PtdIns(3,4)P<sub>2</sub> but potentiates lipid signaling downstream of its product PtdIns(3)P (56)

The targeting of intracellular proteins to membranes plays a major role in cellular processes. These processes include vesicular transport, cytoskeletal reorganization and receptor mediated signaling (4). Proteins are able to accomplish membrane recruitment through a variety of domains that have evolved to provide specific targeting of proteins to

their substrates. These domains include Pleckstrin homology (PH), Phox homology (PX), FYVE domains (Fab1, YTOB, Vac1 and EEA1), and C2 domains. PH, PX, and FYVE domains interact with phosphatidylinositol lipids in a calcium independent mechanism whereas membrane binding of C2 domains can be regulated by calcium.

PH domains were first identified in the protein Pleckstrin and function as polyphosphatidylinositol binding domains. PH domains consist of approximately 120 amino acids and they are frequently found in proteins mediated by PtdIns 3-kinase signaling. These include tyrosine kinases (Bruton's tyrosine kinase), phospholipases (PLC- $\delta$ ), and Ser/Thr kinases (AKT, PDK1) (11).

PX domains have only recently been characterized. They are frequently found on proteins involved in the NADPH oxidative burst ( $p47^{phox}$ ,  $p40^{phox}$ ) but can be found in other processes such as vesicular sorting in yeast (Vam7p) and (sorting nexin family (SNX) in humans (56). They are even located in class II PtdIns 3-kinases. PX domains consist of approximately 130 amino acids and can have lipid specificity for a range of phosphoinositides including PtdIns(3)P ( $p40^{phox}$ ) and PtdIns(3,4)P<sub>2</sub> ( $p47^{phox}$ ) (56)

FYVE domains are small domains that contain approximately 70 amino acids. FYVE domains are found in numerous proteins including human early endosomal antigen 1 (EEA1) and the mammalian ortholog of yeast PtdIns(3)P 5-kinase Fab1, (PIK-fyve) (57). Both of these proteins are involved in vacuolar or endosomal trafficking. FYVE domains typically have lipid specificity for PtdIns(3)P.

### *C2 Domains*

A primary finding of this thesis is the identification of a C2 domain located on the N-terminus of IP4Ps and the characterization of its lipid and calcium binding properties. C2

domains were originally identified and characterized as a calcium dependent lipid-binding domain located in protein kinase C (58). They consist of approximately 130 amino acids that are conserved in a common structure and are the second most common calcium-binding domain in the human genome (59-62). The overall structure of C2 domains consists of eight  $\beta$ -strands that form two  $\beta$ -sheets to create a  $\beta$ -sandwich (63). Three loops on the upper surface and 3 loops on the bottom surface link the eight strands (64). C2 domains are typically classified by their structural properties. There are two structural topologies of C2 domains, topology I and topology II (59,60). A comparison of 109 $\alpha$  carbons of the topology I C2 domain found in synaptotagmin C2A to the topology II C2 domain found in

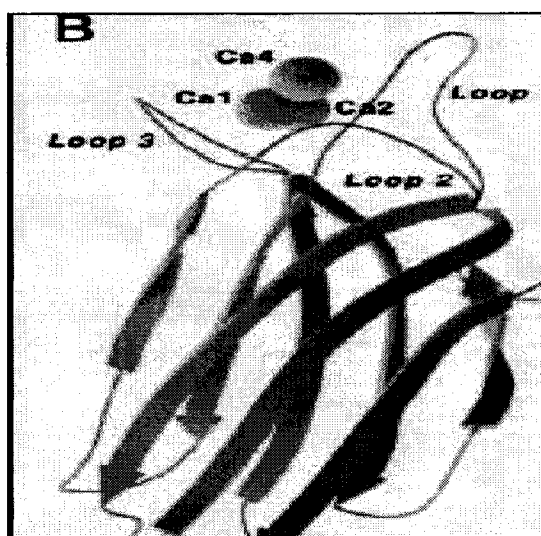


Figure 1. C2 domain of phospholipase C delta (65).

phospholipase C only deviate by a root square mean of 1.4Å (59). The major difference between the two topologies is that if the strands/loops are aligned, strand one in topology I C2 domains is in the same location as strand eight in topology II C2 domains (59).

The  $\beta$ -strands create the platform for the loops to project from (Figure 1). As seen in figure 1, the loops are primarily the site of lipid and calcium interaction (59,65). C2 domains can also be classified due to their function. Proteins that contain single C2 domains frequently are involved in the modification of lipids. These include phospholipases and the

phosphatidylinositol 3 phosphatase PTEN. Proteins that contain two or more C2 domains are frequently involved in vesicular transport such as synaptotagmins (60,66,67).

When an alignment of C2 domains is examined comparing the calcium-dependent C2 domains of Synaptotagmin C2A (Syt C2A), cytosolic Phospholipase A2 (cPLA2), Phospholipase C $\delta$  (PLC $\delta$ 1) and the calcium-independent C2 domain of Protein kinase C epsilon (PKC $\epsilon$ ), the areas in the C2 domain that contain the highest degree of homology are located in the  $\beta$ -strands (59,62,68,69). These residues are primarily hydrophobic residues involved in packing of the sandwich. Within the loops connecting the strands differences can be seen. These differences frequently involve charged amino acids within the loops and determine the ability of C2 domains bind lipids via calcium-dependent or independent mechanisms.

C2 domains can bind lipids in a calcium-dependent or calcium-independent mechanism. To date, only a small fraction of proteins known to contain C2 domains have been characterized with respect to the ability of their C2 domains to bind lipids. SytC2A, PLC $\delta$ 1, Rabphilin3A, Piccolo, and cPLA2 all contain C2 domains that bind lipids in the presence of calcium (9,63,70-72). Protein kinase C epsilon (PKC $\epsilon$ ), and PKC $\delta$  contain a C2 domain that binds lipids in a calcium-independent mechanism (64,68,73).

In Ca<sup>2+</sup>-dependent C2 domains, Ca<sup>2+</sup> is coordinated by amino acid residues located in the calcium binding regions (CBR) linking the top of the  $\beta$ -sheets. Ca<sup>2+</sup> coordination is frequently by conserved Asp residue side chains and by the backbone carbons of other residues located in CBR1 and CBR3 (60). In SytC2A, there are five conserved Asp residues whereas in PLC $\delta$ 1 there are only four (67). Binding of Ca<sup>2+</sup> results in a change in the overall

charge distribution on the surface of the C2 domain making it more positively charged (71,74). This process has often been called an electrostatic switch (75). This allows the C2 domain to interact with the polar head groups of the membrane in an electrostatic mechanism (74). This is especially seen with SytC2A, Rabphilin 3A, Synaptotagmin-like protein 3 (Slp3), PKC $\alpha$ , and a recently characterized C2 domain found in Piccolo (9,71,76,77). Piccolo however, also undergoes a major structural shift with the addition of Ca<sup>2+</sup> allowing it to bind lipids (63). All of the above C2 domains have been shown to prefer negatively charged lipid head groups such as phosphatidylserine and phosphorylated phosphoinositides (9,66,70,78-80). Increasing the ionic strength in solution eliminates electrostatic interactions of calcium dependent C2 domains (68,81). This has been shown for Syt C2A, Piccolo and SLP-3 (61,63,76,77,82).

There are however a class of Ca<sup>2+</sup>-dependent C2 domains that are not sensitive to increasing ionic strength. These C2 domains do not bind PS, instead they interact with PC in a Ca<sup>2+</sup>-dependent manner (71,83,84). This class has been characterized by examining the ability of cPLA2 to interact with lipids. At 1M NaCl, cPLA2 still interacts with PC vesicles indicating that it interacts with PC via hydrophobic residues in its CBR (71). The ability of cPLA2 to translocate to the hydrophobic phase has also been demonstrated using Triton X-114. cPLA2 C2 can be isolated in the detergent phase whereas the SytC2A domain does not (71). Mutations in conserved Asp residues within the CBR of cPLA2 abolished its ability to translocate to membranes in a calcium-dependent mechanism (85).

To date the primary property of C2 domains that has been described is their ability to bind lipids. Overall most C2 domains *in vitro* do not show specificity for individual lipids. Instead they tend to interact with negatively charged lipids or with neutral lipids *in vitro*. One

good example is the localization via calcium-dependent C2 domain of cPLA2 to the Golgi, endoplasmic reticulum or nuclear membranes (85-87). Another is the C2 domain of PKC $\alpha$ . PKC $\alpha$  C2 is directed to the plasma membrane shortly after stimulation with ionomycin (86). The Syt C2A domain localizes to synaptic vesicles but it should be noted that Synaptotagmin I is an integral membrane protein so it does not require its C2 domain for membrane interaction. Its C2 domain mediates the overall ability to bind membranes but it is not involved in translocation. In experiments using GFP, after stimulation with ionomycin SytC2A exhibits a biphasic membrane translocation. First it moves towards the plasma membrane to areas rich in PtdIns (4,5)P<sub>2</sub>, followed by a rapid movement to the trans-Golgi network (85). C2 domain containing proteins also can localize to the nucleus. Double C2 protein  $\gamma$  (Doc2 $\gamma$ ) and Tac2N are recently characterized C2 domains containing proteins that also have a nuclear localization sequence located within CBR3 loops within their C2 domains. Both of these proteins have been shown to localize to the nucleus (88-90). Future studies using GFP tagged C2 domains should provide greater detail on the actual lipid substrates of C2 domains and where they localize.

The ability of Ca<sup>2+</sup>-independent C2 domains to bind lipids has been described for the C2 domains found in PTEN, PtdIns 3-kinase C2 $\beta$  and PKC $\xi$  (18,64,91). The C2 domain of PTEN was originally identified from its crystal structure (91). It does not contain significant homology to other known C2 domains such as SytC2A or PKC $\alpha$ . PTEN appears to prefer Phosphatidylserine over Phosphatidylcholine, however, no other lipid substrates have been tested to date (91). This is also the case for the C2 domain in PtdIns 3-kinase C2 $\beta$  (18). A recent report indicates that the synthetic phosphatidylinositol Di-(C8) PtdIns(4,5)P<sub>2</sub> activates

PTEN in the presence of its substrate PtdIns(3,4,5)P<sub>3</sub> (92). It would be interesting to determine if the C2 domain in PTEN will interact with PtdIns(4,5)P<sub>2</sub>. The C2 domain of PTEN has been proposed to function in the orientation of the active site towards its substrate (93). Positively charged residues located in the CBR regions mediate lipid binding (91). Mutation of these residues abrogates the tumor suppressor function of PTEN in U87-MG glioblastoma cells (94). As previously stated, class I and class II PtdIns 3-kinases contain C2 domains (17,18). These C2 domains are Ca<sup>2+</sup>-independent and bind PtdSer. Deletion of this C2 domain increases the *in vitro* activity using PtdIns as a substrate (18).

The crystal structure of the C2 domain located in PKC $\epsilon$  C2 has been determined. In the presence of Mg<sup>2+</sup>, Mg<sup>2+</sup> is shown to be coordinated within the crystal instead of calcium, this is an interesting finding, although further verification by *in vitro* methods is necessary. Deletion of polybasic residues located in CBR3 decreases the ability to bind phosphatidic acid (68). PKC $\epsilon$  only contains two of five conserved Asp residues found in calcium-dependent C2 domains. Deletion of these residues eliminates lipid binding in calcium-dependent C2 domains but has no effect on PKC $\epsilon$  lipid binding (95).

In summary, C2 domains present the unique ability to recruit or modify protein lipid interactions. This is accomplished by their ability to bind lipids and in the regulation of lipid binding by calcium-dependent or independent interactions. Their roles can vary from the modulation of synaptic vesicle transport by synaptotagmins, the regulation of phospholipase A2 to Golgi vesicles to the orientation of the lipid substrate of PTEN for enhanced enzymatic activity.



### *Immune responses involving PtdIns 3-kinase signaling and IP4P*

Although IP4P has been linked to many different responses its role in the production of superoxide and the oxidative burst is particularly intriguing. In mammals, the NADPH oxidase is essential for host defense against microbial pathogens. This is directly evidenced by the fact that genetic defects in the oxidase complex result in chronic granulomatous disease (CGD)(96-100). Patients with this disease are highly susceptible to infections by pathogens such as *Staphylococcus Aureus*, *Burkholderia cepacea* and *Aspergillus* (101). These microorganisms frequently cause pneumonia in CGD patients that can be very difficult to treat. Another symptom of the disease is the presence of inflammation, which can lead to gastric strictures and chronic inflammation in the lungs (7,101). Over time this can also be fatal.

Lipid signaling plays an important role in the production of reactive oxidative intermediates (ROI) that are subsequently involved in host defense. Three of the proteins involved in superoxide production contain a lipid-binding domain. In p47<sup>phox</sup> and p40<sup>phox</sup> there is a lipid binding PX domain (7,8). The PX domain of p47<sup>phox</sup> has been shown to be able to bind the substrate of IP4P (PtdIns(3,4)P<sub>2</sub>) and the p40<sup>phox</sup> PX domain can bind the product of IP4P (PtdIns(3)P). In addition, Rac2 contains a prenylation site on its C-terminal end that allows for the addition of farnesyl groups that can subsequently be used for membrane recruitment (102).

The function of the NADPH oxidase is to produce ROI such as superoxides that are microbicidal towards bacterial and fungal pathogens (101,103). These intermediates have also been shown to activate proteases that also are involved in destruction of pathogens (100). The production of ROI's can also be detrimental. Overproduction of ROI has been

linked to ischemic injury found in heart attacks and strokes (104,105). There are five major proteins involved in the activated NADPH complex. These include two integral membrane proteins, gp91 and p22<sup>phox</sup> that together form the cytochrome b558 complex (106-108). There are also three cytosolic proteins, p47<sup>phox</sup>, p67<sup>phox</sup> and the small GTPase Rac2 (101,103). Gp91 contains an NADPH binding site, FAD and two non-identical heme groups (108,109). p22<sup>phox</sup> is not stable unless it is co-expressed with gp91, it has been shown to bind phosphorylated p47<sup>phox</sup> upon activation (110,111) (Dinauer personal communication).

The NADPH oxidase complex is regulated by the PtdIns-dependent recruitment of p47<sup>phox</sup>, p67<sup>phox</sup> and p40<sup>phox</sup> to the complex at the membrane upon receptor binding (103). These proteins are held together in a complex by SH3 domain interactions (103,112). Upon activation, p47<sup>phox</sup> is phosphorylated by protein kinase C $\theta$  and possibly by AKT and translocates to the membrane (111,113-115). Beyond its adaptor function, the use of *in vitro* experiments or systems that overexpress p67<sup>phox</sup>, ROI's can still be produced (103,107). Therefore, p47<sup>phox</sup> is not necessary for production of ROI's. p67<sup>phox</sup> however, is thought to be involved in the transfer of electrons to NADPH and there is some evidence that p67<sup>phox</sup> can bind NADPH (55,116). The function of p40<sup>phox</sup> is not understood, it is not however necessary for production of the oxidative burst.

Recent evidence has shed light on the mechanism of NADPH oxidase regulation by Rac2. The GTP bound form of Rac2 will interact with p67<sup>phox</sup> via N-terminal region of p67<sup>phox</sup> binding to Rac2 via its switch region (117). Neutrophils purified from Rac2<sup>-/-</sup> mice are deficient in chemotaxis and superoxide production (118). In a transgenic COS<sup>phox</sup> cell system, it has been shown that the level of superoxide production is dependent upon the amount of activated Rac that is present in the cell (119). In addition, the activation of Rac2

occurs by the guanine nucleotide exchange factor regulated by PtdIns 3-kinase signaling, P-Rex1. It is this activation of P-Rex1 that stimulates the production of superoxide (120). P-Rex1 is activated by both PtdIns(3,4,5)P<sub>3</sub> and by the Gβγ subunits of large heterotrimeric G proteins (120).

PtdIns(3)P lipids have also been shown to have an essential role stimulation of superoxide production. *In vitro* experiments have shown that addition of PtdIns(3)P to neutrophil lysates stimulates superoxide production (55). PtdIns(3)P can be produced from type I PtdIns 3-kinases or it can be produced from the reaction catalyzed by inositol polyphosphate 4-phosphatases (1,51). In a cytosolic purification experiment, the lipid 5-phosphatase SHIP and IP4P were purified by their ability to stimulate superoxide production (26,55). This links the lipid substrate of 4-phosphatases to regulation of the oxidative burst.

#### *Phagocytosis in mature Phagocytes and in Neutrophils*

In mammals, there are two primary cells that are involved in phagocytosis of particles greater than 50μM. These are macrophages and neutrophils (121). Both neutrophils and macrophages contain and undergo phagocytosis via Fcγ receptors and the complement receptor 3 (CR3) (44,121,122). The majority of live cell research on phagocytosis has been performed using GFP labeled proteins and macrophages. The overall mechanism of phagocytosis in neutrophils is unclear due to the inability to manipulate neutrophils. This is due to the fact that they are very short lived as primary cells having an average lifespan of 16 hours. Therefore, phagocytosis via Fcγ receptors in phagocytes will be the primary focus of this review.

The process of phagocytosis can be broken down into several steps. The first is initiation of phagocytosis by Fcγ receptor activation; this is followed by cytoskeletal

reorganization, formation and closure of the phagosomal cup and the maturation of the phagosome (123). Purification and proteomic analysis of phagosomes indicates that there are at least 140 proteins that are involved in the process of phagocytosis and phagosomal maturation (124).

Upon receptor ligation there is a formation of pseudopods that encircle the particle and engulf it in a zipper-like mechanism (125,126). This extension is mediated by the actin cytoskeleton (127,128). The actin cytoskeleton plays an important role in phagocytosis and phosphatidylinositol lipids play a direct role in the organization of the cytoskeleton. After the Fc $\gamma$  receptor is bound by IgG, PtdIns 4-phosphate 5-kinase is activated (128). It produces both PtdIns(4)P and PtdIns(4,5)P<sub>2</sub>. These lipids are able to interact with cytoskeletal proteins such as vinculin and talin. Vinculin and talin will subsequently bind actin (128,129).

Experiments using wortmannin have shown that PtdIns 3-kinase is not involved in this process. Overall this process has been shown in several different experiments.

Overexpression of PtdIns 4-phosphate 5-kinase causes an increase in actin polymerization (128). Also lipid vesicles consisting of PtdIns(4,5)P<sub>2</sub> can recruit actin binding proteins and other regulatory factors including the protein N-WASP (130). N-Wasp binds Arp2/3 and the small G-protein Cdc42 and is involved in nucleation of actin filaments (131).

As actin is recruited to the site of phagocytosis there also are type I PtdIns 3-kinases that are activated to produce PtdIns(3)P and PtdIns(3,4,5) P<sub>3</sub> around the phagosomal cup (23,44). Phagocytosis is almost completely abrogated by the addition of PtdIns 3-kinase inhibitors wortmannin and LY294002 (132). Recruitment of PtdIns 3-kinase is by SH2 domains and tyrosine phosphorylation Syk and by members of the Src family of tyrosine kinases (133-135). The lipid phosphatase SHIP is activated immediately prior to closing of

the phagosomal cup to convert PtdIns(3,4,5) P<sub>3</sub> to PtdIns(3,4)P<sub>2</sub> (44). This process is negatively regulated by the lipid 3-phosphatase PTEN. PTEN<sup>-/-</sup> macrophages display a phenotype of increased phagocytosis compared to control macrophages (136).

