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Departamento de Biologia

Margarida Sâncio da Cruz Fardilha

Caracterização do Interactoma da PP1 do Testículo Humano

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Prof. Doutor Edgar F. da Cruz e Silva, Professor Associado do Departamento de Biologia da Universidade de Aveiro.

o júri

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resumo

A fosforilação de proteínas é um dos principais mecanismos reguladores de cascatas de transdução de sinais em eucariotas. A fosforilação é catalizada por proteínas cinases e é revertida pela acção de proteínas fosfatases. Embora as proteínas fosfatases tenham sido descobertas à mais de sessenta anos, a sua importância central em múltiplos mecanismos celulares só muito recentemente foi reconhecida.

PP1, uma fosfatase específica para serina/treonina, está envolvida em importantes mecanismos celulares, como o ciclo celular, a contracção muscular e a apoptose, entre outros. O seu papel em tão variados processos celulares depende das suas interacções com subunidades reguladoras. Até à data, foram descritas mais de 50 subunidades reguladoras que se ligam à subunidade catalítica da PP1, sendo determinantes para a sua função num local específico da célula.

Das três isoformas da PP1 conhecidas, PP1α, PP1β e PP1γ, a isoforma gama sofre splicing alternativo, originando a PP1γ1, ubíqua e, a PP1γ2 específica de testículo.

Incubação de espermatozóides imaturos com inibidores de fosfatases induz a sua motilidade, sendo a PP1γ2 a fosfatase envolvida. Este facto levou-nos a procurar proteínas de testículo humano capazes de interagir especificamente com a PP1γ2 que possam ser alvos terapêuticos no tratamento da infertilidade, ou na contracepção masculina. Para atingir este objectivo utilizou-se o sistema Dois Híbrido de Levedura no rastreio de uma biblioteca de testículo humano, na busca de novas proteínas que se ligam à PP1 usando como isco a PP1γ1 (no YTH1) ou a PP1γ2 (no YTH2).

Obtivemos 120 clones positivos no YTH1 e 155 no YTH2. Entre eles encontravam-se clones que codificam reguladores da PP1 previamente descritos, como a Nek2 e a NIPP1, assim como novas proteínas. Efectuámos um estudo detalhado de um novo gene, codificando uma nova proteína, que denominamos SEARP-T. Esta proteína de 93kDa é expressa maioritariamente em testículo e, a sua localização intracelular foi determinada por imunocitoquímica de fluorescência. Ambas as proteínas, PP1γ2 e SEARP-T, estão presentes na cauda e no segmento equatorial da cabeça do espermatozóide. Estes resultados clarificam as funções da PP1 no testículo humano e na motilidade do esperma e, indicam que o sistema Dois Híbrido de Levedura é um bom método para compreender o papel da PP1 em múltiplos eventos de regulação celular.

abstract

Protein phosphorylation is a major regulatory mechanism notably of signal transduction cascades in eukaryotic cells. Protein phosphorylation is catalysed by protein kinases and can be reversed by the action of protein phosphatases. Although phosphatases were discovered more than sixty years ago, their importance as central players in multiple cellular mechanisms was only recently recognized.

PP1, a Serine/Threonine specific phosphatase, is involved in important cellular mechanisms such as the cell cycle, muscle contraction and apoptosis, among others. Its role in such diverse cellular processes depends on its interactions with targeting/regulatory subunits. To date, more than 50 regulatory subunits have been identified that bind the catalytic subunit of PP1 determining its function in a specific cellular location.

Several isoforms of PP1 are known, termed PP1α, PP1β and PP1γ. The gamma isoform undergoes alternative splicing to yield a ubiquitously expressed PP1γ1 and a testis-specific PP1γ2 isoform.

Incubation of non-motile immature sperm with phosphatase inhibitors induces sperm motility, and PP1γ2 was implicated in this process. This led us to search for PP1γ2-specific interactors in human sperm that could be targeted for infertility therapeutics or in male contraception. To achieve this goal the Yeast Two Hybrid system was used to screen a human testis library for new PP1 binding proteins using both PP1γ1 (YTH1) and PP1γ2 (YTH2) as baits.

We recovered 120 positive clones in YTH1 and 155 positive clones in YTH2. Among these were clones encoding “bona fide” PP1 interactors such as Nek2 and NIPP1, and also previously uncharacterized proteins. We undertook a more detailed study of a novel gene encoding a novel protein that we termed SEARP-T. This protein of 93KDa is expressed mainly in testis and fluorescence immunocytochemistry was used to determine its intra sperm localization. Both PP1γ2 and SEARP-T proteins are present in the tail and in the equatorial segment of the head.These results provide new insights into PP1 function in human testis and sperm motility, and indicates that the Yeast Two Hybrid System provides a mean to understand the roles PP1 plays in diverse cellular regulatory events.

INDEX

ABBREVIATIONS

aa	Amino acid
AD	Activation domain
Ade	Adenine
Amp	Ampicillin
APS	Ammonium persulfate
BD	Binding domain
BLAST	Basic Local Alignment Search Tool
cAMP	Cyclic AMP (Adenosine 3',5'-monophosphate)
cDNA	Complementary deoxynucleic acid
cds	Protein coding sequence
Chr	Chromosome
CK2	Casein kinase 2
Cys	Cystein
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
DEPC	Diethylpyrocarbonate
dGTP	2'-deoxyguanosine-5'-triphosphate
DMSO	Dimethylsulfoxide
DNA	Deoxynucleic acid
dNTP	Deoxynucleotide triphosphate
dsDNA	Double strand deoxynucleic acid
dTTP	2'-deoxythymidine-5'-triphosphate
EDTA	Ethylenodiaminotetraacetic acid
EST	Expressed sequence tag
GAL4	Gal4 transcription factor
GSK3	Glycogen synthase kinase-3
His	Histidine
I-2	Inhibitor-2
LB	Luria-Bertani Medium (Miller)

Leu	Leucine
LiAc	Lithium acetate
NMDA	N-methyl-D-aspartic acid
nt	Nucleotide
OD	Optical density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PKA	Protein kinase A
PMSF	Phenyl methylsulfoxide
PP1	Protein phosphatase 1
PPM	Mg^{2+}-dependent protein phosphatase
PPP	Phosphoprotein phosphatase
QDO	Quadruple dropout
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase - polymerase chain reaction
SAP	Shrimp alkaline phosphatase
SD	Supplement dropout
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
Ser	Serine
TBS	Tris-buffered saline solution
TDO	Triple dropout
TEMED	N,N,N',N'-tetramethyletylendiamine
Thr	Threonine
Tris	Tryptophan
Trp	Tyrosine
Tyr	Tris (hydroxymethyl)-aminoethane chloride
UAS	Upstream activating sequence
UV	Ultraviolet
X-α-gal	5-bromo-4-chloro-3-indolyl-alpha-D-galactopyranoside
YPD	Yeast extract, Peptone and Dextrose medium for *S. cerevisiae*

YPDA	YPD with adenine
YTH	Yeast Two-hybrid system

I INTRODUCTION

I.1 PROTEIN PHOSPHORYLATION AS A DYNAMIC PROCESS

The reversible phosphorylation of structural and regulatory proteins is a major intracellular control mechanism in eukaryotes. It is involved in almost all cellular functions, from metabolism to signal transduction, cell division and memory. The phosphorylation state of a protein is a dynamic process controlled by both protein kinases and protein phosphatases (Fig. I.1).

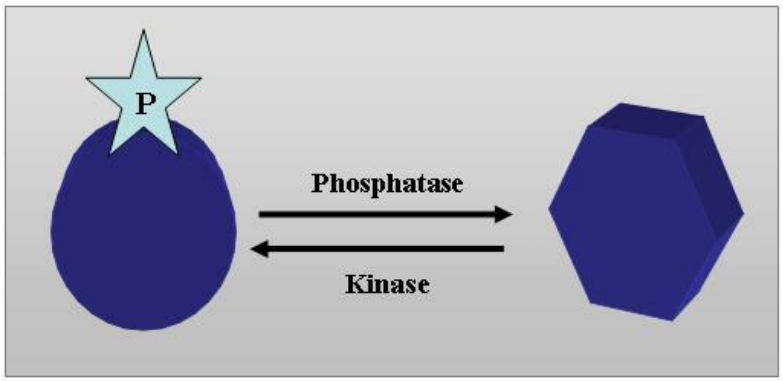


Figure I.1: *The dynamic process of protein phosphorylation.*

The protein kinases and protein phosphatases, the key controlling elements, are regulated by a myriad of extracellular and intracellular signals. Unlike the protein kinases that all belong to a single gene family, the protein phosphatases are divided into several distinct and unrelated protein/gene families. The Ser/Thr-specific protein phosphatases (Ser/Thr-PPs) comprise two distinct families, the PP1/PP2A/PP2B gene family and the PP2C gene family. The Tyr-specific phosphatase family, as well as including the Tyr-specific phosphatases, also comprises the so-called dual specificity phosphatases (capable of dephosphorylating Ser, Thr and Tyr residues). Besides these intracellular phosphatases involved in signal transduction, there are also unrelated non-specific alkaline and acid phosphatases that are usually found either in specialized intracellular compartments or in the extracellular milieu.

The sequencing of entire genomes has revealed that approximately 3% of all eukaryotic genes encode protein kinases or protein phosphatases (Plowman *et al.*, 1999). Surprisingly, there appear to be 2-5 times fewer protein phosphatases than protein kinases. This imbalance is even more pronounced when the analysis is limited to Ser/Thr-PP and kinases, particularly in vertebrates. The human genome, for instance, encodes approximately 20 times fewer Ser/Thr-PP than Ser/Thr-kinases. Thus, whereas the diversity of the Ser/Thr-protein kinases has kept pace with the increasing complexity of evolving organisms, that of Ser/Thr-PP has not. In the past decade it has became apparent that the diversity of Ser/Thr-PP is achieved not only by the evolution of new catalytic subunits, but also by the ability of a single catalytic subunit to interact with multiple regulatory (R) subunits.

I.2 ABNORMAL PHOSPHORYLATION IN DISEASE

Reversible protein phosphorylation is the major metabolic control mechanism of eukaryotic cells. Indeed, cellular health and vitality are dependent on the fine equilibrium of protein phosphorylation systems. Not surprisingly many diseases and dysfunctional states are associated with the abnormal phosphorylation of key proteins (e.g. cancer, diabetes, etc.).

In neurodegenerative diseases such as Alzheimer's Disease there is evidence for abnormal regulation of protein kinases. Altered activities and protein levels of several specific kinases suggest that abnormal phosphorylation contributes to the pathogenesis of these diseases. In Alzheimer's Disease, neurofibrillary degeneration results from the aggregation of abnormally phosphorylated Tau protein into paired helical filaments. Protein kinase A (PKA) and glycogen synthase kinase 3 β (GSK3β) are likely to be key kinases involved (Delobel *et al.*, 2002), and PP2A is thought to be the main tau phosphatase (Planel *et al.*, 2001). Furthermore, activation of protein kinase C (PKC), or inactivation of protein phosphatase 1 (PP1) leads to a relative increase in the utilization of the non-amyloidogenic pathway for Alzheimer's amyloid precursor protein (APP) processing (Gandy and Greengard, 1994; da Cruz e Silva *et al.*, 1995a).

Other neurodegenerative diseases that may result from abnormal phosphorylation include Parkinson's Disease (PD) and Huntington's Disease (HD). Alpha-Synuclein has been implicated in the pathogenesis of PD, and is a major component of Lewy bodies (a

major anatomical hallmark of PD). Alpha-synuclein was demonstrated to be constitutively phosphorylated indicating that its function is regulated by phosphorylation/dephosphorylation mechanisms (Okochi *et al.*, 2000). Motor and cognitive deficits in HD are likely caused by progressive neuronal dysfunction preceding neuronal cell death. Synapsin I is one of the major phosphoproteins regulating neurotransmitter release. In mice expressing the HD mutation, synapsin I is abnormally phosphorylated suggesting that an early impairment in its phosphorylation may alter synaptic vesicle trafficking and lead to defective neurotransmission in HD (Lievens *et al.*, 2002).

Altered phosphorylation has also been implicated in the etiology and/or symptoms of heart failure (Neumann, 2002) as well as in Diabetes (Sridhar *et al.*, 2000).

Therefore, protein phosphorylation systems represent attractive targets for diagnostics and therapeutics of several neurodegenerative and non-neurodegenerative diseases.

I.3 PHOSPHATASES AND THEIR CLASSIFICATION

The Ser/Thr-PPs, based on biochemical parameters, were initially divided into two classes: the type-1 phosphatases (PP1) that were inhibited by two heat-stable proteins, inhibitor-1 (I-1) and inhibitor-2 (I-2), and preferentially dephosphorylated the β-subunit of phosphorylase kinase; and the type-2 phosphatases, insensitive to the heat-stable inhibitors and that preferentially dephosphorylated the α-subunit of phosphorylase kinase (Ingebritsen and Cohen, 1983; Cohen, 1989). Type-2 phosphatases were further subdivided into cation independent (PP2A), Ca^{2+}-dependent (PP2B) and Mg^{2+}-dependent (PP2C) classes. The use of okadaic acid, a specific phosphatase inhibitor, further facilitated the discrimination between different classes (Cohen *et al.*, 1989). Although widely in use, this classification does not reflect the actual phylogenetic relationship between the different Ser/Thr-PP. Molecular cloning revealed that PP2A was in fact much more related to PP1 than to PP2C (Berndt *et al.*, 1987; da Cruz e Silva *et al.*, 1987). From a phylogenetic point of view it is more reasonable to group PP1, PP2A and PP2B in family I or PPP [that also includes the bacteriophage λ, orf221 phosphatase (Cohen *et al.*, 1988)] and PP2C in family II or PPM (Fig. I.2).

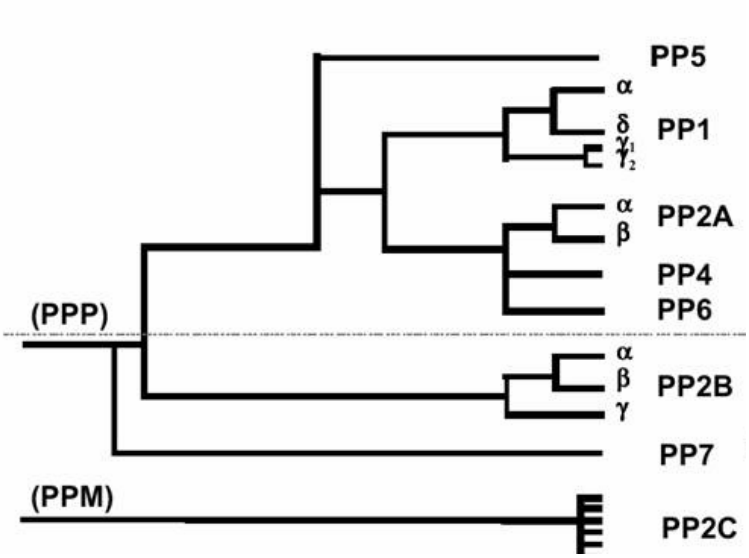


Figure I.2: *Phylogenic tree depicting the degree of similarity between the known phosphatases based on their primary amino acid sequence. PP1-PP7 belong to a single gene family (PPP) that is structurally distinct from the PP2C family (PPM). The phosphatases above the dashed line are highly sensitive to inhibition by the naturally occurring toxins, okadaic acid, mycrocystin and calyculin A (Honkanen and Golden, 2002).*

I.3.1 The PPP Family

The application of recombinant DNA techniques to the field yielded not only the primary structure of all four phosphatase types, but also documented the existence of isoforms for each type and revealed the existence of previously undetected phosphatases in a variety of eukaryotic cells (Berndt *et al.*, 1987; da Cruz e Silva *et al.*, 1987; da Cruz e Silva and Cohen, 1987; Cohen *et al.*, 1988; da Cruz e Silva *et al.*, 1988). Three genes are known to encode type 1 phosphatase catalytic subunits, termed PP1α, PP1β and PP1γ. At least PP1γ is known to undergo tissue-specific processing to yield an ubiquitously expressed PP1γ_1 isoform and a testis-specific PP1γ_2 isoform (da Cruz e Silva *et al.*, 1995b). Two genes are known encoding PP2A catalytic subunits, termed PP2Aα and PP2Aβ, and the three known PP2B catalytic subunit genes (Aα, Aβ and Aγ) are also subject to complex regulation to yield several alternatively spliced isoforms from each.

Perhaps more surprising was the discovery, from a variety of tissues and species, of other previously unknown phosphatase catalytic subunit isoenzymes, that were termed

novel phosphatases (da Cruz e Silva *et al.*, 1988). PP4, PP5 and PP6 (Cohen, 1997) are present in all mammalian tissues examined. In contrast, human PP7 (Huang and Honkanen, 1998), also found in *Arabidopsis thaliana* by Andreeva *et al.* (1998) and two *Drosophila* phosphatases are tissue specific (PPY is testis-specific and RdgC is restricted to photoreceptor organs and a small region in the brain). PP7 has been detected in the human retina and also in specialized sensory cells in plants (Fig. I.3).

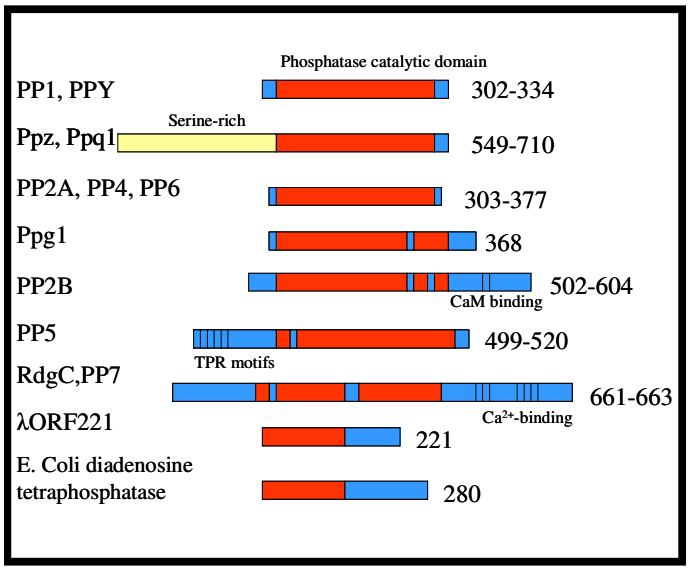


Figure I.3: *Domain organization of PPP family members. The numbers of amino acids in each are indicated on the right.*

I.3.1.1 PP1 - Protein Phosphatase 1

Eukaryotic genomes contain between one (*Saccharomyces cerevisiae*) and eight (*Arabidopsis thaliana*) genes that encode isoforms of PP1 catalytic subunit. These isoenzymes typically show an overall sequence identity of approximately 90% and cannot be distinguished by either their substrate specificity or their ability to interact with R subunits in vitro (Zhang *et al.*, 1993; Schillace and Scott, 1999). The sequence of the catalytic core of PP1 (residues 41-269 of the mammalian PP1α isoform) is almost identical in all isoforms, showing a high degree of similarity with the corresponding fragment of the catalytic subunits of PP2A and PP2B (Egloff *et al.*, 1995; Goldberg *et al.*, 1995). A detailed description of PP1, its properties and binding proteins will be given below.

I.3.1.2 PP2A - Protein Phosphatase 2A

PP2A has one of the most highly expressed catalytic subunits. It has been detected in various cell types and can comprise 0.3-1% of cellular proteins (Ruediger *et al.*, 1991; Lin *et al.*, 1998). The first descriptions of PP2A holoenzymes in the late 1970s and early 1980s demonstrated that the prevalent holoenzymes in rabbit skeletal muscle were heterotrimers composed of a catalytic subunit (C), a structural A subunit and a regulatory B subunit. cDNAs have been identified for two A subunits (α,β), two C subunits (α,β) and over twenty B subunits. For example, PR55 (B subunit), a 54 kDa protein (B' subunit), PR72, a 74 kDa protein (B'' subunit) and PR130. It has becoming increasingly clear that a major function of these regulatory subunits is to target the PP2A holoenzyme to distinct intracellular locations, signaling complexes and substrates. The A subunit is composed almost entirely of 15 imperfect repeats, each of 39 amino acids. These HEAT repeats (so named because they were found in Huntingtin, Elongation factor, PP2A subunit and the TOR kinase) are found in a variety of proteins. It seems that the repeats consist of two α helices connected by an intra-repeat loop, and that these loops are the binding sites for the B and C subunits (Ruediger *et al.*, 1992; Ruediger *et al.*, 1994; Ruediger *et al.*, 1999) (Fig. I.4). The crystal structure of the mammalian Aα subunit (PR65α) has been solved: the subunit is a hook-shaped protein with the C subunit binding to the inner face of the hook; the various B subunits attach to the medial surface of the hook (Fig. I.5) (Cegielska *et al.*, 1994).

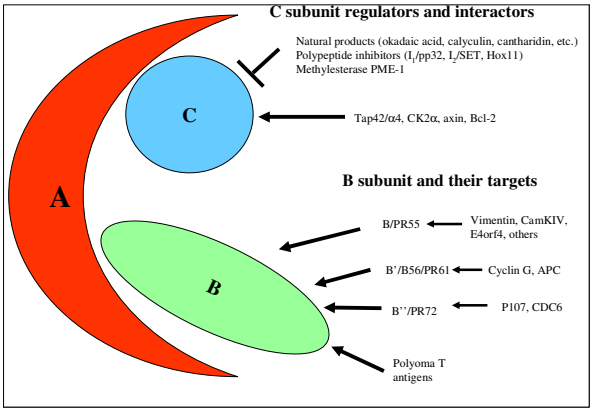


Figure I.4: *The heterotrimeric form of PP2A includes a catalytic C subunit, a structural A subunit and a regulatory B subunit. Several cellular and viral proteins that interact with PP2A components are indicated.*

Other molecular mechanisms that regulate PP2A catalytic subunit function include phosphorylation, carboxylmethylation, inhibition by intracellular protein inhibitors

I(1)PP2A and I(2)PP2A (Li *et al.*, 1995) and stimulation by ceramide. The C subunit of PP2A can be phosphorylated *in vitro* by the tyrosine kinases $p60^{v\text{-}src}$, $p56^{lck}$, epidermal growth factor receptor and insulin receptor (Chen *et al.*, 1992). Phosphorylation occurs at Tyr-307 at the extreme C-terminus of the protein and leads to 90% loss of activity. Dephosphorylation reactivates the phosphatase. This reactivation is prevented by okadaic acid, suggesting an auto-dephosphorylation reaction. A carboxymethyltransferase (Lee and Stock, 1993; Xie and Clarke, 1994a, b) has PP2A C subunit as the major substrate. The methylation of the α-carboxyl group of the C-terminal Leu-309 of PP2A *in vitro* has only moderate stimulatory effects on phosphatase activity and impairs binding of peptide-specific antibodies to the C-terminus of PP2A C subunit (Favre *et al.*, 1994). Interestingly, the C-terminal sequence of PP2A C subunit is conserved from yeast to man suggesting that modifications of the C-terminus may be a conserved regulatory mechanism (Zolnierowicz *et al.*, 1994). This modification may influence the interaction of the C subunit with its regulatory subunits. *In vitro* ceramide activates the trimeric forms of PP2A that contain either the PR55a or the 54kDa (B') subunit. In contrast, PP2A dimmeric form and the isolated C subunit are insensitive to ceramide (Dobrowsky *et al.*, 1993).

Figure I.5: *Crystal structure of the mammalian Aα subunit of PP2A.*

PP2A plays a central role in processes as varied as cell cycle regulation, cell signaling and neuronal function.

Cell Cycle Control and PP2A

PP2A was first implicated in the control of DNA replication when PP2A C subunit was purified as replication protein C on the basis of its ability to stimulate the efficient initiation of simian virus 40 (SV40) DNA replication (Virshup and Kelly, 1989). PP2A C subunit restored SV40 DNA replication activity to extracts from G1 phase cells suggesting that specific PP2A activity was regulated in the cell cycle as well (Virshup *et al.*, 1989). More recently, strong evidence has been obtained for the involvement of PP2A in chromosomal DNA replication (Lin *et al.*, 1998). The immunodepletion of A-subunit-containing forms of PP2A from *Xenopus* egg extracts inhibit subsequent DNA replication. This inhibition was relieved by replenishing the extracts with purified free PP2A C subunit. It was shown that PP2A activity was required for initiation of chromosomal replication rather than elongation. Immunodepletion of A/C dimers alone did not inhibit DNA replication, suggesting that a specific heterotrimeric form of PP2A is required (Lin *et al.*, 1998). The finding of a novel PR72-related B subunit that recruits PP2A to the human replication initiator protein CDC6 suggests that this family of B subunits (the PR72/130/B'' family) may be involved in initiation of both chromosomal and viral DNA replication (Yan *et al.*, 2000). Another novel PR72 family member, PR59, has been isolated in a two hybrid screen using p107, the Rb-related cell-cycle regulatory protein (Voorhoeve *et al.*, 1999a). PR59 may target PP2A to dephosphorylate p107 in response to stimuli such as UV irradiation that causes G1 arrest (Voorhoeve *et al.*, 1999b). So, the PR72-related proteins may be the PP2A regulatory subunit family involved in regulation of the G1/S transition.

Interestingly, a body of data suggests that a subset of PP2A holoenzymes function as tumor suppressors. The PP1/PP2A inhibitor okadaic acid can promote tumor formation suggesting that phosphatase inhibition can function similarly to kinase activation. The most compelling evidence that PP2A is a tumor suppressor remains the finding that DNA tumor virus small and middle T antigens replace B subunits in the PP2A heterotrimer and modulate substrate specificity (Pallas *et al.*, 1990; Walter *et al.*, 1990). Other potentially oncogenic human proteins that inhibit PP2A function have been reported, including HOX11 and SET oncogenes (Li *et al.*, 1996b; Kawabe *et al.*, 1997). Set expression and hence PP2A inhibition has been shown to induce c-JUN and AP-1 activity, whereas increased expression and hence increased activity of PP2A catalytic subunit inhibits ras-

induced transformation (Al-Murrani *et al.*, 1999; Baharians and Schonthal, 1999). Although some studies indicate that PP2A might exert tumor suppressive functions, other findings demonstrate the requirement for PP2A in cell growth and survival, which is not a characteristic of a tumor suppressor. This discrepancy might be due to the fact that PP2A is a multitask enzyme system, rather then a single enzyme. The puzzling observation that PP2A exerts inhibitory as well as stimulatory effects on cell growth could be due to the activity of different PP2A complexes with distinct subcellular location and diverse substrate specificity.

PP2A and neuronal function

The roles of PP2A in dephosphorylation of brain proteins are obscure. PP2A is a major activity that dephosphorylates voltage-sensitive sodium channels (Chen *et al.*, 1995), and is also a major brain phosphatase that dephosphorylates autophosphorylated CaMKII. PP2A selectively dephosphorylates soluble CaMKII rather then CaMKII associated with postsynaptic density, where it becomes a substrate for PP1 (Strack *et al.*, 1997a). The recent demonstrations that PP2A associates with and regulates CaMKIV (Westphal *et al.*, 1998), p70S6 kinase and p21-activated kinase (Westphal *et al.*, 1999a), support the proposal that PP2A might be a general regulator of protein kinases (Barnes *et al.*, 1995). PP2A is also known to be associated with neurofilaments, dephosphorylating NF-M and NF-L (Saito *et al.*, 1995; Strack *et al.*, 1997b). It probably regulates either the stability of neurofilaments or their interactions with other components of the neuronal cytoskeleton. Another potential function of PP2A is the regulation of the state of phosphorylation of microtubulule-associated proteins (MAPs). Neuronal-specific MAPs, including Tau and MAP2, bind to microtubules and regulate their stability. Neural MAPs are phosphorylated at multiple sites by a variety of protein kinases. Phosphorylation causes disassociation of MAPs from microtubules and their loss of stability. Accumulation of hyperphosphorylated Tau in neurofibrillary tangles is a pathological mark of Alzheimer's disease (T. H. Lee, 1995; Mandelkow *et al.*, 1996; Billingsley and Kincaid, 1997). Disruption of the microtubule cytoskeleton as a result of the loss of Tau-mediated stabilization may be one mechanism of neuronal cell death.

Signaling Cascades and PP2A

PP2A also plays an important role in the regulation of specific signal transduction cascades, as witnessed by its presence in a number of macromolecular signaling modules,

where it is often found in association with other phosphatases and kinases. Viral proteins target specific PP2A enzymes in order to deregulate chosen cellular pathways in the host and promote viral progeny. The observation that a variety of viruses utilize PP2A to alienate cellular behavior emphasizes the fundamental importance of PP2A in signal transduction. Other examples of the importance of PP2A in signaling cascades include the Wnt signaling pathway and the TOR (Target Of Rapamycin) signaling pathway.

Wnt signaling regulates vertebrate axis formation in early embryogenesis, and deregulation of the Wnt pathway is found in multiple epithelial cancers, including those of colon, skin and liver. One major effect of Wnt signaling is increased transcription driven by heterodimeric β-catenin/TCF transcription factor (Brown and Moon, 1998). In the absence of Wnt signaling β-catenin is maintained at low levels by phosphorylation-regulated proteolysis. Phosphorylation is mediated by a large multiprotein complex containing the adenomatous polyposis coli protein (APC), the serine/threonine kinase GSK3β and axin. PP2A has also been placed in this complex. It was shown by a two hybrid screen (Seeling *et al.*, 1999) that an amino-terminal domain of APC interacted with all members of the B'/B56 family of PP2A regulatory subunits. It was also found (Hsu *et al.*, 1999) that the carboxyl terminus of axin binds to PP2A via the catalytic subunit. Thus, the role of B56-containing PP2A heterotrimers in Wnt signaling may be to regulate the activity of GSK3β, keeping it dephosphorylated and thus active, leading to lower β-catenin levels *in vivo*. It was shown that PP2A C subunit dephosphorylated and activated GSK3β *in vitro*.

TOR signaling activates a response to nutrients, leading to TOR kinase activation with subsequent phosphorylation and activation of the ribosomal S6 kinase and phosphorylation and inactivation of eukaryotic initiation factor 4E-BP1, an inhibitor of translation initiation factor eIF4E (Gingras *et al.*, 1999). The result is stimulation of mRNA translation. Rapamycin inhibits TOR leading to growth arrest. TOR signaling may be due largely to its effect on PP2A and related phosphatases (Di Como and Arndt, 1996). TOR directly phosphorylates the Tap42 protein of *S. cerevisiae*, which then binds to either the C subunit of PP2A or the related yeast phosphatase Sit4 (Jiang and Broach, 1999). The Tap42-PP2A C dimer does not contain PP2A A or B, subunits representing a novel form of PP2A. In mammals, mTOR is downstream of phosphatidylinositol 3 kinase (PI3K) and can be directly activated by phosphorylation by PKB/Akt. A mammalian protein related to the

yeast Tap42, α4, has been shown to bind PP2A C (Murata *et al.*, 1997) and also the related phosphatases PP4 and PP6 (Jiang and Broach, 1999).

In summary, many proteins have been identified that interact with PP2A. Some of them affect PP2A activity [such as I(1)PP2A, I(2)PP2A, PTPA, Tap42/α4, SV40 small t, polyoma middle T/small t, HIV-1 NCp-7Vpr, adenovirus E4orf4CKIIα, Hox11 and PKR], some are PP2A substrates (such as Bcl2, p70s6 kinase, CaMKIV, vimentin, paxillin and SCR), for some PP2A it self is a substrate (such as caspase-3, JAK2, PME-1 and PKR) and some act as targeting proteins (such as eRF1, axin, APC and CG-NAP). An overview is given in Figure I.6.