Maturation of the phagosome has largely been studied using GFP labeled proteins that are known to bind during different stages of maturation. This includes the small GTPase Rab5a that recruits type III PtdIns 3-kinase and early endosomal antigen 1 which binds PtdIns(3)P produced by type III PtdIns 3-kinase (123,137). There is some evidence however that PtdIns(3)P can be produced before Rab5 is recruited but the mechanism of lipid production is not clear (137). If Rab5 recruits type I PtdIns 3-kinase and Rab5 is not on the phagosomal membrane where does the PtdIns(3)P come from? It could come from a yet unidentified PtdIns 3-kinase or perhaps it could be due to the activation of IP4P. As the phagosome matures, Rab7 and 9 are recruited as well as lysosomal associated membrane protein 1 (LAMP-1) a marker for phagolysosome formation (123). Rab7 is involved in regulation of membrane transport from early to late phagosomes (138-140).

Calcium also plays a role in phagocytosis. As previously stated many of the cytoskeletal proteins involved in the formation of actin filaments are also calpain substrates. This allows for turnover of actin and movement of the vesicle. Calcium is released via a two-stage mechanism. Cleavage of PtdIns(4,5)P<sub>2</sub> by phospholipase C produces diacylglycerol (DAG) and inositol 3,4,5-trisphosphateP<sub>3</sub> (IP<sub>3</sub>). DAG and IP<sub>3</sub> then stimulate release of calcium and activation of protein kinase C. Recent studies have examined the actual location of calcium fluxes during Fcγ mediated phagocytosis. Upon binding of the receptor there is a wave of calcium that is released that travels around the cell. After phagocytosis the wave splits into two separate signals, the first travels around the phagosome. The second continues

around the cell (122,141). Mutational studies examining the role of the receptor identified three residues in the cytoplasmic tail of the receptor (LTL) that when mutated prevented the calcium signal from encircling the phagosome. This prevented phagolysosomal fusion without influencing the recruitment of the small GTPases Rab5 and Rab7 (138). In a separate study, researchers found that calcium controls activation of the NADPH oxidase in neutrophils upon  $\beta 2$  integrin mediated phagocytosis (122).

The role of the oxidative burst in phagosomal killing is controversial. Traditionally, it has been assumed that the production of oxidative intermediates is in itself able to kill engulfed pathogens. Recently evidence has suggested that production of superoxide is involved in the activation of proteases within the phagolysosome (100). This occurs by the rise in anionic charged superoxides resulting in the influx of  $K^+$  into the endocytic vesicle. It is then hypothesized that the rise in ionic strength causes a release of cationic granule proteins such as elastases and cathepsin G, these proteases subsequently kill the bacteria (100).

There are some differences in phagosomes when comparing neutrophils to macrophages. The process of phagocytosis in neutrophils is not fully understood. This is due to the inability to manipulate neutrophils. Neutrophils do produce significantly higher amounts of superoxides, they also have granules that contain proteases such as gelatinase and alkaline phosphatase. Fusion of these granules appears to be mediated by calcium. It is interesting to note that synaptotagmin II associates with specific granules and translocates to the phagosome in a calcium dependent mechanism (142). Synaptotagmins contain calcium dependent C2 domains and are thought to act as calcium sensors during vesicular fusion.

In conclusion, polyphosphatidylinositols are lipid second messengers that mediate signal transduction events by their ability to recruit and localize proteins required for many cellular processes. These processes include cytoskeletal reorganization, vesicular trafficking and production of oxidative intermediates. IP4P are a family of proteins that remove the 4-position phosphate from the lipid second messenger  $\text{PtdIns}(3,4)\text{P}_2$  to produce a new second messenger  $\text{PtdIns}(3)\text{P}$ . These second messengers are able to recruit proteins to the membrane by specificity implied by their headgroup conformation. Understanding the role of membrane recruitment of IP4P family members will give greater insight into the regulation of processes mediated by its substrate and product second messengers.

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# CHAPTER 2. IDENTIFICATION OF A NOVEL SPLICEOFORM OF INOSITOL POLYPHOSPHATE 4- PHOSPHATASE TYPE I EXPRESSED IN HUMAN PLATELETS: GENE STRUCTURE OF HUMAN INOSITOL POLYPHOSPHATE 4-PHOSPHATASE

A paper published in *Biochemical and Biophysical Research Communications*

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## **Abstract**

Inositol polyphosphate 4-phosphatases (IP4Ps) are enzymes involved in the regulation of phosphoinositide 3-kinase (PI3K) signaling. IP4Ps catalyze the hydrolysis of the D-4 position phosphoester of the PI3K generated lipid second messenger, phosphatidylinositol 3,4-bisphosphate. Western blot analysis detected the expression of a novel 110 kDa form of IP4P type I $\alpha$  in mouse spleen, heart, lung, and uterus. In addition, the 110 kDa form of IP4P type I $\alpha$  was found to be the major form of this enzyme expressed in human platelets, MEG-01 megakaryocytes and Jurkat T-cells. RT-PCR analysis of MEG-01 megakaryocytes and Jurkat T-cells indicates that the 110-kDa form of IP4P I $\alpha$  is derived from an alternatively spliced mRNA that encodes an additional internal domain of 40 amino acids not present in the two previously described brain IP4P I $\alpha$  spliceoforms. The predicted molecular mass of this

spliceoform is 109,968 Da, consistent with its apparent molecular mass estimated by Western blot analysis. The novel domain is proline rich and contains a PEST sequence characteristic of proteins that are rapidly degraded by the calpain family of proteases. Analysis of genomic DNA sequence indicates that the IP4P type I gene consists of 25 exons and that this novel spliceoform is obtained as a result of an unusual type of differential splicing involving the use of an alternative 5'-GU donor splice site during the excision of intron 15. In addition, we show that all three known spliceoforms of IP4P I $\alpha$  result from alternative splicing involving exon 15 and 16 indicating that structural variability in this region of the enzyme may be important for its function.

## **Introduction**

Phosphoinositide 3-kinases (PI3Ks) phosphorylate the D-3 position of inositol lipids to produce the second messengers phosphatidylinositol 3-phosphate (PtdIns(3)P), phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P<sub>2</sub>) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>). These second messengers are involved in the regulation of numerous cellular events including growth, differentiation, vesicular sorting, glucose transport and platelet aggregation (1–3). Inositol polyphosphate 4-phosphatases (IP4Ps) are enzymes involved in the regulation of PI3K signaling. IP4Ps are Mg<sup>2+</sup>-independent phosphatases that catalyze the hydrolysis of the D-4 position phosphoester of the second messenger, PtdIns(3,4)P<sub>2</sub> (4, 5). IP4P type I and II are encoded by different genes and have 37% amino acid identity (6). Both IP4P type I and II mRNAs are alternatively spliced to yield transcripts that encode proteins with variable C-terminal domains that are either hydrophilic (IP4P I $\alpha$  and IP4P II $\alpha$ ) or hydrophobic (IP4P I $\beta$  and IP4P II $\beta$ ) (6). IP4P I $\alpha$  has recently been implicated in the regulation of PI3K signaling in human platelets. IP4P I $\alpha$  is

inactivated in response to thrombin stimulation in platelets by calpain-dependent proteolysis (7). In addition, human platelet IP4P I $\alpha$  has been shown to co-immunoprecipitate with PI3K suggesting a direct interaction that may be important for the regulation of PI3K in platelets (8).

Here we report that the major form of IP4P I $\alpha$  expressed in human platelets is a novel 110-kDa spliceform that contains an internal 40 amino acid domain that is not present in the previously described brain isoform. This domain contains a PEST sequence, a region rich in proline, serine, glutamate/aspartate and threonine residues that is characteristic of proteins that are rapidly degraded by the calpain family of proteases (9, 10). Western blot analysis of mouse and human tissues and cells indicates that this 110-kDa spliceform is expressed in a tissue-dependent manner. In addition, we describe the genomic structure of the human IP4P type I gene and demonstrate that the mRNA encoding the 110-kDa spliceform results from an unusual type of differential splicing involving the use of an alternative 5'-GU donor site for the excision of intron 15 that extends this exon by 120 bp. Sequence analysis indicates that three spliceforms of IP4P I $\alpha$  result from alternative splicing involving exons 15 and 16. This variable internal region of IP4P I $\alpha$  spliceforms may be important for tissue-specific function of these enzymes.

## **Materials and Methods**

### *Tissue and cells*

Human brain tissue was obtained from the Harvard Brain Tissue Resource Center. Jurkat T-cells clone E-6 (ATCC#TIB-152), Ramos B cells (ATCC CRL-1596), MEG-01 megakaryocytes (ATCC CRL-2021), NIH 3T3 cells (ATCC CRL-1658) were all purchased from the American Type Culture Collection. The NK 3.3 cells were a generous gift from Dr.

Joan Cunnick. Human platelets were obtained from normal human donors and were purified as described previously (9).

*Cloning of IP4P type I $\alpha_3$  cDNA*

Total RNA was isolated from MEG-01 cells and Jurkat T-cells using Trizol reagent (Life Technologies, Inc.), and first strand cDNA was synthesized using AMV reverse transcriptase (Promega). The antisense IP4P type I specific primer used for the reverse transcription was 5'-TCACTCATCATGGCCACGCAG-3'. PCR reactions were performed in 50  $\mu$ l using 2.5 units of Pfu Turbo™ polymerase (Stratagene) and the 125 ng of each oligonucleotide. The templates used in these reactions were 1  $\mu$ l of first strand cDNA or 1  $\mu$ g of human brain Quick-Clone™ cDNA (Clontech). The oligonucleotides used for PCR were 5'-CTGACTACATTGCCTCCAAG-3' (sense), and 5'-CGCGCTGTCCTGCATGAGC-3' (antisense). PCR reaction mixtures were denatured for 1 minute at 95°C and then cycled 29 times with a 1 min denaturation step at 95 °C, 1 min annealing step at 58°C, 1.5 min extension step at 72°C. Following the PCR cycling, samples were incubated for an additional five minute extension at 72°C to fill in any ends. PCR products were blunt end ligated into Sma I digested pBluescript SK<sup>+</sup> (Stratagene) and sequenced by the Iowa State University DNA Sequencing Facility. The IP4P type I $\alpha_3$  pBluescript SK<sup>+</sup> was then subcloned into IP4P type I $\alpha$  PCDNA3 with Sse 8387-1 (Takara Biomedicals) and Sfi-1 (Promega). The cloned IP4P type I $\alpha_3$  PCDNA3 was used for transfections into NIH 3T3 cells. The genomic sequence (AC010134) was obtained by a BLAST search of the GenBank database using the sequence of IP4P type I $\alpha$ .

### *Transfections of IP4P I $\alpha_3$*

A 0.4  $\mu$ g aliquot of each plasmid was transfected into NIH 3T3 cells with EFFECTENE (Qiagen). One day after transfection, the cells were washed twice with Phosphate Buffered Saline and lysed in 20 mM Tris, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1% Triton X-100, 1 mM Pefabloc, and 1mM leupeptin. The lysates were centrifuged for 10 minutes at 14,000 rpm and the supernatants collected. 16 $\mu$ l of each supernatant was then diluted with 4 $\mu$ l of 5x SDS loading buffer and loaded onto a 6% SDS gel, transferred to a nitrocellulose membrane, and probed with the polyclonal N-terminal IP4P antibody (5). Detection was performed with the SuperSignal West Pico Luminol/Enhancer system (Pierce).

## **Results**

### *Western Blot Analysis of Inositol Polyphosphate4-Phosphatase Type I Expression*

Western blot analysis of mouse tissues using rabbit anti-serum reactive against the C-terminus of IP4P I $\alpha$  detected a novel 110-kDa form of this enzyme expressed in spleen, skeletal muscle, lung and uterus (Fig. 1A). This 110-kDa form was shown to be expressed in a tissue-dependent manner at varying levels relative to previously described 106- and 102-kDa spliceoforms designated IP4P I $\alpha_1$  and IP4P I $\alpha_2$ , respectively. Identical results were obtained for western blots with rabbit antiserum reactive to the N-terminus of IP4P type I confirming that the 110-kDa protein is the novel form of IP4P I $\alpha$  (data not shown). In addition, Western blot analysis detected the expression of the 110-kDa form of IP4P I $\alpha$  in human tissues and cultured cells. The 110-kDa form of IP4P I **a** is the major form expressed in human platelets, MEG-01 megakaryocytes, and Jurkat T-cells whereas only the 106-kDa enzyme is detected in human brain lysate (Fig. 1B). Treatment of Jurkat T-cells lysates with

potato acid phosphatase and calf intestine phosphatase had no effect on the electrophoretic mobility on the 110-kDa form of the enzyme, indicating that the apparent size difference relative to the brain form of the enzyme was not a result of a difference in phosphorylation (data not shown).

#### *Characterization of a Novel Spliceoform of IP4P Type I $\alpha$*

In order to determine if the 110 kDa form of IP4P I $\alpha$  was derived from alternative pre-mRNA splicing, RT-PCR analysis was performed using total RNA isolated from MEG-01 megakaryocytes and Jurkat T-cells. The use of one pair of oligonucleotides resulted in the amplification of two products (Fig. 2). The minor product had the predicted 428-bp sequence of the previously cloned human brain cDNA encoding the 106 kDa form of the enzyme (5). However, the major product had a 548-bp sequence representing a novel alternatively spliced mRNA containing a 120-bp insertion encoding a proline-rich internal domain of 40 amino acids (Fig. 3A). The predicted molecular mass of this IP4P I $\alpha$  spliceoform is 109,968 Da consistent with the molecular weight estimated by western analysis. The 548-bp PCR product was amplified from both megakaryocytes and Jurkat T-cells RNA but not brain cDNA and therefore correlated with the expression of the 110- kDa form of IP4P I  $\alpha$  (Figs. 1B and 2). Western blot analysis of this novel IP4P I  $\alpha$  spliceoform transiently expressed in NIH 3T3 indicates that it has a electrophoretic mobility indistinguishable from that of the 110-kDa form of the enzyme expressed in Jurkat T-cells whereas transient expression of the previously described 106-kDa spliceoform co-migrated with the major enzyme expressed in brain (Fig. 4). These data indicate that the observed 110 kDa enzyme is a novel spliceoform of IP4P I $\alpha$ . This represents the third spliceoform of IP4P I $\alpha$  identified in mammals and is therefore designated IP4P I $\alpha_3$ .



Analysis of the human genome database indicates that BAC clone RP11-12K18 derived from human chromosome 2 (GenBank Accession No. AC010134) contains all of the known IP4P type I exons. Table 1 and Fig. 1B show the intron/exon organization of the IP4P type I gene. The open reading frame for this gene is encoded by 25 exons. The start methionine and the stop for the C-terminus of IP4P I $\alpha$  was found to be 67,559-bp apart. Human IP4P type I cDNA untranslated regions (UTRs) have not been well characterized and the exons that encode 5' and 3'-UTRs are therefore not defined. Exon 1 contains the ATG start codon as well as 103 nucleotides of 5' UTR. Analysis of this genomic sequence indicates that the exon 24 and 25 encode the C-termini of IP4P I $\beta$  and IP4P I $\alpha$  spliceoforms, respectively. Further analysis shows that the insertion characteristic of IP4P I  $\alpha$ 3 results from the use of an alternative 5'-GU splice site during the excision of intron 15 that extends exon 15 by 120-bp (Fig. 1B). Interestingly, all three of the known IP4P I $\alpha$  spliceoforms result from alternatively splicing involving exon 15 and 16. IP4P I $\alpha$ <sub>1</sub> transcripts result from the use of 5'-GU splice site A during the excision of intron 15 whereas IP4P I $\alpha$ <sub>2</sub> transcripts are formed by splicing exon 15 splice site A to exon 17 thereby skipping exon 16. Inspection of exon 15 indicates that between the competing 5'-GU splice sites are three repeats spaced seven bases apart with the nucleotide sequence CCCCTYCW where Y represents C or T and W represents A or T (Fig. 3B). In addition, exon 16 contains the sequence CCCCTCCT with this consensus (Fig. 3B). These pyrimidine-rich elements may represent recognition sites for splicing factors that regulate the tissue-specific alternative splicing of exon 15 and 16.

## Discussion

Inositol polyphosphate 4-phosphatases (IP4Ps) are  $Mg^{2+}$ -independent enzymes that catalyze the hydrolysis of the D-4 position phosphoester of the PI3K generated lipid second messenger, PtdIns(3,4)P<sub>2</sub> (4, 5). Two genes that encode IP4Ps have been identified in mammals. The IP4P type I gene (INPP4A) and IP4P type II gene (INPP4B) map to chromosome 2q11.2 and chromosome 4q28.1-q31.1, respectively (11, 12). IP4P type I and II have the conserved sequence CKSAKDRT that contains the CX<sub>5</sub>R active site consensus sequence of  $Mg^{2+}$ -independent phosphatases (6, 13). Structural diversity of both type I and II IP4Ps results from alternative splicing of pre-mRNAs that results in spliceoforms with hydrophilic or hydrophobic C-terminal domains that are designated  $\alpha$  and  $\beta$ -splice variants, respectively (6). In addition, two spliceoforms of IP4P I $\alpha$  have been identified that differ in an internal region of the enzyme. IP4P I $\alpha$ <sub>1</sub> is a 106 kDa form that represents the major form of the enzyme expressed in human, rat and mouse brain (5, 14). IP4P I $\alpha$ <sub>2</sub> is a 102 kDa form that is a minor species expressed in rat and mouse brain (5). The amino acid sequence of IP4P I $\alpha$ <sub>1</sub> and IP4P I $\alpha$ <sub>2</sub> differs in that the proline-rich sequence DCSPPEESSP encoded by human exon 16 is deleted in IP4P I $\alpha$ <sub>2</sub> (5). In this study, we report the identification of a novel 110 kDa form of IP4P I $\alpha$  which we designate IP4P I $\alpha$ <sub>3</sub>. IP4P I $\alpha$ <sub>3</sub> represents the major form of IP4P I $\alpha$  that is expressed in human platelets, MEG-01 megakaryocytes, and Jurkat T-cells (Fig. 1B). This enzyme is derived from an alternatively spliced mRNA that encodes a proline-rich domain of 40-amino acids in an internal region of the protein not present in the previously described spliceoforms. This domain contains two features that may be important for the function of this enzyme in human platelets. Previously, it was shown that human platelet IP4P I $\alpha$  was inactivated in response to thrombin stimulation by calpain-mediated

proteolysis (7). Evaluation of this 40-amino acid domain using the computer program PEST-FIND (9) indicates a strong PEST score of 7.5 for the sequence RPEDPFCDVPSSPCPSTM-PSTACH. PEST sequences are proline, glutamate/aspartate, serine, threonine rich motifs that are common features of proteins that are rapidly degraded by the calpain family of proteases (9, 10). The presence of this PEST sequence in IP4P  $\text{I}\alpha_3$  may enhance its susceptibility to calpain proteolysis, important for its regulation in platelets. In addition, this domain contains two PXXP consensus sequences for binding sites of Src homology 3 (SH3) domains that are involved in protein complex formation of several signaling proteins (15). Human platelet IP4P  $\text{I}\alpha$  was shown previously to form a complex with PI3K (8). This interaction may be a result of the binding of these PXXP motifs to the SH3 domain of the p85 regulatory subunit of PI3K.

Analysis of exon/intron organization of the human IP4P type I gene has revealed several important features relevant for the alternative splicing of its pre-mRNA. IP4P type I gene consists of 25 known exons (Table 1 and Fig. 3). The alternative splicing occurs in two regions of the pre-mRNA. Firstly, the variable C-terminal domains characteristic of the  $\alpha$  and  $\beta$ -spliceforms result from alternative splicing involving exons 23, 24, and 25 (Fig. 3B). IP4P  $\text{I}\beta$  transcripts are formed by splicing exon 23 to exon 24 whereas IP4P  $\text{I}\alpha$  transcripts are formed by skipping exon 24 and splicing exon 23 to exon 25. Secondly, the variable internal domains of the IP4P  $\text{I}\alpha$  spliceforms result from alternative splicing involving exons 15 and 16 (Fig. 3B). Exon 15 contains two competing 5'-donor splice sites that can be used in intron excision (Fig. 3B). The use of site A during splicing results in IP4P  $\text{I}\alpha_1$  mRNA whereas the use of site B extends this exon by 120-bp and results in mRNA encoding the

additional 40-amino acid domain characteristic of IP4P  $I\alpha_3$ . IP4P  $I\alpha_2$  mRNA results from the use of site A during the splicing of exon 15 to exon 17 thereby skipping exon 16.

The use of alternative 5'-donor splice sites resulting in exons of variable length is unusual and has only been reported for a few alternatively spliced pre-mRNAs including those encoding the mammalian proteins caldesmon (16) and SWAP (17), and the *Drosophila fruitless* protein (18). The regulation of the alternative splicing of the pre-mRNAs of caldesmon and *fruitless* have been studied in detail and has been shown to involve repetitive exonic sequence elements located between the competing 5'-GU donor splice sites that function as splice enhancers (16, 19). Splice enhancers are short RNA sequences that bind members of the SR family of proteins that regulate the choice of splice sites used by the spliceosome (20, 21). A diverse group of sequences have been shown to function as splice enhancers and both purine and pyrimidine-rich classes of splice enhancers have been identified (16, 20). Inspection of the sequence of exon 15 between the competing 5'-donor splice sites indicates the presence of three pyrimidine rich elements with the consensus sequence CCCCTYCW. In addition, a single element with this consensus sequence occurs in exon 16. These sequences may represent splice enhancers important for the tissue-dependent splicing of exons 15 and 16 resulting in the expression of the three IP4P  $I\alpha$  spliceoforms.

### Figure Captions

**Fig. 1:** Western blot analysis of the expression of inositol polyphosphate 4-phosphatase type  $I\alpha$  in mouse and human tissues and cells. Lysates were separated on 6% SDS-PAGE and then analyzed by Western blotting (A) IP4P  $I\alpha$  expressed in mouse brain (lane 1), spleen (lane 2), heart (lane 3), skeletal muscle (lane 4), lung (lane 5), and uterus (lane 6) was detected using rabbit polyclonal antiserum. B. IP4P type  $I\alpha$  expressed in human brain (lane

1), Jurkat T-cells (lane 2), MEG-01 (lane 3), and platelets (lane 4) was detected using rabbit polyclonal serum. Each lane represents approximately 10 µg total protein loaded except for the human and mouse brain lanes which represents 1 mg total protein.

**Fig. 2:** RT-PCR analysis of alternatively spliced mRNAs of inositol polyphosphate 4-phosphatase. RT-PCR products generated as described in the materials and methods were separated on a 1.5% agarose gel and stained with ethidium bromide. Lane 1 contains DNA size markers of 2000, 1200, 800 and 400 bp. Lane 2 contains the PCR products amplified from human brain cDNA. The 548-bp and 423-bp RT-PCR products amplified from RNA isolated from Jurkat T-cells and MEG-01 are shown in lanes 3 and 4, respectively.

**Fig. 3:** Sequence analysis of the novel alternatively spliced RT-PCR product. A. DNA sequence and predicted amino acid sequence is shown for the 548-bp RT-PCR product amplified from total RNA isolated from MEG-01 and Jurkat T-cells. Bars indicate the oligonucleotides used for PCR. The boxes indicate novel amino acid sequence not present in the previously described 106 kDa human brain IP4P type I $\alpha$ . The asterisk indicates the GT representing the 5'-donor site A of exon 15. Dashed lines indicated position of possible splice enhancers with the repeated sequence CCCCTYCW where Y represents C or T and W represents A or T. B. This schematic represents the exon/intron structure of the IP4P type I gene (INPP4A). Exons are represented by boxes and introns by lines. Exons involved in alternative splicing are numbered and connected by angled lines. The exploded diagram shows the position of the alternative 5'-GU donor sites involved in excision of intron 15 labeled A and B. The dark gray box represents the additional 120 bp of exon 15 that encodes the domain of 40 amino acids characteristic of the novel spliceoform IP4P I $\alpha_3$ .

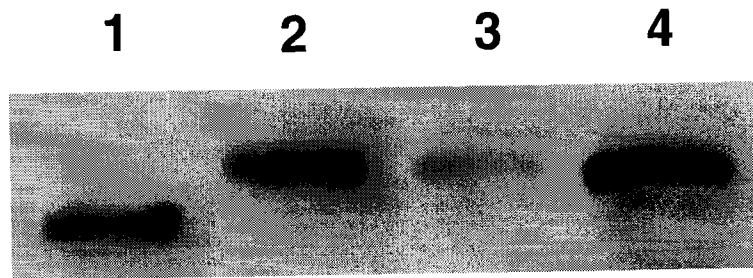
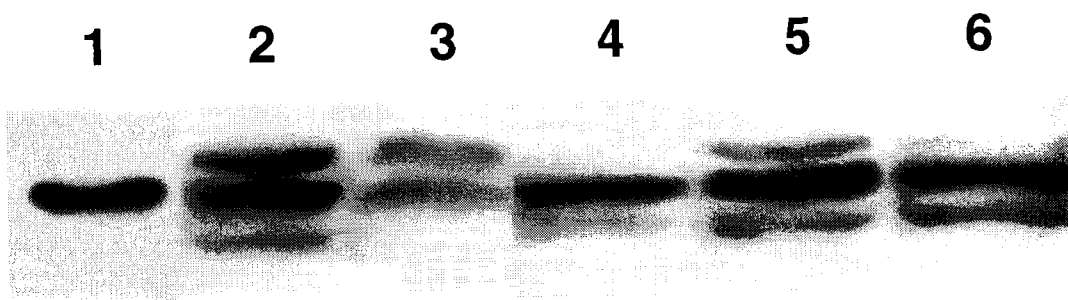
**Fig. 4:** Comparison of the electrophoretic mobility of inositol polyphosphate 4-phosphatase type I $\alpha$  and I $\alpha_3$  to that of the endogenous enzyme. Lysates were separated on 6% SDS-PAGE and then analyzed using rabbit polyclonal serum reactive against the N-terminus of IP4P type I. The endogenous enzyme expressed in Jurkat T-cells (lane 1) and human brain (lane 2) is compared to IP4P I $\alpha_3$  (lane 3) and IP4P I $\alpha_1$  (lane 4) transiently overexpressed in NIH 3T3 cells.

**Table 1:** Organization of the Human Inositol Polyphosphate 4-Phosphatase Type I Gene

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**Figures****Figure 1****A****B**



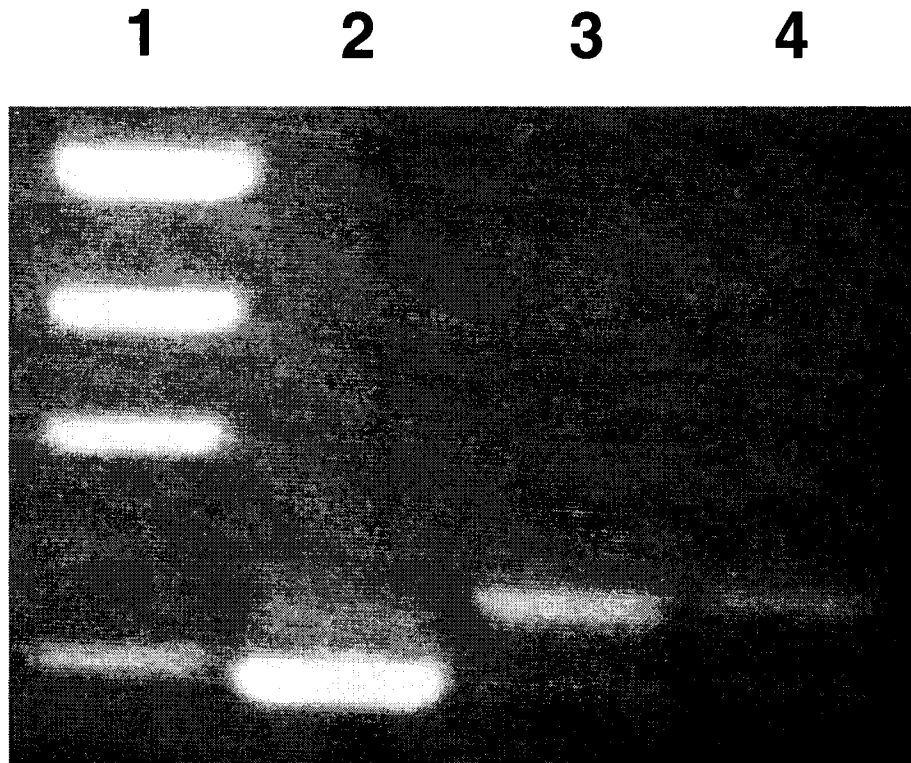
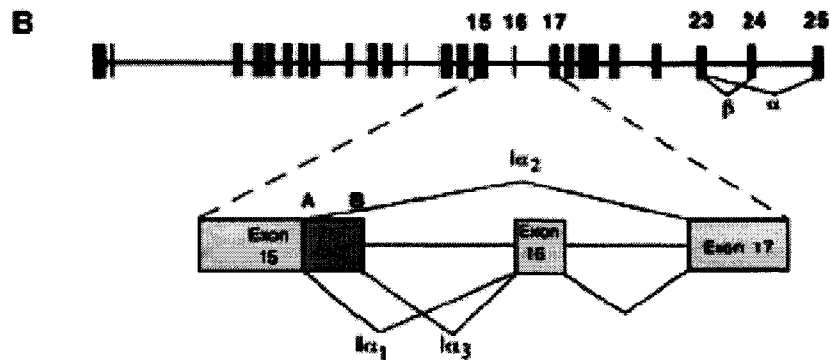
**Figure 2**

Figure 3

**A**

CTGACTACATTGCCTCCAAGGCCTCTCCCACTTCGACTGAGGAGGAGCAGGTGATGCTTA  
 A G H G I P A H A G Q R A E E E Q V M L  
 GAAATGACCAGGACACCCCTCATGGCCCGGTGGACAGGGAGAAACAGCCGATCTTCCCTGC  
 R N D Q D T L M A R W T G R N S R S S L  
 AGGTGGA CTGGCAGGAGGAGTGGGAGAAAGTGTGGCTGAACGTGGACAAGAGCCTAG  
 Q V D W H E E E W E K V W L N V D K S L  
 AGTGCATCATTGCGGTGTGGACAAGCTGCTGCAGAAGGAGCGGCTGCATGGCGAGGGCT  
 E C I I Q R V D K L L Q K E R L H G E G  
 GTGAGGATGTCTTCCCTGTGCAGGCAGCTGCACCAGCAAGAAAGGTAACCCGGACAGCC  
 C E D V F P C A G S C T S K K **G M P D S**  
 ACGCCTACTGGATCAGACCAGAAGACCCCTTCTGTGATGTCCCTCTCACCATGCCCT  
**H A Y W I R P E D P F C D V P S S P C P**  
 CCACCATGCCCTCTACTGCATGCCATCCTCATCTGACCACACATTGCAGTCCCCCTCTG  
**S T M P S T A C H P H L T T H** C S P P P  
 AAGAGTCCAGCCAGGTGAATGGAGTGAGGCCCTTACCCGCTGCTGACCACTCTCACCG  
 E E S S P G E W S E A L Y P L L T T L T  
 ACTGCGTGGCCATGATGAGTGACAAGGCCAAGAAGGCCATGGTATTCTGCTCATGCAGG  
 D C V A M M S D K A K K A M V F L L M Q  
 ACAGCGCG  
 D S A



**Figure 4**

**1                      2                      3                      4**

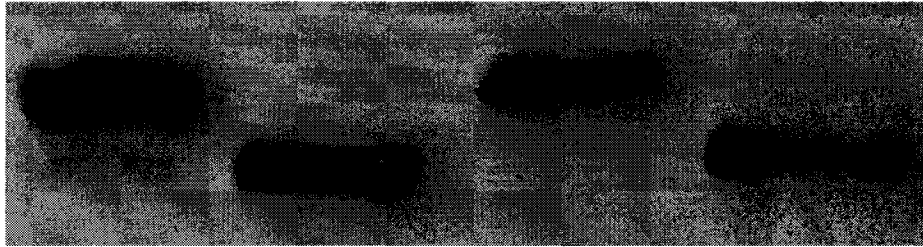


TABLE 1  
Organization of the Human Inositol Polyphosphate 4-Phosphatase Type I Gene

Exon no.	Exon boundary		Exon length	Intron no.	Intron boundary		Intron length
	5'	3'			5'	3'	
1	GGCTAC	TGGCAG	308	1	gtgagcctc	tctattcag	435
2	GAAATA	GCTTAG	42	2	gtagggtatc	attctgtag	12645
3	CTTGCA	ATTGAG	118	3	gtgggtgct	ctgttctag	2232
4	GGAACC	GGAACA	128	4	gttaagtaa	cttctcag	270
5	ATGTAT	ACTAAG	79	5	gttgggtatt	tgatcacag	1653
6	GTCTGC	GGGAGG	111	6	gtgagttac	cattatcag	914
7	ATGGTT	AATCGG	101	7	gtaaaacagc	ccactcag	535
8	TGTTCC	AGCCAG	147	8	gtgaggcca	ctttcagag	4202
9	TGTGTG	ACAGAG	133	9	gtgggtgca	atttcttag	1955
10	GGCCCT	GATCAG	104	10	gtactatt	gtcattcag	510
11	ATCAGA	GAAACA	109	11	gtaaagtagc	ctttagcag	2259
12	TTTTGA	GTGTTG	16	12	gtgagtttt	ctttccag	3813
13	TACATC	GACAAG	183	13	gtaggaggg	caactgcag	1300
14	ACACGG	GAGTGG	218	14	gtgagcttg	atttggag	1062
15a <sup>a</sup>	GAGAAA	AGAAAG	138	15a	gtaacccgg	gcaggaaag	3533
15b <sup>a</sup>	GTAACC	CCACAC	120	15b	gtgggtatg	tctgtgcag	3653
16 <sup>a</sup>	ATTGCA	GCCCAG	32	16	gtacgtggt	gtgcccag	3967
17	GTGAAT	CAGACG	173	17	gtaggcccc	ctcccaag	1001
18	CTGACC	CCTACG	123	18	gtgaggacc	catgtccag	875
19	GGGAGG	AAATCG	126	19	gtagggtgt	tttctccag	260
20	CGACGG	CGAGAG	141	20	gtgggtgac	cttcccaag	2400
21	GTTTGG	AGGATT	97	21	gtaaatatt	ctcttccag	4145
22	GCCTGC	GCTGAG	112	22	gtactgggt	gttggccag	4060
23	ATCTGC	GCGCAG	154	23	gtgagtgac	tttccacag	4431
24 <sup>a</sup>	CATTGG	AAATGA <sup>b</sup>	177	24	ND <sup>a</sup>	tttccacag	
25 <sup>a</sup>	TGAGGG	ACGTGA <sup>b</sup>	129	25	ND <sup>a</sup>		

<sup>a</sup> Alternatively spliced exons.

<sup>b</sup> Available sequence for the 3' UTR does not extend to a recognizable splice junctions and therefore the 3'-end of exons 24 and 25 are defined by their stop codons rather than an intron boundary.

## CHAPTER 3. CHARACTERIZATION OF A C2 DOMAIN IN TYPE I INOSITOL POLYPHOSPHATE 4-PHOSPHATASES

A paper to be submitted to *Biochemistry*

Colin T. Shearn and F. A. Norris

### Abstract

Inositol polyphosphate 4 phosphatases (IP4Ps) are enzymes involved in the regulation of phosphoinositide 3-kinase lipid signaling. They catalyze the hydrolysis of the 4-position phosphate from phosphatidylinositol 3,4-bisphosphate to phosphatidylinositol 3-phosphate. In this paper we have characterized a lipid binding C2 domain located on the N-terminus of type I IP4Ps. We show that this C2 domain binds calcium but calcium interactions lead to the unique finding that calcium inhibits lipid binding. Mutational analysis of the calcium binding loops suggests that Asp 61, 120 and 123 are involved in calcium and lipid binding *in vitro* and Lys122, R124 are involved in binding lipids *in vitro*. This paper provides insight into the mechanism of membrane interaction of IP4Ps and the role of calcium in regulation of IP4Ps.

### Introduction

Inositol polyphosphate 4-phosphatases (IP4Ps) are  $Mg^{2+}$ -independent phosphatases that catalyze the hydrolysis of the D-4 position phosphoester of the second messenger, phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P<sub>2</sub>) to produce PtdIns(3)P (1). IP4P I $\alpha$  have been implicated in the regulation of phosphatidylinositol 3-kinase (PI3K) signaling in platelets and in the regulation of the oxidative burst in human neutrophils (2,3). The

“Weeble” mouse contains a stop mutation in the IP4P I $\alpha$  gene and presents a phenotype of neurological dysfunction and death early in life (4). In *Caenorhabditis elegans*, phosphatidylinositol 3-kinase (PtdIns 3-Kinase) second messengers are involved downstream of the insulin like receptor DAF-2. Recent RNAi evidence suggests that IP4Ps are involved in PtdIns 3-Kinase signaling downstream of the DAF-2 receptor (5). Although IP4Ps were originally purified from soluble fractions in mouse brains, the possible role of these phosphatases in phosphatidylinositol 3-kinase signaling suggests that they must interact with membranes to have access to the lipid substrate. The basis of this mechanism is currently not known.

C2 domains are lipid and protein binding domains first discovered in protein kinase C that are approximately 130 amino acids in length. They consist of a conserved  $\beta$  sandwich containing four antiparallel  $\beta$  strands that form two  $\beta$ -sheets. There are two major types of C2 domains, topology I and topology II. The overall conformation of the two topologies is very similar. Structural studies comparing 109  $\alpha$ -carbons between the two topologies however have shown that the overall structures differ by only 1.4Angstroms (6-8). Some examples of topology I and topology C2 domains include the Synaptotagmin C2A domain and the Phospholipase C $\delta$  C2 (PLC $\delta$  C2) domain. Both of these proteins contain calcium dependent C2 domains that interact with lipids and calcium via the calcium binding regions (CBR) connecting the  $\beta$ -strands (9-13). In addition to calcium-dependent C2 domains there are C2 domains that bind lipids in a Ca<sup>2+</sup>-independent manner (14-18).

Sequence analysis of IP4P I $\alpha$  shows the potential of a previously unrecognized C2 domain located in the N-terminal region of this protein. In this paper we use biochemical

techniques to determine the lipid and calcium binding characteristics of the human type I IP4P C2 domain.