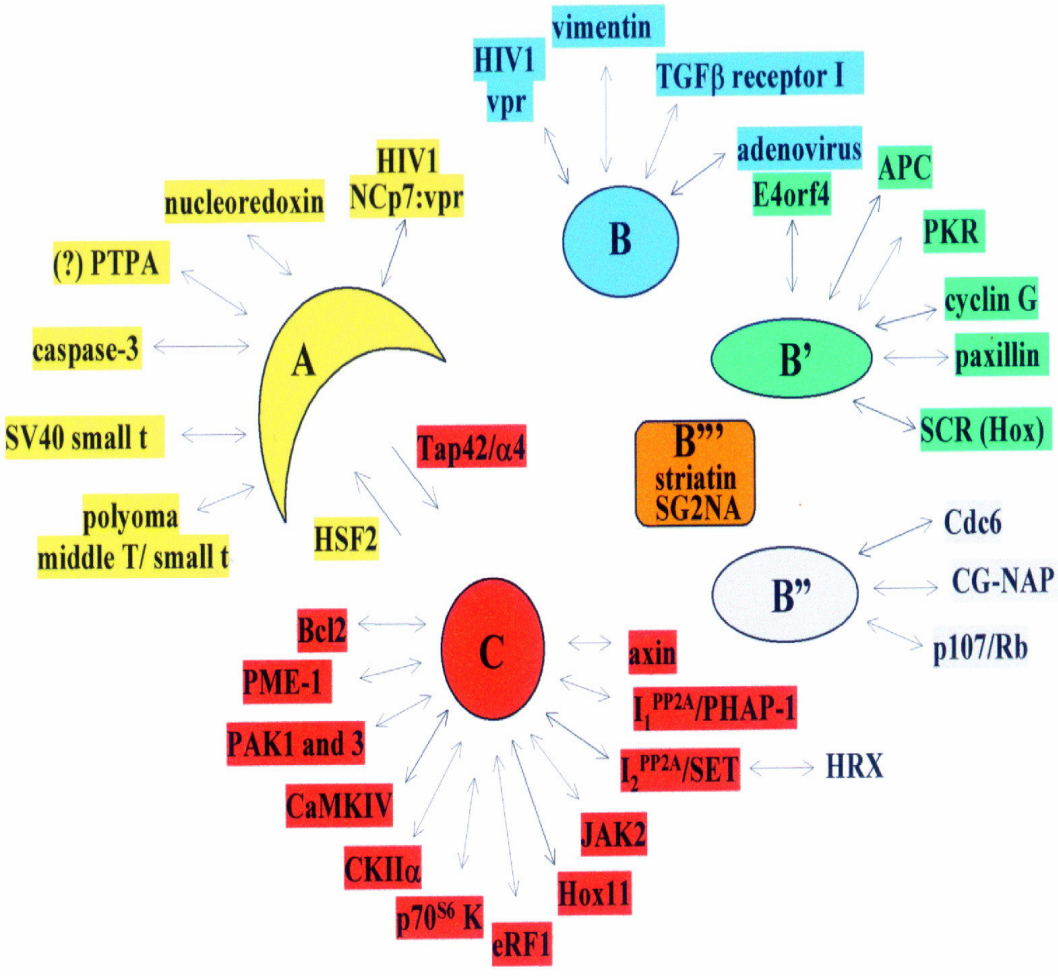


Figure I.6: *PP2A interacts with a variety of proteins (Janssens and Goris, 2001).*

I.3.1.3 PP2B - Protein Phosphatase 2B

PP2B, or calcineurin, is a Ca^{2+}/calmodulin-dependent protein phosphatase. The enzyme consists of two subunits, the catalytic A subunit of 60 kDa (PP2B A) and the myristoylated regulatory B subunit of 19 kDa (PP2B B). Calcineurin is present in nearly all mammalian cells studied but is highly enriched in neural tissue comprising over 1% of the total protein in the brain [a review can be found in (Klee *et al.*, 1998)]. Originally, the term calcineurin referred to the neuronal form of PP2B but, more recently, calcineurin refers to both neuronal and non-neuronal PP2B. The amino acid sequence of PP2B is highly conserved from humans to yeast with over 50% sequence identity (da Cruz e Silva and Cohen, 1989; da Cruz e Silva *et al.*, 1991; Stemmer and Klee, 1994). Cloning from rat brain indicated an A subunit of 521 amino acids. There are three mammalian genes for the A subunit, giving rise to the PP2B Aα, PP2B Aβ and PP2B Aγ isoforms. Aα and Aβ are highly expressed in brain, whereas Aγ is testis specific. Differential splicing of PP2B Aα generates two transcripts (α_1 and α_2), whereas PP2B Aβ gene is alternatively spliced to give three transcripts β_1, β_2 and β_3. PP2B $A\alpha_1$ and PP2B $A\beta_2$ are highly expressed in neuronal tissue (Ueki *et al.*, 1992). The A subunit shows autoinhibition that is relieved by interaction with the B subunit. PP2B, in contrast to PP1 and PP2A, is the only PP directly regulated by Ca^{2+}. The inhibition of B on A is relieved if B binds Ca^{2+}. This explains why the enzyme is dependent on Ca^{2+} for activity. Proteolysis of the autoinhibitory COOH terminus (residues 467-492) of the PP2B A generates a Ca^{2+}-independent form. The B subunit was sequenced at the protein level and found to comprise 168 amino acids, exhibiting a high degree of sequence similarity to calmodulin. Both contain four EF-hand Ca^{2+}-binding loops, both are needed for full PP2B activity, and both bind to unique regions on PP2B A without any cross interference. Two different B subunit genes are known: PP2B Bα and PP2B Bβ. PP2B Bα gives rise to one protein isoform expressed in many tissues termed PP2B $B\alpha_1$ (170 amino acids) and, by means of a different promotor, leads also to another testis-specific isoform PP2B $B\alpha_2$ (216 amino acids). PP2B Bβ (179 amino acids) is only expressed in the testis (Ueki *et al.*, 1992; Chang *et al.*, 1994). The crystal structure of PP2B has been determined (Griffith *et al.*, 1995; Kissinger *et al.*, 1995) and

helped to further clarify the various binding domains and interaction sites of PP2B (Fig. I.7).

The best known *in vitro* and *in vivo* substrates of PP2B are the PP1 inhibitors I-1 and DARPP-32. Thus, PP2B controls PP1 activity and the two together form the first documented phosphatase cascade (Mulkey *et al.*, 1994).

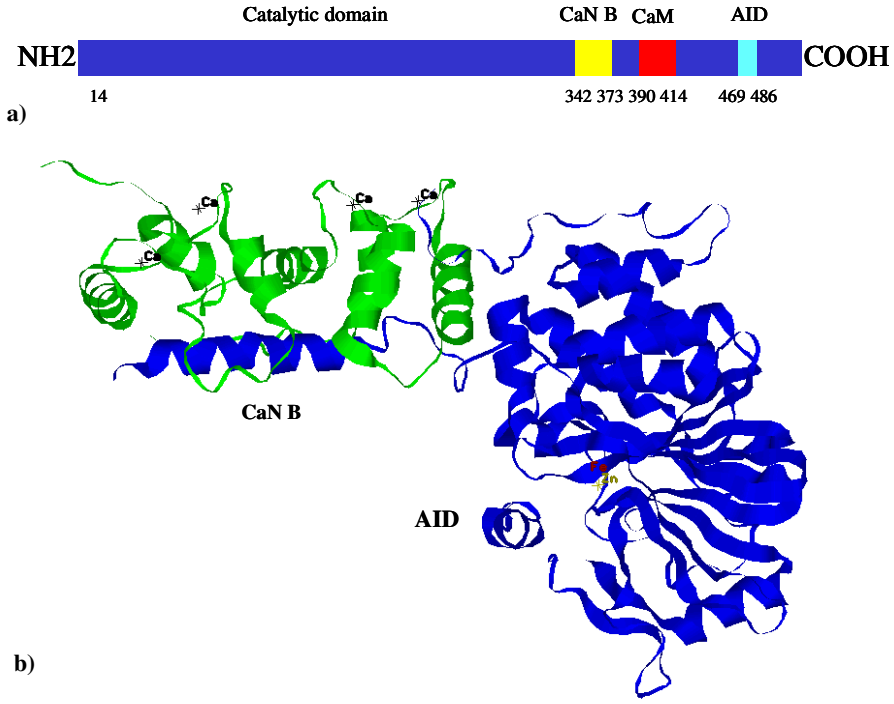


Figure I.7*: Structure of calcineurin.* ***a)*** *Diagrammatic representation of the calcineurin A subunit indicating the catalytic domain, the binding domains for calcineurin B (CaN B), calmodulin (CaM), and the auto-inhibitory domain (AID).* ***b)****, the crystal structure of the human calcineurin heterodimer is shown with secondary structural elements. The calcineurin A subunit is shown in blue and the B subunit in green. Fe (red) and Zn (yellow) are bound in a cleft of the catalytic domain and four Ca (black) are shown binding to the B subunit.*

PP2B and neuronal function

Calcium-stimulated dephosphorylation of target proteins by PP2B modulates numerous neuronal activities. Described functions for PP2B in the brain include Ca^{2+}-dependent dephosphorylation of DARPP-32, inhibition of neurotransmitter release, dephosphorylation of dynamin I, and desensitization of postsynaptic NMDA-receptor-coupled calcium channels (Yakel, 1997; Greengard *et al.*, 1998; Klee *et al.*, 1998). PP2B is also thought to regulate the calcium channel activity of the ryanodine and inositol 1,4,5-

triphosphate receptors via an immunophilin-mediated interaction (Snyder *et al.*, 1998), and plays a role in the neurotoxicity associated with excessive stimulation of NMDA-type glutamate receptors. Immunossuppressants prevent apoptosis and rearrangments of the cytoskeleton in response to excitotoxic glutamate (Halpain *et al.*, 1998). Overexpression of PP2B in transgenic mice revealed a novel phase of long-term potentiation in the CA1 region of the hippocampus that may be normally constrained by PP2B (Winder *et al.*, 1998). These observations provide evidence for a physiological function for PP2B in intact animals. The altered regulation of this component of long-term potentiation by PP2B correlates with reversible deficits in long-term memory that occur during expression of activated PP2B (Mansuy *et al.*, 1998). These observations provide an elegant demonstration of the role of PP2B in memory. Along with PP2A, PP2B is one of the major tau phosphatases *in vitro* and can restore the microtubule-binding ability of hyperphosphorylated tau (Billingsley and Kincaid, 1997). When nerve terminals in the brain are stimulated, a group of phosphoproteins called dephosphins are coordinately dephosphorylated by PP2B. Amazingly, the seven presently known dephosphins are not structurally related, yet each has been independently shown to be essential for synaptic vesicle endocytosis. Nowhere else in biology is there a similar example of the coordinated dephosphorylation of such a large group of proteins each sharing roles in the same biological response.

PP2B and T-cell activation

PP2B plays a crucial role in the Ca^{2+}-signaling cascade of activated T-cells. Increases in Ca^{2+} concentrations in T-cells promoted by antigen presentation to T-cell receptor stimulates PP2B to dephosphorylate the cytosolic subunit of the transcription factor NFAT, Nuclear Factor of Activated T-cells, (Jain *et al.*, 1993). Dephosphorylated NFAT translocates into the nucleus where, in concert with other transcription factors, it induces expression of the IL-2 gene, one of the early genes in the T-cell activation pathway. Inhibition of this Ca^{2+}-signaling cascade by immunosuppressant drugs (such as FK506 and cyclosporin A, when complexed to their intracellular receptors FKBP and cyclophilin, respectively) suppresses T-cell activation.

PP2B and transcription control

Regulation by PP2B of the nuclear import of NFAT has already been mentioned above. It was the first example of the transduction of a signal at the plasma membrane to

the nucleus. More recently, it has been shown that this transcription factor is not only involved in T-cell activation, but also in several other mechanisms. PP2B has been shown to have important roles in axonal guidance (Chang *et al.*, 1995), as well as memory and learning (Mansuy *et al.*, 1998; Winder *et al.*, 1998). In general, these functions in neurons have been thought to be independent of transcription. However, recent evidence indicates that NFAT family members may have critical roles in the development of synaptic connections (Graef *et al.*, 1999). It also seems that the NFAT pathway is involved in the morphogenesis of heart valves. Additionally, it has been shown that severe cardiac hypertrophy can be induced by the overexpression of a truncated constitutively active PP2B A subunit (Molkentin *et al.*, 1998).

NF-κB and other Rel proteins play critical roles in the development of the liver, skin, inflammatory responses and in some aspects of recombinational immune response. Unlike NFAT, which absolutely requires a calcium stimulus, NF-κB can be fully activated by PKC activators in the absence of calcium (Singh *et al.*, 1986). However, suboptimal stimuli can be augmented with a calcium signal (Mattila *et al.*, 1990). This calcium facilitation of NF-κB activity can be mimicked by overexpression of a constitutively active PP2B and can be partially blocked with FK506 or cyclosporin A, indicating that PP2B is required for full induction of NF-κB activity in certain circumstances (Frantz *et al.*, 1994). The AP-1 transcription complex consists of Fos and Jun proteins. Transcription controlled by most AP-1 sites is not sensitive to inhibition by cyclosporin A or FK506 (Mattila *et al.*, 1990). However, a site in the IL-2 promoter that binds junD and perhaps c-jun requires the action of PP2B for full function (Ullman *et al.*, 1993). In addition, an apparent AP-1 site in the collagenase promoter is sensitive to FK506 (Su *et al.*, 1994) and hence likely to be PP2B-dependent.

PP2B inhibitors

Several different classes of PP2B inhibitors have been discovered raising questions about their various roles in Ca^{2+}/PP2B signaling. The inhibitors bind to PP2B and inhibit its ability to dephosphorylate substrates such as NFAT family members, thereby preventing their nuclear localization. One of the most interesting is the DSCR1 gene and its relatives, DSCR2 and ZAK14 (Fuentes *et al.*, 2000). DSCR1 is located on chromosome 21 in the so called critical region (hence its designation, Down's syndrome critical region 1 gene). Some evidences indicate that overexpression of DSCR1 might underlie the

pathogenesis of Down's Syndrome. First, it was shown that DSCR1 protein is overexpressed in the brains of Down's Syndrome patients. Second, some of the symptoms of the Syndrome appear in mice with mutations of the different PP2B-dependent (NFAT) subunits of the transcription complex (Fuentes *et al.*, 2000). It is possible that overexpression of DSCR1 as a result of trisomy leads to inhibition of PP2B and subsequent effects on the development of the brain, immune system, heart and skeleton. However, the Down's Syndrome critical region includes a number of genes and it is possible that the syndrome is due to overexpression of several of these. Recently, yeast has been shown to have a related protein, Pcn1p that binds and inactivates PP2B (Gorlach *et al.*, 2000). Two additional classes of PP2B inhibitors are Cabin/Cain (Lai *et al.*, 1998; Sun *et al.*, 1998), which are novel proteins, and the CHP protein that has similarity to PP2B B subunit (Lin and Barber, 1996; Lin *et al.*, 1999). The CHP proteins appear to compete with PP2B B for binding to the A protein and thereby inhibit the Ca^{2+}-dependent activation of PP2B A. Cabin, or Cain (Calcineurin inhibitor), is a non competitive inhibitor of PP2B phosphatase activity with a Ki of 440nM. A physiologic role for these proteins is still unclear, but they do antagonize NFAT translocation. A fourth class of PP2B inhibitors is found in the genome of certain viruses, (e.g. African swine fever virus). The A238L protein encoded by the virus binds tightly to PP2B and blocks NFAT translocation and function (Miskin *et al.*, 1998). AKAP79 (protein kinase A anchoring protein) was the first PP2B protein inhibitor to be found (Coghlan *et al.*, 1995) and is also a scaffolding protein. AKAP79 binds both calcineurin and protein kinase A, and may anchor PP2B at specific sites that allow the protein to engage the proper substrates when activated. Another inhibitor that constitutes a useful laboratory tool is the auto-inhibitory peptide. This is a 26-residue peptide that interacts with the catalytic core and blocks PP2B activity with an IC50 of 10-15μM (Hashimoto *et al.*, 1990). Immunosuppressant drugs such as cyclosporin A and FK506 (Liu, 1993) belong to a different class of PP2B inhibitors. These are fungal compounds that require binding to their cognate intracellular immunophilins (cyclophilin A and FKB12, respectively) prior to binding to and inhibiting PP2B activity with nanomolar affinity. Being membrane permeable, these compounds have greatly facilitated the *in vivo* study of PP2B function. Yet another class of inhibitors is the type II pyrethroid insecticides, including cypermethrin and deltamethrin (Enan and Matsumura, 1992).

In conclusion, within the past few years PP2B has been implicated in a wide variety of extremely important biological responses including lymphocyte activation, neuronal and muscle development, neurite outgrowth, morphogenesis of vertebrate heart valves, learning and memory (Fig. I.8).

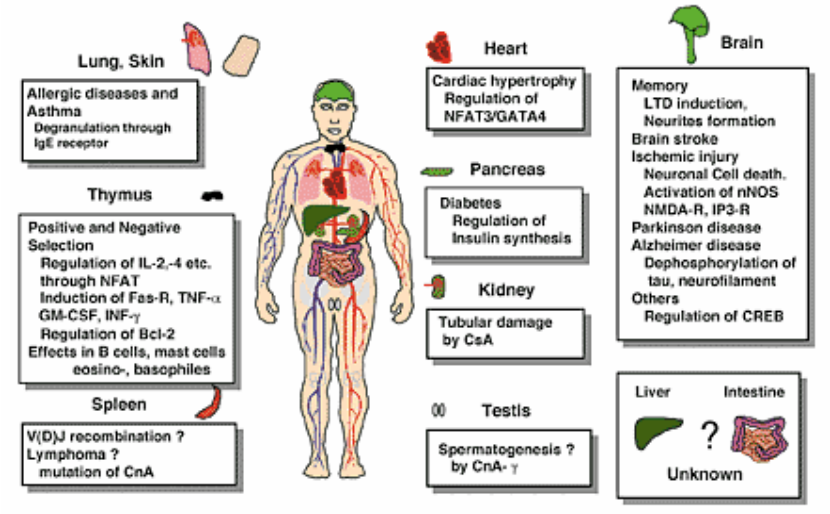


Figure I.8: *Calcineurin as a multifunctional regulator (Shibasaki et al., 2002).*

I.3.2 The PPM Family

The PPM protein phosphatases belong to a large and diversified family of protein phosphatases expressed in both eukaryotes and prokaryotes that dephosphorylate serine and threonine residues, as does the PPP family. PPC relates to the main enzyme subtype of the PPM, including *Arabidopsis* ABI1 (Leung *et al.*, 1994), *Arabidopsis* KAPP-1 (Stone *et al.*, 1994), pyruvate dehydrogenase phosphatase (Lawson *et al.*, 1993) and *Bacillus subtilis* SpoIIE phosphatase (Barak *et al.*, 1996). Within the PPM family, the PP2C domain occurs in numerous structural contexts that reflect functional diversity. For example, PP2C domain of the *Arabidopsis* ABI1 gene is fused with EF- hand motifs (Leung *et al.*, 1994; Meyer *et al.*, 1994), whereas in KAPP-1, a kinase-interaction domain that associates with a phosphorylated receptor precedes the phosphatase domain (Stone *et al.*, 1994). Other examples include the Ca^{2+}-stimulated mitochondrial pyruvate dehydrogenase phosphatase, which contains a catalytic subunit sharing 22% sequence identity with mammalian PP2C (Lawson *et al.*, 1993) and SpoIIE phosphatase, which has ten membrane-spanning regions preceding the PP2C-like catalytic domain (Fig. I.9).

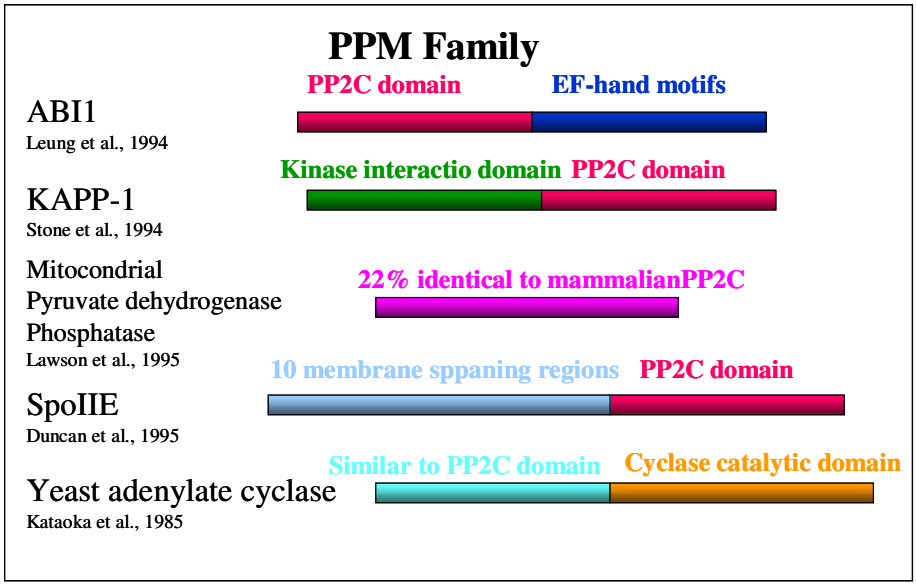


Figure I.9: *The PPM family.*

PP2C was originally identified as a Mg^{2+}-dependent protein phosphatase. The enzyme is monomeric with a molecular mass of 43-48 kDa (Cohen, 1989) and is resistant to okadaic acid. To date the existence of six distinct PP2C genes (PP2Cα, PP2Cβ, PP2Cγ, PP2Cδ, Wip1 and FIN13) has been reported in mammalian cells (Tamura *et al.*, 1989; Wenk *et al.*, 1992; Fiscella *et al.*, 1997; Guthridge *et al.*, 1997; Travis and Welsh, 1997; Tong *et al.*, 1998). In addition, mouse PP2Cβ has been found to have five distinct isoforms (β1, β2, β3, β4, β5), which are splice variants from a single pre-mRNA (Terasawa *et al.*, 1993; Hou *et al.*, 1994; Kato *et al.*, 1995).

Considerable information on the function of mammalian PP2C has accumulated over the last decade. PP2C can be activated by polyunsaturated fatty acids such as arachidonic acid. Upon addition of fatty acids PP2C no longer requires unphysiologically high Mg^{2+} concentrations for activity, and Ca^{2+} ions become inhibitory in the micromolar range. The stimulation of PP2C *in vitro* by fatty acids suggests that this enzyme may be regulated by an endogenous lipid messenger *in vivo* (Klumpp *et al.*, 1998). It was shown (Murray *et al.*, 1999) that PP2Cγ is physically associated with the spliceosome *in vitro* and is required during the early stages of spliceosome assembly for efficient formation of the A complex. PP2C is also involved in the regulation of AMP-activated protein kinase (Moore *et al.*, 1991) and Ca^{2+}/calmodulin–dependent protein kinase II (Fukunaga *et al.*, 1993). PP2C may also selectively inhibit the SAPK pathway through suppression of MKK3b,

MKK4, MKK6b and MKK7 activities in mammalian cells (Hanada *et al.*, 1998). Recent research further revealed that PP2C might be involved in the control of cell apoptosis (Wolf *et al.*, 1997), gene expression and other cellular functions (Bohmann, 1990; Kurosawa, 1994; Schonthal, 1995). More recently, PP2Cα has been implicated in Wnt signaling, being a positive regulator and exerting its effects through the dephosphorylation of axin (Strovel *et al.*, 2000), a role that was first attributed to PP2A (Willert *et al.*, 1999). PP2C influences the dynamic interactions between the actin cytoskeleton and membrane constituents linked to moesin (Hishiya *et al.*, 1999). Members of the moesin protein family are localized membrane structures rich in actin filaments that act as linkers between the plasma membrane and the actin cytoskeleton. Furthermore, PP2Cα and PP2Cβ2 are responsible for the inactivating dephosphorylation of Cdk2/Cdk6 (Cheng *et al.*, 2000b). The dephosphorylation of Cdk2 and Cdk6 by PP2C isoforms is inhibited by the binding of cyclins. PP2Cβ was recently implicated in the regulation of the TAK1 signaling pathway: TAK1 is a stress-activated protein kinase that is activated by interleukin-1, transforming growth factor-β or stress. PP2Cβ negatively regulates the TAK1 signaling pathway by direct dephosphorylation of TAK1 (Hanada *et al.*, 2001).

I.4 PHOSPHATASE INHIBITORS

One of the most significant advances in the study of Ser/Thr-PPs, and the elucidation of the cellular events they control, was the identification of several naturally occurring toxins as powerful and specific phosphatase inhibitors. Among these are okadaic acid (Fujiki and Suganuma, 1993), cantharidin (Laidley *et al.*, 1997), calyculin A (Ishihara *et al.*, 1989), microcystins (Carmichael, 1992; Fujiki and Suganuma, 1993; Carmichael, 1994) and tautomycin (MacKintosh and Klumpp, 1990; Hori *et al.*, 1991) to name a few examples. Another class of phosphatase inhibitors are the protein inhibitors readily available inside the cell. The specificity of these inhibitors for a given phosphatase has placed the former as key tools in the study of phosphorylation dependent processes.

I.4.1 Toxins as natural inhibitors of PPs

I.4.1.1 Okadaic acid and derivatives

Okadaic acid (OA) is a polyether compound with a C-38 structure and was isolated from the black sponge *Halichondria okadai* (Fujiki and Suganuma, 1993) (Fig. I.10).

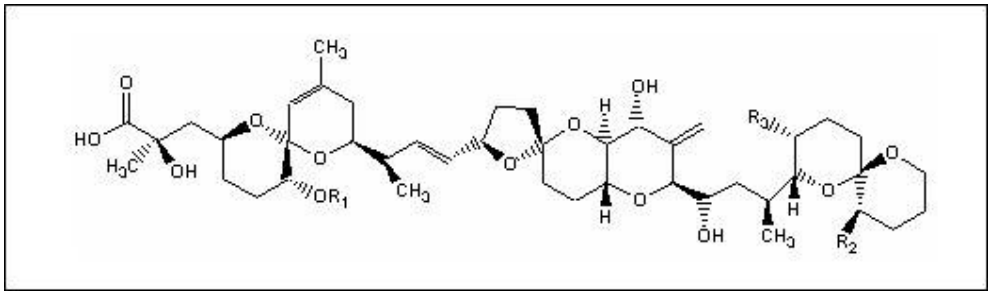


Figure I.10: *Structure of okadaic acid (R_1=H, R_2=H, R_3=CH_3).*

OA is a potent phosphatase inhibitor in smooth muscle preparations (Bialojan *et al.*, 1988), and was reported to inhibite PP1, PP2A and PP2B with IC_{50} values of 272, 1.6, and 3,600nM, respectively (Bialojan and Takai, 1988). PP2C, phosphotyrosyl phosphatase, acid phosphatase and alkaline phosphatase were not inhibited by up to 10µM OA. It is currently thought that tumor promotion by OA and related compounds like calyculin A and microcystin LR is due to inhibition of PP1 and PP2A [a review can be found in (Fujiki and Suganuma, 1993)]. OA in concentrations <2nM inhibits also other PP2A related family members such as PP4, PP5, and PP6 (Cohen, 1997).

I.4.1.2 Cantharidin and analogs

Cantharidin (CT), cantharidic acid, palasonin and endothall are structural analogs (Laidley *et al.*, 1997). CT is the vesicant in blister beetles, palasonin is an anti-helmintic in seeds of a medical tree and endothal is a synthetic herbicide. The toxic action of CT is thought to be due to phosphatase inhibition. Initially, CT was reported to bind to PP2A in mouse liver (Li *et al.*, 1993). However, CT inhibits PP1 and PP2A *in vivo* with IC_{50} values of 500 and 40nM (Honkanen, 1993; Li *et al.*, 1993; Neumann *et al.*, 1995). Palasonin and

cantharidic acid exhibited similar inhibitory activity (Li *et al.*, 1993). Endothal inhibits both PP1 and PP2A with IC_{50} values of 5,000 and 1,000nM (Li *et al.*, 1993). Neither compound inhibits PP2B (>30,000nM) or PP2C (>1mM). CT is a terpenoid and is cell membrane permeable. It is less potent and selective than OA, but much cheaper. Mutational analysis indicates that OA and CA might act on different amino acids on PP1 (Zhang *et al.*, 1994b) (Fig. I.11).

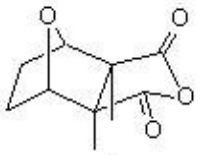


Fig. I.11: *Structure of Cantharidin.*

I.4.1.3 Calyculin A:

Calyculin A (CA) was isolated from another marine sponge, *Discodermia calyx*. It is an octamethylpolyhydroxylated C-28 fatty acid linked to two γ-amino acids and esterified with phosphate. It is cell membrane permeable. CA is equally potent against PP1 and PP2A (Ishihara *et al.*, 1989; Fujiki and Suganuma, 1993) with IC_{50} values from 1 to 14nM. OA and CA seem to compete for the same inhibitory site on PP2A (Takai *et al.*, 1995). Mutational analysis suggests that CA inhibits the enzyme via interaction with amino acid tyrosine-272 on PP1 because its IC_{50} is changed from 0.5 to 3,000nM in the Y272K mutant (J. Zhang *et al.*, 1996) (Fig. I.12).

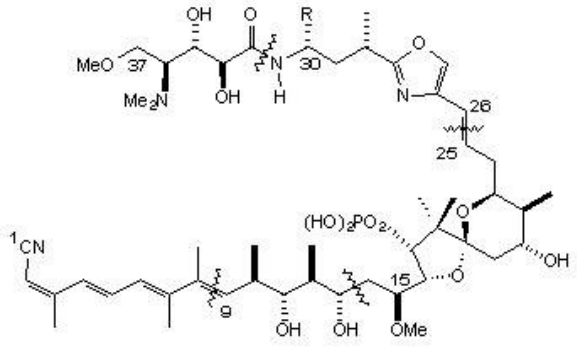


Figure I.12: *Structure of calyculin A (CA A,R=H; CA C, R=Me).*

I.4.1.4 Microcystins

Whereas OA and CA are fatty acid derivatives, microcystins (Mic) and nodularin (Nod) are peptide toxins. Mic are of toxicological and public health relevance. They cause death in cattle and humans exposed to water contaminated by certain algae. These include colonical and filamentous algae and cyanobacteriae like *Microcystis aeruginosa* (Carmichael, 1992; Fujiki and Suganuma, 1993; Carmichael, 1994). Mic are cyclic heptapeptides containing five constant (some of which are unique) and two variable amino acids. The variable amino acids in Mic are indicated in the one-letter code. Hence, their short-hand notation is microcystin-LR, -YR and –RR. Mic-LR inhibits PP1 and PP2A with IC_{50} values of 0.1nM each (MacKintosh *et al.*, 1990). It inhibits PP2B with an IC_{50} of 0.2µM and does not inhibit PP2C up to 4µM (MacKintosh *et al.*, 1990). PP4 and PP5 are inhibited by <2nM Mic. Mic is the most potent (and toxic) PP inhibitor, but being a peptide it is not cell permeable. The 3D structure of Mic-LR bound to PP1 has been determined (Goldberg *et al.*, 1995; Bagu *et al.*, 1997), and mutation of the critical cysteine-273 in PP1 to alanine impeded covalent binding of Mic to PP1 (Fig I.13).

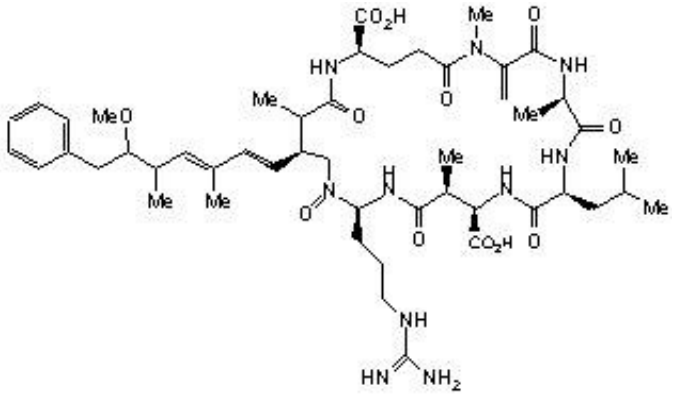


Figure I.13: *Structure of Microcystin.*

I.4.1.5 Nodularin

Nodularin (Nod), a cyclic pentapeptide, was isolated from the toxic cyanobacterium *Nodularia spumigenia* (Carmichael, 1992). Like Mic it is toxic to the liver. Nod R and its derivative motuporin (nodularin V) potently inhibit PP1, as well as PP2A, with IC_{50} values of 1.6 and 0.03nM, respectively (Honkanen *et al.*, 1991). Hence, it inhibits PP1 and PP2A 10 times more potently than OA, being is 70 fold selective for PP2A. It inhibits PP2B with

an IC_{50} of 8.7μM but does not affect PP2C (Honkanen *et al.*, 1991). The IC_{50} values are comparable to those of Mic-LR. In contrast to Mic, Nod R or V do not covalently bind to PP1 or PP2A (Craig *et al.*, 1996). Mutational analysis suggests that Nod also inhibits PP activity via interaction with amino acid Tyr-272 on PP1 because its IC_{50} changed from 0.5 to 150nM in the Y272S mutant (J. Zhang *et al.*, 1996). The 3D solution structure of Nod closely resembles that of Mic-LR (Annila *et al.*, 1996) (Fig. I.14).