## Materials and Methods

### *Constructs*

All vectors were produced using standard procedures and verified by sequencing. Full length IP4P  $I\alpha_3$  was cloned into the baculoviral vector pAcGHLT-A (BD Biosciences Pharmingen). Using the Quick Change Site Directed Mutagenesis kit (SDM)(Stratagene) an Nde-I site was introduced at the 5' end of pCDNA3  $I\alpha_3$ . The oligonucleotides were sense 5'-GCTTGATATCGCTAGCCATATGCACAGCAAGAGCACAGCCC-3' antisense 5'-GGGCTGTGCTCTTGCTGTGCATATGGCTAGCGATATCAAGC-3'. Nde-PCDNA3  $I\alpha_3$  was subsequently cut with Nde-I, Not I and ligated into pAcGHLT-A. This vector was subsequently used as a template for all other constructs. All other constructs were produced using SDM. The initial C2 domain was created by producing a stop codon in pAcGHLT-A- $I\alpha_3$  with the following oligos: sense-5'-GGAGGAGAAGTCAGACTAACGGCCCCCTGTGACC-3', antisense-5'-GGTCACAGGGGGCCGTTAGTCTGACTTCTCCTCC-3'. All other mutants were created from pAcGHLT-A-type I IP4P C2 by SDM using the following oligonucleotides: (R62Q, K63N)-sense-5'-CTGCATACTCCATCGCTAGATCAGAACCCAAATAGTTTTGTTGCG-3', antisense-5'-CGCAACAAAACCTATTTGGGTTCTGATCTAGCGATGGAGTATGCAG-3', (K123N, R125) sense-5'-CTCTCCGTGTATGATGTCAACGATCAATCTCAGGGAACAATG-3', antisense-5'-CATTGTTCCCTGAGATTGATCGTTGACATCATAACGGAGAG-3', (D121A, D124A) sense-5'-CTCTCCGTGTATGCTGTCAAAGCTAGATCTCAGGG-3', antisense-

5'-CCCTGAGATCTAGCTTTGACAGCATACACGGAGAG-3', 9D62A), sense-5'-GCATACTCCATCGCTAGCTCGAAAGCCAAATAG-3', antisense 5'-CTATTTGGCTTTTCGAGCTAGCGATGGAGTATGC-3'. All constructs were verified by sequencing at the Iowa State Sequencing Center. All constructs were transfected into *Sf-9* cells using Effectene transfection reagent (Qiagen) with 0.1 µg of linearized baculoviral DNA (BD-Pharmingen) for every 0.3 µg of plasmid. The virus was amplified as per manufacturer's instructions.

### *Reagents*

D (+)-sn-1,2-di-O-hexadecanoylglycerol, 3-O-phospho linked phosphatidylinositol (3,5)-bisphosphate (PtdIns(3,5)P<sub>2</sub>) was from Echelon Biosciences Inc. Porcine brain L-α-phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P<sub>2</sub>), porcine brain L-α-phosphatidylinositol (4)-phosphate (PtdIns(4)P), L-α-phosphatidylserine, Liver L-α-phosphatidylinositol (PI), L-α-phosphatidylcholine (PtdCho) and L-α-phosphatidic Acid (PA) were from Avanti Polar Lipids, Lissamine Rhodamine B 1,2 dihexadecanoyl sn-glycero-3-phosphatidylethanolaminetriethylammonium salt (Rh-PE) and Fluo 5N was from Molecular Probes. The protease inhibitor cocktail and Fatty acid free BSA were from Sigma. Bacterial Protein Extraction Reagent (B-PER) was from Pierce. Glutathione Sepharose 4B was from Amersham Biosciences.

### *Preparation of lipids*

Fluorescent lipids were prepared as follows: Unless otherwise stated, 20% PtdIns (4,5)P<sub>2</sub>, 79% (PC) and 1% Rh-PE were dried using a Savant SC110 Speed Vacuum the lipids



were subsequently resuspended in 10mM Hepes, 100mM NaCl pH 7.4, sonicated briefly and kept on ice until use.

#### *Lipid Overlay Assay*

The lipid overlay was performed as previously described using a pre-spotted phosphoinositide array (Echelon Biosciences)(19). Briefly the membrane was incubated overnight with 5nM of purified GST-IP4P I $\alpha$  C2 at 4°C. For detection, the primary antibody used was a polyclonal rabbit anti-GST (Molecular Probes) diluted at 1:1000. The secondary antibody was Donkey anti-rabbit coupled to horseradish peroxidase (Pierce) at a dilution of 1:5000 and was detected by Supersignal chemiluminescent system (Pierce).

#### *Fluorescent Lipid Pull down Assay*

Recombinant Synaptotagmin was purified by glutathione Sepharose chromatography. The samples were subsequently mixed at 4°C for 4 hours, washed 3 times in 10mM Hepes, 1% Triton X-100, 500mM NaCl, 2mM EDTA (pH 7.4), 3 times in 10mM Hepes, 1% Triton X-100, 100mM NaCl, 2mM EDTA (pH 7.4) followed by 3 times in 10mM Hepes, 100mM NaCl, 2mM EDTA (pH 7.4). Proteins were then quantified using a Coomassie blue stained 10% SDS PAGE gel. All type I IP4P C2 domain proteins were expressed in *Sf-9* cells as described previously. Flasks were infected for 3 days and subsequently lysed in 10mM Hepes, 1% triton, 100mM NaCl, 2mM EDTA (pH 7.4) plus protease inhibitor cocktail. After lysis, lysates were treated as above. For the lipid pull-down assay equal amounts of protein beads were incubated in 300 $\mu$ l of lipid binding buffer (10mM Hepes, 100mM NaCl pH 7.4) plus the appropriate lipid. Each lipid mixture was 20% lipid, 79% PC and 1% Rh-PE unless otherwise stated. The beads were allowed to shake at 37°C for 1.5 hours. The beads were washed 3 times in lipid binding buffer and read on a Titertek fluoroscan II (EFLAB, Finland)

microtiter plate reader Excitation was at 544nm; emission was at 584nm. All calcium concentrations were determined using Fluo5N and the Max Chelator program (<http://www.stanford.edu/~cpatton/maxc.html>). All graphs were created using Sigma Plot.

#### *Ca<sup>2+</sup> overlay assay*

For each protein 2µg was spotted on a nitrocellulose membrane pre-wetted with Buffer A (50mM Tris, 100mM NaCl, .1mM EDTA pH 7.4) using a Bio-Dot microfiltration apparatus (Biorad). The membrane was incubated for 20 minutes with 70 nM <sup>45</sup>Ca<sup>2+</sup> (Specific Activity 16mCi/mg). Following incubation the membrane was washed briefly twice in Buffer A without EDTA, dried, and subjected to autoradiography overnight on Kodak X-ray film.

## **Results**

### *Sequence analysis of the human type I IP4P C2 domain*

RPS Blast Database searches with the IP4P Iα sequence revealed the presence of a possible C2 domain with an expect value of 0.25. A comparison of the expect values of the known C2 domains of Synaptotagmin C2A (Syt C2A) and Phospholipase Cδ1 (PLCδ1) (expect values of  $7 \times 10^{-40}$ ,  $5 \times 10^{-37}$  respectively) revealed this expect value to be relatively weak. Taking the data from the RPS Blast search a manual alignment was constructed using known C2 domain alignments (Fig1) (8,20). Based on this homology, we hypothesized that there is sufficient sequence similarity between conserved hydrophobic residues located in the β-strands and the overall alignment of conserved residues within the calcium binding regions (CBR) to suggest this domain is a C2 domain. The absence of homology with β-strand 1 in

Syt C2A and the presence of homology with  $\beta$ -strand 8 in PLC $\delta$ 1 indicates that the IP4P C2 domain is topology II.

Type I IP4P C2 contains some of the Asp residues (D61, D120) located in CBR1 and CBR3 that are known to coordinate calcium in other C2 domains such as synaptotagmin (12). In addition, Asp123 is only shifted one position from the Synaptotagmin Asp residue. Surrounding these Asp residues are positively charged Lys and Arg residues (R62, K63, K123, R125). In Syt C2A and PLC $\delta$  C2 the positively charged residues are involved in lipid binding (9,10). This suggests that type I IP4P C2 might bind lipid and calcium by a similar mechanism as typical calcium binding C2 domains.

#### *Lipid binding properties of type I IP4P C2-like domains*

A lipid-binding assay was performed using a phosphatidylinositol lipid overlay blot to characterize the lipid specificity of the type I IP4P C2 domain. A GST fusion of this domain interacted with the lipids on the array in the following order of specificity: PtdIns(3,5)P<sub>2</sub> > PtdIns(3,4,5)P<sub>3</sub> > PtdIns(4,5)P<sub>2</sub> > PtdIns(3,4)P<sub>2</sub> > PtdIns(4)P. There was very little or no binding observed for PtdIns(3)P, PtdIns(5)P or PI (Fig 2A). In addition, a vesicle pull-down assay with Rhodamine labeled vesicles was performed. In this assay, negatively charged lipids such as phosphatidic acid, PtdIns(4,5)P<sub>2</sub>, PtdIns(3,5)P<sub>2</sub> and PtdIns(4)P all bound type I IP4P C2 with similar affinity, whereas PC and PI did not detectably bind. In separate experiments, PE and PS did not bind (Fig 2b).

Increasing the mol percent of PtdIns(4)P and PtdIns(4,5)P<sub>2</sub> revealed a dose dependent lipid binding response (Fig2c). IP4P C2 bound PtdIns(4,5)P<sub>2</sub> slightly better than PtdIns(4)P. This interaction strongly indicates that type I IP4P C2 domains functions as a lipid-binding

domain. Moreover, these data suggest that IP4P I $\alpha$  C2 interacts primarily with phosphatidylinositols containing primary phosphates and phosphatidic acid.

#### *Calcium binding of type I IP4P C2*

Many C2 domains such as Syt C2A and PLC $\delta$  C2 bind calcium however, there are calcium-independent C2 domains (9,10,13,14,17). To examine the ability of the type I IP4P C2-like domain to bind calcium we performed calcium overlay assays (Figure 3a). A nitrocellulose membrane was spotted with type I IP4P C2 with Syt C2A (positive control) and GST (negative control). Type I IP4P C2 binding with  $^{45}\text{Ca}^{2+}$  was similar to that of Syt C2A. Previous work has shown that the calcium ions are coordinated with conserved Asp residues located in the calcium binding regions (7). However, a comparison of these Asp residues in type I IP4P C2 to other C2 domains only three of the five are conserved and the second Asp found in loop 2 is shifted one position in type I IP4P C2 (See Figure 1). A D61A mutation putatively located in CBR1 was subsequently examined in the overlay system. Mutation of D61A significantly decreased binding (Fig 3b). This result supports the hypothesis that the type I IP4P C2-like domain interacts with calcium by a mechanism characteristic of calcium binding C2 domains.

#### *Effects of calcium on IP4P type I C2 lipid binding*

In order to evaluate the effect of calcium on lipid binding, we performed a lipid pull-down in the presence of EDTA or increasing amounts of calcium (Fig 4). Calcium concentrations were determined using fluo5N and the Max Chelator program. Surprisingly, chelation of calcium using 0.2mM EDTA did not affect lipid binding. Moreover, as calcium concentrations were increased, there was a significant decrease in lipid binding. The effects of  $\text{Mg}^{2+}$  were also examined, increasing concentrations of  $\text{Mg}^{2+}$  also decreased lipid binding

but binding was only reduced by 40% compared to approximately 80% using  $\text{Ca}^{2+}$  (data not shown). We also examined the effects of EDTA compared to EGTA and saw no difference in lipid binding (data not shown). As a positive control, the Synaptotagmin C2A (Syt C2A) domain was also tested. Lipid binding of Syt C2A was strongly inhibited by EDTA and was not inhibited by increasing concentrations of consistent with data previously reported (Fig 4 inset)(21). To our knowledge, this is the first report of an example of a C2 domain inhibited by calcium.

To further test the role of the CBRs in lipid binding, mutations were constructed to examine the effects of mutations of the conserved Asp residues located in CBR1 and CBR3 (Fig 5). In the lipid pull-down assay, the mutant D61A affecting CBR1 (a mutation shown to abolish calcium binding) increased lipid binding by approximately 1.5-fold of the WT C2 domain. A double mutant D121A, D124A located in the predicted CBR3 increased lipid binding by approximately 6-fold when compared to wild type. Thus, mutations that abolish calcium binding enhance the ability to bind lipid, consistent with a lipid binding mechanism where calcium acts in an inhibitory manner.

#### *Characterization of the mechanism of electrostatic lipid interaction of type I IP4P C2 domains*

Previous evidence has shown that C2 domains can interact with lipid bilayers via several different mechanisms. These include electrostatic interactions involving basic residues, calcium-dependent electrostatic interactions and hydrophobic interactions. (6,21). In order to clarify the role of electrostatic interactions, we performed a lipid pull-down assay with increasing concentrations NaCl (Figure 6a). Binding of type I IP4P C2 to  $\text{PtdIns}(4,5)\text{P}_2$  was decreased by approximately 50+/-18% when the concentration of NaCl increased to 1M.

This result indicates lipid binding is partially through electrostatic interactions. Other calcium-dependent anionic lipid binding C2 domains such as SytC2A and Piccolo exhibit primarily electrostatic lipid interactions (21,22).

In order to further characterize the electrostatic interaction, we examined the role of positively charged residues present in the CBRs. Lipid binding properties of the double mutant R62N, K63Q (CBR1) and the double mutant K123N, R125Q (CBR3) were examined in the presence of increasing mol percent lipids (Fig 6b). The CBR1 double mutant had very little effect but the CBR3 double mutant showed a decreased ability to bind lipids by approximately 50+/-12%. This is in agreement with the 50% decrease seen in figure 6a when examining the effects of high salt. This data provides evidence of the role of K123 and R125 in lipid binding.

## **Discussion**

The majority of C2 domains bind lipids in a calcium-dependent mechanism (6). However, there are examples of C2 domains such as those found in the lipid phosphatase PTEN, Protein kinase C $\delta$  (PKC $\delta$ ) and PKC $\epsilon$ , that do not require calcium to bind lipids (14,16-18). In this report we describe a novel calcium binding C2 domain whose lipid binding capability is inhibited by increasing concentrations of calcium.

Analysis of the sequence of the type I IP4P C2 domain suggests that it is a topology II C2 domain. Although this domain only contains three out of five Asp residues frequently involved in calcium binding, we show that it binds calcium and that calcium binding can be abolished by a mutation of Asp residues within the predicted CBRs. This is consistent of a mechanism that is similar to other calcium binding C2 domains such as Syt C2A and PLC $\delta$ 1 (6,8).

Most C2 domains bind anionic lipids such as PI, PS or zwitterionic lipids such as PC (6). In the lipid array, there was a clear preference for PtdIns(3,5)P<sub>2</sub>. However, there was no clear preference between PtdIns(4)P, PtdIns(4,5)P<sub>2</sub> and PtdIns(3,5)P<sub>2</sub> in the lipid pull-down assay. From both assays we conclude that a primary phosphate is necessary for binding.

The addition of 1M NaCl to the C2A domain of synaptotagmin I and other calcium dependent C2 domains has been shown to bind lipids primarily through electrostatic interactions as the addition of NaCl has been shown to block lipid binding (6,12,21,22). Lipid binding to in the presence 1M NaCl reduced binding by only 50% suggesting that the mechanism of lipid interaction is both electrostatic combined with a non-electrostatic interactions. To evaluate which amino acids involved in the lipid-binding interface, we mutated positively charged amino acids located in CBR1 and CBR3. Mutation of these residues in calcium-dependent and calcium-independent C2 domains reduces the ability to bind lipid (6,14). When we mutated R62N, K63Q (CBR1) and the K123N, R125Q (CBR3) lipid binding was only decreased in the CBR3 mutation. This data demonstrates that not all of the positively charged residues located in the loops are involved in lipid binding, however, positive residues located in CBR3 are important for forming a positively charged electrostatic surface for lipid interaction.

This study shows that lipid binding of the type I IP4P C2 domain does not require calcium and surprisingly, calcium is inhibitory. To our knowledge this is the first C2 domain with this biochemical property. To gain additional insight concerning the role of calcium binding, mutations of Asp residues located within CBR1 and CBR3 were constructed. Analogous mutations of Asp residues have also been shown to abolish lipid binding in other C2 domains (9). When the lipid binding characteristics of D61A (CBR1), and the D121A,

D124A (CBR3) double mutant was examined we see enhanced lipid binding. The single mutation D61A in CBR1 was experimentally demonstrated to abolish calcium binding, however due to poor expression we were unable to evaluate the ability of the double mutant to bind calcium. The greatest effect was seen in the CBR3 mutation indicating that it plays a major role in lipid interactions consistent with the earlier result of K123N, R125Q also in CBR3 exhibiting decreased lipid binding.

Calcium has been shown to negatively regulate IP4P stability and phosphatase activity in the regulation of IP4P has previously been reported (2). This regulation has been is performed by the calpain family of calcium-dependent proteases, which cleave type I IP4P after platelet stimulation (2,23). Platelets exhibit calcium fluxes upon stimulation activating calpains (23). This study suggests that in addition to calpain mediated proteolytic regulation of type I IP4P, calcium could also influence the ability of type I IP4P to target to membranes.

Phosphatidylinositol 3-phosphate (PtdIns(3)P) has been shown to be involved in the production of reactive oxidative species by the NADPH complex in neutrophils (3). Intracellular calcium release also occurs during these cellular processes. The use of calpain inhibitors has been shown to block production of the oxidative burst (24,25,26). During  $\beta 2$  integrin (CR3) mediated phagocytosis in neutrophils there are calcium fluxes that control the sequential closing of the phagosomal cup and production of ROI (26,27). The production of PtdIns(3)P by IP4P could be a mechanism of controlling phagosomal cup closure and the production of ROI. Regulation of calcium fluxes could be a means of controlling these processes by regulating the ability of IP4P to interact with membranes.

In summary, based on sequence analysis in combination with biochemical analysis we have identified a novel C2 domain in the type I IP4P family of proteins. Our results also



suggest that this C2 domain has the unique feature of being able to bind calcium but the binding of calcium inhibits lipid binding. IP4P I $\alpha$  catalyzes the conversion of PtdIns(3,4)P<sub>2</sub> to PtdIns(3)P. In order to accomplish this function access to membranes is necessary. These data provides insight on the mechanism of membrane targeting of type I IP4P.

### Acknowledgements

We would like to thank Phytodyne Inc for the use of the Titertek fluorimeter, Dr. Marit Nilsen-Hamilton for graciously allowing us the use of the Dot-blot and Dr. Yeon-Kyun Shin for the GST-SytC2A.

### Figure Legends

**Fig. 1:** Sequence alignment of Synaptotagmin I (Syt C2A), Phospholipase  $\delta$ 1 and type I IP4P C2.  $\beta$  strands for each topology are shown above and below the sequences. Calcium and lipid binding loops (CBRs) are boxed. Residues that interact with calcium or are mutated in type I IP4P C2 are shown in bold.

**Fig. 2:** Type I IP4P C2 binds to PtdIns (4,5)P<sub>2</sub>, PtdIns (3,5)P<sub>2</sub> and PtdIns (4)P with equal specificity. A. Lipid Array using 5nM IP4P C2 domain, lipids were spotted at a range of 100pmol to 1.56pmol. B. Effects of total lipid content on binding of GST type I IP4P C2 beads compared to GST control. B. Fluorescent lipid pull-down assay demonstrating lipid specificity of IP4P C2 domains. 5 $\mu$ g of GST-IP4P C2 or GST beads was used for each assay, lipids were prepared as 79% PC, 20% indicated lipid and 1% Rhodamine labeled PI as described in Materials and Methods. A total of 5 $\mu$ g of GST fusion IP4P C2 was used for each assay. C. Effects of Increasing mol percent of PtdIns(4)P (circles), PtdIns(4,5)P<sub>2</sub> (squares), PtdSer (triangles) and PtdIns (diamonds) were used in a pull-down assay. The remaining

lipid consisted of 1% Rhodamine labeled PE and PC. The data are mean with S.E. using three independent experiments.

**Fig. 3:** Calcium binding of type I IP4P C2. A. Overlay assay of  $^{45}\text{Ca}^{2+}$  binding of GST-IP4P C2 compared to GST and GST-Synaptotagmin C2A. 2 $\mu\text{g}$  of each protein was spotted on a nitrocellulose membrane and incubated with  $^{45}\text{Ca}^{2+}$ . Following autoradiography exposure, the membrane was stained with Ponceau Red. B. Overlay assay of  $^{45}\text{Ca}^{2+}$  binding of GST-IP4P C2 compared to D61A mutation under the same conditions.

**Fig. 4:** Effects of EDTA and calcium on type I IP4P C2 lipid binding. Lipid vesicles were prepared as described previously, for each assay five micrograms of protein was used. Type I IP4P C2 binding shown in black bars GST binding shown in white. In the inset is GST-Synaptotagmin as a positive control under the same conditions. The data are mean with S.E. using three independent experiments.

**Fig. 5:** Mutational analysis of type I IP4P C2 lipid binding. Lipid binding/microgram of protein of CBR1 and CBR3 Asp mutants. Five micrograms of (IP4P C2-WT, (D61A), (D120A, D123A) or GST control beads were used for each assay. PtdCho binding is in gray and PtdIns(4)P binding is in black. The data are mean with S.E. using three independent experiments.

**Fig. 6:** Type I IP4P C2 binding to phospholipid vesicles, effects of NaCl. Effects of NaCl concentration on GST IP4P C2 beads lipid binding using 20% PtdIns(4,5) $\text{P}_2$  vesicles compared to GST beads. Lipid vesicles were prepared as described previously, for each assay five micrograms of protein was used. IP4P C2 binding shown in black bars, GST binding shown in white. B. Lipid binding/microgram of protein of CBR1 and CBR3 mutants. Five

micrograms of IP4P C2, (R62N, K63Q), (K123N, R125Q) beads were used for each assay.

The data are mean with S.E. using three independent experiments.

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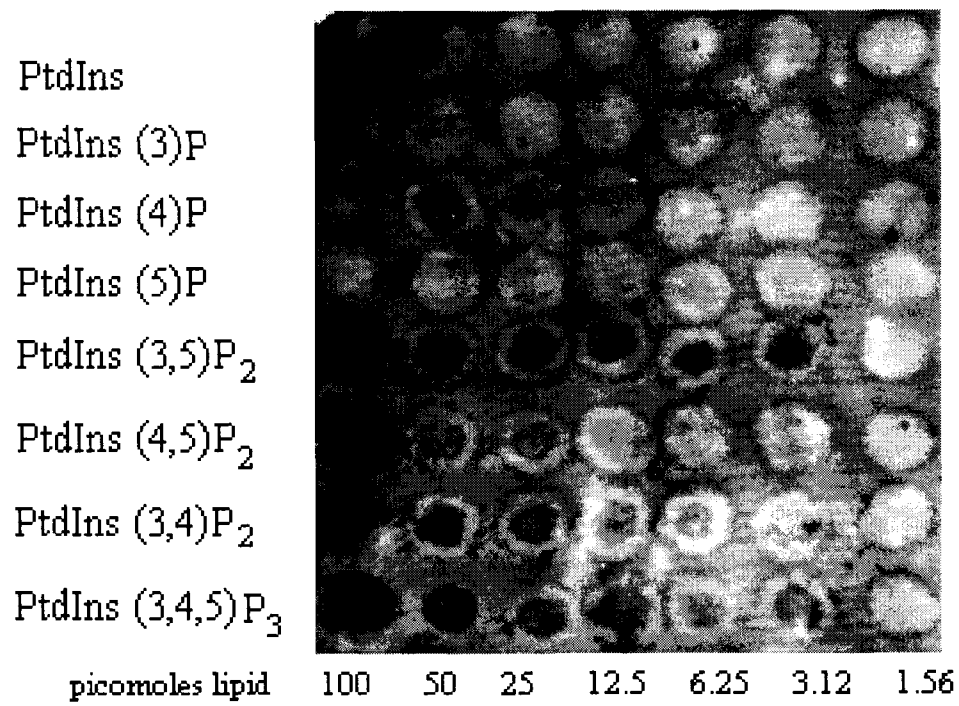
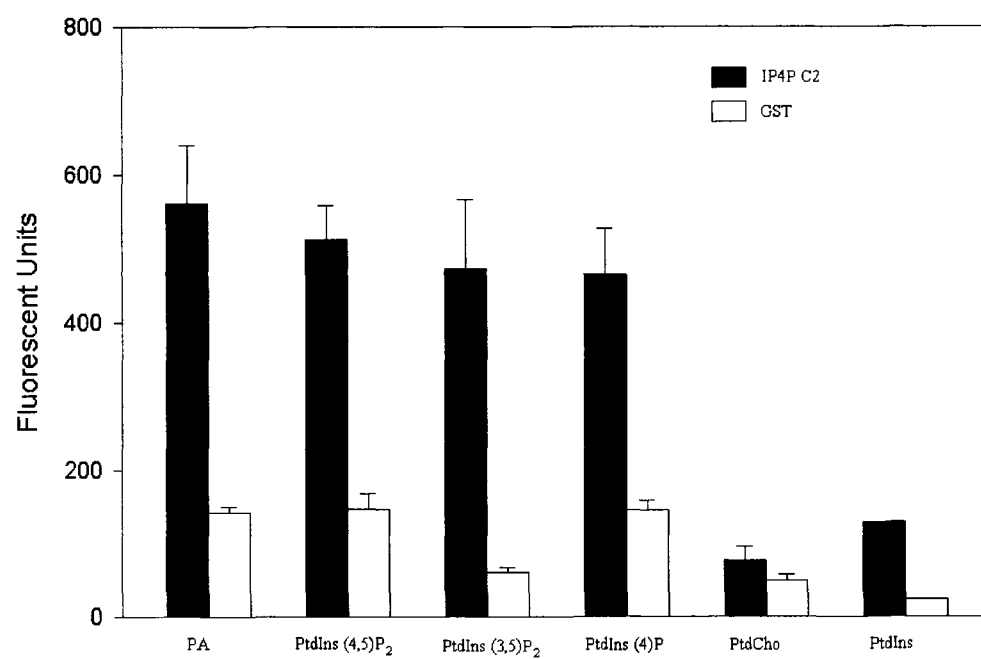
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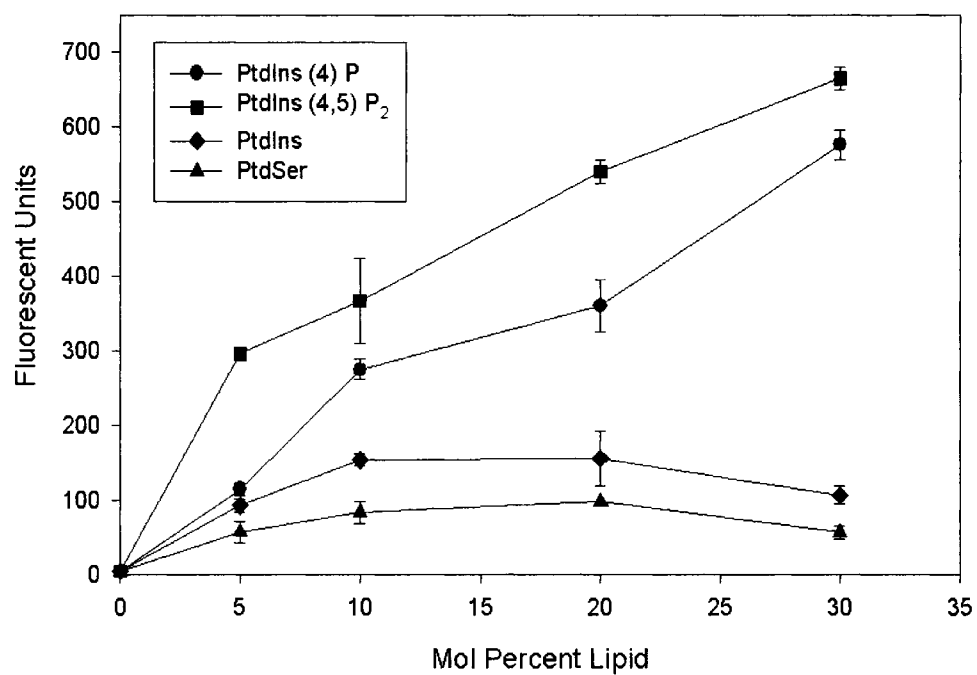
Figure 1

<b>Topology I</b>	<b>[<math>\beta</math>-Strand 1]</b>	<b>[<math>\beta</math>-Strand 2]</b>	<b>[<math>\beta</math>-Strand 3]</b>
Syt C2A (141-265)	KLGLQYSLDYDFQN----	NQLLVGIIQAALPAI---	DMCGTSDPYVKVFLLP----D----
PLC $\delta$ C2 (4-132)	-----	KRLNIRVISGQQLPRV---	MKNSIVDPKVTVEIHGVS--R----
IP4P C2 (44-177)	-----	PILEFSLACS-ELHTPSL-	DRKPNSFNAVSVTTPP----QAFWT
<b>Topology II</b>		<b>[<math>\beta</math>-Strand 1]</b>	<b>[<math>\beta</math>-Strand 2]</b>
		CBR1	
<b>Topology I</b>	<b>[<math>\beta</math>-Strand 4]</b>	<b>[<math>\beta</math>-Strand 5]</b>	<b>[<math>\beta</math>-Strand 6]</b>
Syt C2A (141-265)	KKKKFETKVHRK---TLNPVFNEQ-FTFKV----	PYSELGG-KTLVMAYV	DFD--RFSK--HD
PLC $\delta$ C2 (4-132)	DVASRQTAVITNN--GFNPWWDTE-FAFEV----	VVPDL---ALIRFLV	EDYD--ASSK--ND
IP4P C2 (44-177)	KHA--QTEIIEC---TNPIFLSS-IAFFQ----	DSLINQMTQVKLSVY	DVK--DRSQGTMY
<b>Topology II</b>	<b>[<math>\beta</math>-Strand 3]</b>	<b>[<math>\beta</math>-Strand 4]</b>	<b>[<math>\beta</math>-Strand 5]</b>
	CBR2		CBR3
<b>Topology I</b>	<b>[<math>\beta</math>-Strand 7]</b>	<b>[<math>\beta</math>-Strand 8]</b>	
Syt C2A (141-265)	IIGEFKVPMTNTVDFGH----	VT EEWRLQSAE-----	
PLC $\delta$ C2 (4-132)	FIGQSTIPLNSLK-----	QGYRHVELMSKNGDQHPS--	ATLFVKISLQS---
IP4P C2 (44-177)	LLGSGTFIVKDLL-----	QDRHRLHLTLRSAESDR-	VGNI TVIGWQMEKS
<b>Topology II</b>	<b>[<math>\beta</math>-Strand 6]</b>	<b>[<math>\beta</math>-Strand 7]</b>	<b>[<math>\beta</math>-Strand 8]</b>

**Figure 2****A****B**



C



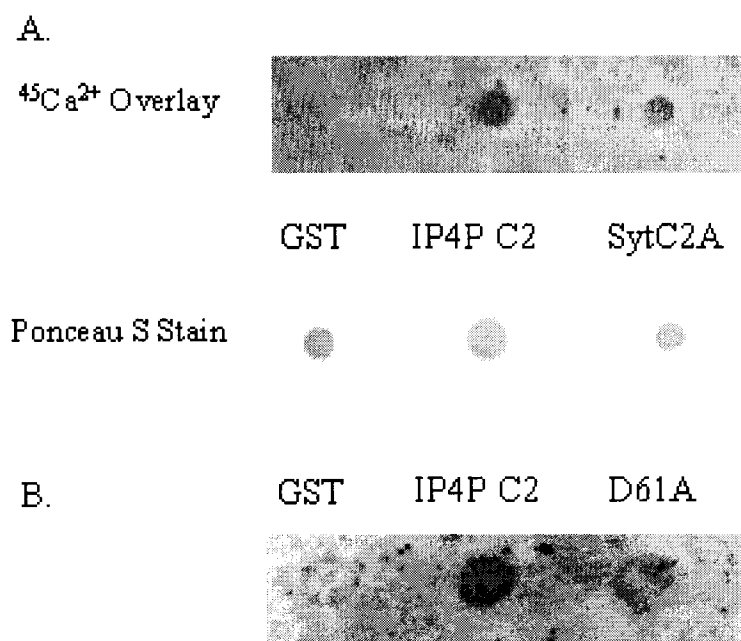
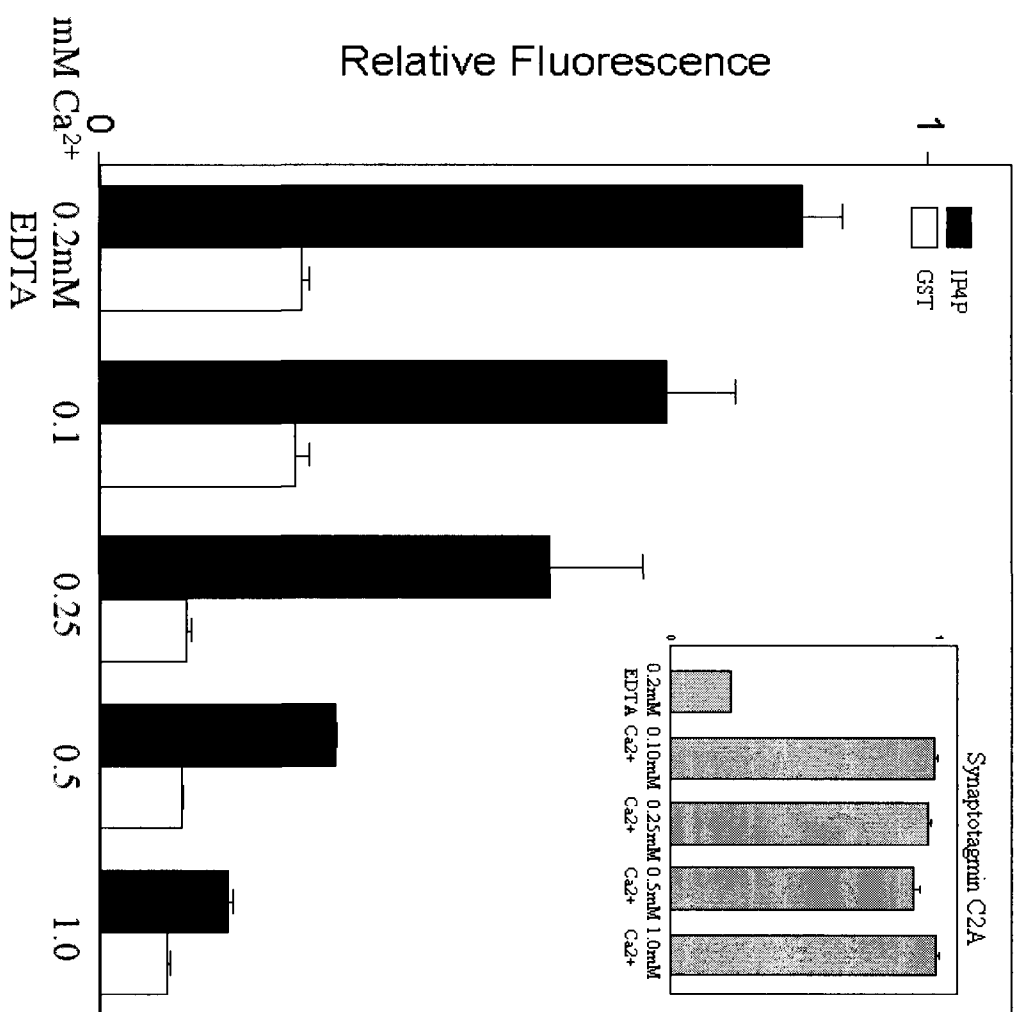
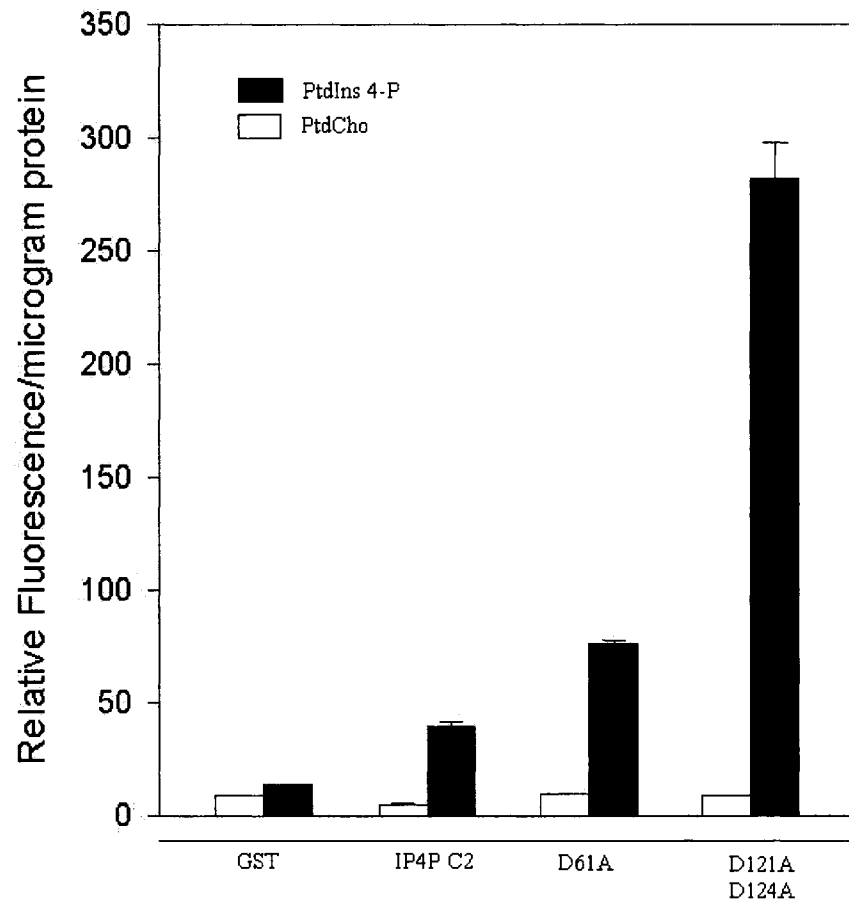
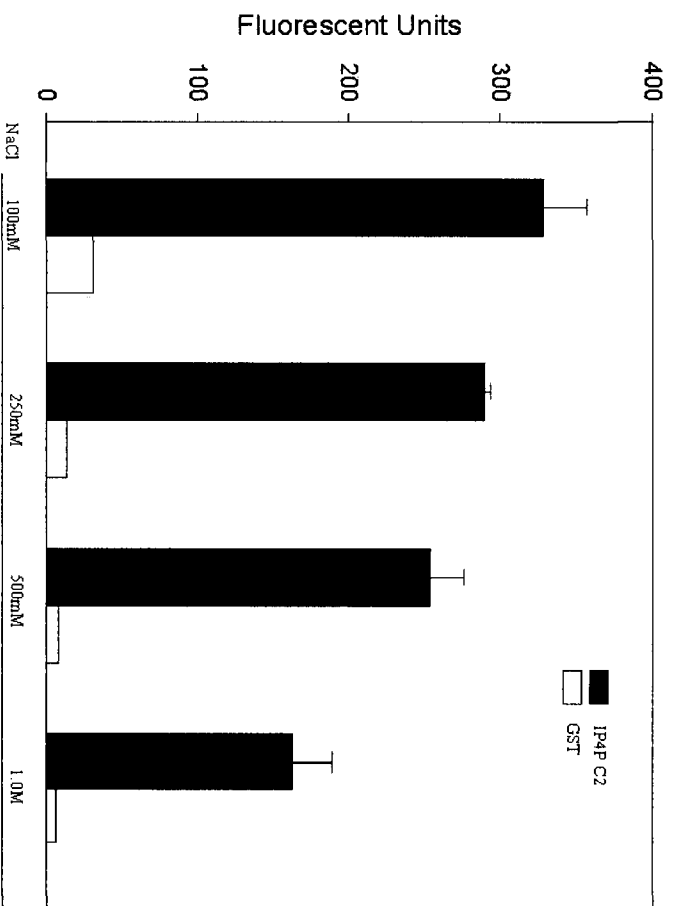
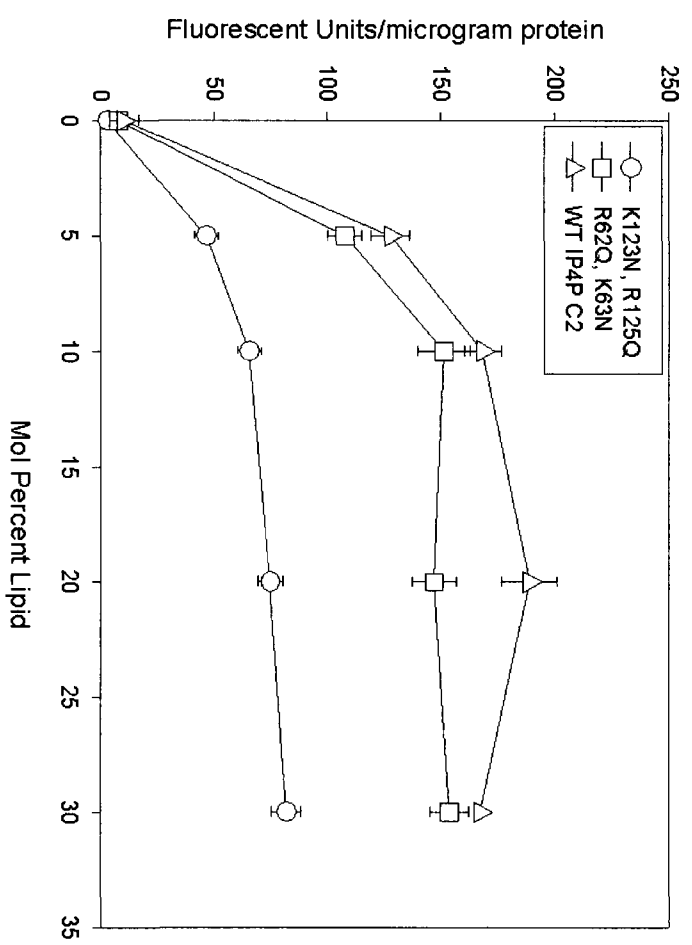
**Figure 3**