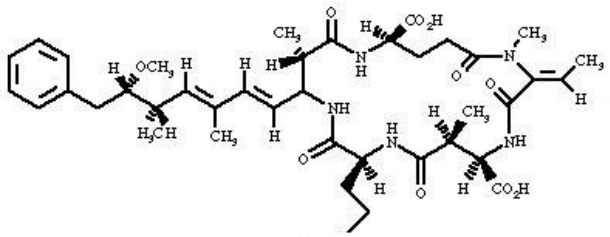


Figure I.14: *Structure of Nodularin.*

I.4.1.6 Tautomycin

Tautomycin (Tau) was isolated from *Streptomyces spiroverticillatus* as an antibiotic because it is toxic to yeast and fungi. Its structure as a polyketide resembles somewhat that of OA (MacKintosh and Klumpp, 1990; Hori *et al.*, 1991). It inhibits PP1 and PP2A with IC_{50} values of 0.2-0.7 and 0.4-0.65nM, respectively (MacKintosh and Klumpp, 1990; Fujiki and Suganuma, 1993). Others reported IC_{50} values of 0.4 and 34nM for PP1 and PP2A, respectively (Takai *et al.*, 1995). It does not inhibit PP2C and its IC_{50} for PP2B is 100μM. OA prevents the interaction of Tau with the catalytic subunit of PP2A (Fig. I.15).

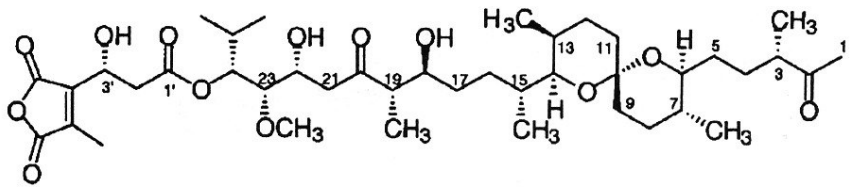


Figure I.15: *Structure of Tautomycin.*

I.4.1.7 Cyclosporin

Ciclosporin (CsA) or cyclosporin A (a cyclic undecapeptide), and FK506 (a macrocyclic lactone) inhibit PP2B (Parsons *et al.*, 1994). This inhibition is not direct as was already mentioned. First CsA and FK506 bind to cyclophilin and a FK506-binding protein (FKBP), respectively. Then, they interact with the latch region of PP2B B (Clipstone *et al.*, 1994; Milan *et al.*, 1994), leading to inhibition of PP2B A. The 3D structure of the PP2B-FK506 and FKBP complex supports this notion (Griffith *et al.*, 1995); (Kissinger *et al.*, 1995). Rapamycin, an immunosupressant fungal metabolite, also binds to FKBP but does not inhibit PP2B because it cannot interact with PP2B B for steric reasons (Griffith *et al.*, 1995). In contrast, it targets a rapamycin-associated protein (FRAP) that leads to inactivation of some specialized protein kinases like $p70^{s6k}$ (Price *et al.*, 1992; Alberts *et al.*, 1993).Work with CsA is complicated by its poor aqueous solubility. However, it is cell membrane permeant (Fig. I.16).

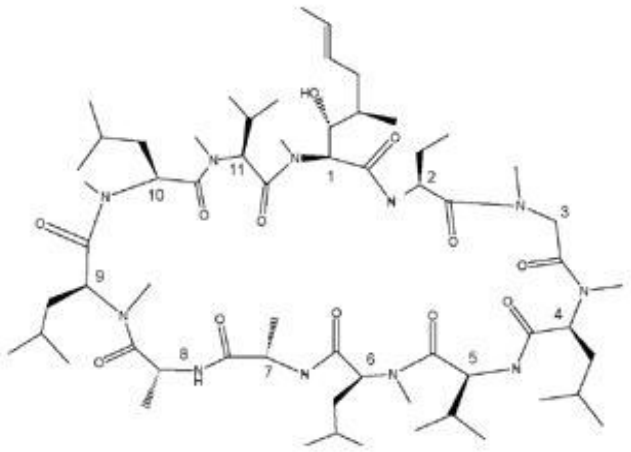


Figure I.16: *Structure of Cyclosporin.*

I.4.1.8 Cypermethrin

Type 2 pyrethroids like cypermethrin are used as insecticides because they modulate ion channel activity, but they also have been reported to inhibit PP2B at sub-nanomolar concentrations (Enan and Matsumura, 1992), independently of mediator proteins like cyclophilin. Subsequent reports that up to 1mM cypermethrin did not affect

PP2B, PP1 or PP2A (MacKintosh and MacKintosh, 1994), are thought to be due to artifactual reasons.

I.4.1.9 Apomorphine

Apomorphine and SKF-38393 inhibited PP2A from rat brain with IC_{50} values of 1 and 50µM, respectively. In contrast, apocodeine was inactive. It has apparently not been reported to inhibit other PPs (Kawai, 1991). Apomorphin is cell membrane permeant.

I.4.1.10 Fostriecin

The antitumor antibiotic fostriecin (FT) is a type II DNA topoisomerase-directed anticancer drug, like doxorubicin or etoposide (Boritzki *et al.*, 1988). FT is a naturally occurring compound from *Streptomyces pulveraceus* subspecies fostreus, an actinomycete found in a Brazilian soil sample. It is a water soluble polyene lacton with a phosphate ester. FT inhibited both PP1 and PP2A with IC_{50} values of 4µM and 40nM, respectively, but it did not inhibit tyrosine phosphatases (Roberge *et al.*, 1994). It has been suggested that the PP inhibition at least contributes to its efficacy against solid tumors in humans.

I.4.1.11 Heparin

Heparin binds and inhibits to PP1 but not PP2A (Gergely *et al.*, 1984; Erdodi *et al.*, 1985a, b). Heparin can actually stimulate PP2A. This property led to a procedure to separate these phosphatases using a heparin-based affinity column chromatography. Spermine inhibits both PP1 and PP2A with similar potency (Shenolikar and Nairn, 1991).

I.4.1.12 Thyrsiferyl 23-acetate

This compound is, like OA, a polyether fatty acid and contains a squalene carbon skeleton. It was isolated from the red alga *L. obtuse*. It is unique because it is a selective inhibitor of PP2A. It does not inhibit PP1, PP2B, PP2C or tyrosine phosphatase activity at concentrations up to 1mM. Its IC_{50} for PP2A is 4µM. It is several orders of magnitude less potent than OA or CA. It is expected to be cell membrane permeant (Matsuzawa *et al.*, 1994).

I.4.1.12 Oscillamide B and Oscillamide C

Two new phosphatase inhibitors, oscillamide B and C, were isolated from the cyanobacteria *Planktothrix* (*Oscillatoria*) *agardhii* and *P. rubescens*. These inhibitors are ureido-containing cyclic peptides and inhibit PP1 and PP2A (Sano *et al.*, 2001).

I.4.2 Protein inhibitors

I.4.2.1 Inhibitor 1 (I-1)

I-1 and I-2 were identified by Huang and Glinsmann (F. L. Huang and W. H. Glinsmann, 1976; Huang *et al.*, 1976). They share unusual physical properties: both are heat stable and are not precipitated by 1% trichloroacetic acid, in contrast to most other proteins. I-1 comprises 171 amino acids (Aitken *et al.*, 1982; Elbrecht *et al.*, 1990; Endo *et al.*, 1996). Only in the C-terminal were differences between rabbit, rat and human noted (Endo *et al.*, 1996). I-1 from rabbit skeletal muscle and human brain has a calculated molecular mass of 18.7 and 19.2kDa, respectively (Aitken *et al.*, 1982; Endo *et al.*, 1996), but the apparent molecular mass on SDS-PAGE is 26kDa. This discrepancy has been explained by a low degree of order in the protein. I-1 binds to and inhibits PP1 only after being phosphorylated on Thr-35 by cAMP-dependent protein kinase or cGMP-dependent protein kinase (Hemmings *et al.*, 1984b). It is highly selective for PP1, inhibiting PP1 and PP2A with IC_{50} values of 1.1 and 21,000nM, respectively (Endo *et al.*, 1996). I-1 is a cytosolic protein; it has been used as a tool to study whether a process involves PP1. Amino acids 9-12 KIQF are conserved in rat, rabbit and human and seem to be crucial for binding and inhibition of PP1 (Egloff *et al.*, 1997).

I.4.2.2 Inhibitor 2 (I-2)

I-2 from rabbit skeletal muscle comprises 204 amino acids and has a calculated mass of 22.9kDa (Holmes *et al.*, 1986a). Similarly to I-1, its apparent molecular mass on SDS-PAGE is much larger (31kDa). It binds to and inhibits PP1 regardless of phosphorylation. Mutational analysis suggests that I-2 inhibits via interaction with amino acid Tyr-272 on PP1 because its IC_{50} changed from 13 to 180ng/ml in the Y272K mutant

(L. Zhang *et al.*, 1996). I-2 inhibition of PP1 can be reversed by GSK3 phosphorylation of I-2.

I.4.2.3 DARPP-32

DARPP-32 (dopamine and cyclic AMP-regulated phosphoproteins, Mr 32,000Da) is similar to I-1 in function but derived from a different gene, and is mainly expressed in the brain (Hemmings *et al.*, 1984a). It is cytosolic and a predicted molecular mass of 22.6kDa, but again a higher apparent molecular mass of 32kDa on SDS-PAGE (Williams *et al.*, 1986). The same Thr residue on DARPP-32 is phosphorylated by cAMP-dependent protein kinase and also by cGMP-dependent protein kinase. Phosphorylation of DARPP-32 changes its IC_{50} for PP1 from 1μM to 2nM (Desdouits *et al.*, 1995a; Desdouits *et al.*, 1995b), underscoring its high selectivity. Under physiological conditions (mM Mn^{2+} in the assay) I-1 and DARPP-32 are dephosphorylated and inactivated by PP2A and even better by PP2B (Hemmings *et al.*, 1984a; Hemmings *et al.*, 1990; Desdouits *et al.*, 1995c). The dephosphorylation by PP2B is dependent on the presence of calcium. It was suggested that this might be a way for Ca^{2+} levels to control protein phosphorylation (Hubbard and Cohen, 1989). Thiophosphorylated I-1 or DARPP-32 is not dephosphorylated and has been successfully used to study the physiological role of PP1-mediated phosphorylation in muscle contraction.

I.4.2.4 Inhibitor 1 PP2A

Inhibitor 1 PP2A has been isolated from bovine kidney. It is thermostable and not inactivated by 1% trichloroacetic acid. Its apparent molecular mass is 30kDa (Li *et al.*, 1995). Identified as putative class II human histocompatibility leukocyte-associated protein (PHAP) I (Li *et al.*, 1996a), it seems to inhibit the catalytic subunit directly. It is not known whether its activity is regulated by a posttranslational modification like I-1 of PP1 (Li *et al.*, 1995).

I.4.2.5 Inhibitor 2 PP2A

This inhibitor has been isolated from bovine kidney. It is also thermostable and not inactivated by 1% trichloroacetic acid. Its apparent molecular mass was initially reported as 20kDa (Li *et al.*, 1995). Protein sequencing revealed that the protein had been described before as SET (Li *et al.*, 1996b), PHAP II (Vaesen *et al.*, 1994) and template activating factor-1β (Nagata *et al.*, 1995). These last two inhibitors might be useful tools to study the physiological function of PP2A. It has been speculated that I-1 and I-2 of PP2A might be involved in signal transduction, particularly to mediate the effects of insulin on PP2A (Li *et al.*, 1995).

I.4.2.6 Simian virus 40 small tumor antigen (SV40)

SV40 is a member of the papova family of small DNA tumor viruses. Its lytic cycle takes place in permissive monkey cells. SV40 infection leads to the production of proteins that are immunogenic and that were called tumor antigens. One such antigen is the SV40 small tumor antigen. It can inhibit PP2A activity with an IC_{50} of 10-15nM. SV40 inhibits PP2A trimeric complex (Yang *et al.*, 1991). In cells the interaction of small tumor antigen with PP2A leads to deinhibition and thus activation of MAP kinase and MEK which induces cell proliferation (Sontag *et al.*, 1993).

I.4.2.7 Inhibitor 4

Kikuchi and coworkers isolated a human cDNA for a novel PP1 inhibitory protein, named I-4, from a cDNA library of germ cell tumors (Shirato *et al.*, 2000). I-4, composed of 202 amino acids, is 44% identical to I-2. I-4 conserves functionally important structure of I-2 and exhibited similar biochemical properties. I-4 inhibited activity of the catalytic subunit of PP1 with IC_{50} of 0.2nM, more potently than I-2 with an IC_{50} of 2nM. Gel overlay experiments showed that I-4 binds PP1c directly through a multiple-point interaction.

I.5 DISTRIBUTION AND EXPRESSION OF PROTEIN PHOSPHATASES

I.5.1 PP1

By using specific antibodies raised against the different PP1 isoforms (α, β and γ_1) it was shown that all the three isoforms were expressed in a variety of mammalian cells tested although they localize within these cells in a distinct and characteristic manner. All the isoforms were present both in the cytoplasm and nucleus during interphase. Within the nucleus PP1α associates with the nuclear matrix, PP1γ_1 concentrates in nucleoli in association with RNA, and PP1β localizes to non-nucleolar chromatin. During mitosis PP1α is localized to the centrosomes, PP1γ_1 is associated with microtubules and PP1β associates with chromosomes (Andreassen *et al.*, 1998).

In the brain the mRNAs for PP1α, PP1β and PP1γ_1 were found to be particularly abundant in hippocampus and cerebellum (da Cruz e Silva *et al.*, 1995b). At the protein level PP1α and PP1γ_1 were found to be more highly expressed in brain than in peripheral tissues (Table I.1), with the highest levels being measured in the striatum, where they were shown to be relatively enriched in the medium-sized spiny neurons (da Cruz e Silva *et al.*, 1995b). At the electron microscopic level, PP1 immunoreactivity was demonstrated in dendritic spine heads and spine necks and possibly also in postsynaptic density (Ouimet *et al.*, 1995). PP1 immunoreactivity has also been reported in human hippocampal neuronal cytoplasm (Pei *et al.*, 1994). In addition, most neuronal nuclei were not immmunoreactive for PP1γ_1 but were usually strongly immunoreactive for PP1α (Ouimet *et al.*, 1995).

I.5.2 PP2A

Both PP2A catalytic subunit isoforms α and β are ubiquitously expressed, and very high levels are found in brain and heart. However, PP2A α is about 10 times more abundant than PP2A β (Khew-Goodall and Hemmings, 1988). PP2A is mainly cytoplasmic (Waelkens *et al.*, 1987), although it has also been found in the nucleus (Turowski *et al.*, 1995). Of all tissues tested, its activity was highest in brain extracts

(Ingebritsen and Cohen, 1983). The catalytic subunit was found to have a wide regional distribution in brain, with the highest immunoreactivity being present in neurons and particularly enriched in the cytosolic and synaptosolic subcellular fractions (Saitoh *et al.*, 1989).

I.5.3 PP2B

PP2B is present in nearly all mammalian cells studied but it is most abundant in the brain (Wallace *et al.*, 1980). In brain, 50-70% of PP2B is bound to membrane or cytoskeleton elements, and is found in most cells, although its concentration varies from one area to another (Yakel, 1997). PP2B $A\alpha_1$ and PP2B $A\beta_2$ isoforms are highly expressed in neuronal tissues whereas PP2B $A\gamma$ is testis specific (Ueki *et al.*, 1992). PP2B $B\alpha$ gives rise to an isoform expressed in many tissues named PP2B $B\alpha_1$ (170 amino acids) and by means of a different promoter leads to another testis-specific isoform PP2B $B\alpha_2$ (216 amino acids). Similarly, PP2B $B\beta$ (179 amino acids) is only expressed in the testis (Ueki *et al.*, 1992; Chang *et al.*, 1994).

The PP2B $A\alpha$ isoform is the most abundantly expressed in neurons. PP2B activity has been detected in both the soluble and particulate fractions of brain homogenates (Tallant *et al.*, 1983). A dual cytoplasmic and membrane distribution was revealed by a series of immunohistochemical studies. PP2B has been detected in association with postsynaptic densities (Carlin *et al.*, 1981; Grab *et al.*, 1981), enriched at postsynaptic loci, plasma membranes and dendritic microtubules, with a more diffuse cytoplasmic distribution (Wood *et al.*, 1980), and has also been reported in axons (Kincaid and Coulson, 1985). PP2B is either absent or present at very low levels in glia (Goto *et al.*, 1986). Loss of PP2B immunoreactivity has been reported in the brains of both Huntington's disease (Goto *et al.*, 1989b) and Parkinson's disease (Goto *et al.*, 1989a) patients.

Developmentally, PP2B is undetectable in rat brain at a very young age, then PP2B expression increases to a peak at around 5 weeks of age, after which it remains at a high level throughout adulthood (Takahashi *et al.*, 2000). PP2B levels in rat brain increase in parallel with synaptogenesis (Tallant and Cheung, 1984).

Table I.1: *Tissue distribution of the main protein phosphatases catalytic subunits of the PPP family.*

	PP1α	PP1γ1	PP1γ2	PP1β	PP2Aα,β	PP2BAα	PP2BAβ	PP2BAγ
Brain	++	+++	-	+++	+	+++	+++	-
Heart	+	+		+	+	+	+	
Liver	+	+		-	+	+	+	
Intestine	+	+	-	+++	+			
Kidney	+	+	-	+	+	+	+	
Spleen	+	++	-	+	+	+		
Adrenal gland	+	++	-	+				
Lung	++	++		+++	+	+	++	
Skeletal muscle	+	+		-	+	++	++	-
Testis	++	+	+++	++		-	-	+++

-, below detection limit.

I.6 PP1 STRUCTURE AND FUNCTION

Protein phosphatase 1 (also known as phosphorylase phosphatase) has been studied since the 1940s as the enzyme responsible for the conversion of phosphorylase a to phosphorylase b (Cori and Green, 1943). The discovery that PP1 was actually a phosphatase came at the same time as the discovery of phosphorylase kinase (Keller and Cori, 1955; Sutherland and Wosilait, 1955). These findings marked the beginning of an era of the study of protein phosphorylation/dephosphorylation as a regulatory mechanism. Investigation of PP1 in the following three decades focused on defining its enzymology and role in glycogen metabolism [a review can be found in (Shenolikar and Nairn, 1991; Bollen and Stalmans, 1992; Brautigan, 1994; Shenolikar, 1994; E. Y. C. Lee, 1995)] and the progress in the isolation and characterization of PP1 activity was very slow. The study of the enzymology of this enzyme is still incomplete and PP1 continues to bring surprises as well as new questions about its cellular functions.

I.6.1 Historical background

The key steps that led to the beginning of the understanding of the enzymology of PP1 came with the isolation of the catalytic subunits (PP1c) from liver and muscle (Brandt *et al.*, 1975a; Gratecos *et al.*, 1977). Underlying this success was the discovery that treatment of tissue extracts with denaturants (alcohol or urea) led to the release of a 35kDa catalytic subunit which could be readily isolated (Brandt *et al.*, 1974). This 35kDa subunit was derived from a 37kDa form by proteolytic cleavage near the C-terminus (Cohen, 1989; E. Y. C. Lee, 1995). The denaturant treatment released the catalytic subunit from its regulatory subunits, so that the enzyme activity behaved in a monodisperse manner and was amenable to isolation by conventional chromatographic methods. Following this treatment (ammonium sulfate precipitation followed by 95% ethanol at room temperature) the PP1 activity could be extracted from the pellet of denatured proteins. At the time it was clear that while phosphorylase a was the best PP1c substrate, both glycogen synthase and phosphorylase kinase were also dephosphorylated. Later studies (Cohen, 1989) showed that this enzyme was relatively non-specific when compared, for example, to the protein kinases. PP1c isolation from rabbit skeletal muscle led to the isolation of another catalytic subunit of 35kDa, which differed in its ability to act on p-nitrophenyl phosphate (Silberman *et al.*, 1984). This activity turned out to be PP2A catalytic subunit (Cohen, 1989). Ethanol or trypsin treatment of tissue extracts led to large increases in PP1 activity, concomitant with the reduction in apparent size to the free catalytic subunit (Brandt *et al.*, 1974). These findings led to the discovery of the heat-stable PP1 inhibitor proteins (Brandt *et al.*, 1975b).

The existence of these inhibitors led to two important considerations about the enzymology of PP1: the role of the inhibitor proteins in regulating phosphatase activity and the existence of holoenzyme forms. Subsequent studies (F. L. Huang and W. H. Glinsmann, 1976) identified two such inhibitors: inhibitor-1 (I-1) and inhibitor-2 (I-2) [a review can be found in (Cohen, 1989; Shenolikar and Nairn, 1991; Bollen and Stalmans, 1992)]. I-1 and I-2 are small proteins (165 and 204 amino acids, respectively) with estimated molecular mass of 23kDa and 19kDa. I-1 is only inhibitory when phosphorylated at a threonine residue by a cAMP-dependent kinase (Cohen, 1989). The concept of a holoenzyme complex of the catalytic subunit with regulatory subunits was supported by

the findings that PP1 activity was generally associated with high molecular weight complexes recovered by gel filtration, but that could be reduced to a low molecular weight form by proteolysis, urea or ethanol treatment (Killilea *et al.*, 1979; Mellgren *et al.*, 1979). A high molecular weight form of PP was first isolated as a myosin light chain PP (Pato *et al.*, 1993). However, studies of an inactive form of PP1 that could be activated by ATP and that had been studied by Merlevede and co-workers for many years, led to its isolation and the surprising finding that it consisted of a 1:1 complex of the 37kDa PP1c and I-2 (Yang *et al.*, 1980; Merlevede *et al.*, 1984). In muscle, early studies had shown the existence of a "glycogen particle" in which glycogen sedimented from muscle extracts was shown to be associated with phosphorylase phosphatase, PP1 and phosphorylase kinase (Haschke *et al.*, 1970; Meyer *et al.*, 1970). The purification of the glycogen-bound form of muscle PP1 revealed it to be a heterodimer of the catalytic subunit and a glycogen binding subunit, GM (Stralfors *et al.*, 1985). GM bound both glycogen and PP1, and also modified its substrate specificity in that its activity toward glycogen synthase was enhanced. These studies led Cohen (Cohen, 1989; Hubbard and Cohen, 1993) to propose that the catalytic subunit could associate with different targeting subunits, which serve to direct the enzyme to specific subcellular locations in addition to serving regulatory functions. This finding was followed by isolation of the myosin bound PP1 which contained two other subunits, one of which, M110, binds to myosin (Chen *et al.*, 1994). The targeting hypothesis is substantiated by the recent discovery of a large number of PP1 binding proteins, and the current view that PP1c exists in many heterodimeric forms provides an understanding of some of the original difficulties in the isolation of PP1c.

I.6.2 Recombinant PP1c

I.6.2.1 Molecular cloning and expression of the PP1c

Attempts to express PP1 as a recombinant protein were initially unsuccessful as it was readily overexpressed in the pET3a vector, but as an inactive, insoluble protein in inclusion bodies (Browner *et al.*, 1991). Attempts to renature the solubilized PP1 protein were only partially successful and required the presence of Mn^{2+}, dithiothreitol, high salt and high dilution. The renatured enzyme exhibited only 5% of the expected specific

activity. So another vector system was tried: the pTACTAC vector, which had been used for the expression of rabbit muscle phosphorylase in a soluble form (Browner *et al.*, 1991). This vector allowed the expression of PP1 in an active soluble form (Zhang *et al.*, 1992). Expression was dependent on induction at a lowered temperature of 26-28ºC, rather than at 37ºC and was also dependent on the presence of Mn^{2+} in the culture media, as had been used for the expression of phosphorylase (Browner *et al.*, 1991).

I.6.2.2 Enzymatic properties of recombinant PP1

The striking feature of the recombinant protein which marks it as distinct from the catalytic subunit isolated from muscle and liver is that its activity is dependent on the presence of added Mn^{2+} (Zhang *et al.*, 1992). Muscle PP1 is normally isolated in the presence of buffers containing EDTA, and such preparations are also active in the presence of EDTA (Silberman *et al.*, 1984; Bollen and Stalmans, 1992). The exceptions are the catalytic subunits released from the PP1-inhibitor-2 complex (Bollen and Stalmans, 1992) or from higher molecular weight forms of PP1 by trypsin (Brautigan *et al.*, 1982). In addition, a form of cardiac myofibrillar PP1 that requires activation by Co^{2+} has been reported (Chu *et al.*, 1994). It has been suggested by Lee and coworkers (Zhang *et al.*, 1993) that the recombinant enzyme may be related to the form present in the PP1-inhibitor-2 complex. The existence of the metal-dependent and –independent activity forms of PP1 suggests that there exist two stable conformations of PP1 (discuss below in terms of the metalloprotein nature of PP1). The specific activities and general enzymatic properties of recombinant PP1 toward phosphorylase a are similar to those of the muscle enzyme with some exceptions: it is much less sensitive to inhibitor-1 (Alessi *et al.*, 1993; Zhang *et al.*, 1993) and dephosphorylates phosphotyrosine containing substrates (MacKintosh *et al.*, 1996). Recombinant PP1 actively dephosphorylates p-nitrophenol phosphate (Zhang *et al.*, 1992), a property that makes it useful for the assay of toxins (by inhibition of PP1 activity) such as microcystin by simple colorimetric methods (Ward *et al.*, 1997).

I.6.3 PP1c crystal structure and catalytic mechanism

During the past decade there has been major progress in the elucidation of the atomic structures of the Ser/Thr-PPs in general. Crystal structures for PP1-microcystin (Goldberg *et al.*, 1995), PP1-tungstate (Egloff *et al.*, 1995), PP1-GM peptide complexes (Egloff *et al.*, 1997) and more recently PP1-okadaic acid (Maynes *et al.*, 2001) have been determined. Two structures for PP2B have been solved, the auto-inhibited enzyme (Fig. I.7) and a ternary complex of a truncated PP2B with FKBP12/ FK506 (Griffith *et al.*, 1995; Kissinger *et al.*, 1995). These structures show that the molecular architecture of the catalytic cores [a review can be found in (Barford, 1996)] of PP1 and PP2B are conserved, and that both contain a bimetal center at the active site which is structurally similar (Strater *et al.*, 1995) to that present in the purple acid phosphatase. PP1, like PP2B, is a metalloprotein that possess a bimetal center that is bridged by a water molecule at the active site. The use of proton induced X-ray emission spectroscopy revealed the presence of Mn and Fe in the ratio 1 to 0.5 in PP1 (Egloff *et al.*, 1995). The nature of the metal ions in the wild type muscle PP1 is unknown, although it can be speculated that this may be a Fe/Zn pair as in PP2B (King and Huang, 1984; Kissinger *et al.*, 1995). The current views of the catalytic mechanism (reviewed in Barford, 1996) for PP1 are that the metals serve as ligands for the phosphate oxygens and for the generation of a hydroxide ion which serves as the nucleophile that is involved in the catalysis, while H125 serves as a proton donor for the leaving alcohol group (Fig I.17). Other residues in the active site which serve to stabilize the proposed pentacoordinate state of the phosphate intermediate are R96, N124 and R221. Mutation of the metal ligands (J. Zhang *et al.*, 1996) H66, D64, D92 and H248 led to severe loss of catalytic function. Mutation of H125 and of H173 did not result in readily expressed proteins, although small amounts of the H125S and H125A mutants could be isolated. These mutants were inactive. Mutation of D95, which is proposed to stabilize the protonation of H125 by a salt bridge in PP1, resulted in significant reduction in catalytic activity. Mutations of R96 and N124 have also supported their proposed roles in phosphate binding.

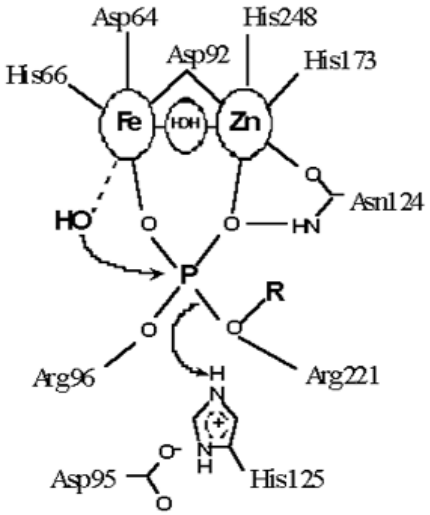


Figure I.17: *Active site of PP1. The active site of PP1 contains a bimetal center (shown as Fe and Zn) bridged by a water molecule which is displaced on binding of phosphate (Egloff et al., 1995). Ligands for the metal ions are shown in heavy lines. The hydrogen bonds of the phosphate oxygens to N124, R96 and R221 are shown in thin lines. The catalytic mechanism, based on studies of PP1 (Egloff et al., 1995), purple acid phosphatase (Strater et al., 1995) and PP2B (Griffith et al., 1995) is indicated in the diagram. The proposed reaction mechanism involves nucleophilic attack by a hydroxide ion, with the H125 providing the proton for the leaving alcohol group, and is supported by mutagenesis of the active site residues (J. Zhang et al., 1996).*

I.6.3.1 Binding region for toxins

Several mutants of PP1 which exhibit a general loss of sensitivity towards several natural toxins show that these toxins have a common binding region on PP1. It was shown that a mutated PP2A (Cys269 to Gly) had reduced sensitivity to OA (Shima *et al.*, 1994). By comparing the sequences of PP1 and PP2A in this region (Fig. I.18) it was noted that the region was well conserved except for a four residue difference, YRCG in PP2A (267-270) and GEFD (274-277) in PP1.

```
                                                  274     277
PP1   254   GYEFFAKRQ------LVTLFSAPNYCGEFDNAGAMMSVDE
PP2A  247   GYNWCHDRN------VVTIFSAPNYCYRCGNQAAIMELDD
PP2B  287   GYRMYRKSQTTGFPSLITIFSAPNYLDVYNNKAAVLKYEN
                                                  313     316
```

Figure I.18: *Alignment of C-terminal regions of PP1, PP2A and PP2B. The boxed region shows the loop regions that connect beta sheets 12 and 13 in the structures of PP1 and PP2B.*

The chimeric PP1 mutant in which GEFD was changed to YRCG resulted in increased sensitivity to okadaic acid (Zhang *et al.*, 1994a), consistent with the fact that PP2A is more sensitive to okadaic acid than PP1. The same occurred when F276 was mutated to Cys (J. Zhang *et al.*, 1996). It was also shown that Y272 is important for the binding of all of the inhibitors tested, as its conservative mutation to phenylalanine caused decreases in PP1 toxin sensitivity. These mutagenesis studies indicate that binding of the toxins must all involve some common contacts on PP1, and that Y272 is particularly important in this context. Y272 is located close to the active site with its hydroxyl within several angstroms from the Fe ion (Egloff *et al.*, 1995); the mutation of Y272 without deleterious effects on its catalytic activity suggests that it is not involved in the catalysis. This region of PP1 represents the loop region connecting beta strands 12 and 13 in PP1 structure (Fig I.19).

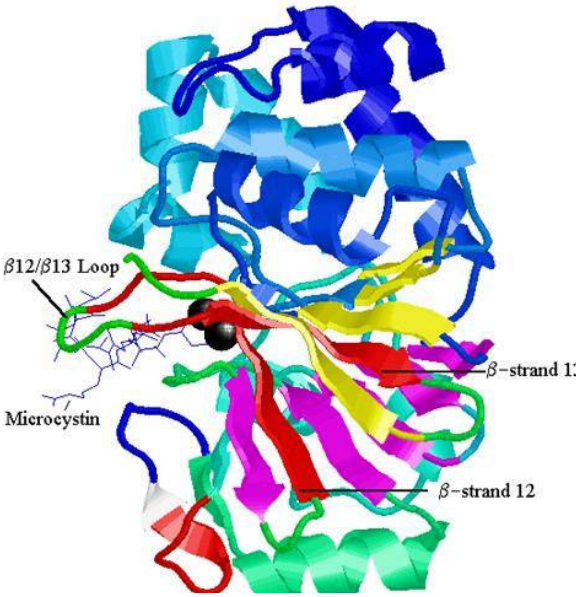


Figure I.19: *The β12/β13 loop of PP1. The diagram shows a ribbon model of the PP1 structure (Goldberg et al., 1995). The two beta sheets that are the scaffold for the active site are shown in yellow (beta sheet 1) and magenta (beta sheet 2). Beta strands 12 and 13 are shown in red. Microcystin is shown in wireframe and the two metal ions as black spheres.*

If the toxins bind to the same site on PP1 this must reflect the possibility that these diverse molecules must present topographically similar surfaces at the points of interaction with PP1. This idea has been supported by molecular modeling studies (Bagu *et al.*, 1997; Gauss *et al.*, 1997; Lindvall *et al.*, 1997).