Figure 4



**Figure 5**

**Figure 6****A****B**

## CHAPTER 4. CHARACTERIZATION OF STUBBY: A NATURAL C2 DOMAIN CONTAINING SPLICEOFORM OF TYPE II INOSITOL POLYPHOSPHATE 4-PHOSPHATASES

A paper to be submitted to the *Biochemical Journal*

Colin T. Shearn, Raji E. Joseph, and F. A. Norris

### Abstract

Inositol polyphosphate 4 phosphatases (IP4Ps) are enzymes involved in the regulation of phosphoinositide 3-kinase lipid signaling. They catalyze the hydrolysis of the 4-position phosphate from phosphatidylinositol 3,4-bisphosphate to phosphatidylinositol 3-phosphate. In this paper we have identified a novel spliceoform of type II IP4P which we denote Stubby. Stubby is comprised solely of the N-terminal C2 domain of type II IP4P. In a comparison between the type II C2 domain and Stubby, we show that both proteins will bind calcium but that calcium will inhibit the polyphosphatidylinositol lipid binding. We further show that Stubby and the type II IP4P C2 domain have different lipid preferences and that the overall mechanism of lipid binding is primarily electrostatic. This paper provides insight into the mechanism of membrane interaction of IP4Ps and the role of calcium in regulation of type II IP4Ps.

### Introduction

The family of phosphoinositide 3-kinases (PI3K) phosphorylates the D3 position of inositol lipids to produce the lipid second messengers phosphatidylinositol 3-phosphate (PtdIns(3)P), phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P<sub>2</sub>), and phosphatidylinositol

3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>) (1). These lipids are known to be involved in many cellular processes including cell growth, phagocytosis and the production of the NADPH oxidative burst (2-4). Inositol polyphosphate 4-phosphatases (IP4P) are enzymes that are involved in the regulation of PI3K signaling in thrombin stimulated platelets and in production of oxidative intermediates during the NADPH oxidative burst (5,6)

IP4Ps are Mg<sup>2+</sup>-independent enzymes that catalyze the hydrolysis of the D-4 position of the lipid second messenger PtdIns(3,4)P<sub>2</sub> converting it to PtdIns(3)P (7). The mammalian family of IP4Ps consists of two types, type I and type II. Type I and type II are located on different chromosomes and have 37% amino acid identity between them (8). Both type I and type II IP4P's are alternatively spliced. Type I IP4P can be spliced at both the C-terminus to yield variable C-terminal domains that are either hydrophilic (type I $\alpha$ ) or hydrophobic (type I $\beta$ ), or it can be spliced within the variable region to yield three different spliceoforms (I $\alpha$ <sub>1</sub>, I $\alpha$ <sub>2</sub> and I $\alpha$ <sub>3</sub>)(9,10). Type II can be alternatively spliced at its C-terminus to yield types II $\alpha$  and II $\beta$  which are homologous to type I $\alpha$  and I $\beta$  (10).

We have recently identified a novel C2 domain on the N-terminus of type I IP4P (11). This C2 domain prefers to bind PtdIns(3,5)P<sub>2</sub> and in *in vitro* pull-down assays, calcium inhibits its ability to bind lipids (11). C2 domains are domains of approximately 130 amino acids that are found in proteins involved in the regulation of vesicular processes and also in proteins involved in the modification of lipid second messengers (12). C2 domains contain 8  $\beta$ -strands that form a  $\beta$ -sandwich. The strands are linked on the top by loops that can bind lipid and Ca<sup>2+</sup> (13). These regions of C2 domains are also known as Ca<sup>2+</sup> binding regions (CBR). Currently the function of the loops linking the strands on the other face of C2 domains is unknown function (13). A possible function for the bottom loops could be in

protein/protein interactions. In this paper we report the cloning of an alternatively spliced variant of type II IP4P that consists only of a truncated C2 domain. We compare the lipid and calcium binding properties of this unique human protein with the C2 domain that we have identified in type II IP4P.

## **Materials and Methods**

### *Reagents*

D (+)-sn-1,2-di-O-hexadecanoylglycerol, 3-O-phospho linked Phosphatidylinositol (3,5)-bisphosphate (PtdIns (3,5)P<sub>2</sub>) was from Echelon Biosciences Inc. Dipalmitoyl phosphatidylinositol tris-3,4,5-phosphate ammonium salt was from Matreya Inc. Porcine brain L- $\alpha$ -phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)-P<sub>2</sub>) and L- $\alpha$ -phosphatidylcholine and were from Avanti Polar Lipids, Lissamine Rhodamine B 1,2 dihexadecanoyl sn-glycero-3-phosphatidylethanolaminetriethylammonium salt (Rh-PE) and Fluo5N was from Molecular Probes. The protease inhibitor cocktail and Fatty acid free BSA were from Sigma. Bacterial Protein Extraction Reagent (B-PER) was from Pierce. Glutathione Sepharose 4B was from Amersham Biosciences. Lipids were prepared as described previously (11).

### *Cloning of Stubby*

BLAST searches using IP4P Type II sequence revealed the presence of two EST clones, AA759168 and AI634119 encoding human Stubby (14). These two EST clones were obtained from the I.M.A.G.E. consortium through ATCC. The clones were sequenced from both directions using the flanking T7 and Universal (M13) vector primers. The bacterial expression construct for Stubby was created by PCR amplification and cloned into the BamHI/SmaI sites of pGEX4T-1 (Amersham Pharmacia Biotech). pAcGHLT-A Stubby was



PCR amplified using oligonucleotides 5'-

GGCCGCGTCGACTAACCATATGGGAAATCAAAGAGGAAGG-3' (sense) and 5'-

CGGGAGCTGCATGTGTCAGAG-3' (antisense), using pGEX 4T-1 Stubby as template.

Following amplification, the fragment was TOPO-cloned into pCR 2.1-TOPO (Invitrogen) as

per manufacturers instructions and sequenced. pCR 2.1-TOPO Stubby was digested with

Nde-I/Not-I and ligated into pAcGHLT-A. Full length IP4P type II was cloned into

pAcGHLT-A as follows. Using the Quick Change Site Directed Mutagenesis kit

(SDM)(Stratagene) an Nde-I site was introduced at the 5' end of type II pCDNA3, with

oligonucleotides Sense 5'-

GGCCGCGTCGACTAACCATATGGGAAATCAAAGAGGAAGG-3', and antisense-5'-

CCTTCCTCTTTGATTTCCCATATGGTTAGTCGACGCGGCC-3'. Nde-IP4P type II was

subsequently digested with Nde-I and Not-I and ligated into Nde-I/Not-I digested

pAcGHLT-A to produce type II IP4P pAcGHLT-A. To produce IP4P II C2, a stop mutation

was introduced into type II using SDM and the following oligonucleotides, sense 5'-

CACAACAGACGTGCAGGGATAAAAGTGTGCCCTAGTG-3', antisense-5'-

CACTAGGGCACACTTTTATCCCTGCACGTCTGTTGTG-3'. All constructs were

verified by sequencing at the Iowa State sequencing center. All constructs were transfected

into *Sf-9* cells using Effectene transfection reagent (Qiagen) and with 0.1 µg of linearized

baculoviral DNA (BD-Pharmlingen) for every 0.3 µg of plasmid. The virus was amplified as

per manufacturers instructions. All oligonucleotides were purchased from Integrated DNA

Technologies.

*Northern Blot*

Two Clontech multi-tissue northern blots were probed for Stubby as per the manufacturers instructions. The probe 5'-CCTCAAGATCCACTTATACTCAAATTCTGGATGAGTTAGCTATG-3' was prepared by 5' end labeling using  $\gamma$ ATP (American Radiochemicals) and T4 polynucleotide kinase (Promega) according to the manufacturers protocol.

#### *Fluorescent Lipid Pull down Assay*

Recombinant Synaptotagmin was purified by glutathione Sepharose chromatography as described previously (11). Stubby and all type II IP4P C2 domain proteins were expressed in *Sf-9* cells as described previously (11). Each assay was read on a Titertek fluoroscan II (EFLAB, Finland) microtiter plate reader Ex-544nm, Em-584nm. All  $\text{Ca}^{2+}$  concentrations were determined using Fluo5N and by using the Max Chelator program (<http://www.stanford.edu/~cpatton.maxc.html>). All graphs were created using Sigma Plot.

#### *Lipid Overlay Blot*

The lipid overlay was carried out as described earlier (15). The lipid blot was incubated overnight at 4°C with 10nM purified GST-tagged Stubby or 10nM purified GST-tagged IP4P type II C2. The blot was washed and incubated with an anti-GST antibody (Molecular Probes). The blot was washed extensively and developed using an HRP-tagged anti-rabbit secondary antibody, and a chemiluminescent system (Pierce).

#### *$\text{Ca}^{2+}$ overlay assay*

The calcium overlay assay was performed as previously described (11).

## **Results**

### *Cloning and Gene Structure of Stubby*

The domain architecture of IP4P Type II consists of an N-terminal C2 domain and a C-terminal phosphatase domain. Database searches for proteins similar to IP4P type II revealed the presence of two human testis EST clones encoding a novel alternate splice variant of IP4P Type II, which we have named Stubby. Stubby consists of 131 amino acids, predicting a protein of molecular mass 14,739 Da (Fig. 1A). The domain architecture of Stubby consists of an N-terminal partial C2 domain followed by a novel C-terminus consisting of just seven amino acids. Stubby is therefore much smaller than IP4P Type II (109 kDa), as it lacks the C-terminal phosphatase domain.

Analysis of the human genomic database revealed that the gene for IP4P Type II is located on chromosome 4q28.1-q31.1. The gene for IP4P Type II is unusually large and is spread across >300 kb of DNA. The gene consists of 25 exons that can be utilized as coding sequence (Table 1). Stubby is formed by using exons 1 through 5 (Fig. 1B). IP4P Type II $\alpha$  is formed using exons 1 through 25, excluding exons 5 and 24, while IP4P Type II $\beta$  is formed using exons 1 through 24 excluding exon 5 (Fig. 1B). While the size of the exons vary from 24 bp to 204 bp, the size of the introns vary more widely from 366 bp (intron 11) to 51,728 bp (intron 23) (Table 1). Moreover, the IP4P Type II gene is nearly five times as large as the human IP4P Type I gene which is approximately 67 kb long (9).

#### *Northern blotting*

Northern blot analysis of the expression of Stubby is shown in (Fig 2). There is one primary transcript of 5.2 kilobases that is expressed in pancreas. No detectable expression of Stubby was seen in heart, brain placenta, lung, liver skeletal muscle or kidney. In addition, another multi-tissue northern blot was examined containing spleen, thymus, prostate, testis

ovary, small intestine, colon and peripheral blood leukocyte RNA and there was no detectable expression (data not shown).

*Comparative analysis of the human type II IP4P C2 domain and Stubby*

RPS Blast database searches with the IP4P II $\alpha$  sequence and Stubby revealed the presence of a conserved C2-like domain with an expect value of 0.18 for the N-terminus of IP4P type II and 0.019 for stubby. These values are weak when compared to values of known C2 domains such as synaptotagmin C2A and phospholipase C $\delta$ 1 ( $7 \times 10^{-40}$ ,  $5 \times 10^{-37}$ ). To map the potential  $\beta$ -strands and connecting loops, a sequence alignment was performed against PLC  $\delta$ 1 C2 domain (Fig 3a). The initial alignment was constructed by RPS-BLAST of each C2 domain followed by a manual alignment based on known C2 sequence alignments (11,13). Based on this homology, we hypothesize that there is sufficient sequence similarity between conserved hydrophobic residues located in the  $\beta$ -strands and the overall alignment of  $\beta$ -strands domain to conclude that the type II IP4P C2 domain is a topology II C2 domain. In this model, we have placed the additional sequence from Stubby as  $\beta$ -strand 6. From this homology surprisingly, Stubby does not contain  $\beta$ -strands 7 and 8 and has a shorter CBR3 compared to the type II C2 domain (Figure 3b). This suggests that Stubby is a unique variant of a C2 structure which may have different biochemical lipid and calcium binding properties.

Type II IP4P C2 and Stubby contain three of the Asp residues (D56, D116, D123) located in CBR1 and CBR 3 that are known to coordinate calcium in other C2 domains such as phospholipase C $\delta$ -1 (PLC $\delta$ 1)(13,16,17). In addition, Asp119 is shifted one position from the PLC $\delta$ 1 Asp residue. Surrounding the Asp residues are positively charged Lys and Arg residues R55, K57, K58 in CBR1 and K118, K120 in CBR3. In PLC $\delta$  C2 the positively

charged residues are involved in lipid binding (17). This suggests that type II IP4P C2 and Stubby might bind lipid and calcium by a similar mechanism as typical calcium binding C2 domains.

### *Lipid Binding Analysis*

To determine whether the lipid binding property of Stubby was different from that of the Type II C2 domain alone, we tested its lipid binding property using a lipid overlay. As shown in Fig. 4A, Stubby interacted preferentially with PtdIns(3,5)P<sub>2</sub>, PtdIns(3,4)P<sub>2</sub>, PtdIns(3,4,5)P<sub>3</sub>. This is similar to the profile found for the type I IP4P C2 domain (11). Interestingly, when we performed the overlay using the type II C2 domain, it preferentially interacted almost exclusively with PtdIns(3,4,5)P<sub>3</sub> (Fig 4B). This suggests that the variation in the type II C2 domain, Stubby and the type I IP4P C2 domains interacts with different biochemical signaling pathways. To confirm the lipid binding properties in a separate assay, lipid pull-down assays were used. In Fig 4C, both Stubby and type II IP4P C2 domain prefer anionic phosphatidylinositol lipids. Stubby however, binds more lipid per microgram protein suggesting a higher affinity.

### *Calcium overlay of type II C2 and Stubby*

Type I IP4P C2 domains have been shown to bind calcium by a <sup>45</sup>Ca<sup>2+</sup> overlay assay (11). To examine the ability of Stubby and the type II IP4P C2 domain to bind calcium, we performed calcium overlays using Synaptotagmin C2A (Syt C2A), a known calcium binding C2 domain as a positive control and GST as a negative control (13). In Figure 5, both Stubby and type II IP4P C2 domain bind calcium. Interestingly, Stubby is lacking  $\beta$ -strands 7 and 8 within its structure but still binds calcium indicating that the amino acid residues necessary

for calcium binding are not located within the missing strands. This is consistent with the hypothesis that the CBRs remain intact and are involved in binding calcium.

*Effects of calcium on lipid binding of type II C2 and Stubby*

We have shown previously that the IP4P I $\alpha$  C2 domain exhibits a calcium dependent inhibition of lipid binding (11). Both IP4P type II C2 and Stubby are 37% homologous to the type I IP4P C2 domain (8,10). In order to evaluate the effect of calcium on lipid binding, we performed a lipid pull-down in the presence of calcium chelators and increasing amounts of calcium (Figure 6a). In agreement with type I C2 domains both the IP4P type II C2 domain and Stubby did not show a decrease in lipid binding in the presence of the calcium chelator EDTA and as calcium concentrations are increased, significant decreases in lipid binding occur as shown in Figure 6. The SytC2A domain was also tested using this assay as a positive control. Using our assay, SytC2A did not bind lipids in the presence of EDTA and did not show a calcium dependent inhibition consistent with data previously reported (data not shown)(11,16). The effects of EGTA on lipid binding were similar to EDTA (data not shown). Surprisingly, similar effects were also seen for the lipid binding properties in the presence of Mg<sup>2+</sup>. 1.0mM Mg<sup>2+</sup> decreased binding by approximately 50 percent less than calcium (Figure 6b). This data suggests that the IP4P family of proteins represent a biochemically unique class of C2 domains whose lipid binding is inhibited by divalent cations.

*Effects of increasing ionic strength on lipid binding*

Calcium-dependent C2 domains such as Synaptotagmin and Piccolo are known to interact with the lipid head groups by primarily an electrostatic mechanism (16,18-20). This has also been shown for the type I IP4P C2 domain (11). There are some exceptions however

including cytosolic phospholipase A2 (20). To further examine the mechanism of lipid interaction, we used a lipid pull-down assay and increasing ionic strength. From Figure 7, lipid binding for both Stubby and the type II IP4P C2 domain are reduced by approximately 70% upon addition of .5M NaCl. This is consistent with the mechanism of lipid binding of the type I C2 domain suggesting that the mechanism of lipid binding is maintained in the IP4P family of C2 domains (8,11,18,21,22). Lipid binding however did not go to background, this suggests that there are additional processes involved besides an electrostatic interactions.

## Discussion

The IP4P family of proteins are phosphatidylinositol lipid phosphatases that catalyze the conversion of the lipid second messengers PtdIns(3,4)P<sub>2</sub> to PtdIns(3)P (8). IP4P consists of two isoforms, type I and type II both of which are alternatively spliced. The gene for human IP4P Type II is enormous, spread across >320 kb of DNA. It therefore belongs to a rare category of genes as less than 2% of mammalian genes are >100kb in length (23). Comparison of this gene to the IP4P Type I gene which is located on chromosome 2q11.2, shows that the Type II gene is almost five times bigger than the Type I gene, although both encode 4-phosphatases of similar size (9). Interestingly, the open reading frames for both these genes are derived from 25 exons. Moreover, the order of exons 24 and 25, which encode the  $\beta$  and  $\alpha$  splice variants respectively of IP4P, are maintained in the Type II gene as well. However, the hypervariable region of the IP4P Type I gene (exons 15,16 and 17), which gives rise to the Type I  $\alpha_1$ ,  $\alpha_2$  and  $\alpha_3$  splice forms are missing in the Type II gene (9). Conversely, exon 5 of the IP4P Type II gene which gives rise to Stubby, is missing in the Type I gene.

Although the original EST clone for Stubby was from human testis, Northern blot analysis detected a 5.3-kilobase transcript of Stubby solely in pancreas. IP4P type II was previously shown by Northern blot to be primarily expressed in heart and skeletal muscle with lower abundance transcripts been located in brain, placenta, and pancreas respectively (10). The difference in expression profiles indicates that Stubby may have unique tissue specific functions.

The analysis of the sequences of PLC  $\delta 1$  C2 domain suggests that both Stubby and the type II IP4P C2 domains are similar to topology II C2 domains. Other proteins that contain topology II C2 domains include PI3-kinase class IB (PI3K $\gamma$ ) and phospholipase A2 (24,25). Stubby is an unusual protein as it consists of only a partial C2 domain lacking  $\beta$ -strands seven and eight. To our knowledge, it is the first member of the C2 domain family that consists of only a single C2 domain with very little additional sequence. Both Stubby and type II IP4P C2 domains can interact with lipids however, suggesting that the mechanism of interaction does not involve strands 7 and 8. The structural variation may influence lipid specificity as lipid overlay blot showed a dramatic preference, however using a lipid pull-down assay a broader interaction was indicated under these conditions. They both also interact with the lipids head groups by a similar mechanism as evidenced by the effects of increasing ionic strength.

Calcium overlays indicated that both Stubby and the type II C2 domain bind calcium. Interestingly, both the type II C2 domain and Stubby showed a calcium dependent inhibition of lipid binding. Synaptotagmin C2A and other calcium dependent C2 domain containing proteins do not have this property and have been shown to bind lipids in the presence of calcium (16,26). This unusual property was previously shown to be a feature of the type I



IP4P C2 domain. Moreover both type II and Stubby are shown to be inhibited by  $Mg^{2+}$  to a lesser degree (Figure 6b)(data not shown). Combined, this suggests that the IP4P family of proteins is uniquely regulated by divalent cations.

PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> are lipids frequently found in vesicular membranes and have been implicated in processes such as phagosomal maturation and the oxidative burst (1,6,27,28). To complete the hydrolysis of their substrates, IP4Ps must be able to interact with membranes. Other phosphatases such as the PtdIns 3-phosphatase PTEN have been shown to contain C2 domains that are involved in coordination of their lipid substrate for activity (29,30). Calcium has already been shown to negatively regulate type I IP4P activity in platelets by the calcium-dependent protease calpain (5). Calcium inhibition of membrane association could potentially augment the inhibition of this enzyme under increased calcium conditions. The existence of Stubby, a spliceoform of type II IP4P, implies that the C2 domain can function independently. Possible functions of this C2-like protein could be to interact with an effector to modulate the ability of type II IP4P or their signaling partners to function. It also could influence the recruitment of other associated proteins to the membrane.

### **Acknowledgements**

We would like to thank Phytodyne Inc for the use of the Titertek fluorimeter, Dr. Marit Nilsen-Hamilton for graciously allowing us the use of the Dot-blot, Dr. Yeon-Kyun Shin for the GST-SytC2A and David Senchina for providing the use of Sigma Plot.

### **Figure Legends**

**Fig. 1:** Structure of Stubby. A. Nucleic acid and amino acid sequence of Stubby. The alternate spliced region is boxed. B. Chromosome organization of IP4P Type II. Schematic of

the gene structure of human IP4P Type II with 25 exons. The alternate splice variant, Stubby is formed using exon 5.

**Fig. 2:** Northern Analysis of Stubby mRNA expression. A human Multi-tissue Northern blot from Clontech was probed for the presence of Stubby in the issues indicated.

**Fig. 3:** Secondary Structure of Stubby A. Sequence alignment of Phospholipase  $\delta 1$  C2, type II IP4P C2 and Stubby.  $\beta$  strands for each topology are shown below the sequences. Calcium and lipid binding loops (CBR) are shown below in bold. Residues that interact with calcium in PLC  $\delta 1$  and their homologous residues in type II IP4P C2 and Stubby are shown in bold. Conserved residues are shaded in gray. B. Block diagram of  $\beta$ -strands in Stubby (shaded white) plus  $\beta$ -strands 7 and 8 that are not found in Stubby (Shaded gray).

**Fig. 4:** Lipid binding properties of IP4P Type II and Stubby. Phospholipid overlay blots using (A) Purified GST-tagged Stubby or (B) purified GST-tagged IP4P Type II C2 domain. Binding was detected using an anti-GST antibody coupled with a chemiluminescent detection system. C. Fluorescent lipid pull-down assay demonstrating lipid binding properties of Stubby and type II IP4P C2 domains. GST-type II IP4P C2 (black bars), Stubby (gray bars) or GST beads (white bars). The data are mean with S.E. using three independent experiments.

**Fig. 5:** Calcium binding of type II IP4P C2 and Stubby. Overlay assay of  $^{45}\text{Ca}^{2+}$  binding of GST-type II IP4P C2 and Stubby compared to GST and GST-Synaptotagmin C2A.  $2\mu\text{g}$  of each protein was spotted on a nitrocellulose membrane and incubated with  $^{45}\text{Ca}^{2+}$ . Proteins were quantified using Coomassie blue staining with a 10% SDS-PAGE gel.

**Fig. 6:** Effects of EDTA and divalent cations on type II IP4P C2 and Stubby lipid binding. A. Effects of increasing calcium on lipid binding. IP4P type II C2 binding shown in black bars, Stubby is shown in gray and GST binding shown in white. B. Effects of increasing magnesium concentrations on lipid binding. Type II IP4P C2 binding shown in black bars, Stubby is shown in gray bars. Calcium concentrations were verified using Max Chelator and Fluo 5N. The data are mean with S.E. using three independent experiments.

**Fig. 7:** Effects of ionic strength on Stubby and type II IP4P C2 binding to phospholipid vesicles. Lipid vesicles were prepared as described previously with increasing amounts of NaCl as shown. Type II IP4P C2 binding shown in black bars, Stubby is shown in gray and GST binding shown in white. The data are mean with S.E. using three independent experiments.

**Table 1:** Gene structure of human IP4P Type II. Table showing the intron-exon boundaries of IP4P Type II gene. A. 5' exon boundary is defined as the start ATG; B. alternate spliced exons; C. 5' intron boundary is not determined (ND) and is defined by the stop codon.

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Figures

Figure 1

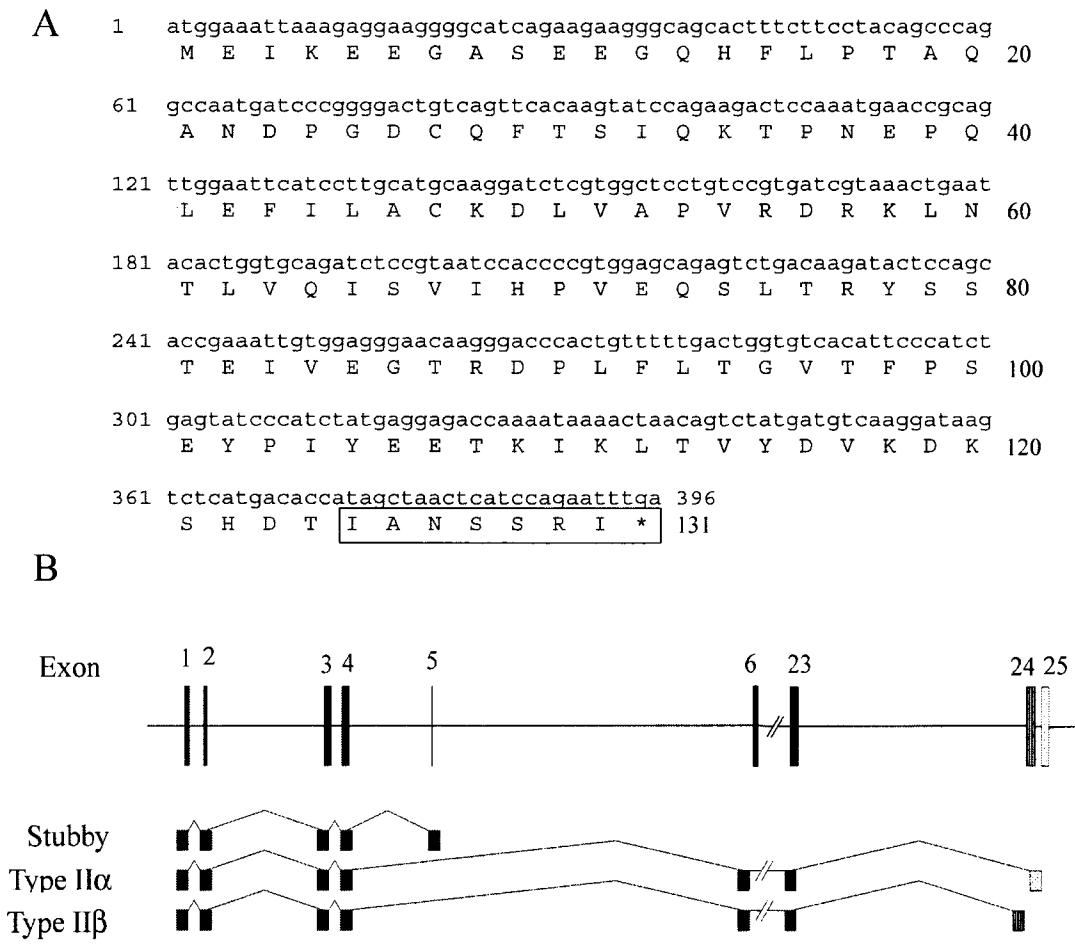
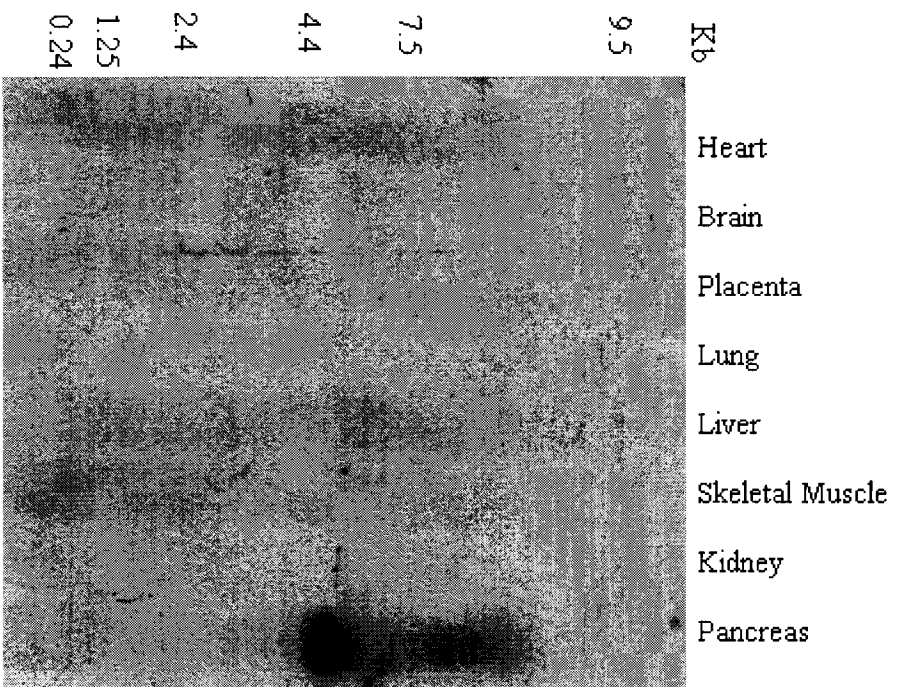


Figure 2

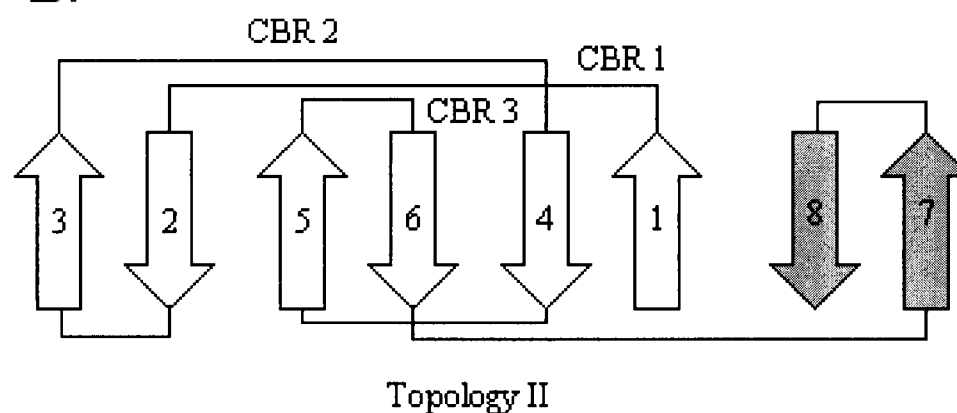


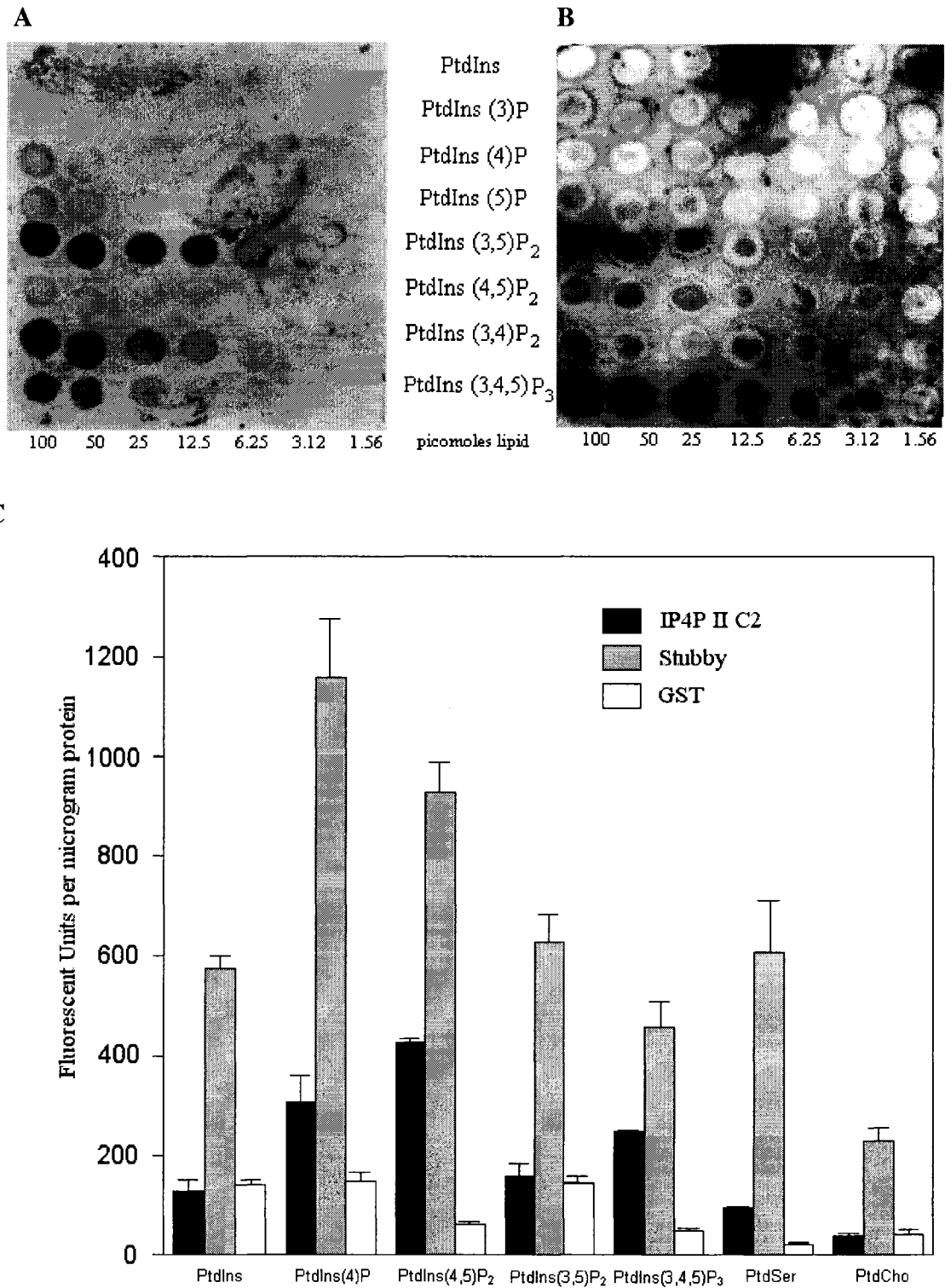


A.

PLC $\delta$ C2 (4-132)	KRLNIRVISGQQLPRVNR---NKNSIVDPKVTVEIHGVSR---DVASRQTAVITN
IP4P II (39-192)	PQLEFILACKDLV----APVRDRKLNILVQISV-IHPVEQSLTRYSS--TEIVEGT
Stubby (39-132)	PQLEFILACKDLV----APVRDRKLNILVQISV-IHPVEQSLTRYSS--TEIVEGT
Topology II	<div> <div>[<math>\beta</math>-Strand 1]</div> <div> <div>CBR 1</div> <div>CBR 2</div> </div> </div> <div> <div>[<math>\beta</math>-Strand 2]</div> <div>[<math>\beta</math>-Strand 3]</div> </div>
PLC $\delta$ C2 (4-132)	GFPWWDTE-FAFEV--VVPDLAL--IRFLVEDYD--ASSKND-----
IP4P II (39-192)	-RDLPLFTG-VTFPS--EYPIYEETKIKLTWYDVKDK---SHDTVRTSVLPEHKDPPPEV
Stubby (39-132)	-RDLPLFTG-VTFPS--EYPIYEETKIKLTWYDVKDK---SHDT-----
Topology II	<div> <div>[<math>\beta</math>-Strand 4]</div> <div> <div>[<math>\beta</math>-Strand 5]</div> <div>CBR 3</div> </div> </div>
PLC $\delta$ C2 (4-132)	----FIQSTIPLNSLK----QGYRHVELMSKNGDQHPS-ATLF---VKISLQS
IP4P II (39-192)	VGRSFLGYASFVGEEL--KSKEQLLVLSLRTSDGGRVVGTIIEVSVMKGEIE
Stubby (39-132)	----IANSSRI
Topology II	<div> <div>[<math>\beta</math>-Strand 6]</div> <div>[<math>\beta</math>-Strand 7]</div> <div>[<math>\beta</math>-Strand 8]</div> </div>

B.