The structure of PP1 bound to okadaic acid (Maynes *et al.*, 2001) is remarkably similar to the two structures of PP1 and PP2B determined previously (Fig I.20). Even with only the phosphate-mimic tungstate present the architecture of the active site of the

tungstate-bound PP1 structure is virtually identical to OA-bound PP1 complex. In contrast, the microcystin-bound structure reveals large changes in the conformation of the active site. These changes are mainly restricted to the β12/β13 loop. The loop in the microcystin-bound PP1 structure folds back on itself causing significant shifting of residues 273-278. One critical difference between microcystin and OA is the presence of a dehydroalanine residue in microcystin that covalently alkylates the Sγ of Cys273 in a time dependent reaction (Dawson and Holmes, 1999). This covalent linkage is not the primary cause of inhibition of PP1 by microcystin (Goldberg *et al.*, 1995). Given the strong similarity of the PP1-interacting domains of OA and microcystin it is likely that the primary mode of inhibition of PP1 by microcystin is similar to that of OA and that the movement of the β12/β13 loop in the microcystin complex is a secondary event accompanying the covalent binding reaction. An important interaction between PP1 and microcystin that is not present in the PP1-OA complex is the hydrogen bond that occurs between Arg96 (PP1) and the acid of the methyl-aspartate residue (microcystin). This interaction may account for the 100-fold greater inhibition of PP1 by microcystin over OA (Holmes and Boland, 1993). The structure of PP1-OA is very similar to the structure of PP2B despite the fact that OA does not strongly inhibit PP2B.

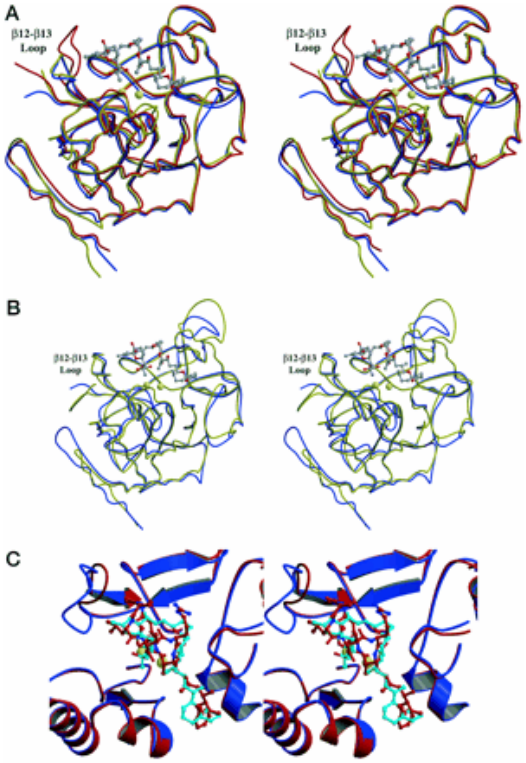


Figure I.20: *A, stereo representation of the backbone carbon alignment of tungstate-bound PP1 (gold), PP1-OA complex (blue) and PP1-microcystin LR complex (red). OA is shown in ball and stick representation with carbon atoms in gray and oxygen atoms in red. The metals are from the PP1-OA structure and are shown as spheres. B, stereo representation of the backbone carbon alignment of PP1-OA (blue) and PP2B (gold). C, stereo representation of the backbone carbon alignment between the PP1-OA complex (blue) and the PP1-microcystin LR complex (red). OA is shown as ball and stick representation and is colored light blue, microcystin LR is shown as ball and stick representation and is colored red (adapted from (Maynes et al., 2001).*

I.6.3.2 Substrate binding

The active site of PP1 lies at the confluence of three shallow grooves, a C-terminal groove, an acidic groove and a hydrophobic groove, which are potential binding sites for substrates and inhibitors (Egloff *et al.*, 1995; Goldberg *et al.*, 1995). Microcystin binds in a manner such that it occupies the active site, while its extended ADDA side chain occupies the hydrophobic groove. The hydrophobic groove forms the obvious binding site for peptide substrates. The two PP1 inhibitors, I-1 and DARPP-32 both carry four basic residues N-terminal to the phosphothreonine residue and its binding to PP1 has been hypothesized to be that of a pseudosubstrate (Goldberg *et al.*, 1995). Binding of peptide/polypeptide substrates to PP1 can be considered to be composed of three elements: interaction of the basic residues N-terminal to the phosphoserine with the acidic residues in the acidic groove, binding of the phosphoserine to the active site and an interaction of the region C-terminal of the phosphoserine (or phosphothreonine) to the hydrophobic groove. In the active site region the structure of the PP1-tungstate complex has shown that R96, N124 and R221 are involved in the binding of the phosphate oxygens (Egloff *et al.*, 1995).

R221 and R96 are well positioned to form salt bridges with two of the phosphate oxygens while the amino group of N124 can be hydrogen bound to the third oxygen. D208 was hypothesized to be important for the orientation of R221 via a salt bridge interaction. It was also pointed out (Egloff *et al.*, 1995) that W206 and Y134 are well positioned to interact with the Ser or Thr carrying the phosphate residue. These interactions and the location of the acidic and hydrophobic grooves are shown in Figure I.21.

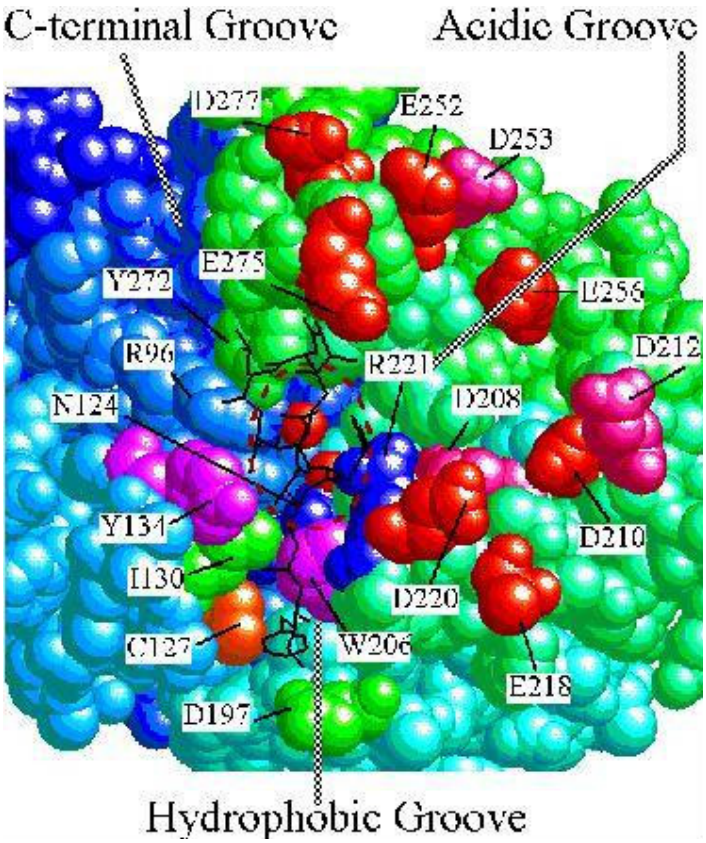


Figure I.21: *The C-terminal, acidic and hydrophobic grooves of PP1. The diagram shows a space filled model of PP1 with a view of the face of the molecule containing the active site. The dashed red oval shows the location of the active site, with the two metal ions as red spheres. Microcystin is shown in wireframe. The location of the hydrophobic groove is delineated by the binding of the ADDA group of microcystin. The hydrophobic, acidic and C-terminal grooves radiate from the active site. Residues which are labeled are those which were analyzed by mutation (Zhang and Lee, 1997).*

I.7 PP1 TARGETING/BINDING PROTEINS

During the last decade evidence has accumulated, that the substrate specificity of PP1 is achieved by the interaction with other proteins that can act as targeting subunits or activity modulators. Targeting, as the requirement for the molecular juxtaposition of proteins for the generation of signaling events, is well established as a paradigm in a number of growth regulated signaling systems involving tyrosine phosphorylation (Lemmon and Schlessinger, 1994; Pawson, 1994; Kuriyan and Cowburn, 1997), as well as in the anchoring of Ser/Thr-protein kinases by A-kinase anchoring proteins (AKAPs), one of which also binds PP2B (Lemmon and Schlessinger, 1994; Rubin, 1994). The concept of targeting as it relates to PP1, however, has a major twist in terms of the large number of PP1 binding proteins that have been reported during the past years, as it expands the number of PP1 heterodimers that may exist and consequently the repertoire of cellular functions that involve PP1.

Genetic studies of yeast mutations that affect glycogen metabolism and cell cycle regulation, and the use of the yeast two hybrid system have revealed over a dozen genes that encode putative PP1-binding proteins (reviewed in (Stark, 1996). These include GAC1, REG1, REG2, SCD5, GIP1, SHP1, GIP2 and SDS22 in *S. cerevisiae*. These genes are required for the control of glycogen metabolism, protein synthesis, glucose repression, meiosis, sporulation and mitotic cell cycle regulation. Rigorous biochemical demonstration that these PP1-binding proteins actually interact with PP1 or the nature of the targeting function or the substrates have not been shown in all cases. A key element of the targeting hypothesis is that the cellular activity of PP1 is only expressed when it is targeted. This would explain why the PP1 catalytic subunit exhibits a relatively nonspecific phosphatase activity. The strongest experimental support for a targeting function of a PP1 regulator (R) has come from genetic and biochemical studies of yeast glycogen metabolism. The glycogen-deficient yeast mutant *glc7-1* was found to express a PP1 with a R73C point mutation (Peng *et al.*, 1990). This did not affect PP1 activity but resulted in loss of its ability to bind to the yeast homolog (Gac1p) of the mammalian glycogen binding protein (Stuart *et al.*, 1994). The activation of glycogen synthase requires its dephosphorylation by PP1 and it has been shown that glycogen synthase in this mutant is largely in the inactive phospho-form. Overexpression of Gap1p, on the other hand, led to increased glycogen

accumulation. These findings demonstrated that the physiological functioning of PP1 in glycogen metabolism was dependent on it being targeted to the appropriate micro-environment and that other cellular functions were not affected when targeting to glycogen was disrupted.

Several mammalian PP1-binding proteins have also been isolated either by biochemical methods or by yeast two hybrid analyses (Table I.2). Based on their effect on PP1c the best characterized R subunits can be divided into three groups. The first group is represented by activity-modulating proteins, including true inhibitors such as I-1 (Connor *et al.*, 1999) and CPI-17 (Koyama *et al.*, 2000) that in their phosphorylated form block the activity of PP1c towards all substrates. Other members of this group act instead as substrate-specifiers of PP1c. For example, I-1 PP2A/PHAP-I and I-2 PP2A/PHAP-II, which are potent inhibitors of PP2A, promote the dephosphorylation of specific substrates by PP1c (Katayose *et al.*, 2000). A second group of R subunits contains the targeting proteins, which bind both PP1c and one of its substrates. For example, MYPT1 binds PP1c as well as specific substrates such as myosin (Fukata *et al.*, 1998; Hartshorne and Hirano, 1999; Toth *et al.*, 2000a). Other targeting subunits do not bind the substrate directly but instead associate with a subcellular structure that contains the substrate. For example, the G subunits target PP1 to glycogen particles which also bind the substrate glycogen synthase (Liu and Brautigan, 2000). The targeting proteins of PP1 also include scaffolding proteins that mediate the formation of protein complexes. Often, these complexes function as signaling modules that contain both protein kinases and phosphatases and are localized in close proximity to the substrates of these enzymes. The third group of proteins that directly and tightly associates with PP1c defines a subset of its substrates. Some of these substrates also function as targeting proteins. Thus, the centrosomal protein kinase Nek2 not only binds its substrate C-Nap1 but also PP1c and both Nek2 and C-Nap1 are proposed substrates of the associated PP1c (Helps *et al.*, 2000). Some PP1c-bound substrates also function as activity modulators. For example, the retinoblastoma protein interacts with PP1c both as a substrate and as a noncompetitive inhibitor (Tamrakar and Ludlow, 2000).

Table I.2: *Classification of the R subunits of PP1*

Activity modulators	I-1; DARPP-32
	CPI-17; PHI-1; KEPI
	G-substrate;
	I-2; I-4
	I-1 PP2A/PHAP-I; I-2 PP2A/SET/PHAP-II/TAF-1β
	GADD34
Targeting proteins	AKAPs (AKAP149, AKAP220; Yotiao)
	G subunits (GM/RGL; GL; R5/PTG/U5; R6)
	M subunits (M110/M130/MYPT1; MYPT2/PP-1bp55)
	Neurofilament-L
	Neurabin-I; spinophilin/neurabin-II
	NIPP1
	sds22
	Tau
Substrates	Bad
	Nek2
	Phosphofructokinase
	Retinoblastoma protein
	Ryanodine receptor
Unclassified	BH-protocadherin-c
	HCF (host cell factor)
	HOX11
	Inhibitor-3/HCGV
	p53BP2
	PP-1bp80
	PSF
	R111/p99/PNUTS
	ribosomal proteins (L5, RIPP1)
	GRP-78
	NCLK
	Myr8
	FAK
	Bcl-2
	Herpes virus γ1 34.5 protein

AKAPs, A-kinase anchoring proteins; G subunits, glycogen-targeting subunits; M subunits, myosin-targeting subunits; GRP, glucose related protein; NCLK, nuclear cdc-2 like kinase; FAK, focal adhesion kinase. The names of structurally related proteins are on the same line and are separated by a semicolon. Synonyms are separated by a slash.

I.7.1 "RVxF motif"

Members of all three groups of R subunits have been shown to bind to PP1c via a short sequence that is now referred to as the "RVxF motif" (Barford *et al.*, 1988; Egloff *et al.*, 1997; Zhao and Lee, 1997a). Although the sequences that correspond to the RVxF motif are degenerate (the consensus sequence is [K/R]-X_{0-1}-[V/I/L]-{P}-[F/W] (Wakula *et al.*, 2003), the evidence for their role in the binding of PP1c is convincing. First, the co-crystallization of PP1c with a synthetic fragment of the muscle-type G subunit revealed that the RVxF sequence binds tightly in an extended conformation to a hydrophobic channel of PP1c that is remote from the catalytic site (Egloff *et al.*, 1997) (Fig. I.22). Modelling suggested that the variant RVxF motif of I-2 (KLHY) can be accommodated in a similar way in this channel (Yang *et al.*, 2000). Second, it has been demonstrated for various R subunits that mutations of the hydrophobic (V/I/L) and/or aromatic (F/W/Y) residue in this motif is sufficient to disrupt or weaken their interaction with PP1c (Johnson *et al.*, 1996; Egloff *et al.*, 1997; Kwon *et al.*, 1997b; Beullens *et al.*, 1999; Yang *et al.*, 2000).

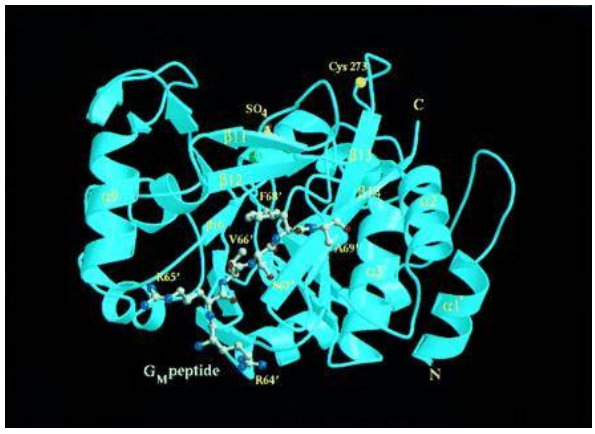


Figure I.22: *Structure of the PP1-GM peptide complex. A ribbon diagram of PP1c indicates the position of the peptide-binding channel at the interface of two β-sheets, the GM peptide atoms are represented as ball and stick and the position of one of the metal ions at the catalytic site is indicated as a green sphere. The Cα of Cys273, whose side chain forms a covalent bond with the MDHA side chain of microcystin is shown as a yellow sphere.*

The residues of PP1c that are necessary for binding to the RVxF motif (in particular residues 287-293) are invariant in all isoforms from all species (Barford *et al.*, 1988; Egloff *et al.*, 1997). However, they are not conserved in the catalytic subunits of PP2A or PP2B, which explains why most regulators of PP1 do not interact with these structurally related phosphatases. Conversely, the R subunits of PP1 (HOX11, I-1 PP2A/PHAP-I and I-2 PP2A/PHAP-II) that are also able to interact with the catalytic subunit of PP2A do not contain an RVxF sequence (Kawabe *et al.*, 1997; Katayose *et al.*, 2000).

The binding of the RVxF sequence does not cause important conformational changes in the catalytic subunit (Egloff *et al.*, 1997) and does not have major effects on the activity of the phosphatase (Endo *et al.*, 1996; Kwon *et al.*, 1997a; Beullens *et al.*, 1999). Studies on MYPT1 and I-2 have indicated that the RVxF motif can function as an anchor for PP1c and enables these R subunits to make additional contacts with the phosphatase in an ordered and cooperative manner (Toth *et al.*, 2000b; Yang *et al.*, 2000). For example, four phosphatase-interaction sites, in addition to the RVxF motif, have been identified for MYPT1 and I-2 (Hartshorne and Hirano, 1999; Toth *et al.*, 2000b; Yang *et al.*, 2000). Another recurring theme is that the R subunits have common or overlapping binding sites on PP1c in addition to the RVxF-binding channel. For example, the inhibition of PP1c by phosphorylated I-1 (Endo *et al.*, 1996), DARPP-32 (Kwon *et al.*, 1997a) and MYPT1 (Hartshorne and Hirano, 1999) have all been attributed to the binding of the phosphorylated residue at or near the catalytic site as a pseudo-substrate. The sharing of interaction sites is also in accordance with findings that various point mutants of PP1c show altered affinity for multiple R subunits (Baker *et al.*, 1997; Ramaswamy *et al.*, 1998). As expected from the unusually high conservation of residues on the surface of PP1c, mutagenesis studies in yeast have identified many surface residues as being essential for the binding of R subunits (Baker *et al.*, 1997; Ramaswamy *et al.*, 1998). A site that lies adjacent to the RVxF–binding groove has been identified as a binding pocket for the N-terminal "IKGI" motif of inhibitor-2 (Connor *et al.*, 2000).

The picture that emerges shows that the binding of the R subunits to PP1c is mediated by multiple, degenerate, short sequence motifs and that the R subunits can share interaction sites. It should be pointed out that this combinatorial control (Bollen, 2001) of PP1c does not rule out the possibility that some R subunits might have unique binding sites. The combinatorial control of PP1 allows the exquisite physiological regulation of

PP1 holoenzymes by hormones, growth factors and metabolites at the molecular level. Work on the various holoenzymes has demonstrated that their physiological regulation involves modulation of subunit interactios, which is mediated by the reversible phosphorylation or allosteric regulation of the R subunits. For three unrelated R subunits it has been shown that the phosphorylation of Ser residue(s) within or close to the RVxF motif disrupts the binding of this motif to PP1c (Beullens *et al.*, 1999; McAvoy *et al.*, 1999; Liu *et al.*, 2000). This results in altered activity of the holoenzyme or the release of the catalytic subunit. In contrast phosphorylation of other subunits strengthens their interaction with PP1c. Examples include I-1 and DARPP-32, in which an additional binding site for PP1c is created by phosphorylation. A different type of regulation involves the binding of allosteric regulators. The allosteric binding of phosphorylase a to the C-terminal tail of the liver-type G subunit (GL) abolishes the activity of the associated PP1c towards glycogen synthase (Armstrong *et al.*, 1998). An additional level of regulation of the PP1 holoenzymes is provided by targeting of these enzymes to specific substrates or subcellular structures. Bollen and coworkers, by a combination of bioinformatics tools and mutagenesis studies have delineated the consensus sequence and function of three PP1 binding motifs as being [K/R]-X_{0-1}-[V/I/L]-(P)-[F/W], were X denotes any residue and P any residue except proline (Wakula *et al.*, 2003). This sequence is very similar to a consensus sequence proposed by Zhao and Lee (Zhao and Lee, 1997a), [R/K]-[K/R]-X_{0-2}-V-[R/H]-[F/W]-X-[DE], by panning of a random peptide display library. The main differences are the presence of an N-terminal basic residue and a C-terminal acidic residue in the second.

The RVxF-consensus sequence is present in about one third of all eukaryotic proteins but only a small fraction are PP1-binding proteins. It seems that RVxF-consensus sequences function as PP1 interaction sites only when they are present in a flexible and exposed loop that can be modeled into a β-strand. Additionally, other low affinity regions on the PP1 regulators strengthen the binding. Thus, the RVxF-consensus sequence functions like an anchor and other low affinity interactions have to occur that have regulator-specific effects on PP1 activity and specificity (Wakula *et al.*, 2003).

Recently, another PP1 binding motif has been described, F-X-X-R-X-R, that also appears to exist in several PP1 interactors (Ayllon *et al.*, 2002). A combined bioinformatics and mutagenesis approach could also be used to study this new consensus motif.

The existence of common binding sites for the R subunits explains why a relatively small protein such as PP1c can interact with numerous different R subunits and why the binding of most R subunits is mutually exclusive.

I.8 PP1 IN TESTES AND SPERM

Post-translational modifications of proteins are essential to the viability of all eukaryotic cells, and reversible protein phosphorylation is recognized as the major regulatory mechanism. In recent years the serine/threonine-specific protein phosphatases have received increasing attention, and the nature of their physiological substrates and regulation is the subject of intensive study. As previously mentioned, of the three known PP1 genes (alpha, beta and gamma), PP1γ undergoes tissue-specific splicing to yield a poorly characterized testis-specific isoform, PP1γ2 (Kitagawa *et al.*, 1990; Sasaki *et al.*, 1990; da Cruz e Silva *et al.*, 1995b).

Since sperm are terminally differentiated cells essentially devoid of transcriptional and translational activity, they are a relatively simple model system to study the regulation of PP1 in relation to motility and metabolism. The PP1 driven endogenous regulation of protein phosphorylation and sperm motility could represent an important mechanism for physiological regulation of a cell that encounters dramatically different environments as it journeys through the seminiferous tubules and the female reproductive tract. Previous results (Smith *et al.*, 1996; Vijayaraghavan *et al.*, 1996) provide strong support for a novel unifying hypothesis based on the observation that PP1 is present in sperm and that pharmacological modulation of its activity profoundly affects sperm motility. In other cell types PP1 has been implicated in the control of very diverse processes of cell metabolism, muscle contraction, mitosis, neurotransmitter release, etc. This regulation is the result of complex intracellular pathways, initiated by activation of distinct receptors and second messenger systems. However, the precise role played by phosphatases and their regulation have only recently started to be elucidated. The available data demonstrate their highly regulatable nature, contrary to previous views. One particularly interesting mechanism for controlling PP1 activity involves its inhibition by heat-stable protein phosphatase inhibitors I-1, and I-2, phosphoproteins whose state of phosphorylation controls their inhibitory activity. I-1 is phosphorylated by cAMP-dependent protein kinase and dephosphorylated by the phosphatase calcineurin (PP2B). Thus, PP1 is involved in the cross-talk between the

intracellular messengers calcium and cAMP. I-2 is also capable of inhibiting the catalytic subunit of PP1 leading to the production of a stable PP1-I-2 complex. GSK-3 is capable of phosphorylating the I-2 in the PP1-I-2 complex, relieving the inhibition and producing active PP1.

This biochemical scheme is likely to be operative in mammalian sperm since preliminary studies have identified an I2-like activity and also GSK-3 in mammalian sperm. Immotile bovine caput epididymal sperm contain twofold higher levels of protein phosphatase activity, identified as being PP1γ2, and six fold higher GSK-3 activity than do mature motile caudal sperm. Thus, the complex PP1γ2/ I2-like is inactive in motile caudal sperm and the phosphatase activity is re-established in immotile sperm by the higher GSK-3 activity (Smith *et al.*, 1996; Vijayaraghavan *et al.*, 1996). In addition, the phosphatase inhibitors okadaic acid and calyculin A, were able to induce motility in completely immotile bovine caput epididymal sperm and to stimulate the kinetic activity of mature caudal sperm.

Besides the mechanism of protein phosphatase activity regulation described above others may exist. It is now well established that in somatic cells PP1 activity relies on its binding to several phosphatase regulators, thus the same is likely to occur in germ cells. Other hypothesis will be evaluated in Chapter V.

I.8.1 PP1γ knockout mice

The PP1γ gene was disrupted by targeted insertion in murine embryonic stem cells to address the importance of PP1γ1 and PP1γ2 functions. Mice derived from these cells were viable and homozygous females were fertile. On the contrary, disruption of the PP1γ gene in mice causes sterility in males due to arrest of spermatogenesis at the spermatid stage (Varmuza *et al.*, 1999). Histological examination revealed severe impairment of spermiogenesis beginning at the round spermatid stage. Histopathological evidence shows that meiosis may be disrupted: the presence of polyploidy spermatids suggests a failure of one of the reductional divisions. These observations show that other isoforms of PP1 can compensate for the lack of PP1γ1 in somatic cells and for the lack of PP1γ2 in germ cells until the final stages of spermatogenesis were PP1γ2 becomes indispensable (Varmuza *et*

al., 1999). PP1γ knockout mice raised very interesting questions about the importance of protein phosphatases in spermiogenesis.

I.9 SIGNAL TRANSDUCTION THERAPEUTICS

Cellular health and vitality are dependent on the fine equilibrium of protein phosphorylation systems. Not surprisingly many diseases and dysfunctional states are associated with the abnormal phosphorylation of key proteins (e.g. cancer, diabetes, etc.). Thus, protein phosphorylation systems represent attractive targets for diagnostics and therapeutics. However, unlike the myriad of known protein kinases that all belong to a single gene superfamily, the protein phosphatases belong to several unrelated families. Furthermore, relatively few protein phosphatase catalytic subunits exist, exhibiting broad and overlapping substrate specificities *in vitro*. From a medical perspective, non-selective or marginally-selective phosphatase inhibitors have broad biological activity and are highly toxic to eukaryotic cells due to the inhibition of a number of critical cellular processes. Therefore, the development of non-specific inhibitors (i.e. calyculin A, microcystin and cantharidin) into therapeutic agents for systemic use seems unlikely. However, the development of type and isoform specific inhibitors seems very promising. Both ISIS 15534 (Zuo *et al.*, 1998) and ISIS 14435 (Cheng *et al.*, 2000a) have been employed to specifically suppress the expression of human PP5 and PP1γ1, respectively.

More interesting, however, is the data that indicates that *in vivo*, as phosphatases possess exquisite specificities, both in terms of substrates and localization, the key control mechanism must reside in the nature of the proteins to which they bind. An increasing number of proteins are being identified in diverse cell types that are responsible for regulating the catalytic activity of protein phosphatases. Indeed, the diversity of such phosphatase regulatory subunits explains not only the need for few catalytic subunit types, but also make them attractive targets for pharmacological intervention. The functional diversification of PP1 is controlled via its interaction with regulatory proteins. A major question remaining is how the regulators exert their discrimination between virtually identical mammalian PP1 isoforms.

The importance of PP1 and its binding proteins as potential targets for signal transduction therapeutics is further strengthened by the work of Greengard and co-workers

demonstrating the central role played by DARPP-32 in mediating many of the most important neuronal signaling pathways (Greengard *et al.*, 1999). To date more than twenty primary signaling cascades have been shown to be under the regulation of the PP1/DARPP-32 system in the striatum, and the PP1/I-1 system in other brain regions. PP1 may also have a central role to play in the molecular mechanisms of the actions of drugs of abuse. Furthermore, several lines of evidence also link PP1 to the basic processes thought to underlie memory and learning, such as LTP (long term potenciation) and LTD (long term depression). In fact, the relevance of PP1 within the context of aging and memory loss was recently given a rather intriguing boost. PP1 has been linked to the efficacy of learning and memory by limiting the acquisition of new knowledge and favouring memory decline (Genoux *et al.*, 2002). PP1 inhibition prolongs memory when induced after learning, suggesting that PP1 promotes forgetting. These findings may account for aging-related cognitive decline and emphasize the physiological importance of PP1 as a suppressor of learning and memory. Thus, at least in mice, the molecular machinery is not completely deteriorated with aging and the results show that near normal cognitive functions can be restored simply by inhibiting PP1. Altered PP1 activity may therefore be associated not only with the normal cognitive decline during aging, but may also explain the accelerated decline observed in AD patients and in other neurodegenerative diseases.

I.10 AIMS

Taking into account all the new roles that have been attributed to PP1 based on its binding subunits, it is extremely important to identify novel PP1-binding proteins in order to address cellular functions to PP1. Moreover, relatively little is known about isoform-specific PP1 regulators. Serine/threonine phosphatase PP1γ2 is a testis-specific protein phosphatase present in spermatozoa. This enzyme has been shown to play a key role in sperm motility. Immotile spermatozoa contain higher activity levels of PP1γ2 compared with motile spermatozoa. Inhibition of protein phosphatase activity by okadaic acid and calyculin A initiates motility in caput epididymal sperm without requirement for a change in cAMP levels (Vijayaraghavan *et al.*, 1996). Based on this data a role for PP1γ2 in sperm motility was suggested.

PP1γ2 is expressed during germ cell differentiation in testis (Kitagawa *et al.*, 1990; Sasaki *et al.*, 1990; da Cruz e Silva *et al.*, 1995b). Of the four PP1 isoforms known, PP1γ2

is the only isoform present in spermatozoa and has been involved in the regulation of all mammalian spermatozoa studied so far. It has also been involved in the onset of hyperactivated motility and acrosome reaction (Smith *et al.*, 1996; Vijayaraghavan *et al.*, 1996; Khare *et al.*, 1999; Si, 1999; Si and Okuno, 1999; Smith *et al.*, 1999). The evolutionary conservation and the importance of serine/threonine phosphatases in regulating flagellar motility is highlighted by the involvement of a PP1 homolog in the regulation of rooster sperm motility (Ashizawa *et al.*, 1994; Ashizawa *et al.*, 1995) and by the involvement of a serine/threonine phosphatase in the regulation of microtubule sliding velocity in *Paramecium* and *Chlamydomonas* (Klumpp *et al.*, 1990; Klumpp and Schultz, 1991; Habermacher and Sale, 1995, 1996, 1997).

Spermatozoa leave the testis incapable of progressive motility an unable to fertilize an egg. Motility is acquired during transit through the epididymis (O'Brien *et al.*, 1994) and the ability to fertilize is achieved after incubation within the female reproductive tract, a process referred to as capacitation (Yanagimachi, 1994). There, an acrosome reaction occurs and the spermatozoa acquire a hyperactivated state. A variety of experimental approaches have involved a huge number of proteins in mediating the interaction between a spermatozoon and its environment (Wassarman *et al.*, 2001). However, the nature of the molecular events behind acquisition of progressive motility, capacitation, hyperactivation of motility, directed motility or induction of acrosome reaction remains unresolved.

A combination of biochemical and molecular approaches will be used, together with the unique PP1 tools available in our laboratory to expand the preliminary observations (Smith *et al.*, 1996; Vijayaraghavan *et al.*, 1996) that identified PP1γ2 as a key component and suggested that PP1γ2 activity may ultimately regulate spermatozoa motility and fertility. Thus, the objective of this study is to determine the identity and define the properties of the protein regulators of human testis PP1γ2. Therefore, in order to identify the sperm proteins that interact with PP1, we performed two separate yeast two-hybrid screens of a human testis library using the baits PP1γ1 and PP1γ2. These results should provide new insights into PP1 function in human testis and sperm motility and point to possible targets for pharmacological intervention, particularly regarding infertility and contraception. We will undertake an exhaustive characterization of the PP1γ2 interactome from human testis.

II YEAST TWO HYBRID SCREENS

II.1 INTRODUCTION

Since sperm are terminally differentiated cells essentially devoid of transcriptional and translational activity, they are a relatively simple model system to study the regulation of PP1 and its relation to motility and metabolism. The PP1 driven endogenous regulation of protein phosphorylation and sperm motility could represent an important mechanism for physiological regulation of a cell that encounters dramatically different environments as it journeys through the seminiferous tubules and the female reproductive tract. Previous results (Smith *et al.*, 1996; Vijayaraghavan *et al.*, 1996) provide strong support for a novel unifying hypothesis based on the observation that PP1 is present in sperm and that pharmacological modulation of its activity profoundly affects sperm motility. On the other hand, PP1 activity towards different substrates appears to be mediated via binding to specific regulatory proteins. Such proteins play critical regulatory and targeting roles for PP1. In this context we have undertaken an in-depth survey using the yeast two-hybrid approach to identify the proteins expressed in human testis capable of interacting specifically with the alternatively spliced isoforms of PP1γ.

The yeast two hybrid system (YTH) was originally devised as a simple means to probe protein-protein interactions (Fields and Song, 1989). YTH uses the fact that most eukaryotic transcription activators have two functionally independent domains, the DNA-binding domain (BD) that recognizes a specific DNA sequence in the promoters of different genes and a DNA-activation domain (AD) that brings the transcriptional machinery to the promoter vicinity. This independence was demonstrated by fusing the AD of yeast GAL4, a yeast transcription factor involved in galactose metabolism, with the BD of *E. coli* LexA to create a functional transcription factor in yeast (Brent and Ptashne, 1985). Fields and Song (1989) took advantage of this property of GAL4 to prove the interaction between two known proteins. They fused GAL4-AD to a protein X (prey) and GAL4-BD [recognizing the UASG sequence, (Weaver, 2002)] to a protein Y (bait). These two elements were cointroduced into yeast with one or more reporter genes (the bigger the number of reporter genes used the lower the number of false positives obtained) that were made to be transcriptionally dependent on activation through a binding site to the BD.

Interaction of the BD fusion with the AD fusion positions the AD in the proximity of the reporter gene, thus activating its transcription (Fig. II.1). The reporter genes that are most commonly used are the MEL1 gene (encoding α-galactosidase that is secreted into the culture medium), LacZ gene (encoding β-galatosidase) and auxotrophic genes (like HIS3 and ADE2) that allow the yeast to grow in medium lacking histidine and adenine, respectively). This system can also be used to find new interacting proteins by fusing to the AD a collection of cDNAs from an expression library.

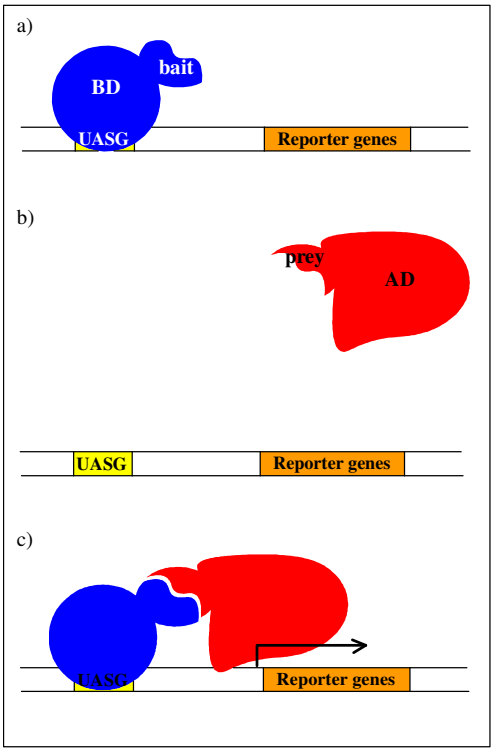


Figure II.1: The yeast two-hybrid system. Two chimeric proteins are expressed in yeast: ***a)*** *GAL4 DNA-binding domain (BD) fused to a bait protein and GAL4 activation domain (AD) fused to a prey protein. The BD-bait hybrid protein can bind to upstream activation sites (UASG) but cannot activate transcription).* ***b)*** *The AD-prey protein cannot recognize the UASG, thus, alone is not capable of initiating transcription.* ***c)*** *When the bait and the prey interact, the BD and AD are brought together and can activate reporter gene transcription.*

Larger scale two-hybrid approaches typically rely on interaction mating (Finley and Brent, 1994). In this method a yeast strain expressing the bait protein is mated with another yeast strain of opposite mating type pretransformed with the cDNA library. Interaction between two proteins can then be determined by the activation of one or more reporter genes in the diploid strain. The advantages of this approach are the possibility of using frozen aliquots of pretransformed yeast cells saving time and resources, and that diploid cells are more tolerant to expression of toxic proteins and less false positives will appear since the diploids have reporter genes less sensitive to transcription (Kolonin and Finley, 1998).

Over the 14 years since its introduction this system has been modified greatly expanding its biological and technological applications. The YTH has been developed first as an agent of biological discovery, second as a tool in proteomics and finally as a means towards engineering novel pharmaceutical agents. Some YTH alternative systems have been developed and in many cases resulted in remarkably elegant hybrid systems (a review can be found in (Serebriiskii *et al.*, 2001).

II.2 MATERIALS AND METHODS

For the complete composition of all reagents, media and solutions used, see the list presented in Appendix I. All reagents were cell culture grade or ultrapure.

II.2.1 Isolation of plasmids from bacteria

Method 1 – alkaline lysis "mini-prep"

A single bacterial colony was transferred into 2ml of LB medium containing ampicilin (100µg/ml) and incubated overnight at 37ºC with vigorous shaking. 1.5 ml of this culture were transferred into a microtube and centrifuged at 14,000g for 1min at 4ºC. The medium was removed by aspiration. The bacterial pellet was resuspended in 100µl of ice-cold solution I [50mM glucose/ 25mM Tris.HCl (pH 8.0)/ 10mM EDTA] by vigorous vortexing. Then, 200µl of freshly prepared solution II (0.2N NaOH, 1%SDS) were added to the microtube that was mixed by inverting several times. Keeping the microtube on ice, 150µl of ice-cold solution III (3M potassium acetate/ 2M glacial acetic acid) were added and the microtube gently vortexed. The microtube was then left on ice for 5min, centrifuged at 14,000g for 10min at 4ºC and the supernatant transferred to a clean microtube. The DNA was precipitated by adding 2 volumes of ethanol at room temperature (RT) and vortexed. The mixture was allowed to stand for 2min at RT. After centrifugation at 14,000g for 5min at 4ºC the supernatant was completely removed and the pellet washed with 70% ethanol. After further centrifugation, the pellet was allowed to air-dry for 10min. The DNA was dissolved in H_2O containing DNAase-free pancreatic RNAase (20µg/ml) and stored at -20ºC.

Method 2 - QIAGEN "miniprep"

The bacterial pellet was obtained as described above. The pellet was then resuspended in 250µl of buffer P1, 250µl of buffer P2 were added and the microtube was mixed by gently inverting until the solution became viscous and slightly clear. Afterwards, 350µl of buffer N3 were added and the microtube was repeatedly inverted until the solution became cloudy. The microtube was centrifuged for 10min and the resulting supernatant was applied to a QIAprep spin column placed in a microtube. After a 1min centrifugation the flow-through was discarded. The column was washed by adding 0.75ml of buffer PE and centrifuging 1min to discard the flow-through. The column was centrifuged for an additional 1min to remove residual wash buffer. Finally, the column was placed in a clean microtube and 50µl of H_2O were added to elute the DNA by centrifuging for 1min after letting it stand for 1min. This method gives a cleaner DNA preparation than Method 1 with better yields. This method was used when the DNA was subsequently used for DNA sequencing. For enzymatic restriction the first method was commonly used.

Method 3 - PROMEGA "Megaprep"

1000ml of cell culture were pelleted by centrifugation at 1,500g for 20min at RT. The cell pellet was resuspended in 30ml of cell resuspension solution [50mM Tris-HCl (pH 7.5)/ 10mM EDTA/ 100µg/ml RNAase A] by manually disrupting the pellet with a pipette. 30ml of cell lysis solution (0.2M NaOH/ 1%SDS) were added to the cells and the solution mixed gently by inverting until it became clear and viscous. Then, 30ml of neutralization solution [1.32M potassium acetate (pH 4.8)] were added and the tube immediately mixed by inverting. After centrifugation at 14,000g for 15min at RT the clear supernatant was transferred by filtering through gauze swabs to a new tube and the volume of supernatant was measured. At this stage 0.5 volumes of RT isopropanol were added and the solution mixed by inversion. This solution was centrifuged at 14,000g for 15min at RT, the supernatant was discarded and the pellet resuspended in 4ml of TE buffer. 20ml of Wizard ™ Megapreps DNA purification Resin were added to the DNA and mixed by swirling. A Wizard ™ Megacolumn was inserted into the vacuum manifold port and the DNA/resin mix was transferred into the Megacolumn. Vacuum was applied to pull the mix through the Megacolumn. Two washes with 25ml of column wash solution (80mM potassium acetate/ 8.3mM Tris-HCl/ 40µM EDTA/ 55% ethanol) were performed and the

resin was rinsed with 10ml of 80% ethanol. The Megacolumn was inserted into a 50ml screw cap tube and centrifuged at 2,500rpm for 5min using a swinging bucket rotor centrifuge. The Megacolumn was placed in a clean tube and 3ml of pre-heated water (70ºC) were added to the column. After waiting 1min the DNA was eluted by centrifugation at 2,500rpm for 5min. The DNA was stored at –20ºC. This method can yield more than 1mg of plasmid DNA from 1,000ml of culture.

This procedure was used to prepare large amount of plasmid DNA for storage and subsequent cloning, transfection, probe labelling, and other purposes.

II.2.2 DNA digestion with restriction enzymes

For a typical DNA digestion the manufacturer's instructions were followed. In a microtube the following components were added to the following concentrations:

- 100µg/ml DNA
- 1X reaction buffer (specific for each restriction enzyme)
- 1U/µg DNA of restriction enzyme

The mixture was incubated at the appropriate temperature for a few hours (or overnight if convenient). The restriction enzyme should contribute less than 10% of the final volume. When sequential digestions with different enzymes were carried out the DNA was purified between the two reactions.

II.2.3 DNA purification

Method 1 - QIAGEN DNA Purification kit

This kit was used to purify DNA fragments from PCR and other enzymatic reactions. It allowed purification from primers, nucleotides, polymerases and salts by using QIAquick spin columns. Briefly, 5 volumes of buffer PB were added to 1 volume of the solution to be purified and mixed. The spin column was placed in a collection microtube and the sample was applied to the column and centrifuged for 1min at 14,000rpm to bind the DNA. The flow-through was discarded and the column was washed with 0.75ml of buffer PE, centrifuged for 1min at 14,000rpm and the flow-through discarded. The column was placed back in the same microtube and centrifuged again to remove traces of washing buffer. Then, the column was placed in a clean microtube, 50µl of H_2O were added and

allowed to stand for 1min. To elute the DNA the column was centrifuged for 1min at 14,000rpm. The DNA was stored at –20ºC.

Method 2: DNA precipitation with ethanol

This method is used to concentrate nucleic acids as well as to purify them. Approximately 1/10 volume of 3M sodium acetate (pH 5.2) was added to the DNA solution to adjust the salt concentration, followed by 2 volumes of ice-cold ethanol. The solution was well mixed and stored at –20ºC for 30min to allow the DNA precipitate to form DNA was recovered by centrifugation at 4ºC for 15min at 14,000rpm. The supernatant was carefully removed without disturbing the pellet. The microtube was half filled with ice-cold 70% ethanol and recentrifuged using the same conditions as above for 5min. The supernatant was again removed and the pellet allowed to dry before being resuspended in sterile water.

Method 3: DNA purification by extraction with phenol/chloroform

This approach can be used to remove proteins from DNA solutions, for instance to inactivate and remove enzymes. An equal volume of phenol/chloroform was added to the DNA sample. The contents were mixed until an emulsion was formed. The emulsion was then centrifuged at 14,000rpm for 1min at RT until the aqueous and organic phases were well separated. The aqueous phase was transferred to a clean tube and an equal volume of chloroform was added and mixed until an emulsion was formed. Centrifugation was performed using the same conditions as above, the aqueous phase was recovered and the DNA recovered by precipitation with ethanol (see above).

II.2.4 DNA ligation

Ligation of cohesive termini and blunt ended DNA

The plasmid and the DNA fragment to be inserted were digested with the appropriate restriction enzymes. Digested fragments were separated by gel electrophoresis (see below) and purified from low melting temperature agarose. 0.1µg of vector DNA were transferred to a microtube with an equimolar amount of insert DNA. H_2O was added to 7.5µl, 1µl of 10X bacteriophage T4 DNA ligase (PROMEGA) buffer and 1µl of

bacteriophage T4 DNA ligase were added to the reaction mix and incubated for 4h at 16ºC. Two additional control reactions were set up that contained the plasmid vector alone and the insert fragment alone. To ligate blunt-ended DNA the protocol followed was exactly the same as above.

Alkaline phosphatase treatment

In order to prevent self ligation of vector molecules, shrimp alkaline phosphatase (SAP) (ROCHE), was used according to the manufacturer's suggestions. The reaction mixture was adjusted with 1/10 volume 10X concentrated dephosphorylation buffer, and incubated with 1µl of SAP at 37ºC for 1h. Finally, SAP was inactivated by heating the reaction mixture at 65ºC for 15min.

II.2.5 Preparation of competent cells

A single colony of *E. coli* XL1-Blue was incubated in 10ml SOB medium at 37ºC overnight. Then, 1ml of this culture was used to incubate 50ml SOB until OD_{550}=0.3. The culture was incubated on ice for 15min and centrifuged at 4,000rpm at 4ºC for 5min. The supernatant was discarded and 15ml of Solution I were added. After allowing to stand on ice for 15min the cells were centrifuged at 4,000rpm for 5min at 4ºC and 3ml of Solution II were added to the cell pellet. The cells were immediately divided in 0.1ml aliquots and stored at –80ºC.

II.2.6 Bacteria transformation with plasmid DNA

Competent cells (100µl) were thawed on ice and 0.1-50ng of DNA were added to the cells and gently swirled. The microtube was incubated on ice for 20min and heat shocked at 42ºC for 90sec. The microtubes were then incubated on ice for 2min before adding 0.9ml of SOC medium. The tubes were incubated at 37ºC for 30min with shaking at 220rpm. The cells were then plated on the appropriate antibiotic agar medium and incubated at 37ºC for 16h until colonies appeared. Control transformations were also performed in parallel. These always included a negative control transformation without

DNA and a positive control transformation with 0.1ng of a control plasmid, for instance pUC19.

II.2.7 Electrophoretic analysis of DNA

The electrophoresis apparatus was prepared and the electrophoresis tank was filled with enough 1X TAE to cover the agarose gel. The appropriate amount of agarose was transferred to an Erlenmeyer with 50ml 1X TAE. The slurry was heated until the agarose was dissolved and allowed to cool to 60ºC before adding ethidium bromide to a final concentration of 0.5µg/ml. The agarose solution was poured into the mold and the comb was positioned. After the gel was completely set the comb was carefully removed and the gel mounted in the tank. The DNA samples were mixed with the 6X loading buffer (LB) (0.25% bromophenol blue/ 30% glycerol in water) and the mixture was loaded into the slots of the submerged gel using a micropipette. Marker DNA (λ-Hind III fragments or 1kb ladder) of known size was also loaded into the gel. The lid of the gel tank was closed and the electrical leads were attached so that the DNA migrated towards the anode. The gel was run until the bromophenol blue had migrated the appropriate distance through the gel. At the end, the gel was examined by UV light and photographed or analysed on a Molecular Imager (Biorad).

II.2.8 DNA purification from low melting point agarose gels

To purify DNA after restriction digestion, the mixture was ran on a low melting temperature agarose gel. The procedure is the same as described in the section above except for the use of low melting temperature agarose. In this type of agarose hydroxyethyl groups are present in the polysaccharide chain. This substitution causes the agarose to gel at around 30ºC and to melt at approximately 65ºC, below the melting point of dsDNA. After preparing the gel as described above, the gel was ran in a cold room (4ºC) at 40V for 4h to ensure that the gel does not melt during the run. After, the desired DNA band was localized on the gel with a long-wavelength UV lamp, it was excised using a sharp scalpel and transferred to a clean, pre-weighted microtube. To extract the DNA from the agarose

slice two methods were used: agarose digestion with agarase (NEB) or purification through a QUIAGEN column.

II.2.9 DNA sequencing

All the DNA samples to be sequenced followed the same protocol. In a microtube the following components were added:

- 500ng dsDNA
- 4µl of Ready Reaction Mix*
- 3.2pmol primer
- H_2O to a final volume of 20µl

* Ready Reaction Mix is composed of: dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA polymerase, FS, rTth pyrophosphatase, magnesium chloride and buffer (Applied Biosystems).

This reaction mixture was vortexed and spun down for a few seconds. PCR was then performed using the following conditions:

96ºC 10sec
50ºC 5sec } 25 cycles
60ºC 4min
hold at 4ºC

Afterwards, the samples were purified by ethanol precipitation (see above). Briefly, 2.0µl of 3M sodium acetate (pH4.6) and 50µl of 95% ethanol were added to the reaction microtube. The microtube was vortexed and incubated at RT for 15min to precipitate the extension products. The microtube was then centrifuged at 14,000rpm for 20min at RT. After discarding the supernatant 250µl of 70% ethanol were added, the microtube was briefly vortexed and recentrifuged for 5min at 14,000rpm at RT. The supernatant was discarded and the pellet dried. After this procedure the DNA was ready to be applied in an Applied Biosystems Automated DNA Sequencer.

II.2.10 Yeast transformation with plasmid DNA

Preparation of competent yeast cells

The appropriate selection media and agar plates were prepared in advance. One colony of yeast strain AH109 was inoculated into 1ml of YPD medium in a 1.5ml microtube and vortexed vigorously to disperse cell clumps. The culture was transferred into a 250ml flask containing 50ml of YPD and incubated at 30ºC with shaking at 230rpm overnight until it reached stationary phase with OD_{600} > 1. Enough of this culture (20-40ml) was transferred into 300ml YPD in a 1L flask to produce an OD_{600} = 0.2-0.3. The culture was incubated for 3h at 30ºC with shaking at 230rpm, centrifuged at 2,200rpm for 5min at RT and the supernatant was discarded and the cells resuspended in 25ml H_2O. The cells were recentrifuged and the pellet was resuspended in 1.5ml of freshly prepared, sterile 1XTE/LiAc.

Yeast transformation- LiAc method

In a microtube 0.1µg of plasmid DNA were added to 100µg of herring testes carrier DNA. Then, 100µl of freshly prepared competent cells were added to the microtube, followed by 600µl of sterile PEG/LiAc (40% PEG 4000/ 1X TE/ 1X LiA). The solution was incubated at 30ºC for 30min with shaking (200rpm). After adding 70µl of DMSO the solution was mixed gently and then heat shocked for 15min in a 42ºC water bath. The cells were pelleted after being chilled on ice, centrifuged for 5sec at 14,000rpm and resuspended in 0.5ml of 1X TE buffer. 100µl of these cells were then plated in the appropriate selection media, and incubated at 30ºC for 2 days.

II.2.11 Expression of proteins in yeast

Preparation of yeast cultures for protein extraction

For each transformed yeast to be assayed by immunoblotting, 5ml overnight cultures in SD (supplement dropout) selection medium were prepared by inoculating a colony of the previously transformed yeast (see above). As a negative control an untransformed yeast colony (AH109) was inoculated in YPD. The overnight cultures were

vortexed and added to 50ml of YPD. These cultures were incubated at 30ºC with shaking (220rpm) until OD_{600} reached 0.4-0.6 (this took 4-8h). The culture was quickly chilled by pouring it into a prechilled 50ml centrifuge microtube halfway filled with ice. The microtube was immediately centrifuged at 1,000g for 5min at 4ºC. The supernatant was discarded and the pellet washed in 50ml of ice-cold water. The pellet was recovered by centrifugation at 1,000g for 5min at 4ºC and immediately frozen by placing the microtube in liquid nitrogen.

Preparation of protein extracts

Complete cracking buffer was prepared and pre-warmed to 60ºC. 100µl of cracking buffer were used per 7.5 OD_{600} units of cells. The cell pellets were quickly thawed by resuspending in the pre-warmed cracking buffer. The samples were briefly placed at 60ºC to hasten melting. An additional aliquot (1µl of 100X PMSF per 100µl of cracking buffer) of the 100X PMSF stock solution was added to the samples every 7min during the procedure. Each cell suspension was transferred into a 1.5ml microtube containing 80µl of glass beads per 7.5 OD_{600} units of cells. The samples were heated at 70ºC for 10min to release the membrane-associated proteins. Then, the microtubes were vortexed vigorously for 1 min and the debris pelleted at 14,000rpm for 5min at 4ºC. The supernatants were transferred to fresh microtubes and placed on ice. The pellets were boiled for 5min, vortexed for 1min and centrifuged again. The supernatants were combined and the samples were boiled and stored at –70ºC (or loaded immediately on a SDS-PAGE gel).

II.2.12 SDS-PAGE

In SDS polyacrylamide gel electrophoresis (SDS-PAGE) separations were carried out using well stablished methods (Laemmli, 1970).

The percentage and size of the gel used depended on the molecular weight of the proteins being separated in the gel (Table II.1). For instance, when we wanted to visualize PP1 (with molecular weight around 40kDa) 12% gels were used.

Table II.1: *Composition of the running and stacking gels for SDS-PAGE (mini gels).*

Components	Running gel (7.5%)	Running gel (12%)	Running gel (15%)	Stacking gel (3.5%)
Water	4.9 ml	3.45 ml	2.45 ml	6.6 ml
30%Acryl./8%Bisacryl.	2.5 ml	4.0 ml	5.0 ml	1.2 ml
4X LGB	2.5 ml	2.5 ml	2.5 ml	-------
5X UGB	-------	-------	-------	2.0 ml
SDS 10%	-------	-------	-------	100 µl
10% APS	50 µl	50 µl	50 µl	100 µl
TEMED	5 µl	5 µl	5 µl	10 µl

The 12% running gel was prepared by sequentially adding the components indicated on Table II.1 (APS and TEMED were the last added, as they initiate the polymerising process). The solution was then carefully pipetted down the spacer into the gel sandwich, leaving some space for the stacking gel. Then, water was carefully added to cover the top of the gel and the gel was allowed to polymerise for 1h. The stacking gel was prepared according to Table II.1. The water was poured out and the stacking gel was added to the sandwich; a comb was inserted and the gel allowed to polymerise for 30min. Then, the samples were prepared by adding to the protein sample (when appropriate the amount of protein loaded on the gel will be described) ¼ volume of LB (Loading Buffer). The microtube was boiled and centrifuged, the combs removed and the wells filled with running buffer. The samples were carefully loaded into the wells that were filled with running buffer, and the samples were electrophoresed at 90mA until the bromophenol blue from the LB reached the bottom of the gel.

II.2.13 Staining gels with Coomassie Blue

Coomassie Blue staining is based on non-specific binding of the Coomassie Blue dye to proteins. The separated proteins are simultaneously fixed and stained in the gel, and

then destained to remove the background prior to drying and photographing. The proteins are detected as blue bands on a clear background.

The staining was performed at room temperature. Covered plastic trays were used to minimize exposure to acetic acid and methanol vapours. The gel was placed in staining solution (enough volume was used so that the gel could float) and agitated slowly for 30min. After, the gel was placed in destaining solution until the bands were visible. The gel was then dried using a vacuum gel dryer. To this end the gel was placed on a sheet of filter paper of the same size, on top of a larger sheet of filter paper covering the metal screen on the dryer. The gel was covered with plastic wrap, then the silicon dryer cover flap was lowered and the vacuum applied. Finally, the heat was turned on to 65ºC for 1h. Alternatively, gels were scanned wet with a GS-710 calibrated imaging densitometer (Biorad) and analyzed with Quantity One software (version 4.2.1).

II.2.14 Immunoblotting

For immunoblotting tank transfer system was used. 3MM blotter paper was cut to fit the transfer cassette and a nitrocellulose membrane of the gel size was also cut. The gel was removed from the electrophoresis device and the stacking gel removed and discarded. The transfer sandwich was assembled under transfer buffer to avoid trapping air bubbles. The cassette was placed in the transfer device filled with transfer buffer. Transfer was allowed to proceed for 4h at 100mA, for mini gels, or overnight at 200mA for larger gels. Afterwords, the transfer cassettes were disassembled, the membrane carefully removed and allowed to air dry prior to further manipulations.

II.2.15 Immunodetection by enhanced chemiluminescence (ECL)

ECL ™ is a light emitting non-radioactive method for the detection of immobilised antigens, conjugated directly or indirectly with horseradish peroxidase-labelled antibodies. First, the membrane was soaked in 1X TBS for 10min. Non-specific binding sites were blocked by immersing the membrane in 5% low fat milk in TBST for 1h. Then, the membrane was incubated with a solution of the appropriate primary antibody diluted in 5% low fat milk in TBST for 1h with shaking. After three washes of 10min each in 1X TBST

the membrane was incubated with a solution of the appropriate secondary antibody diluted in 5% low fat milk in TBST for 1h with shaking. Depending on the origin of the primary antibody (mouse, rabbit or sheep), the secondary antibody was anti-rabbit or anti-mouse, diluted 1:5000, or anti-sheep, diluted 1:1000, all conjugated to peroxidase. The membrane was washed, three times for 10min, and incubated for 1min at RT with the ECL detection solution (a mixture of equal volumes of solution 1 and solution 2 from the ECL kit, approximately 0.125ml/cm^2 membrane). Excess solution was drained by touching the edge of the membrane against tissue paper and the membrane was gently wrapped with cling-film, eliminating all air bubbles. In the dark room, the membrane was placed in a film cassette and an autoradiography film (XAR-5 film, KODAK) was placed on the top. The cassette was closed and the blot exposed over a certain period of time. The film was then removed and developed in a developing solution, washed in water and fixed in fixing solution. If needed, a second film was exposed more or less time according to the first result.

To reuse the same membrane with another antibody, the membrane was sometimes stripped with stripping solution for 45min at 50ºC, washed with 1X TBST for 15min three times and then dried until needed.

II.2.16 cDNA library screening by yeast mating

A concentrated overnight culture of the bait strain (AH109+insert) was prepared by inoculating a colony of the bait strain into 50ml of SD/-Trp and incubating it at 30ºC overnight with shaking at 250rpm. The next day, the culture was centrifuged at 1,000g for 5min and the pellet was resuspended in the residual liquid (5ml) by vortexing. The cells were counted using a haemocytometer (the concentration was > $1X10^9$ cells/ml). Just prior to use, a frozen aliquot (1ml) of the library culture (Pretransformed Human Testis MATCHMAKER cDNA Library) was thawed in a room temperature water bath. The library was gently mixed and 10µl were set aside for later tittering (see below). The entire bait strain culture was combined with the 1ml library culture in a 2L sterile flask, 45ml of 2X YPDA were added and gently swirled. This culture was incubated at 30ºC for 20-24h with shaking at 40rpm. After 20h of mating a drop of the mating culture was checked under a phase-contrast microscope to check for the presence of zygotes (Figure II.7). If so,

the mating was allowed to proceed for four more hours. The mating mixture was transferred to a sterile 50ml tube and the cells spun down at 1,000g for 10min. The mating flask was rinsed twice with 2X YPDA (50ml) and the rinses were combined and used to resuspend the first pellet. The cells were centrifuged again at 1,000g for 10min, the pellet resuspended in 10ml of 0.5X YPDA and the total volume (cells + medium) was measured. Half of the library mating mixture was plated on SD/QDO (SD without Leu, Trp, Ade and His), and the other half on SD/TDO (SD without Leu, Trp and His), at 200µl per 150mm plate. For mating efficiency controls, 100µl of 1:10,000, 1:1,000; 1:100 and 1:10 dilutions of the mating mixture were plated in 100mm SD/-Leu, SD/-Trp and SD/-Leu/-Trp plates. All plates were incubated at 30ºC until colonies appeared, generally 3-8 days on TDO and 8-21 days on QDO medium. Then, growth of the control plates was scored and the mating efficiency and number of clones screened were calculated. All positive clones were replated twice in SD/QDO medium containing X-α-Gal and incubated at 30ºC for 3-8 days. True positives formed blue colonies. The master plates were sealed with parafilm and stored at 4ºC. Glycerol stocks were prepared for all the positive clones.

II.2.17 Library titering

A library aliquot (10µl) was transferred to 1ml of YPDA in a 1.5ml microtube – dilution A (dilution factor 10^{-2}). 10µl from dilution A were added to 1ml of YPDA in another microtube and mixed gently – dilution B (dilution factor 10^{-4}). 10µl of dilution A were added to 50µl of YPDA in another microtube and mixed gently. This solution was spread onto a SD/-Leu plate. From dilution B, 50 and 100µl were spread onto separate SD/-Leu plates. All the plates were incubated at 30ºC for 3 days after which the number of colonies was counted. The titer of the library was calculated using the following expression: [# colonies] /[plating volume (ml)x dil factor] = cfu/ml.

II.3 RESULTS

Two separate yeast two-hybrid screens were performed to identify proteins from a human testis cDNA library capable of interacting with the alternatively spliced isoforms of PP1γ, PP1γ1 (YTH1) and with PP1γ2 (YTH2). These two isoforms are identical, except for the C-terminus (Fig. II.2):

```
PP1gamma1       GGGCGCGAGCCGGCGGCGGCGCCGCTGCGGGAGGGTCGGCGGTGGGAAGGCGATGGCGGA 60
PP1gamma2       GGGCGCGAGCCGGCGGCGGCGCCGCTGCGGGAGGGTCGGCGGTGGGAAGGCGATGGCGGA 60
                ************************************************************

PP1gamma1       TTTAGATAAACTCAACATCGACAGCATTATCCAACGGCTGCTGGAAGTGAGAGGGTCCAA 120
PP1gamma2       TTTAGATAAACTCAACATCGACAGCATTATCCAACGGCTGCTGGAAGTGAGAGGGTCCAA 120
                ************************************************************

PP1gamma1       GCCTGGTAAGAATGTCCAGCTTCAGGAGAATGAAATCAGAGGACTGTGCTTAAAGTCTCG 180
PP1gamma2       GCCTGGTAAGAATGTCCAGCTTCAGGAGAATGAAATCAGAGGACTGTGCTTAAAGTCTCG 180
                ************************************************************

PP1gamma1       TGAAATCTTTCTCAGTCAGCCTATCCTACTAGAACTTGAAGCACCACTCAAAATATGTGG 240
PP1gamma2       TGAAATCTTTCTCAGTCAGCCTATCCTACTAGAACTTGAAGCACCACTCAAAATATGTGG 240
                ************************************************************

PP1gamma1       TGACATCCATGGACAATACTATGATTTGCTGCGACTTTTTGAGTACGGTGGTTTCCCACC 300
PP1gamma2       TGACATCCATGGACAATACTATGATTTGCTGCGACTTTTTGAGTACGGTGGTTTCCCACC 300
                ************************************************************

PP1gamma1       AGAAAGCAACTACCTGTTTCTTGGGGACTATGTGGACAGGGGAAAGCAGTCATTGGAGAC 360
PP1gamma2       AGAAAGCAACTACCTGTTTCTTGGGGACTATGTGGACAGGGGAAAGCAGTCATTGGAGAC 360
                ************************************************************

PP1gamma1       GATCTGCCTCTTACTGGCCTACAAAATAAAATATCCTGAGAATTTTTTTCTTCTCAGAGG 420
PP1gamma2       GATCTGCCTCTTACTGGCCTACAAAATAAAATATCCTGAGAATTTTTTTCTTCTCAGAGG 420
                ************************************************************

PP1gamma1       GAACCATGAATGTGCCAGCATCAACAGAATTTATGGATTTTATGATGAATGTAAAAGAAG 480
PP1gamma2       GAACCATGAATGTGCCAGCATCAACAGAATTTATGGATTTTATGATGAATGTAAAAGAAG 480
                ************************************************************

PP1gamma1       ATACAACATTAAACTATGGAAAACTTTCACAGACTGTTTTAACTGTTTACCGATAGCAGC 540
PP1gamma2       ATACAACATTAAACTATGGAAAACTTTCACAGACTGTTTTAACTGTTTACCGATAGCAGC 540
                ************************************************************

PP1gamma1       CATCGTGGATGAGAAGATATTCTGCTGTCATGGAGGTTTATCACCAGATCTTCAATCTAT 600
PP1gamma2       CATCGTGGATGAGAAGATATTCTGCTGTCATGGAGGTTTATCACCAGATCTTCAATCTAT 600
                ************************************************************

PP1gamma1       GGAGCAGATTCGGCGAATTATGCGACCAACTGATGTACCAGATCAAGGTCTTCTTTGTGA 660
PP1gamma2       GGAGCAGATTCGGCGAATTATGCGACCAACTGATGTACCAGATCAAGGTCTTCTTTGTGA 660
                ************************************************************

PP1gamma1       TCTTTTGTGGTCTGACCCCGATAAAGATGTCTTAGGCTGGGGTGAAAATGACAGAGGAGT 720
PP1gamma2       TCTTTTGTGGTCTGACCCCGATAAAGATGTCTTAGGCTGGGGTGAAAATGACAGAGGAGT 720
                ************************************************************

PP1gamma1       GTCCTTCACATTTGGTGCAGAAGTGGTTGCAAAATTTCTCCATAAGCATGATTTGGATCT 780
PP1gamma2       GTCCTTCACATTTGGTGCAGAAGTGGTTGCAAAATTTCTCCATAAGCATGATTTGGATCT 780
                ************************************************************

PP1gamma1       TATATGTAGAGCCCATCAGGTGGTTGAAGATGGATATGAATTTTTTGCAAAGAGGCAGTT 840
PP1gamma2       TATATGTAGAGCCCATCAGGTGGTTGAAGATGGATATGAATTTTTTGCAAAGAGGCAGTT 840
                ************************************************************
```

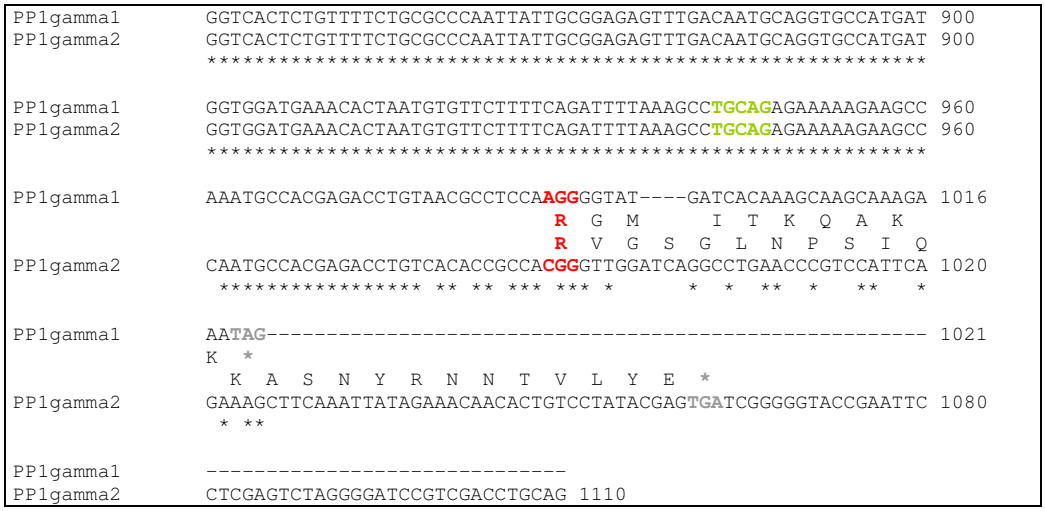


Figure II.2: *Comparison of the human alternatively spliced PP1γ1 and PP1γ2. The red letters mark the splice site, the grey letters mark the stop codon and the green letters represent the PstI restriction site.*

II.3.1 Construction of the bait plasmids

The vector used to clone the bait cDNAs encoding PP1γ1 and PP1γ2, was Clontech's GAL4 binding domain expression vector pAS2-1 (Appendix IV). pAS2-1 has several characteristics that make it suitable for YTH. It has the GAL4-BD, two independent yeast and bacteria replication origins, it confers ampicilin and cyclohexamide resistance and allows the yeast to grow without tryptophan in the culture media. It also has a multiple cloning site used to clone the bait cDNA.