**Figure 4**

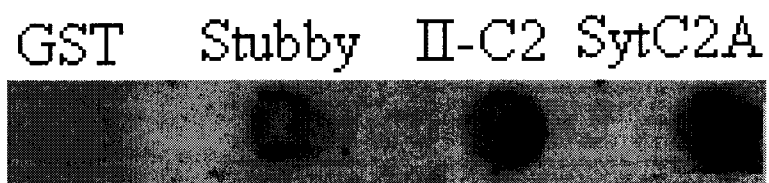
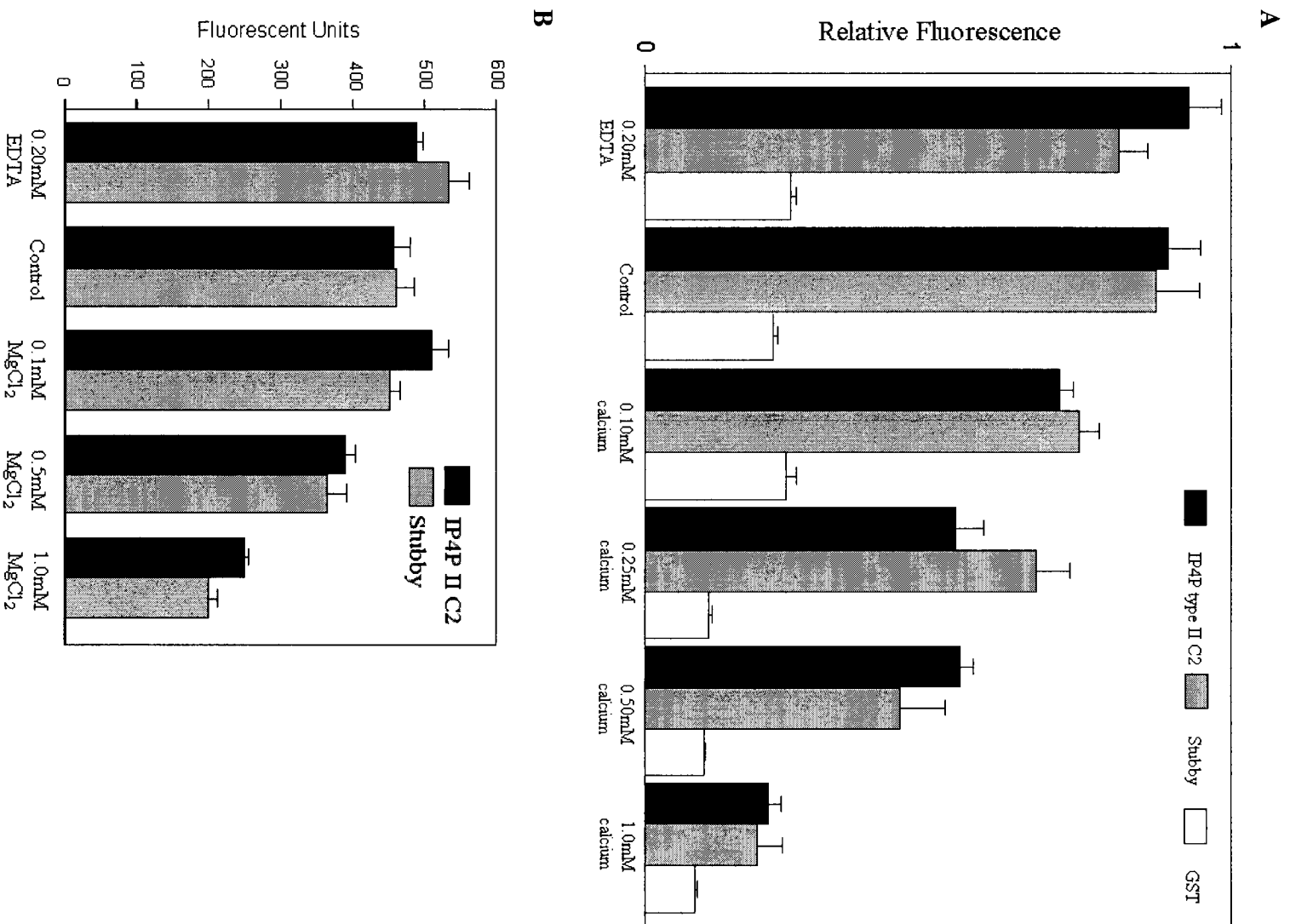
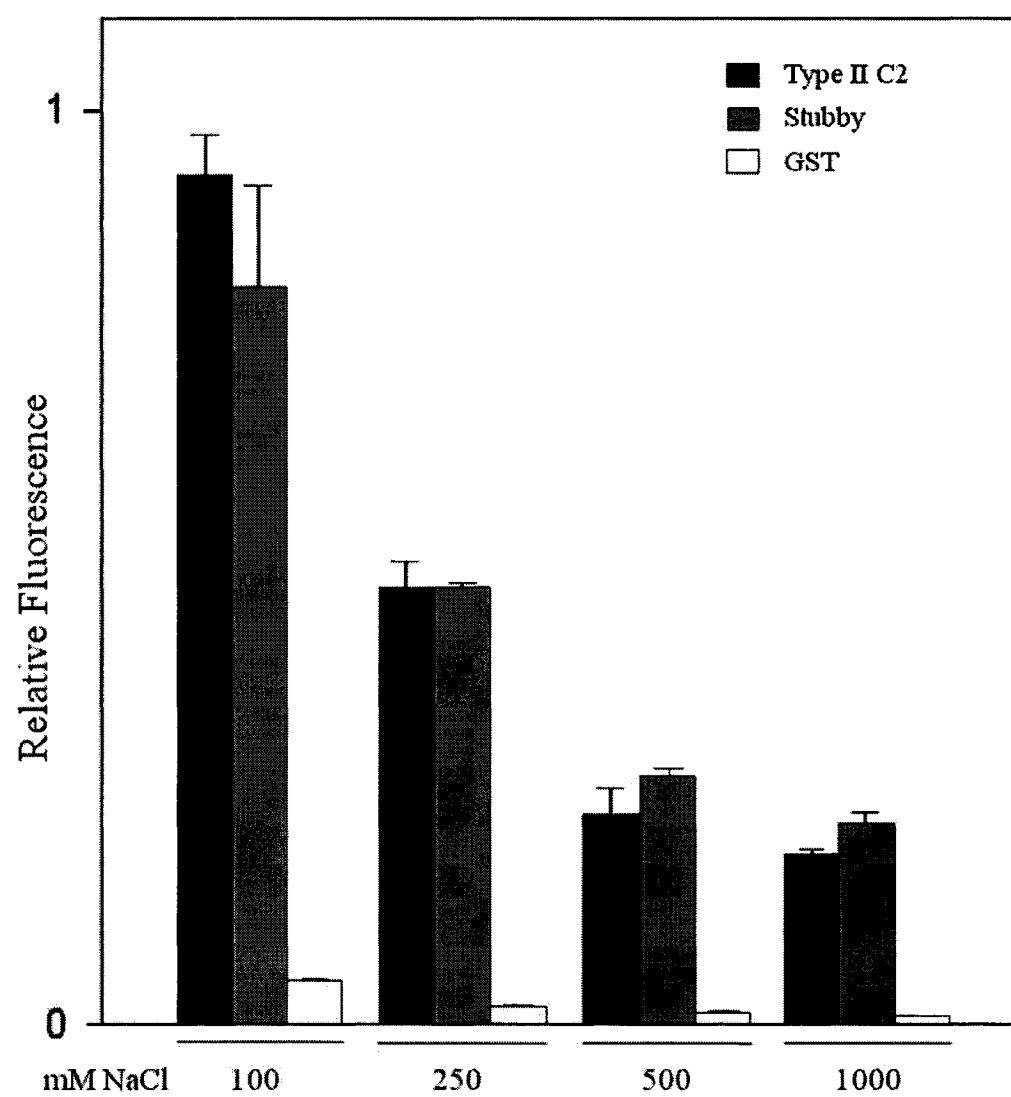
**Figure 5**

Figure 6



**Figure 7**

**Table 1**

Organization of the Human Inositol Polyphosphate 4-Phosphatase Type II Gene							
Exon no.	Exon boundary		Exon length	Intron no.	Intron boundary		Intron length
	5'	3'			5'	3'	
1 <sup>a</sup>	ATGGAA	TCACAA	91	1	gtaagtatg	tatttatag	1951
2	GTATCC	TCCTTG	45	2	gtaagaaat	cgttgccag	23848
3	CATGCA	GTGGAG	119	3	gtgagatgc	ctgttacag	2151
4	GGAACA	GACACC	117	4	gtaagtact	cttcccaag	18821
5 <sup>b</sup>	ATAGCT	ATTTGA	24	5	ND <sup>c</sup>	tctccacag	-
6	GTTCGA	GTTGGG	51	6	gtaagtga	tatttcag	9174
7	CGAAGT	CCTGAG	80	7	gtgggtctc	ttgtgcag	34683
8	AACTTC	CAAAAG	112	8	gtaggatct	tttctctag	10098
9	TGTGCC	ATTCAG	73	9	gtatgtaac	ttttccag	22480
10	TGTTAA	GTGCAG	148	10	gtaagagaa	tcttaacag	28837
11	AAACCA	AAACAG	131	11	gtgtggaag	ttccatcag	366
12	GGTCCT	TGAAAG	105	12	gtatcataa	ctactccag	15229
13	ATGCTC	AAGAAA	109	13	gtaagtaaa	ttgtgcag	19277
14	TACCGG	GAAAAA	178	14	gtaagatcc	tctttccag	13070
15	ACAGAG	GAGTGG	204	15	gtaagtctc	tttttcag	14361
16	GACAGG	CAAGAG	157	16	gtaagaaat	ttcccccag	21079
17	AGGACT	CAAGCA	173	17	gtagggtgcc	tttttcag	1172
18	CTTGCT	CATACA	124	18	gtgagtatt	attgtttag	1046
19	GCGATG	AAGACG	118	19	gtaagcagt	cttctttag	9445
20	AGAACA	TGAAAG	141	20	gtaagcttt	tgttttag	4351
21	GTTTGG	CTGATT	98	21	gtaagtatg	ccccttcag	21836
22	ATATTT	GCAACG	113	22	gtaagcact	atgttacag	3958
23	ATTTGC	GAGAAG	155	23	gtatctcac	atttaacag	51728
24 <sup>b</sup>	CAGACA	CAATAA	174	24	ND <sup>c</sup>	tttcaccag	-
25 <sup>b</sup>	AGAAGG	ACCTAA	133	25	ND <sup>c</sup>		-

## CHAPTER 5. GENERAL CONCLUSIONS

### Summary

The primary focus of the Norris lab is the study of Inositol polyphosphate 4-phosphatases (IP4P). IP4Ps are lipid phosphatases that remove the 4-position phosphate from the lipid second messengers phosphatidylinositol 3,4 bis-phosphate converting it to phosphatidylinositol 3 phosphate (1). There are two families of IP4Ps that are 37% homologous to each other denoted type I and type II. IP4P are lipid phosphatases, therefore they must be able to get to membranes to have access to their substrate (2). Currently the mechanism of membrane recruitment and binding is unknown.

So, is there a domain within the IP4P family that is capable of binding lipids in addition to the active site? To answer this question, various lipid binding assays have been used to identify a C2 domain in both type I and type II IP4Ps that binds calcium and phosphatidylinositols. In addition, both calcium and magnesium inhibit the ability of IP4P C2 domains to bind lipids.

Previously several different isoforms of both type I and type II IP4P had been identified. These included the  $\beta$ -spliceoforms of both type I and II that contained a hydrophobic tail instead of a hydrophilic one and a novel deletion spliceoform found in brain tissue that was approximately 1kDa smaller than type I $\alpha$  characterized in mouse brain tissue called type I $\alpha_2$  (3). When examining the gel, there was an additional band running higher than type I $\alpha$  on an SDS PAGE gel when lysates from Jurkat T-cells were run. So is this an additional spliceoform or is it a post-translational modification of type I $\alpha$ ? In answering this question a novel spliceoform was identified that is the predominant IP4P not only in

lymphocytes but in platelets and neutrophils as well. IP4P type I $\alpha_3$  is an 110kDa splice variant of type I IP4P's that contains an additional 40 amino acids creating a PEST region. This protein could have specialized roles in immune related functions.

The second is a short spliceoform of type II IP4P denoted Stubby that only contains a lipid binding C2 domain. The C2 domain of Stubby is not complete however, by sequence homology only  $\beta$ -strands 1-6 however, all three calcium-binding loops are present. With this in mind, can Stubby still interact with lipids and calcium even though it does not contain a full C2 domain? Comparison of the lipid binding properties of Stubby and the type II C2 domain revealed different substrate specificity. In a lipid overlay the IP4P type I C2 domain and Stubby prefer to bind phosphatidylinositol 3,5-bisphosphate whereas the type II C2 domain prefers phosphatidylinositol 3,4,5-trisphosphate. This suggests that different forms of IP4's are localized to different places in the cell. In addition, the fact that Stubby is still able to bind calcium and lipids gives greater insight on the mechanism of IP4P C2 domain function.

### **Future Study**

An important part of research is that when questions begin to be answered, these answers stimulate new questions that can be asked. This research opens new doorways for the study of biochemical and structural regulation of IP4P family members.

For starters, preliminary evidence exists for the presence of the PEST sequence having an effect on calpain mediated proteolytic susceptibility of the longer isoform. It would be interesting to examine different permutations of the three type I $\alpha$  isoforms in the context of the removal of part or all of the PEST domains. Would the proteins still get cleaved? Where are they cleaved by calpains, we have preliminary evidence that at least two of the sites of



calpain cleavage are near the variable region and hence near PEST sequences. Currently there is only evidence that type I $\alpha_3$  is cleaved *in vivo*, are type I $\alpha$  and type I $\alpha_2$  also cleaved *in vivo*? In fact are type II IP4Ps also cleaved by calpains? They do not have a PEST homology domain.

Type I IP4P has been implicated in the regulation of the oxidative burst in neutrophils by the production of PtdIns(3)P (5). All of this work was performed *in vitro* however. There are now systems that can use the ability of transfections to examine individual components during the production of the reactive oxidative burst. One of these systems is the COS<sup>phox</sup> system. This system uses COS cells that have been stably transfected with the components of the NADPH oxidase (p91, p22, p47<sup>phox</sup>, p67<sup>phox</sup>, Rac2) (6). Upon stimulation the cytosolic components will translocate to the membrane and produce superoxides. This system is ideal for the examination of the role of IP4P in the production of the oxidative burst. To date numerous mutants including the catalytically inactive CS and C2 domain mutants have been created that can be examined in this system. Is type II IP4P capable of stimulating superoxide production. In recent years other tissues have been shown to express homologs of the phox proteins (7,8). It is unknown if type I or type II is the predominant form in these tissues. Also, *in vitro* activity has not been demonstrated for type I $\beta$  IP4P. Can this activity be determined using the COS phox system or perhaps examination of lysates prepared from IP4P I $\beta$  infected *Sf-9* cells?

Now that there is evidence of a C2 domain on the N-terminus of IP4Ps, does it get recruited to the membrane, where does it go, and does it react to stimuli? Using GFP tagged constructs, preliminary evidence suggests that the type I C2 domain could be localized at vesicular bodies. Further research needs to be completed examining this in the context of

colocalization with other proteins known to localize to endosomes or lysosomes such as EEA1 or LAMP1.

The role of IP4P in phagocytosis can also be examined. Using the RAW cell phagocytic system, different GFP tagged versions of IP4P can be examined under the context of phagosomal fusion (9). Does the inactive mutant block this process?

The presence of C2 domains in both type I and II IP4Ps gives rise to some interesting questions. First, if a mutation of the C2 domains blocks lipid binding what are its effects on overall protein activity? Could the C2 domain also play a role in orientation of the active site? Second what is the role of CBR3 in lipid binding. Although there is little difference in lipid specificity using the pull-down system there is a clear difference using the overlay system. Is this difference due to the longer CBR3 in type II when compared to Stubby and type I C2 domains? The crystal structures of many C2 domains have been solved; to date IP4Ps are the first C2 domains that are negatively regulated by calcium. Can crystal structures be obtained for IP4P family C2 domains? These structures would provide insight into the mechanism of lipid binding. They would also verify that the overall predicted structure of the  $\beta$ -strands and CBRs are correct. Also can calcium coordination be determined from the crystal structures? This could help answer why calcium negatively regulates lipid binding. It would also help answer if amino acids in the longer CBR3 loop found in type II C2 domains are properly coordinated to interact with lipid head groups. From the data obtained from the crystals, additional mutations can be made and their effects on lipid binding evaluated. How do the CBR loops of IP4P interact with membranes, do they penetrate the membrane or is the interaction primarily due to surface interactions? NaCl does not completely eliminate lipid-binding, this suggests that there is some association with the

hydrophobic tails within the membrane. Using techniques such as Surface Plasmon Resonance this question can be addressed. In addition, what are the dissociation constants for lipid binding and for calcium binding? Does calcium interact with IP4P C2 domains in the physiological range?

Stubby is a natural spliceoform of type II IP4P that only consists of the first 2/3 of the C2 domain. What is the role of Stubby? Could Stubby be involved as an adaptor protein which can bind other proteins once it is on the membrane? There is preliminary evidence that Stubby will associate with the type II C2 domain. This could give rise to the association of protein complexes using Stubby or the type II IP4P. Or is Stubby involved in the regulation of type II lipid binding. An interesting experiment would be to determine using a lipid competition assay if Stubby could supplant the type II C2 domain in binding lipids. Also where in a cell is Stubby located?

### **Biological significance**

The discovery of two new spliceoforms within the IP4P family provides additional tools for the understanding of how IP4P tissue specificity can lead to differential regulation. The first spliceoform (type I $\alpha_3$ ) is present primarily in hematopoietic cells. The fact that it contains an additional PEST element provides clues to how it is regulated. Gaining a better understanding of how type I IP4P family members are proteolytically regulated will provide clues to their overall function within the cell.

Second and most importantly is the significance of a lipid-binding domain within the IP4P family. Although all of this work is *in vitro*, the biological significance of the findings from this thesis is several fold. Reports have shown that regulation of IP4P occurs in many cellular processes. These range from platelet activation to production of superoxides in

neutrophils, including development of Purkinje cells in the brain (13,14). For the first time a lipid-binding domain has been characterized that could recruit IP4Ps to their substrates. The lipid binding property of this domain is negatively regulated by calcium. This research provides clues on how IP4P family members bind to membranes and how they are regulated within the cell. With this knowledge, a better understanding of cellular processes such as phagocytosis and the production of reactive oxidative intermediates will be gained. The production of ROI's is known to cause damage during diseases such as heart attacks and strokes (15). In fact, superoxide has been shown to be produced even in platelets (16). In addition, inhibition of calpain proteases has been shown to decrease damage during ischemia-reperfusion injury (17-19). Recall that mutant alleles of IP4P are more resistant to oxidative stress in a *C. elegans* model (Walker, J, Peterson, K, Norris, F.A.-unpublished data). The involvement of IP4P in oxidative stress resistance could be regulated by calpain. IP4P could be an integral protein involved in the regulation of such processes, gaining better insight in the regulation of IP4P will provide possible pathways of treatment in the future.

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## ACKNOWLEDGEMENTS

I would like to thank many people who have assisted me during my process in completing this dissertation. First, without a doubt I would like to thank my parents Don and Tina Shearn. They taught me early in life that I would have to work hard to obtain my goals. They have been there whenever I needed them both emotionally and physically and have always given me their support throughout this difficult process.

Second, I would like to thank my transplant surgeons Dr. Micheal Wachs and Dr. Igal Kam along with the nurses and other support staff who work in the West side of the seventh floor at University Hospital in Denver, Colorado. Who would have thought that 6 years after their saving my life that I would be able to complete a dissertation and have a chance to give back to the community by doing research in the field of Immunobiology. The experience of receiving a new liver has proved to be a difficult one to handle and their support throughout the process was tremendous.

I would also like to thank the rest of my family, my twin brother Kevin who always let me stay at his house when I went home and was a great break from studying when we played golf. My older brother and sister Don and Chrissie Shearn who were there whenever I needed them. Besides my immediate family, I am eternally grateful for my dog Princeton who passed away 12 days before my defense, he had a great knack for bringing a smile back on my face after a long day in the lab.

There are too many people to thank at Iowa State but I would especially like to thank Dr. Jan Buss for her help with reviewing my data, answering my many questions and her advice in difficult situations, Raji Joseph, my lab partner for 6 years who was always there for technical questions that I might have, Lynn Summers, who taught me patience, Alisabeth

Ackerman who made these final days absolutely wonderful and finally my Major professor Dr. Andy Norris who took a chance on me and helped me learn the world of phosphatidylinositol signaling.