II.3.1.1 Construction of pAS-PP1γ1

The cDNA for human PP1γ1 (da Cruz e Silva, 1995) was removed from pBluescript (Appendix IV) by restriction digestion with *Xma*I/*Sal*I and directionally subcloned into *Xma*I/*Sal*I-digested pAS2-1. The resulting plasmid was sequenced to check the correctness of the PP1γ1 sequence and if the fusion protein was in the proper reading frame (Fig. II.3).

```
atgaagctactgtcttctatcgaacaagcatgcgatatttgccgacttaaaaagctcaag
 M  K  L  L  S  S  I  E  Q  A  C  D  I  C  R  L  K  K  L  K
tgctccaaagaaaaaccgaagtgcgccaagtgtctgaagaacaactgggagtgtcgctac
 C  S  K  E  K  P  K  C  A  K  C  L  K  N  N  W  E  C  R  Y
tctcccaaaaccaaaaggtctccgctgactagggcacatctgacagaagtggaatcaagg
```

```
 S  P  K  T  K  R  S  P  L  T  R  A  H  L  T  E  V  E  S  R
ctagaaagactggaacagctatttctactgatttttcctcgagaagaccttgacatgatt
 L  E  R  L  E  Q  L  F  L  L  I  F  P  R  E  D  L  D  M  I
ttgaaaatggattctttacaggatataaaagcattgttaacaggattatttgtacaagat
 L  K  M  D  S  L  Q  D  I  K  A  L  L  T  G  L  F  V  Q  D
aatgtgaataaagatgccgtcacagatagattggcttcagtggagactgatatgcctcta
 N  V  N  K  D  A  V  T  D  R  L  A  S  V  E  T  D  M  P  L
acattgagacagcatagaataagtgcgacatcatcatcggaagagagtagtaacaaaggt
 T  L  R  Q  H  R  I  S  A  T  S  S  S  E  E  S  S  N  K  G
caaagacagttgactgtatcgccggtattgcaatacccagctttgactcatatggccatg
 Q  R  Q  L  T  V  S  P  V  L  Q  Y  P  A  L  T  H  M  A  M
gaggccgaattcccgggcgcgagccggcggcggcgccgctgcgggagggtcggcggtggg
 E  A  E  F  P  G  A  S  R  R  R  R  R  C  G  R  V  G  G  G
aaggcgatggcggatttagataaactcaacatcgacagcattatccaacggctgctggaa
 K  A  M  A  D  L  D  K  L  N  I  D  S  I  I  Q  R  L  L  E
gtgagagggtccaagcctggtaagaatgtccagcttcaggagaatgaaatcagaggactg
 V  R  G  S  K  P  G  K  N  V  Q  L  Q  E  N  E  I  R  G  L
tgcttaaagtctcgtgaaatctttctcagtcagcctatcctactagaacttgaagcacca
 C  L  K  S  R  E  I  F  L  S  Q  P  I  L  L  E  L  E  A  P
ctcaaaatatgtggtgacatccatggacaatactatgatttgctgcgacttttgagtac
 L  K  I  C  G  D  I  H  G  Q  Y  Y  D  L  L  R  L  F  E  Y
ggtggtttcccaccagaaagcaactacctgtttcttggggactatgtggacaggggaaag
 G  G  F  P  P  E  S  N  Y  L  F  L  G  D  Y  V  D  R  G  K
cagtcattggagacgatctgcctcttactggcctacaaaataaaatatcctgagaatttt
 Q  S  L  E  T  I  C  L  L  L  A  Y  K  I  K  Y  P  E  N  F
tttcttctcagagggaaccatgaatgtgccagcatcaacagaatttatggattttatgat
 F  L  L  R  G  N  H  E  C  A  S  I  N  R  I  Y  G  F  Y  D
gaatgtaaaagaagatacaacattaaactatggaaaactttcacagactgttttaactgt
 E  C  K  R  R  Y  N  I  K  L  W  K  T  F  T  D  C  F  N  C
ttaccgatagcagccatcgtggatgagaagatattctgctgtcatggaggtttatcacca
 L  P  I  A  A  I  V  D  E  K  I  F  C  C  H  G  G  L  S  P
gatcttcaatctatggagcagattcggcgaattatgcgaccaactgatgtaccagatcaa
 D  L  Q  S  M  E  Q  I  R  R  I  M  R  P  T  D  V  P  D  Q
ggtcttctttgtgatcttttgtggtctgaccccgataaagatgtcttaggctggggtgaa
 G  L  L  C  D  L  L  W  S  D  P  D  K  D  V  L  G  W  G  E
aatgacagaggagtgtccttcacatttggtgcagaagtggttgcaaaatttctccataag
 N  D  R  G  V  S  F  T  F  G  A  E  V  V  A  K  F  L  H  K
catgatttggatcttatatgtagagcccatcaggtggttgaagatggatatgaatttttt
 H  D  L  D  L  I  C  R  A  H  Q  V  V  E  D  G  Y  E  F  F
gcaaagaggcagttggtcactctgttttctgcgcccaattattgcggagagtttgacaat
 A  K  R  Q  L  V  T  L  F  S  A  P  N  Y  C  G  E  F  D  N
gcaggtgccatgatggtggatgaaacactaatgtgttcttttcagattttaaagcctgca
 A  G  A  M  M  V  D  E  T  L  M  C  S  F  Q  I  L  K  P  A
gagaaaaagaagccaaatgccacgagacctgtaacgcctccaaggggtatgatcacaaag
 E  K  K  K  P  N  A  T  R  P  V  T  P  P  R  G  M  I  T  K
caagcaaagaaatag
 Q  A  K  K  *
```

Figure II.3: *Partial sequence of the pAS-PP1γ1 construct. Human PP1γ1 sequence is in green and blue; Sequence marked in green is a non-coding sequence in the human PP1γ1; pAS2-1 sequence that is fused to PP1γ1 is in black; splice site of PP1γ is in red; Pst I restriction site is in orange; Initiantion codon is in pink; Stop codon is in grey.*

II.3.1.2 Construction of pAS-PP1γ2

The cDNA for mouse PP1γ2 was a generous gift from E.Y.C. Lee (Zhang *et al.*, 1993). This cDNA was inserted into the expression vector pTACTAC. However, the mouse PP1γ2 cDNA provided could not be used since sequencing of this plasmid revealed that it contained a mutation that resulted in a premature stop, thus yielding a C-terminally truncated protein (Fig. II.4). Therefore, the carboxyl terminus of PP1γ2 was taken from this vector by digestion with *Pst*I and subcloned into *Pst*I-digested pAS-PP1γ1giving the pAS-PP1γ2 plasmid (the N-terminal of this cDNA was from human origin and the C-terminus from mouse). The resulting plasmid was sequenced to check the sequence of the insert cDNA PP1γ2 and also to check if the fusion protein was in the correct reading frame (Fig. II.5).

```
ratPP1gamma2      AGCTCCTCCTCTCGCCACTGGAACCACGAGAAGAGGAGGAAGCCGGGAGCGGGGCGGCTG 60
LeePP1gamma2      AGCTCCTCCTCTCGCCACTGGAACCACGAGAAGAGGAGGAAGCCGGGAGCGGGGCGGCTG 60

ratPP1gamma2      GGGGGGGGACCCGCCGCGGCTGCTGCTGCCACCGCCGCCGCCACCACCGCTCGTGGGGCT 120
LeePP1gamma2      GGGGGGGGACCCGCCGCGGCTGCTGCTGCCACCGCCGCCGCCACCACCGCTCGTGGGGCT 120

ratPP1gamma2      CGTGGCGTGAAGAAGGAGGACGAGTGAGACCCGGGCGCGACGGGGCGCGTGCGGGAGGGT 180
LeePP1gamma2      CGTGGCGTGAAGAAGGAGGACGAGTGAGACCCGGGCGCGACGGGGCGCGTGCGGGAGGGT 180

ratPP1gamma2      CGGCGGCGGGACGGCGATGGCGGATATCGATAAACTCAACATCGACAGTATCATCCAACG 240
LeePP1gamma2      CGGCGGCGGGACGGCGATGGCGGATATCGATAAACTCAACATCGACAGTATCATCCAACG 240

ratPP1gamma2      GCTGCTGGAAGTGAGAGGGTCCAAGCCAGGCAAGAATGTCCAGCTCCAGGAGAATGAAAT 300
LeePP1gamma2      GCTGCTGGAAGTGAGAGGGTCCAAGCCAGGCAAGAATGTCCAGCTCCAGGAGAATGAAAT 300

ratPP1gamma2      CCGGGGACTGTGCTTGAAGTCTCGGGAGATCTTCCTCAGTCAGCCTATCCTTTTAGAACT 360
LeePP1gamma2      CCGGGGACTGTGCTTGAAGTCTCGGGAGATCTTCCTCAGTCAGCCTATCCTTTTAGAACT 360

ratPP1gamma2      TGAAGCACCACTCAAGATATGTGGTGACATCCACGGGCAGTACTATGATTTGCTCCGTCT 420
LeePP1gamma2      TGAAGCACCACTCAAGATATGTGGTGACATCCACGGGCAGTACTATGATTTGCTCCGTCT 420

ratPP1gamma2      GTTTGAATACGGTGGCTTTCCTCCAGAAAGCAACTATTTGTTTCTCGGGGACTATGTGGA 480
LeePP1gamma2      GTTTGAATACGGTGGCTTTCCTCCAGAAAGCAACTATTTGTTTCTCGGGGACTATGTGGA 480

ratPP1gamma2      CAGGGGCAAACAGTCACTAGAGACGATCTGCCTCTTGCTGGCCTACAAAATCAAGTATCC 540
LeePP1gamma2      CAGGGGCAAACAGTCACTAGAGACGATCTGCCTCTTGCTGGCCTACAAAATCAAGTATCC 540

ratPP1gamma2      GGAGAACTTTTTTCTTCTTAGAGGGAACCATGAGTGTGCCAGCATCAATAGAATCTACGG 600
LeePP1gamma2      GGAGAACTTTTTTCTTCTTAGAGGGAACCATGAGTGTGCCAGCATCAATAGAATCTACGG 600

ratPP1gamma2      ATTTTATGATGAGTGTAAGAGAAGATACAACATTAAGCTGTGGAAAACGTTCACAGACTG 660
LeePP1gamma2      ATTTTATGATGAGTGTAAGAGAAGATACAACATTAAGCTGTGGAAAACGTTCACAGACTG 660

ratPP1gamma2      TTTTAACTGCTTACCGATAGCAGCCATCGTGGACGAGAAGATATTCTGCTGTCATGGAGG 720
LeePP1gamma2      TTTTAACTGCTTACCGATAGCAGCCATCGTGGACGAGAAGATATTCTGCTGTCATGGAGG 720

ratPP1gamma2      TTTATCACCAGATCTTCAATCTATGGAGCAGATTCGGCGAATTATGAGACCAACTGATGT 780
LeePP1gamma2      TTTATCACCAGATCTTCAATCTATGGAGCAGATTCGGCGAATTATGAGACCAACTGATGT 780

ratPP1gamma2    ACCAGATCAAGGTCTTCTTTGTGATCTTTTGTGGTCTGACCCCGATAAAGATGTCTTTGG 840
                  P  D  Q  G  L  L  C  D  L  L  W  S  D  P  D  K  D  V  F  G
                  P  D     R  S  S  L  *
LeePP1gamma2    ACCAGAT--AGGTCTTCTTTGTGATCTTTTGTGGTCTGACCCCGATAAAGATGTCTTAGG 838

ratPP1gamma2      CTGGGGTGAAAATGACAGAGGAGTGTCCTTCACATTTGGTGCAGAAGTGGTTGCAAAATT 900
LeePP1gamma2      CTGGGGTGAAAATGACAGAGGAGTGTCCTTCACATTTGGTGCAGAAGTGGTTGCAAAATT 898
```

```
ratPP1gamma2      TCTCCATAAGCATGATTTGGATCTTATATGTAGAGCCCATCAGGTGGTTGAAGATGGATA 960
LeePP1gamma2      TCTCCATAAGCATGATTTGGATCTTATATGTAGAGCCCATCAGGTGGTTGAAGATGGATA 958

ratPP1gamma2      TGAGTTTTTTGCAAAGAGGCAGTTAGTCACTCTGTTTTCTGCACCCAACTACTGTGGCGA 1020
LeePP1gamma2      TGAGTTTTTTGCAAAGAGGCAGTTAGTCACTCTGTTTTCTGCACCCAACTACTGTGGCGA 1018

ratPP1gamma2      GTTTGACAATGCGGGCGCCATGATGAGTGTGGATGAGACCCTCATGTGCTCCTTCCAGAT 1080
LeePP1gamma2      GTTTGACAATGCGGGCGCCATGATGAGTGTGGATGAGACCCTCATGTGCTCCTTCCAGAT 1078

ratPP1gamma2      TTTAAAGCCTGCAGAGAAAAAGAAGCCCAATGCCACGAGACCTGTCACACCGCCACGGGT 1140
LeePP1gamma2      TTTAAAGCCTGCAGAGAAAAAGAAGCCCAATGCCACGAGACCTGTCACACCGCCACGGGT 1138

ratPP1gamma2      TGGATCAGGCCTGAACCCGTCCATTCAGAAAGCTTCAAATTATAGAAACAACACTGTCCT 1200
LeePP1gamma2      TGGATCAGGCCTGAACCCGTCCATTCAGAAAGCTTCAAATTATAGAAACAACACTGTCCT 1198

ratPP1gamma2      ATACGAGTGATCGATAATGCTTTCTTTGGCTACATTCTTTATTCTGCGGTGACATTGAGG 1260
LeePP1gamma2      ATACGAGTGATCGATAATGCTTTCTTTGGCTACATTCTTTATTCTGCGGTGACATTGAGG 1258

ratPP1gamma2      CTTATAAATCAAAAGGAACTAACTTGCCGTCCACCGGTTTATACAGAACTCACAGTATCT 1320
LeePP1gamma2      CTTATAAATCAAAAGGAACTAACTTGCCGTCCACCGGTTTATACAGAACTCACAGTATCT 1318

ratPP1gamma2      ATGACTTTTTTAAACTACGACCTGTTAAAATGAATCTGTTTCCACAGATGCCGTGTACAA 1380
LeePP1gamma2      ATGACTTTTTTAAACTACGACCTGTTAAAATGAATCTGTTTCCACAGATGCCGTGTACAA 1378

ratPP1gamma2      TGCCATGTGCTAAGAATGATTTCAGACTTATTAAATGCGAGCTTGTT 1427
LeePP1gamma2      TGCCATGTGCTAAGAATGATTTCAGACTTATTAAATGCGAGCTTGTT 1425
```

Figure II.4: *Alignment of the rat PP1γ2 (Gi:3236123) versus the sequence of rat PP1γ2 provided by E.Y.C. Lee. The PstI site is marked in green, the splice site is marked in red, the stop codon in grey . The cDNA provided by E.Y.C. Lee has a substitution T838-A836 and a two bases deletion (C788A789) marked in blue. The last mutation alters the open reading frame leading to a premature stop codon (in pink) that originates a 22kDa protein (data not shown).*

```
atgaagctactgtcttctatcgaacaagcatgcgatatttgccgacttaaaaagctcaag
 M  K  L  L  S  S  I  E  Q  A  C  D  I  C  R  L  K  K  L  K
tgctccaaagaaaaaccgaagtgcgccaagtgtctgaagaacaactgggagtgtcgctac
 C  S  K  E  K  P  K  C  A  K  C  L  K  N  N  W  E  C  R  Y
tctcccaaaaccaaaaggtctccgctgactagggcacatctgacagaagtggaatcaagg
 S  P  K  T  K  R  S  P  L  T  R  A  H  L  T  E  V  E  S  R
ctagaaagactggaacagctatttctactgatttttcctcgagaagaccttgacatgatt
 L  E  R  L  E  Q  L  F  L  L  I  F  P  R  E  D  L  D  M  I
ttgaaaatggattctttacaggatataaaagcattgttaacaggattatttgtacaagat
 L  K  M  D  S  L  Q  D  I  K  A  L  L  T  G  L  F  V  Q  D
aatgtgaataaagatgccgtcacagatagattggcttcagtggagactgatatgcctcta
 N  V  N  K  D  A  V  T  D  R  L  A  S  V  E  T  D  M  P  L
acattgagacagcatagaataagtgcgacatcatcatcggaagagagtagtaacaaaggt
 T  L  R  Q  H  R  I  S  A  T  S  S  S  E  E  S  S  N  K  G
caaagacagttgactgtatcgccggtattgcaatacccagctttgactcatatggccatg
 Q  R  Q  L  T  V  S  P  V  L  Q  Y  P  A  L  T  H  M  A  M
gaggccgaattcccgggcgcgagccggcggcggcgccgctgcgggagggtcggcggtggg
 E  A  E  F  P  G  A  S  R  R  R  R  R  C  G  R  V  G  G  G
aaggcgatggcggatttagataaactcaacatcgacagcattatccaacggctgctggaa
 K  A  M  A  D  L  D  K  L  N  I  D  S  I  I  Q  R  L  L  E
gtgagagggtccaagcctggtaagaatgtccagcttcaggagaatgaaatcagaggactg
 V  R  G  S  K  P  G  K  N  V  Q  L  Q  E  N  E  I  R  G  L
tgcttaaagtctcgtgaaatctttctcagtcagcctatcctactagaacttgaagcacca
 C  L  K  S  R  E  I  F  L  S  Q  P  I  L  L  E  L  E  A  P
ctcaaaatatgtggtgacatccatggacaatactatgatttgctgcactttttgagtac
 L  K  I  C  G  D  I  H  G  Q  Y  Y  D  L  L  R  L  F  E  Y
ggtggtttcccaccagaaagcaactacctgtttcttggggactatgtggacaggggaaag
 G  G  F  P  P  E  S  N  Y  L  F  L  G  D  Y  V  D  R  G  K
cagtcattggagacgatctgcctcttactggcctacaaaataaaatatcctgagaatttt
 Q  S  L  E  T  I  C  L  L  L  A  Y  K  I  K  Y  P  E  N  F
```

```
tttcttctcagagggaaccatgaatgtgccagcatcaacagaatttatggattttatgat
 F  L  L  R  G  N  H  E  C  A  S  I  N  R  I  Y  G  F  Y  D
gaatgtaaaagaagatacaacattaaactatggaaaactttcacagactgttttaactgt
 E  C  K  R  R  Y  N  I  K  L  W  K  T  F  T  D  C  F  N  C
ttaccgatagcagccatcgtggatgagaagatattctgctgtcatggaggtttatcacca
 L  P  I  A  A  I  V  D  E  K  I  F  C  C  H  G  G  L  S  P
gatcttcaatctatggagcagattcggcgaattatgcgaccaactgatgtaccagatcaa
 D  L  Q  S  M  E  Q  I  R  R  I  M  R  P  T  D  V  P  D  Q
ggtcttctttgtgatcttttgtggtctgaccccgataaagatgtcttaggctggggtgaa
 G  L  L  C  D  L  L  W  S  D  P  D  K  D  V  L  G  W  G  E
aatgacagaggagtgtccttcacatttggtgcagaagtggttgcaaaatttctccataag
 N  D  R  G  V  S  F  T  F  G  A  E  V  V  A  K  F  L  H  K
catgatttggatcttatatgtagagcccatcaggtggttgaagatggatatgaatttttt
 H  D  L  D  L  I  C  R  A  H  Q  V  V  E  D  G  Y  E  F  F
gcaaagaggcagttggtcactctgttttctgcgcccaattattgcggagagtttgacaat
 A  K  R  Q  L  V  T  L  F  S  A  P  N  Y  C  G  E  F  D  N
gcaggtgccatgatggtggatgaaacactaatgtgttcttttcagattttaaagcctgca
 A  G  A  M  M  V  D  E  T  L  M  C  S  F  Q  I  L  K  P  A
gagaaaaagaagcccaatgccacgagacctgtcacaccgccacgggttggatcaggcctg
 E  K  K  K  P  N  A  T  R  P  V  T  P  P  R  V  G  S  G  L
aacccgtccattcagaaagcttcaaattatagaaacaacactgtcctatacgagtgatcg
 N  P  S  I  Q  K  A  S  N  Y  R  N  N  T  V  L  Y  E  *
ggggtaccgaattcctcgagtctaggggatccgtcgacctgcag
```

Figure II.5: Partial s*equence of the pAS-PP1γ2 construct. PP1γ2 sequence is in green and blue; Sequence marked in green is a non-coding sequence in the human PP1γ2; pAS2-1 sequence that is fused to PP1γ1 is in black; splice site of PP1γ is in red; Pst I restriction site is in orange; Initiantion codon is in pink; Stop codon is in grey.*

II.3.2 Expression of the bait proteins

In order to verify the ability of the recombinant constructs to drive PP1 expression, they were transformed into yeast strain AH109, the transformed cells were grown on the appropriate media and PP1 expression was confirmed by immunobloting of the corresponding protein extracts (Fig. II.6). To detect the fusion protein PP1γ1/GAL4-BD the immunoblot was performed using an anti-PP1γ1 antibody (da Cruz e Silva *et al.*, 1995b), and an anti-rabbit secondary antibody conjugated to peroxidase. A band of the expected molecular mass was detected only in protein extracts from yeast cells containing the pAS-PP1γ1 plasmid (Fig. II.6A). A non-specific band was detected both in the controls (protein extracts from AH109 yeast cells and from AH109 yeast cells transformed with the vector pAS2-1 alone) and in the protein extracts from yeast cells containing the pAS-PP1γ1 plasmid. Detection of the PP1γ2/GAL4-BD protein used an anti-PP1γ2 antibody prepared essentially as described in (da Cruz e Silva *et al.*, 1995b; Vijayaraghavan *et al.*, 1996), that recognizes the C-terminal sequence of PP1γ2. A protein extract from human

testis was used as a positive control, and as a negative control the protein extract from untransformed AH109 yeast cells was used (Fig. II.6B).

None of the constructs activated transcription from the UAS (the DNA sequence that is recognized by GAL4-BD) as detected by lack of growth of AH109 yeast cells containing the different bait plasmids on selective media (data not shown).

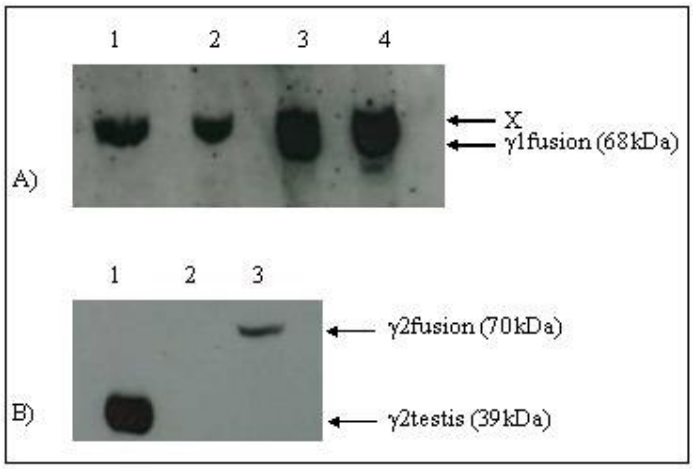


Figure II.6: *Immunoblot analysis of protein extracts from AH109 yeast transformed with pAS-PP1γ1 (A) or pAS-PP1γ2 (B).* ***A)****, The blot was probed with anti-PP1γ1 and X represents a non-specific immunoreactive yeast protein. Lane 1, untransformed yeast control; Lane 2, pAS2-1 vector control; Lanes 3 and 4, two independent pAS-PP1γ1 clones.* ***B)****, The blot was probed with anti-PP1γ2. Lane 1, protein extract from rat testis; Lane 2, untransformed yeast control; Lane 3, pAS-PP1γ2 extract. The calculated molecular masses (in kDa) of the fusion proteins is indicated.*

Once yeast cell expression of the fusion proteins GAL4-BD/PP1γ1 and GAL4-BD/PP1γ2 was confirmed, the yeast two hybrid screens were performed.

II.3.3 Identification of PP1 interacting clones

After transforming the bait plasmids into the appropriate yeast strain, AH109 (mat a), the next step was to obtain the desired library pretransformed in a yeast strain of the opposite mating type. In our case we used a pretransformed pACT-2 library in the yeast strain Y187 (mat α) containing human testis cDNA sequences fused to the GAL4 transactivation domain (AD). By using a pretransformed library the most costly and time-

consuming steps of the library screening are already done. These include, constructing the library, amplifying the library in *E. coli*, isolating the library DNA, performing the library-scale transformation of yeast strain Y187, plating the transformation mixture and harvesting the transformants at high viability and density in freezing medium.

The YTH screens were performed by yeast mating, instead of the more commun cotranformation protocol. By using the yeast mating protocol more unique positive clones are obtained due primarily to the "jump-start" that the new diploids receive before being plated on selective medium. Besides that, diploid yeast cells are more vigorous than haploid cells and can better tolerate the expression of toxic proteins. Also, in diploids, the reporters are less sensitive to transcription activation than they are in haploids, reducing the incidence of false positives from transactivating baits.

The human testis cDNA library was screened to identify new interacting partners for PP1γl (YTH1) and PP1γ2 (YTH2). Both screens were performed by mating as described in the methods section. The mating culture was checked under a phase-contrast microscope to check for the occurrence of zygotes (Fig. II.7), indicative that mating was occurring as expected.

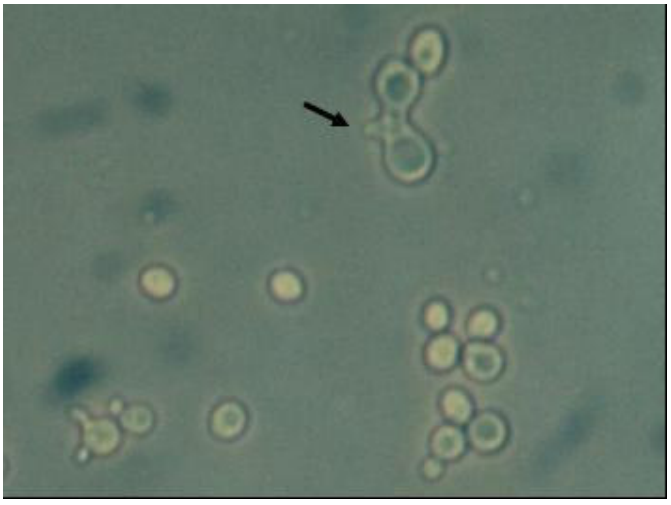


Figure II.7: *Zygote formation in the mating mixture with its typical three-lobed shape. The arrow is pointing the budding diploid cell. The other two lobes are the two haploid (parental) cells. This picture was taken using an inverted microscope, during the mating procedure (40X magnification).*

After plating the mating mixture in the appropriate selective media and waiting several days for colonies to appear, the growth on the control plates was scored and the mating efficiency and number of clones screened were calculated (Table II.2).

Table II.2: *Results from the yeast two hybrid screens.*

YTH screen	Bait	Positive clones	Mating efficiency (% diploids)	Clones screened
YTH1	PP1γ1	120	5	9×10^7
YTH2	PP1γ2	155	5	5×10^7

To calculate de number of clones screened the following equation was used [# cfu/ml of diploids X resuspension volume]. To calculate the mating efficiency the following equation was used [# cfu (in SD –Leu/-Trp) X 1000µl/ml/ volume plated (µl) x dilution factor] / [# cfu (in SD -Trp) X 1000µl/ml/ volume plated (µl) X dilution factor] X 100.

For YTH1 268 clones were colected and for YTH2 333 clones were isolated (Fig. II.8) in SD/QDO media. This media tests the expression of the nutritional reporter genes HIS3 and ADE2. These clones were further tested for MEL1 expression, another reporter gene, by growing these putative primary positive clones in SD/QDO media with X-α-Gal. True positive clones turn a blue colour (Fig. II.9). 120 and 155 true positive clones were thus identified for the YTH1 and the YTH2, respectively.

A)

1Q	3Q	4Q	5Q	7Q	8Q	9Q	12Q	13Q	17Q	20Q	26Q
27Q	29Q	30Q	31Q	32Q	33Q	34Q	35Q	36Q	37Q	39Q	40Q
41Q	44Q	45Q	46Q	47Q	48Q	49Q	53Q	57Q	55Q	58Q	4T
12T	13T	20T	22T	27T	29T	31T	32T	33T	36T	37T	39T
42T	43T	48T	50T	55T	58T	60T	62	101T	113T	118T	120T
121T	125T	127T	129T	131T	133T	135T	136T	137T	138T	141T	143T
144T	146T	152T	153T	155T	156T	157T	158T	161T	162T	164T	201T
208T	210T	212T	214T	219T	220T	223T	224T	226T	227T	228T	229T
232T	23T	236T	240T	244T	245T	246T	249T	251T	252T	254T	255T
256T	257T	258T	259T	260T	261T	302T	303T	304T	305T	311T	316T

B)

C)

21	22	24	25	26	212	213	214	215	216	2165	2168	2174	2175	2176	2178	2179	2183	2186	2187
217	218	219	220	225	226	229	231	232	239	2188	2189	2192	2194	2198	2202	2204	2205	2209	2210
244	245	247	253	260	261	262	263	264	265	2211	2215	2216	2217	2218	2220	2223	2224	2226	2227
266	268	269	270	271	272	273	275	276	278	2229	2231	2232	2234	2235	2236	2237	2238	2239	2240
279	280	284	285	287	289	291	294	296	298	2247	2248	2250	2252	2254	2255	2257	2258	2259	2261
2100	2101	2104	2109	2110	2123	2124	2133	2138	2140	2263	2264	2267	2268	2269	2271	2272	2275B	2276	2277
2141	2142	2143	2144	2145	2146	2147	2149	2150	2151	2278	2283	2287	2285	2296	2305	2307	2308	2324	2325
2152	2153	2155	2158	2159	2160	2161	2162	2163	2164	2326	2327	2329	2331	2332					

D)

Figure II.8: *Positive clones identified in the yeast two hybrid screens using PP1γ1 as bait (A and B) or PP1γ2 (C and D). The numbers in B and D reflect their recovery from the original SD/TDO or SD/QDO plates.*

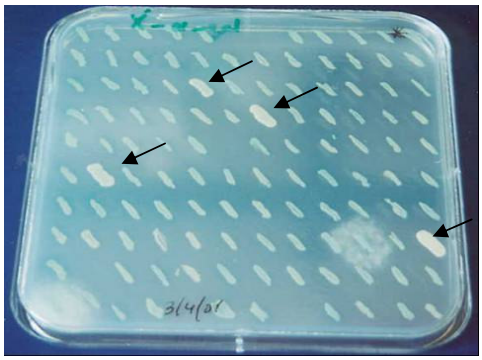


Figure II.9: *MEL-1 expression test of the positive clones obtained in the YTH1 screen. White colonies (arrows) represent false positives that grew on SD/QDO medium but could not turn blue in the presence of X-α-GAL.*

II.4 DISCUSSION

As with all detection methods, the YTH system is known to result in the detection of some false positives. This was a relatively serious problem in the early days of the YTH method but the elimination of such false positive results has been greatly improved nowadays. False positive signals result from cells in which the reporter genes are active even though the bait and prey do not interact. There are several classes of false positives. For example, false positives may arise from preys that interact with DNA upstream of the reporter genes or with proteins that interact with promoter sequences. These two classes of false positives can be eliminated by the use of more than one reporter gene under the control of different promoters, as was the case with the present work. Another inherent problem with the system is that not all proteins will be efficiently folded and/or post-translationaly modified in the yeast nucleus, which may result in the protein not interacting with the true partner. In the same way, the protein may adopt a different tertiary structure when expressed as fusions with the transcription factor domains. Also, some proteins may be toxic when expressed as fusions in yeast, inhibiting growth when expressed at high levels. This can be circumvented to some extent by the use of inducible expression plasmids. Other false positive results include interactions that occur in the YTH screen but not in a physiological context, because the partners are not expressed in the same cellular or subcellular environment at the same time.

By screening 9 X 10^7 clones from a human testis cDNA library with PP1γ1 as bait we obtained 120 positive clones as accessed by their ability to grow on SD/ODO selective media and to turn blue in the presence of X-α-Gal. Similarly, the screening of 5 X 10^7 clones with PP1γ2 as bait originated 155 positive clones. We observed multiple colonies (268 and 333 for the YTH1 and YTH2, respectively) that were able to induce the expression of the nutritional reporters (thus growing on SD/QDO) although they were not considered as true positives since they do not induce the expression of another reporter gene, MEL-1.

In conclusion, supplementary data from other sources should be used to evaluate the credibility of interactions in an YTH screen. Thereby, the verification of a putative interaction can be achieved in a variety of ways. One approach is to mix the recombinant

proteins and verify binding *in vitro* through a variety of biochemical assays. Another approach is to express both proteins in cells by transfection and analyse interactions by immunoprecipitation studies. However, even if co-immunoprecipitation is successful, there is still the possibility that the proteins only interact under the conditions used. So, a crucial validation of the two hybrid results is to prove that the two proteins exist in the same subcellular environment, by doing immunoprecipitation in the tissue of interest.

III CHARACTERIZATION OF THE POSITIVE CLONES

III.1 INTRODUCTION

The vast majority of the PP1 binding proteins that have been identified to date were discovered using the yeast two hybrid (YTH) method. Often, when performing an YTH screen, only a few clones are selected and further characterized. Using such an approach many of the rarer positive clones are never analyzed, and some important potential interactors may be missed.

In our screens we obtained 120 positive clones for the interactions with PP1γ1 and 155 for the interactions with PP1γ2. We decided to analyse all the positive clones in order to identify not only the most abundant clones but also the more interesting ones, even though they may have been detected only once or twice in the screen. This does not mean they are not important but may simply reflect the low abundance of the mRNA in the library used or its low abundance in the tissue from which the library was made.

All positive clones were partially sequenced and some of the clones were further analyzed.

III.2 MATERIALS AND METHODS

For the complete composition of all reagents, media and solutions used, see the list presented in Appendix I. All reagents were cell culture grade or ultrapure.

III.2.1 Plasmid isolation from yeast and transformation into bacteria

Yeast plasmid DNA was extracted by resuspending cells in 0.2ml of breaking buffer [2% Triton X-100/ 1% SDS/ 100mM NaCl/ 10mM Tris-HCl (pH 8.0)], adding 0.3g of 0.5mm acid-washed glass beads plus 0.2ml 25:24:1(v/v/v) phenol/chloroform/isoamyl alcohol and vortexing for 2min before centrifuging for 5min. DNA was ethanol precipitated.

An alternative method was also used for isolating yeast plasmid DNA and turned out to be more efficient: 3ml of yeast cells were pelleted and the DNA extracted using the QUIAGEN kit (Chapter II).

Both types of DNA were used to transform *E. coli* XL1-Blue and plasmid DNA was isolated from single colonies as described in Chapter II.

III.2.2 Analysis of the positive plasmids by restriction digestion, sequencing and database searching

Plasmid DNA was digested with the restriction endonuclease *Hind*III and fragments produced were separated by agarose gel electrophoresis as described in Chapter II.

The cloning vector used (pACT-2) produced a characteristic pattern of fragments that allowed its differentiation from colonies resulting from transformation by the bait vector. Plasmids generating DNA fragments characteristic of the pACT-2+library insert digested with *Hind* III were further analysed. The DNA was purified (500ng) for the sequencing PCR (Chapter II) with 3.2 picomole GAL4-AD primer (1µl), 4µl of Term Ready Reaction Mix (Applied Byosystems) to a final volume of 20µl with dH_2O. PCR conditions were as follow:

96ºC 10sec
50ºC 5sec } 25 cycles
60ºC 4min

The PCR products were ethanol precipitated before being sequenced using an Applied Byosystems automated DNA sequencer, according to the manufacturer's instructions, using Taq dye terminator cycle sequencing. A search for similar sequences in the Genbank database was performed using the BLAST algorithm (Altschul *et al.*, 1997).

III.2.3 Yeast colony hybridization

A nitrocellulose filter was placed on an agar plate containing the selective media (SD/QDO). Isolated yeast colonies were patched onto the filter and incubated overnight at 30ºC. For each filter to be processed one piece of 3MM Whatman paper was cut, placed in a 150mm petri dish and soaked with 6 ml of freshly prepared SCE/DTT/Lyticase solution.

The filter was lifted from the growth medium and carefully placed, cell side up, onto the saturated paper, removing all air bubbles beneath the filter. The Petri dish containing the membrane was closed, placed into a plastic bag and incubated overnight at 30ºC. Then, a fresh sheet of plastic wrap was lined on the bench and pieces of 3MM Whatman paper were soaked with the solutions described below.

1) 10% SDS, 5min

2) 0.5N NaOH, 10min

3) 200mM Tris(pH 7.5)/ 2X SSC, 5min (repeated 3X)

The nitrocellulose filters were then placed on a 3 MM Whatman sheet to air dry for 1h and baked for 2h in a 80ºC vacuum oven between pieces of Whatman. Finally, they were stored between pieces of Whatman 3MM filter paper until being used for hybridization. Filter hybridization was performed using specific oligonucleotides.

Before use each oligonucleotide was 5' end-labelled using T4 polynucleotide kinase (Roche) and [γ-^{32}P] ATP (3000Ci/mmol, 10mCi/ml) from Amersham Pharmacia as follows: 30picomole (90µCi) of [γ-^{32}P] ATP (9µl) were added to 37.5µl 60mM Tris.HCl (pH 7.8)/ 10mM $MgCl_2$ / 14mM 2-mercaptoethanol containing 30picomole of oligonucleotide (10µl). The reaction was initiated by the addition of 15 units of T4 polynucleotide kinase (1.5µl) and incubated for 30min at 37ºC. 2µl of 0.5M EDTA (pH 8.0) were added to stop the reaction and the resulting labelled oligonucleotide was added directly to the pre-hybridization solution.

The baked nitrocellulose filters were wetted in dH_2O and transferred to 3X SSC/ 0.1% SDS/ 1mM EDTA at 45ºC for 15min. The cellular debris was removed from the membrane by gently rubbing with fingertips over a plastic sheet. The membranes were left in the same solution for another 15min with shaking. The procedure was repeated and the membranes transferred to a glass hybridization bottle containing approximately 0.1ml of prehybridization buffer per cm^2 of membrane. Prehybridization was carried out 5 degrees below the Tm of the oligo for 4h to overnight in a rotary hybridization oven. The radiolabelled oligonucleotide was then added to a final concentration of 0.8pmol/ml and hybridization was performed at the same temperature for a minimum of 12h with constant shaking. At the end of the hybridization period the membrane was transferred to a plastic box containing 6X SSC prewarmed to the hybridization temperature. The membrane was washed with constant shaking for 15min and the wash solution was replaced and the

membrane washed for further 15min. A final wash was performed for 10min. Then, the membrane was removed from the solution, sealed in a plastic bag and autoradiographed overnight at –70ºC using a Kodak cassette and intensifying screens. Autoradiographs on X-Omat S film were developed as described in Chapter II.

III.2.4 Isolation of total RNA from mammalian tissues

The frozen tissues (~500mg) were placed into 9ml of Denaturing Solution (Promega kit Z5110), previously chilled on ice, and disrupted with a manual homogenizer. Then, 900µl 2M Sodium Acetate (pH 4.0) was added and mixed thoroughly by inverting the tube 4-5 times. After, the 9ml of Phenol: Chloroform: Isoamyl Alcohol was added to the tube. The tube was capped and the contents mixed by inverting the tube 3-5 times and then shaking vigorously for 10sec. The tube was chilled on ice for 15min and then, the mixture was transferred to DEPC-treated tube and centrifuged at 10,000g for 20min at 4ºC. The top aqueous phase, that contains the RNA, was carefully removed and transferred to a fresh DEPC-treated tube. An equal volume of isopropanol was added to the aqueous phase and incubated at –20ºC for 20min to precipitate the RNA. The RNA was then pelleted by centrifugation at 10,000g for 10min at 4ºC. The RNA pellet was resuspended in 5ml of Denaturing Solution and vortexed until the RNA was dissolved. An equal volume of isopropanol was added and the RNA was precipitated again by incubating at –20ºC for 20min and centrifuging at 10,000g for 10min at 4ºC. The resulting pellet was washed by adding 10ml of 75% ethanol, resuspended by pipeting with a sterile, RNase-free tip and centrifuged at 10,000g for 10min at 4ºC. The pellet was air-dried, resuspended in Nuclease-Free Water and stored at –20ºC.

III.2.5 Electrophoretic analysis of RNA

An agarose gel was prepared by melting the appropriate amount of agarose in water, cooling it to 60ºC, and adding 5X formaldehyde gel-running buffer and formaldehyde to produce a final concentration of 1X and 2.2M, respectively. The gel was then cast in a chemical hood and allowed to set for 30min at room temperature.

The RNA samples were prepared by mixing the following components in a sterile microtube:

- RNA (≈10µg) 4.5µl
- 5X formaldehyde gel-running buffer 2.0µl
- 17.5% formaldehyde 3.5µl
- 50% formamide 10.0µl

The samples were incubated for 15 min at 65ºC, chilled on ice and centrifuged to deposit all of the fluid in the bottom of the tubes. 1µl of ethidium bromide (1mg/ml) and 2µl of formaldehyde gel-loading buffer were added to the samples before electrophoresis.

Before applying the samples the gel was pre-run for 5 min at 5V/cm submerged in 1X formaldehyde gel-running buffer. After loading the samples the gel was run at 3V/cm for 3h. the integrity of the RNA samples was checked by photographing the gel under UV illumination.

The gels that were transferred to nitrocellulose were not stained with ethidium bromide because it can reduce the efficiency of Northern hybridization.

III.2.6 Northern blot analysis

Probe isolation - The DNA to be labelled was cut with the appropriate restriction enzymes in order to release the appropriate fragment. Then the fragments were separated by electrophoresis on 1% low melting agarose. After staining the gel in ethidium bromide (0.2µg/ml) for 30min, the desired band was cut out of the gel and placed in a pre-weighted microtube. After, 3ml of dH_2O were added per gram of gel slice and placed in a boiling water bath for 7 min. The sample was stored at –20ºC. Prior to using the DNA in a labelling reaction the DNA was denatured by boiling for 10 min and kept at 37ºC.

Labelling reaction - 25 ng of template DNA was dissolved in a final volume of 8µl dH_2O and denatured in a boiling water bath for 10min. Then, the following components were added to the DNA: 4µl of High Prime reaction mixture (Roche) containing random primer mixture, Klenow polymerase and reaction buffer followed by 1µl of dATP, 1µl of dGTP and 1µl of dTTP and 5µl (50µCi) [α^{32}P]dCTP (3000Ci/mmol). This mixture was

incubated for 1h at 37ºC. The reaction was stopped by adding 2µl 0.2M EDTA (pH 8.0) and heating at 65ºC for 10min.

Stratagene's NucTrap probe purification columns were used to rapidly separate unincorporated nucleotides from the radiolabeled DNA, according to the manufacturer's instructions.

Membrane hybridization - ExpressHyb Solution Clontech was warmed at 65ºC to completely dissolve any precipitate. The membrane, a Clontech rat Multiple Tissue Northern (MTN) membrane, was then prehybridized in 10ml of ExpressHyb Solution with continuous shaking at 68ºC for 2h. Meanwhile, the radioactively labelled DNA probe was denatured at 100ºC for 5min and chilled on ice. After, the probe was added to the prehybridized solution and the membrane was incubated overnight.

The blot was then rinsed at RT in solution I (2X SSC/ 0.05% SDS), 3 times for 30min. Then, 2 washes with Solution I at 50ºC for 30min were performed. Finally, the blot was covered with plastic wrap and exposed for 1day in a K screen and visualised in a Phosphor Imager.

To remove the probe the blot was placed in 0.5% SDS at 90-100ºC for 10min with shaking. The solution was allowed to cool for 10min before removing the blot. Then the blot was slipped into a plastic bag and stored at –20ºC until needed. Complete removal of the probe was verified as described above prior to use.

III.2.7 PCR (polymerase chain reaction)

Amplification reactions were performed according to the manufacturer's instructions. Depending on the experiment, two different polymerases were used: Taq and Pfu (Promega). The latter is more accurate due to its proofreading capacity. Standard amplification reactions were performed in 0.2ml microtubes by adding10X reaction buffer, a mixture of the four dNTPs, two primers and the DNA template.

The mixtures were heated for 5 min at 94ºC to denature the DNA completely. After, the DNA polymerase (1µl) was added to the reaction. Typical conditions used are indicated below in Table III.1:

Table III.1: *Standard PCR conditions.*

Cycle	Denaturation	Annealing	Polymerization
First cycle	5 min, 94ºC	2 min at 50ºC	3 min at 72ºC
24 cycles	1 min, 94ºC	2 min at 50ºC	3 min at 72ºC
Last cycle	1 min, 94ºC	2 min at 50ºC	10 min at 72ºC

The annealing temperature varied with the primers in the reaction.

To verify the success of the PCR an aliquot was analysed by agarose gel electrophoresis.

III.2.8 cDNA synthesis and RT-PCR

cDNA synthesis - The control and experimental reactions were prepared by adding the following components to separate microtubes in order:

5.9µl RNase-free water (not DEPC-treated water)

1.0µl 10X StrataScript RT buffer (Strategene)

0.6µl oligo(dT) primer (100ng/ µl)

1.0µl dNTP mix (40mM)

1.0µl RNA (100ng) or 1.0µl control mRNA

Reactions were incubated at 65ºC for 5min and then cooled at RT for 5min to allow the primers to anneal to the RNA. After, 0.5µl of StrataScript Reverse Transcriptase (20U/µl) were added to each reaction. The tubes were incubated for 30min at 42ºC. At the end the completed first-strand cDNA synthesis reactions were placed on ice for subsequent use in the PCR amplification. The PCR conditions were as follows:

95ºC, 5 min

95ºC, 30 sec
55ºC, 30 sec } 25 cycles
68ºC, 3 min

All the resulting PCR products were sequenced to confirm the nucleotide sequence.

III.2.9 Overlay Blot

The partial cDNAs obtained for Nek2 and Nek2-T were used to express the corresponding proteins in bacteria. The purified protein samples were run on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then overlaid with purified PP1γ2 protein (Watanabe *et al.*, 2003) in TBST/BSA for 1h. After washing with TBST to remove excess protein, the bound PP1γ2 was detected by incubating the membrane with anti-PP1γ2 antibody (1:1000 in 5% milk in TBST). Immunoreactive bands were revealed by incubating with horseradish peroxidase conjugated secondary antibody (1:5000 in 5% milk in TBST) and developed by ECL (Amersham).

III.3 RESULTS

III.3.1 Preliminary analysis of the positive clones

In order to identify the library insert present in a given positive clone, the plasmid DNA must first be isolated from yeast. Thus, a mixture of different plasmidic DNAs can be isolated from a single yeast clone: the bait plasmid (Figure III.1, lanes 3 or 4) and one, or more, library plasmids (Figure III.1, lane 6), because each yeast cell can incorporate more than one library plasmid. So, in order to obtain single plasmids and pure DNA for sequence analysis, the plasmid DNA isolated from yeast cells was used to transform *E. coli* XL1-Blue. The plasmid DNA obtained from the resulting transformants was further analysed by restriction digestion with endonuclease *Hind*III. The restriction fragments were then separated by agarose gel electrophoresis. Figure III.1 exemplifies a typical result obtained after this procedure:

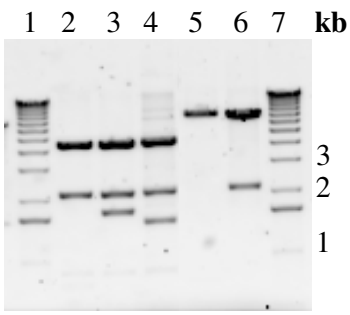


Figure III.1: *HindIII restriction analysis of yeast two hybrid plasmids. Lanes 1and 7, 1Kb ladder marker; Lane 2, pAS2-1 vector (4.6+2.2+0.9Kb); Lane 3, pASPP1γ1 bait plasmid (4.6+2.2+1.7+0.9 Kb); Lane 4; pASPP1γ2 bait plasmid (4.6+2.2+1.5+0.9+0.3Kb); Lane 5, pACT-2 vector (7.4+0.7Kb); Lane 6, pACT-2+library insert (7.4+(0.7+ ~1.5 insert)Kb).*

The same strategy was followed for each positive clone. After identifying transformants carrying the cDNA library plasmids, their respective inserts were sequenced with the GAL4-AD primer (Appendix II). Figure III.2 is a representative example obtained with one of the positive clones:

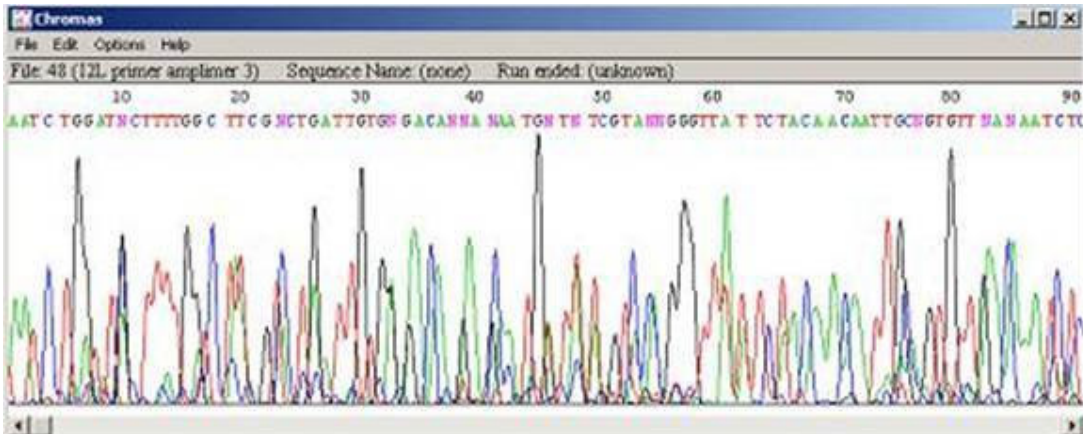


Figure III.2: *Nucleotide sequence of the positive clone 48.*

The nucleotide sequence of each clone was then converted to FASTA format (Fig. III.3). In this format, in the first line the signal > precedes a name or additional information on the sequence and the sequence itself starts on the second line.

>48 seq chrom sequence exported from chromatogram file (l2like)
CTCTGCTGTGCCGACCCTTCTCTTCGCGGACCCCACGCCAAGCAGCGACCCTGAGGCG
ACAGCCGGAGCGCCCGGCAATGGCGGCCTCGACGGCCTCCCACCGGCCCATCAAGGG
GATCTTGAAGAACAAGACCTCTACGACTTCCTCTATGGTGGCGTCGGCNNANCACCCCA
NNCCGANTGTNTANAAAANNCTTNANCAAAAATTCCATAANTGCGNATGAAANTNANNTC
TTGGNCNTCCNAANNTCCANANCAANAAGGCNTTGNGTNANTGANAATANATNACCNAC
CCTTCNTACCATAAAATNNATGGTGANNTTNANATCCNTNTNCGGCCTTGNCNNCCTTGN
AACATGGGNCTNAANTCNANTCATAATTTNNNCTNTTCCGCTTGANACCCANCNNCNNTC
NCNATNNCTCCCTNNNGANTCANNNNNAANNTTNCNTNAANACAAAAAATACGACATNTN
CAAANNGTACNTTACTNNGGNGANNANNNNTNNTNCNAATATATNTTANGCATCANNCCA
NANTNATNGTTTTCCTANGGNANTANCACNNAATTCNCGGTCTNTTNCTCCNAAANACNN
NNTNACAATTNNCACNACTACNAAANNNTNNNTATCGCTCATCACTANAATCACANNNCT
NGCAACGGNATTAATNANCNTGTNATCACGNTNACGGCATTTGGTGNGTAGTTGCAAAT
CNCTNCNNCACTTCNNCNNNACACCNNACTTACTANNG

Figure III.3: *Sequence of clone 48 in the FASTA format.*

Then, the GenBank sequence was copied to the BLAST window (Fig. III.4A) to be compared with the GenBank Database of nucleotide sequences. Figure III.4B shows a representative example of the results obtained.

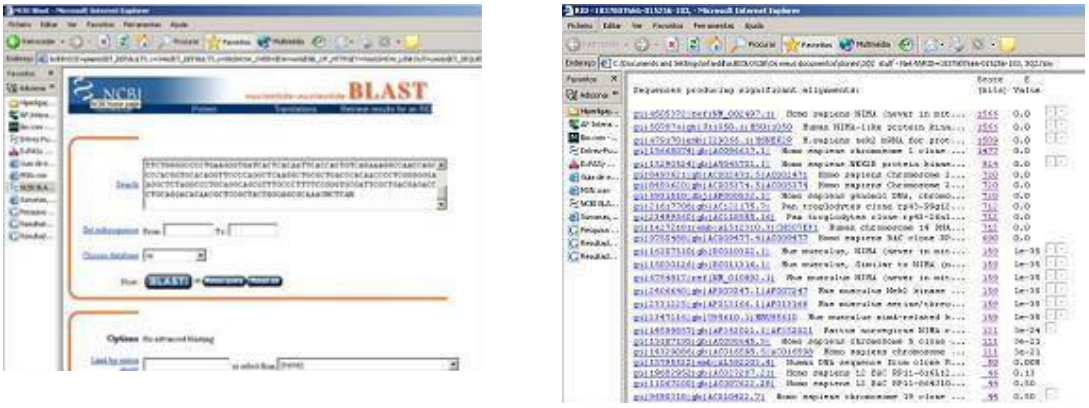


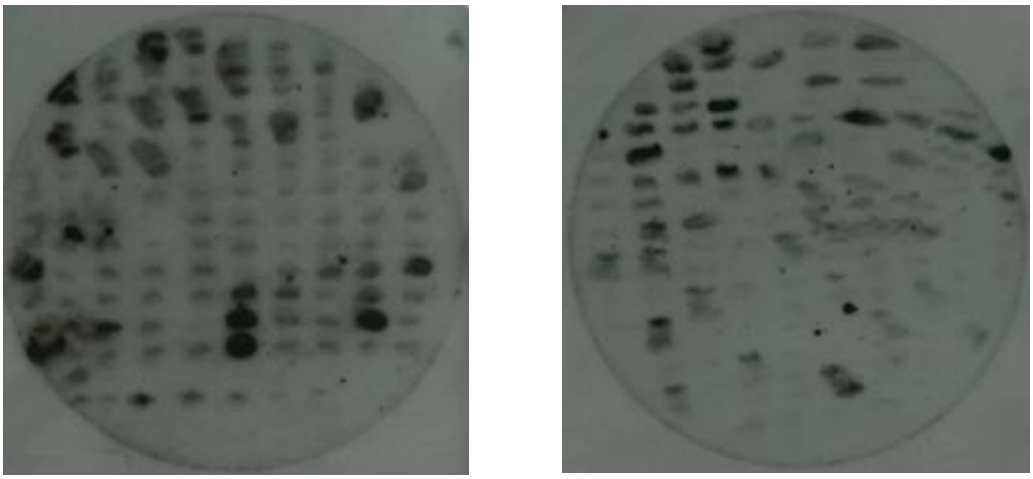
Figure III.4: *A, Blast window to introduce the query sequence; B, Blast results.*

The identity of the positive clones identified was thus obtained. After identifying around 30 positive clones from both screens, a large number was identified as a known PP1 regulator, named Nek2 by Cohen and coworkers (Helps *et al.*, 2000). Therefore, in order to reduce the number of clones that needed to be sequenced, all positives resulting from both screens (YTH1 and YTH2) were subjected to colony hybridization using appropriate oligonucleotides.

III.3.2 Yeast Colony hybridization analysis

Yeast colony hybridization is an efficient way to screen a large collection of library transformants for the presence of an abundant cDNA insert. Transformants carrying the same or overlapping library plasmid can be easily identified.

To quickly identify putative positive Nek2 clones among the large number of transformants obtained, a Nek2 specific oligonucleotide (OLIGONEK, Appendix II) was radiolabeled with ^{32}P-ATP and hybridised to the full collection of yeast positive colonies arrayed in nitrocellulose membranes. The results obtained are shown bellow in Fig. III.5 and summarized in Table III.2:



A) B)

Figure III.5: *Identification of putative Nek2 positives by colony hybridization:* ***A)*** *YTH1,* ***B)*** *YTH2.*

Table III.2: *Putative positive clones coding for Nek2.*

YTH1	1Q, 8Q, 33Q, 44Q, 45Q, 49Q, 53Q, 12T, 13T, 60T, 155T, 208T, 210T, 212T, 226T, 227T, 229T, 233T, 236T, 240T, 246T, 252T, 255T, 260T
YTH2	21, 239, 244, 261, 263, 264, 265, 269, 270, 272, 289, 291, 294, 296, 2123, 2146, 2147, 2159, 2160, 2204, 2220, 2235, 2254, 2263, 2269, 2283

This approach allowed the identification of several different clones in a short period of time, not only for Nek2 but also for other abundant positives. Such positives were set aside until all the others were sequenced.

III.3.3 Identification of the positive clones

Of the 120 positives identified in YTH1 and 155 positives identified in YTH2, 114 and 153, respectively, were recovered and definitively identified by partial or full DNA sequence analysis. The results obtained are summarized in Table III.3:

Table III.3: *Complete list of positives.*

Clone ID	YTH1	YTH2	PP1 BM	chr	Data base ID	Insert	Nº positives
Nek2A	7Q,8Q,30Q,31Q,36Q, 37Q,44Q,45Q,53Q, 57Q,4T,13T,60T,113T, 227T,236T,240TC,246T, 251T,252T,254TB,254TC, 257T,259TB,260T,261TB, 302T,311T,316T	21,24,25,212,225,226,239,244, 262,263,264,265,270,272,278, 284,289,291,294,296,2101,2104, 2109,2123,2140,2141,2142A,2144, 2146,2147D,2151,2152,2153,2158, 2159,2160,2163,2165,2168,2174, 2175,2176,2186,2187,2198,2204, 2209,2210,2215,2220,2234,2247, 2248,2252,2254,2258,2261,2263, 2267,2269,2276,2278,2283,2326 2329	KVHF	1	NM_002497	1170(3Q2)	94
Nek2A-T	3Q,5Q,12Q,13Q,17Q,20Q, 26Q,27Q,49Q,55QB,12T, 13TA,155T,208T,229T, 256T,303T,304TB	26,215,218,232,268,269	KVHF	1	NM_002497	1340(25)	24
PPP1R15B	39Q,48QA	214,229,245,260,266,275,276, 2110,2138,2142C,2147A,2192, 2226,2227,2271,2272,2308	KVTF	1	NM_032833	2331(275)	19
PPP1R13B (WRF) ASPP1	47Q,48QB,29T,36T,37T, 43T,101T,137T,223T	2178,2229	RVRF	14	NM_015316	≈2500	11
KPI-2	46Q,58Q,39T,201T,254TA		VTF	7	NM_014916	≈1900	5
PPP1R13A (WRF) ASPP2	129T,153T	2189	RVKF	1	NM_005426	≈2500 (2200)	3
PPP1R8-T NIPP1-T		273	RVTF	1	NM_138558	710(273)	1
C1QA (WRF)		216,217,287,2155,2223,2231, 2259,2277	RSLGF KGLF	1	NM_015991	540(217)	8
ZAP3	42T,214T,255T		KEVEF	14	XM_085151	≈1700	3
DAPPER 1		2183	KILRF	14	NM_016651	≈1100	1
RANBP2-like	20T		KQLF/RGVIF	10	Gi:30146988	≈400	1
JAK2 kinase	33T		after stop	9	Gi:3068751	≈1200	1
RANBPM		2232	RMIHF	6	AB055311	≈2800	1
PPP1R2 -L(WRF) I2-L	48T		KLHY	5	XM_018216	≈400	1
AXUD1		261	KRKF/RVAF/ RILSF/RVEF	3	NM_033027	≈1100	1
FLJ35803	1Q,9Q,41Q,50,55,120T,125T,12 7T,133T,135T,212T,233T,245T, 258T	22,220,247,253,285,2133,2143, 2145,2239	RVRF	9	AK093122	≈1000	23
DKFZp434D193 (WRF)	32Q,55QA,31T,58T,62T, 144T,146T,157T,210T, 244TC	2164,2179,2194A,2202,2205,2216,2218A,2250,2 324,2325,2327	RVSF	2	XM_114297	≈2500	19
KIAA1949	4Q,29Q,33Q,34Q,35Q,22T,27T,3 2T,141T	213,2238C	KISF	6	XM_166376	1269 (4Q1)	11
FLJ90254 (WRF)	143T,162T,224T,249T		KISF	9	RP11-350014	≈2300	4
MGC22793	131T	2161,2162,2305	RQVRF	7	NM_145030	≈900	4
MGC35144	152T	2124,2149,2150,2332	KTVRF	5	Gi21595061	≈300	4
IMAGE5581122	240TB,259TC			MIT	Gi:22028276	≈1300	2
FLJ32766 (testis clone)		271,2188	absent	2	AK057328	≈1800	2
LOC51315 (WRF)		2211,2217	after stop	2	NM_016618	117	2
DKFZp434N0650 (WRF)		280	KVCF	21	NM_032261	≈900	1
FLJ11904	244TA		absent/short peptide	18	AK021966	≈1300	1
FLJ14069	261TC		RVTF	17	AK024131	≈3000	1
FLJ20373		2257	RVHF	17	BC045694	≈1100	1
LOC388135 (WRF)	219T		RVRF	15	XM_301534	≈1200	1
SARP	40Q		KVHF	11	AK095193	2416	1
FLJ32771		2307	KVHF	11	NP_659454	≈1500	1
MGC16664 (WRF)	156T		absent	1	NM_173509	≈2100	1
FLJ39908 (part of)		298	absent	1	NM_144569	≈3850	1
FLJ32001		279	RVRF	1	NM_152609	≈3700	1
RP11-114H24	161TA,161TC,220T,228T	2218B,2236,2237,2268,2285, 2287	RVWW	15	Gi27545119	161	10
RP11-383C5		2194B,2235,2255	KVRF	10	Gi:16416159	≈200	3
RP11-282K6		231,2224,2275B	absent	1	Gi21954023	≈800	3
RP11-13J14	244TB,304TA		absent/short peptide	1	AL596217	≈900	2
CTD-2036P10	121T		RVSF	15	Gi:18249987	≈2400	1
RP11-77G23	20		KVTF	10	Gi:20334535		1
RP11-792D24		2296	RVRF	10	Gi:20177216	≈800	1
RP13-39P12		2238A		10	Gi:026953	≈2300	1
RP11-405I21	240TA		absent	7	Gi:18042433	≈800	1
RP1-223E5	305T		KFVSF	6	Gi:11830787	≈1400	1
RP11-294018		2100	absent	3	Gi:22657521	437	1
RP11-84A19		219	absent	1	Gi:28467537	≈3200	1

WRF (wrong reading frame) when shown indicates that the reading frame of the isolated clone differs from that of the identified protein. The occurrence of a consensus PP1 binding motif (PP1 BM) is indicated by the corresponding sequence.The size of the inserts is indicated in base pairs.

The identified clones were divided in four groups: proteins already known to be PP1 regulators (in purple), proteins known in other contexts (in yellow), proteins of unknown function present in the database (in blue) and clones present in the database only at the genomic level (in orange). All clones were subject to the same analysis: they were partially or completely sequenced, special features of the amino acid sequence were searched using Motif search databases and the amino acid sequence was searched for a consensus PP1 binding motif ([K/R]-X_{0-1}-[V/I/L]-X-[F/W]). The existence of ESTs (expressed sequence tags) was also ascertained by database searching. Inasmuch as several positives corresponded to independent hits on the same protein, the PP1 interactomes comprised 28 and 29 different proteins for the YTH1 and the YTH2, respectively. Interestingly, the most abundant interactions detected for both PP1 isoforms were with the same kinase. Thus, 47 positives out of the 120 detected with PP1γ1 and 71 positives out of the 155 detected with PP1γ2 encoded Nek2A (Table III.3). Another abundant interaction detected with PP1γ2 (17 positives) was with a protein identified only as R15B in the GenBank database. It was thus named since it shares a region of homology with R15A (also known as GADD34). Only 2 positives were obtained for R15B with PP1γ1. The next known PP1 binder more abundant was PPP1R13B. For the YTH1 9 clones were isolated while for the YTH2 only 2 clones were obtained. Also, 3 clones were obtained for a related protein, PPP1R13A, 2 with PP1γ1 as bait and 1 with PP1γ2. KPI-2, a recently identified PP1 inhibitor, was also isolated in the YTH1 in five independent clones. Finally, 1 positive coding for NIPP-1 was isolated as a PP1γ2 binding protein.

Some of the positives identified were analyzed more thoroughly: Nek2A-T, NIPP1-T, R15B, I2-L, KIAA1949, and SEARP. These will be further discussed below (SEARP will be addressed in Chapter IV).

III.3.4 Nek2 (NIMA- related kinase 2)

Nek2 is a protein kinase that localizes to the centrosomes throughout the cell cycle (Fry *et al.*, 1998b) and regulates their structure during the G2/M transition. It is known that Nek2 interacts with PP1, that dephosphorylates Nek2 itself and other Nek2 substrates

(Helps *et al.*, 2000). Surprisingly, Nek2 was the most abundant interacting protein identified in both screens (47 and 71 positives respectively).

By completely sequencing the Nek2 clones with the primers GAL4-AD, OLIGONEK, OLIGONEK2, OLIGONEK3 and Amplimer 3' (Appendix 4), it was discovered that two differently spliced isoforms of Nek2 had been identified. One of the isoforms was identical to the previously reported sequence (Fig. III.6) (Helps *et al.*, 2000). For this isoform several independent clones were obtained (Table III.4) whereas for the novel spliced isoform only two independent clones were isolated.

Table III.4: *Independent Nek2 and Nek2-N clones in the YTH1 and YTH2.*

5' Sequence	First nt	YTH1	YTH2	
ACGAGTTTTG	642		296	Nek2A
CCTGAACAAA	687	24TC	294	
ATGAATCGCA	696		225,226,2158,2159,2163, 2258,2263	
GCTTTTAGCC	786	227T,236T	291,2101,2151	
ATTCAGGCGA	830		239,2104,2140,2141,2142A, 2144,2147,2152, 2153,2254	
TCTGATGAAT	855		289, 2123	
GAATTGAATG	861	113T		
CCATCGACCT	905	13		
CATCGACCTT	906		212,262,2146,2269,2326	
GCAGATTTGG	948		265	
GAGCAAAGAA	966	44Q,45Q,57Q,4T,246T, 251T,252T,254T,254TC, 257T,259TB,260T,261TB, 302T,311T,316T	21,244,263,272,278,2109, 2165,2168,2175,2186,2187, 2198,2204,2209,2210,2215, 2234,2248,2261,2267,2276, 2278,2283	
GAGAGAAGAG	984	7Q,8Q,31Q,36Q,37Q		
GAAAAATCGC	1014	30Q, 53Q	24,25,270,284,2160,2174, 2176	
TTTACAGCTT	780	3Q,5Q,12Q,13Q,20Q,27Q, 55QB,12T,229T,303T, 304TB	26, 215, 218, 232, 268, 269	Nek2A-T
CAGGATTCCA	1023	17Q,26Q,49Q,136TA,155T, 208T,256T		

The novel, alternatively spliced isoform was termed Nek2A-T and missed 24 nucleotides, corresponding to amino acids 371 to 378, that we called phosphorylatable

domain (PD) (in red in Fig. III.6 and Fig. III.7). From all clones from the YTH1 and YTH2, 47 and 71 coded for Nek2A/Nek2A-T, respectively. By sequencing all the Nek clones we could state that 94 were Nek2A and 24 were Nek2A-T (Table III.4).

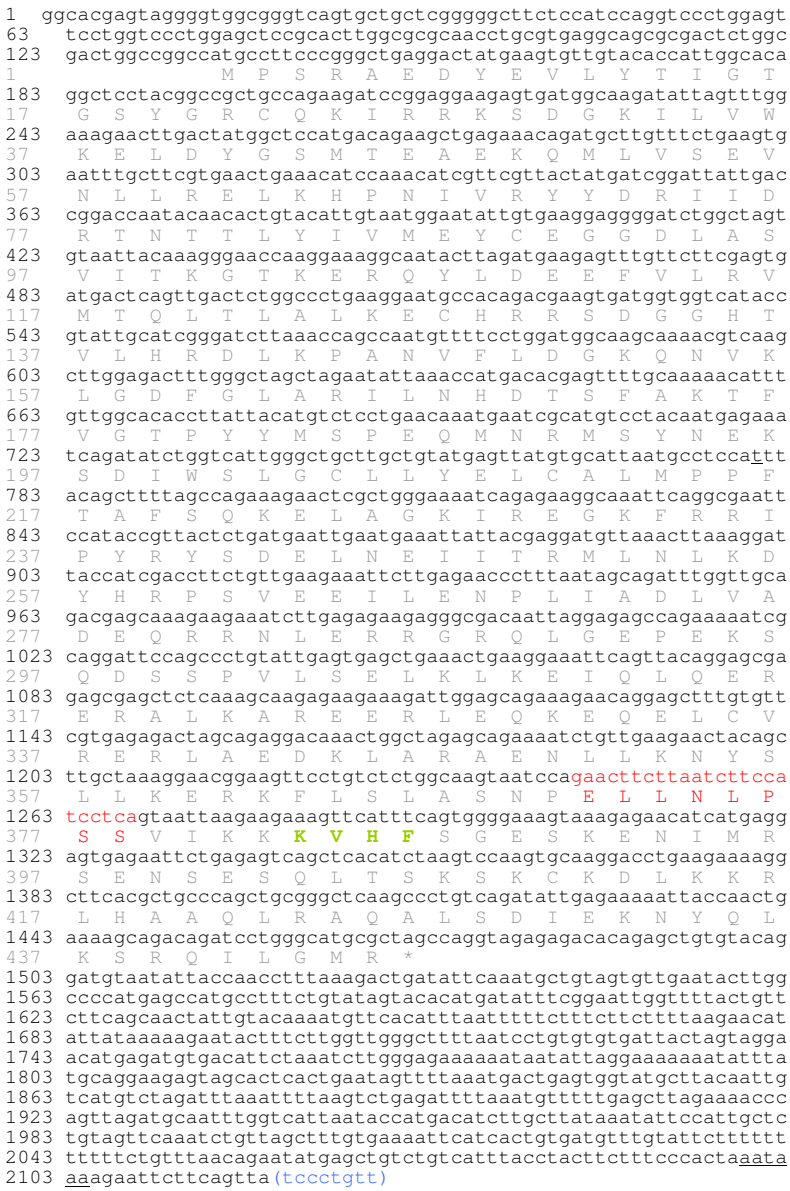


Figure III.6: *Sequence of Nek2 (NM_240097). The Nek2A-T clone that we have used for further study was the 3Q clone that starts at the nucleotide 780. This clone has an extra 8 nucleotides (in blue) after the polyadenylation signal (underlined) and misses the 24 nucleotides that characterize this new alternatively spliced isoform (in red, phosphorylatable domain). The PP1 binding motif (KVHF) is indicated in green.*

Another spliced variant of Nek2 exists derived by the use of alternative polyadenylation signals (Hames and Fry, 2002). The two isoforms were called Nek2A and Nek2B. Although both isoforms localize to centrosomes, only Nek2A can induce centrosome splitting upon overexpression.

Besides the PP1 binding motif, amino acid sequence analysis of Nek2A identified the protein kinase domain (residues 8-271) and the protein kinase Serine/Threonine active site (residues 137-149), an interesting putative leucine-zipper domain (306-327) and a couple of serines whose phosphorylation may be very important for Nek2 function (Fig. III.7). Removal of the phosphorylatable domain (PD) leads to the appearance of a new nuclear targeting motif (residues 361-377).

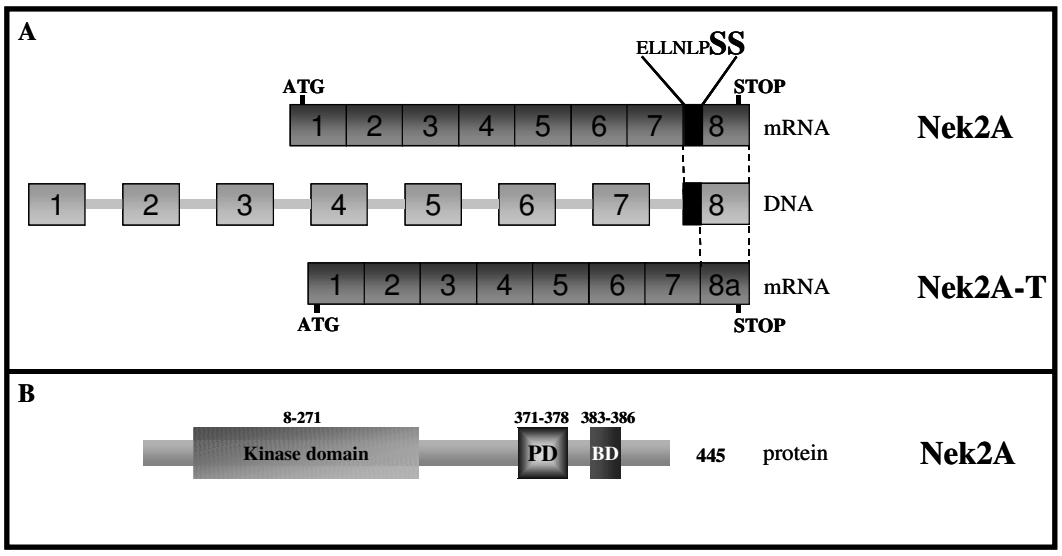


Figure III.7: (A) *Genomic structure of the Nek2 gene, showing the exon-intron organization (not to scale). The 8 amino acid sequence corresponding to the phosphorylatable domain (PD) that is specifically spliced out in the testis (exon 8) is shown. The two phosphorylated serines are shown enlarged. (B) Schematic representation of the encoded domain of the Nek2 protein. Amino acids missing in Nek2A-T are indicated by the PD box and the PP1 consensus binding motifs by the BD box (not drawn to scale). The numbers denote the exact amino acid localization of the various domains.*

The Nek2 gene maps to human chromosome 1 (Hames and Fry, 2002). By aligning the Nek2 cDNA with the corresponding genomic sequence the intron-exon boundaries can

be identified (Fig. III.7, III.8 and Table III.5). Nek2 is encoded by 8 exons spread over 13Kb on chromosome 1. The novel alternative splice identified occurs in exon 8.

```
ggcacgagtaggggtggcgggtcagtgctgctcgggggcttctccatccaggtccctgga
gttcctggtccctggagctccgcacttggcgcgcaacctgcgtgaggcagcgcgactctg
gcgactggcc ggcc
atgccttcccgggctgaggactatgaagtgttgtacaccattggcacaggctcctacggc
 M  P  S  R  A  E  D  Y  E  V  L  Y  T  I  G  T  G  S  Y  G
cgctgccagaagatccggaggaagagtgatggcaagatattagtttggaaagaacttgac
 R  C  Q  K  I  R  R  K  S  D  G  K  I  L  V  W  K  E  L  D
tatggctccatgacagaagctgagaaacagatgcttgtttctgaagtgaatttgcttcgt
 Y  G  S  M  T  E  A  E  K  Q  M  L  V  S  E  V  N  L  L  R
gaactgaaacatccaaacatcgttcgttactatgatcggattattgaccggaccaataca
 E  L  K  H  P  N  I  V  R  Y  Y  D  R  I  I  D  R  T  N  T
acactgtacattgtaatggaatattgtgaaggaggggatctggctagtgtaattacaaag
 T  L  Y  I  V  M  E  Y  C  E  G  G  D  L  A  S  V  I  T  K
ggaaccaaggaaaggcaatacttagatgaagagtttgttcttcgagtgatgactcagttg
 G  T  K  E  R  Q  Y  L  D  E  E  F  V  L  R  V  M  T  Q  L
actctggccctgaaggaatgccacagacgaagtgatggtggtcataccgtattgcatcgg
 T  L  A  L  K  E  C  H  R  R  S  D  G  G  H  T  V  L  H  R
gatcttaaaccagccaatgttttcctggatggcaagcaaaacgtcaagcttggagacttt
 D  L  K  P  A  N  V  F  L  D  G  K  Q  N  V  K  L  G  D  F
gggctagctagaatattaaaccatgacacgagttttgcaaaaacatttgttggcacacct
 G  L  A  R  I  L  N  H  D  T  S  F  A  K  T  F  V  G  T  P
tattacatgtctcctgaacaaatgaatcgcatgtcctacaatgagaaatcagatatctgg
 Y  Y  M  S  P  E  Q  M  N  R  M  S  Y  N  E  K  S  D  I  W
tcattgggctgcttgctgtatgagttatgtgcattaatgcctccatttacagcttttagc
 S  L  G  C  L  L  Y  E  L  C  A  L  M  P  P  F  T  A  F  S
cagaaagaactcgctgggaaaatcagagaaggcaaattcaggcgaattccataccgttac
 Q  K  E  L  A  G  K  I  R  E  G  K  F  R  R  I  P  Y  R  Y
tctgatgaattgaatgaaattattacgaggatgttaaacttaaaggattaccatcgacct
 S  D  E  L  N  E  I  I  T  R  M  L  N  L  K  D  Y  H  R  P
tctgttgaagaaattcttgagaacccttaatagcagatttggttgcagacgagcaaaga
 S  V  E  E  I  L  E  N  P  L  I  A  D  L  V  A  D  E  Q  R
agaaatcttgagagaagagggcgacaattaggagagccagaaaaatcgcaggattccagc
 R  N  L  E  R  R  G  R  Q  L  G  E  P  E  K  S  Q  D  S  S
cctgtattgagtgagctgaaactgaaggaaattcagttacaggagcgagagcgagctctc
 P  V  L  S  E  L  K  L  K  E  I  Q  L  Q  E  R  E  R  A  L
aaagcaagagaagaaagattggagcagaaagaacaggagctttgtgttcgtgagagacta
 K  A  R  E  E  R  L  E  Q  K  E  Q  E  L  C  V  R  E  R  L
gcagaggacaaactggctagagcagaaaatctgttgaagaactacagcttgctaaaggaa
 A  E  D  K  L  A  R  A  E  N  L  L  K  N  Y  S  L  L  K  E
cggaagttcctgtctctggcaagtaatccagaacttcttaatcttccatcctcagtaatt
 R  K  F  L  S  L  A  S  N  P  E  L  L  N  L  P  S  S  V  I
aagaagaaagttcatttcagtggggaaagtaaagagaacatcatgaggagtgagaattct
 K  K  K  V  H  F  S  G  E  S  K  E  N  I  M  R  S  E  N  S
gagagtcagctcacatctaagtccaagtgcaaggacctgaagaaaaggcttcacgctgcc
 E  S  Q  L  T  S  K  S  K  C  K  D  L  K  K  R  L  H  A  A
cagctgcgggctcaagccctgtcagatattgagaaaaattaccaactgaaaagcagacag
 Q  L  R  A  Q  A  L  S  D  I  E  K  N  Y  Q  L  K  S  R  Q
atcctgggcatgcgctagccaggtagagagacacagagctgtgtacaggatgtaatatta
 I  L  G  M  R  *
ccaacctttaaagactgatattcaaatgctgtagtgttgaatacttggccccatgagcca
tgcctttctgtatagtacacatgatatttcggaattggttttactgttcttcagcaacta
ttgtacaaaatgttcacatttaatttttctttcttctttttaagaacatattataaaaaga
atactttcttggttgggcttttaatcctgtgtgtgattactagtaggaacatgagatgtg
acattctaaatcttgggagaaaaaatataattaggaaaaaaatatttatgcaggaagagt
agcactcactgaatagttttaaatgactgagtggtatgcttacaattgtcatgtctagat
ttaaattttaagtctgagattttaaatgtttttgagcttagaaaacccagttagatgcaa
tttggtcattaataccatgacatcttgcttataaatattccattgctctgtagttcaaat
ctgttagctttgtgaaaattcatcactgtgatgtttgtattctttttttttttctgttta
acagaatatgagctgtctgtcatttacctacttctttcccactaaataaaagaattcttc
agtta
```

Figure III.8: *The exon-intron boundaries of Nek2 are indicated by the different colours in the cDNA sequence. The alternatively spliced sequence missing from Nek2A-T is indicated in grey.*

Table III.5 describes the exon-intron boundaries of Nek2A-T showing in bold the canonical 3' splice acceptor and the 5' splice donor.

Table III.5: *Exon-intron boundaries of Nek2A-T. The canonical exon-intron boundaries sequences are in bold.*

INTRON 3' splice acceptor	EXON	No.	5' splice donor	INTRON
YNCURACYNNAG	**G**		**AG**	**GUAAGU**
	GGCACG	1	GGCA**AG**	**GT**GAGC
TTCTTATTTC**AG**	ATATTA	2	GGAA**AG**	**GT**AAGC
CTTTGGTTTT**AG**	**G**CAATA	3	TCTCCT	**GT**AAGT
TTCCCTGTAT**AG**	**G**AACAA	4	ATTAAT	**GT**AAGT
TTTTAACCCT**AG**	**G**CCTCC	5	TTAA**AG**	**GT**AAAG
TTTTTTCCCC**AG**	**G**ATTAC	6	TGGAGC	**GT**AAGT
CTCTTTTTAA**AG**	AGAAAG	7	ATCC**AG**	**GT**ATGA
TTCCTTCTAA**AG**	TAATTA	8A		

The isolation of two independent Nek2A-T cDNAs from the library used indicated that this novel isoform was unlikely to be the result of an artifact. However, in order to ascertain the occurrence of the corresponding mRNA in human testis, RT-PCR was performed using appropriate oligonucleotide primers. Using mRNA from human testis as template and two different sets of primers, OLIGONEK/OLIGONEK2 and OLIGONEK/OLIGONEK3 (Appendix II), DNA fragments of 224nt (Nek2A-T) and 238nt (Nek2A) were obtained (Fig. III.9). DNA sequence analysis of the Nek2A-T product confirmed the alternatively spliced sequence (data not shown).

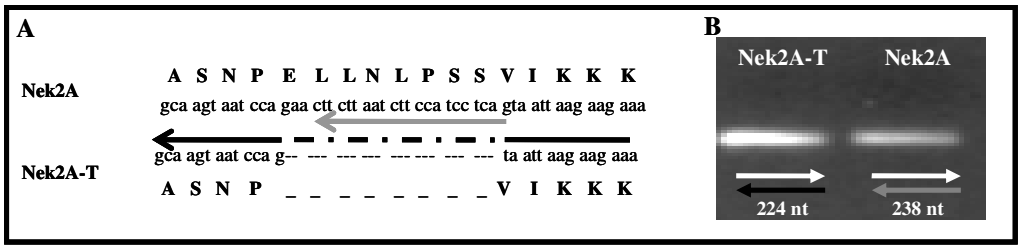


Figure III.9: *RT-PCR analysis of Nek2 expression in human testis using isoform-specific primers.* ***A)*** *Alignment of the amino acid and nucleotide sequences of Nek2A and Nek2A-T. The arrows indicate the specific oligonucleotide primers used.* ***B)*** *Agarose gel electrophoretic analysis of RT-PCR products obtained from human testis using the indicated primers.*

The expression of Nek2 in different tissues was analysed by Northern blot hybridization using a Rat Multiple Tissue Northern (MTN) Blot and the cDNA from clone 3Q as probe (Fig. III.10):

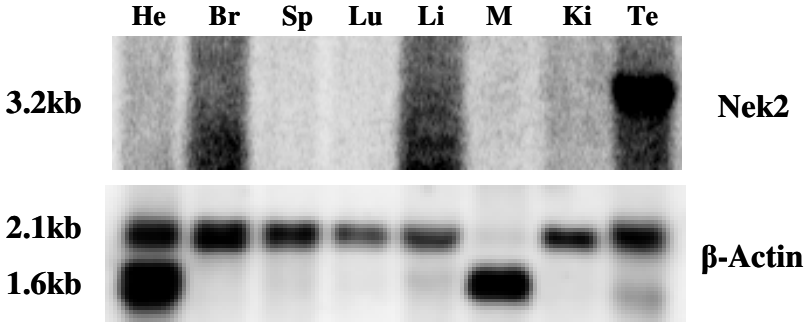


Figure III.10: *Northern blot analysis of different rat tissues.He, heart; Br, brain; Sp, spleen; Lu, lung; Li, liver; M, skeletal muscle; K, kidney; Te, testis.*

Nek2 expression was only detected in testis, where a single transcript of 3.2kb hybridized with the probe used (Fig. III.10, top panel). As a control the same blot was also hybridized with β-actin probe (Fig. III.10, lower panel). Densitometry analysis of both blots allowed for correction of Nek2 expression in terms of the β-actin 2.1kb band (muscle was excluded from this analysis due to the absence of the β-actin species). The results obtained are summarized in Table III.6:

Table III.6: *Relative abundance of Nek2 mRNA.*

Tissue	Heart	Brain	Spleen	Lung	Liver	Kidney	Testis
Nek mRNA	2	0	2	0	0	0	100

The value for testis was set at 100 and other tissues are expressed in relative terms.

In order to confirm the interaction of Nek2A and Nek2A-T with PP1γ1 and PP1γ2, blot overlay analysis was performed. For this purpose, the cDNAs of clones 25 and 3Q, respectively, were used to express the corresponding N-terminally truncated proteins (work performed in our laboratory by Wu Wenjuan). The proteins were separated by SDS-PAGE gel, transferred to nitrocellulose and the membrane was incubated with PP1γ2 (also purified by Wu Wenjuan). Then, the membrane was immunobloted with Anti-PP1γ2 and developed by ECL (Fig. III.11).

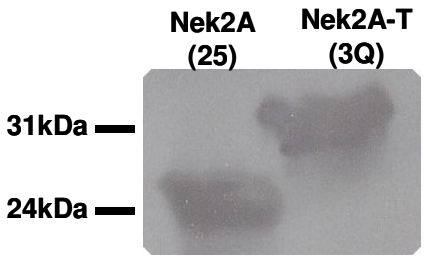


Figure III.11: *Overlay assay of Nek2 A and Nek2A-T The size of the proteins detected corresponds to the calculated molecular weights of the proteins encoded by the partial cDNAs used (25 and 3Q, respectively).*

Additionally, sequential transformation of yeast with the bait and the prey was performed in order to confirm the YTH interaction (Fig. III.12):

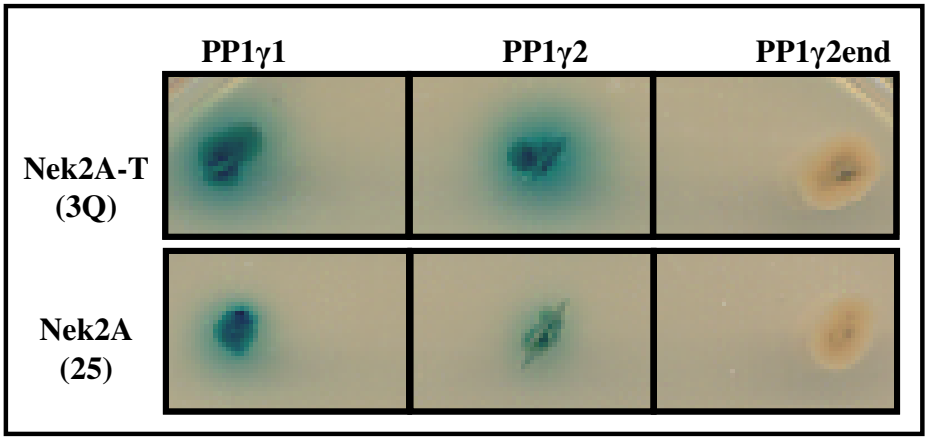


Figure III.12: *Sequential transformation of yeast AH109 a bait plasmid (either pAS2PP1γ1,or pAS2PP1γ2 or pAS2PP1γ2end) and the prey plasmid (pACT2-3Q, or pACT2-25).*

The results indicate that both Nek2A and Nek2A-T interact with PP1γ1 and PP1γ2. They also appear to interact with the PP1γ2 C-terminal, although the interaction is weaker since the blue colour takes longer to develop. This was expected since the C-terminal PP1 peptide expressed did include the sequence thought to be responsible for binding to the consensus binding motifs in other proteins.

III.3.5 NIPP1-T (Nuclear Inhibitor of Protein Phosphatase 1 in Testis)

NIPP1 (Beullens *et al.*, 1992) is a 38.5kDa nuclear inhibitor of PP1 that is ubiquitously expressed in multicellular eukaryotes (Ceulemans *et al.*, 2002). Biochemical analysis of NIPP1 indicates that it is composed of several functional domains. The basic NH_2-terminal domain contains a forkhead associated domain (FHA). The acidic central third, expressed in bacteria, is a potent inhibitor of PP1 (Van Eynde *et al.*, 1995). This domain contains the consensus PP1 binding motif "RVXF", an additional inhibitory PP1 binding site (Beullens *et al.*, 1999) and phosphorylation sites for PKA (Ser^{199}) and CK2 (Ser^{204}). The basic COOH-terminus is identical to the polypeptide Ard-1 (Activator of RNA decay) that was shown to be a rare splice variant of NIPP1 (Van Eynde *et al.*, 1999). The inhibitory potency of NIPP1 is regulated by multisite phosphorylation and by the binding of RNA (Vulsteke *et al.*, 1997; Beullens *et al.*, 2000). NIPP1 is targeted through its FHA to the speckles, or splicing-factor compartments, that are storage sites for splicing

factors (Trinkle-Mulcahy *et al.*, 1999; Jagiello *et al.*, 2000). This FHA domain interacts with the phosphorylated forms of the essential splicing factors CDC5L (Boudrez *et al.*, 2000) and SAP155 (unpublished observation of Bollen M. and coworkers). Since these splicing factors are *in vitro* substrates for PP1, it has been speculated that NIPP1 might target them for dephosphorylation by PP1. This function of PP1 may be very important, since toxins that inhibit Ser/Thr-protein phosphatases often inhibit pre-mRNA splicing (Mermoud *et al.*, 1992). NIPP1, besides being targeted to the speckles, is a component of the spliceosomes and has an essential role in a late step of spliceosome assembly (Beullens and Bollen, 2002). Surprisingly, this NIPP1 function seems to be unrelated to its ability to bind PP1. So, NIPP1 can be thought of as a bifunctional protein, having a PP1-independent function in spliceosome assembly and, after splicing, involved in the regeneration of splicing factors by recruiting PP1 to dephosphorylate them. Recently, it was shown that PP1 is required for Tat-induced HIV-1 transcription and for viral replication in cultured cells (Ammosova *et al.*, 2003). By inhibiting PP1 through overexpression of NIPP1, Tat-induced HIV-1 transcription was inhibited. By contrast, a mutant of NIPP1 that could not bind PP1 did not have this effect. These important results indicate that nuclear PP1 may represent a novel target for anti-HIV-1 therapeutics.

The structure of a human NIPP1 pseudogene and the authentic NIPP1 gene was reported by Van Eynde *et al.* (Van Eynde *et al.*, 1999), who also reported the existence of various NIPP1 mRNAs, including the Ard-1 transcript, that are generated by alternative 5'-splice site selection, exon skipping and/or alternative polyadenylation. By completely sequencing the NIPP1 clone 273, with the primers GAL4-AD, OLIGONIPP, OLIGONIPP2 and Amplimer 3' (Appendix 4), we verified that we had isolated a previously unknown alternatively spliced isoform of NIPP1. A single clone isolated from the YTH2 screen, it started at nucleotide 854 (NM_138558), was 710bp long and differs from all the other known NIPP1 isoforms (Fig. III.13):

Figure III.13: *Nucleotide and corresponding amino acid sequence of NIPP1β (NM_138558). Highlighted regions correspond to the sequence of clone 273 (the PP1 binding motif is underlined). The dark blue area indicates the C-terminal domain specific for NIPP1-T isoform that is not translated in the previously known variants.*

This new isoform was named NIPP1-T and uses the same polyadenylation site as NIPP1β. NIPP1β (NM_138558) is the longer NIPP1 isoform and is encoded over 7 exons. Our clone starts at exon 4, and contains exons 5, 6 and 7. Nevertheless, exon 7 in our clone

is different from that previously described for NIPP1β. Interestingly, in NIPP1-T the polyadenylation signal overlays the stop codon. At the protein level, NIPP1-T differs from NIPP1β in that it has a longer C-terminal extension (Fig. III.14). It is interesting to note that whereas NIPP1β possesses the sequence –KKPTP- in the spliced domain (a proline directed kinase consensus acceptor site), a related sequence also occurs in NIPP1-T (-NAPSP-). Both motifs are located exactly 4 amino acids from the C-terminus of the two proteins.

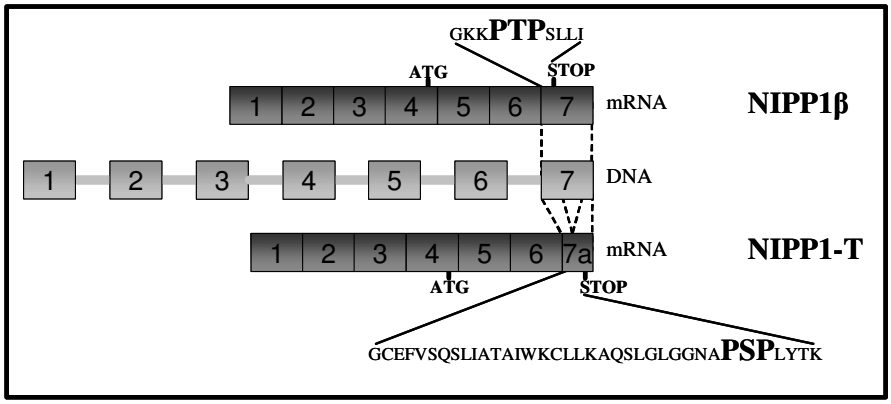


Figure III.14: *Genomic structure of the NIPP-1 gene (not to scale). The alternative splicing of exon 7 shown produces the different C-terminal amino acid sequences shown. ATG, location of the initiator methionine codon; PP1-BD, PP1 consensus binding domain; STOP, location of the stop codon.*

Table III.7 summarizes the intron-exon organization of clone 273 encoding NIPP1-T.

Table III.7: *Exon-intron organization of clone 273 (NIPP1-T).*

Exon No.	Nt No.* NIPP1-T	Exon size (bp)	Intron No.	Intron Nt No.*	Intron size (Kb)	Nt No.* NIPP1β
4	2777243385-27772433 (854-901)	48	4	27772434-27774384	1951	27772213-27772433 (681-901)

5	27774385-27774529 (902-1046)	145	5	27774530-27778226	3697	27774385-27774529 (902-1046)
6	27778227-27778291 (1047-1111)	65	6	27778292-27781291	3000	27778227-27778291 (1047-1111)
7	27781292-27781727 (1112-1548)	436				27781292-27782871 (1112-2691)

*Clone 273 starts at exon 4 from NIPP1β. Exon 5 and 6 are the same as in NIPP1β but exon 7 is different. *, corresponding to sequence from chromosome 1 (NC_000001). In column 2 and 7 sequences in brakets correspond to sequence NM_138558.*

RT-PCR was performed using mRNA from human testis and the primers OLIGONIPP and OLIGONIPP2 (Appendix II). These primers are specific for NIPP1-T, amplifying a PCR product of 208nt (Fig. III.15). The correct sequence of the PCR product was determined by automatic sequence analysis, thus confirming the position of this alternative splice (data not shown).

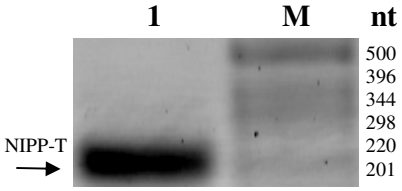


Figure III.15: *RT-PCR analysis of mRNA from human testis using the primers OLIGONIPP/OLIGONIPP2 (lane 1). A single band of the expected size (208bp) was obtained M, molecular weight markers.*

Interestingly, searching of the EST database identified a single EST (from kidney) corresponding to this novel splice variant (gi|3890874|gb|AI271707.1|AI271707). The expression of NIPP1 mRNA in different rat tissues was also verified by Northern analysis of a Multiple Tissue Northern (MTN) Blot, using the cDNA from clone 273 as probe (Fig. III.16).The results from densitometric analysis of the resulting autoradiogram, corrected for the actin control, are shown in Table III.8. Muscle was excluded from this analysis since it does not express the 2.1kb β-actin message.

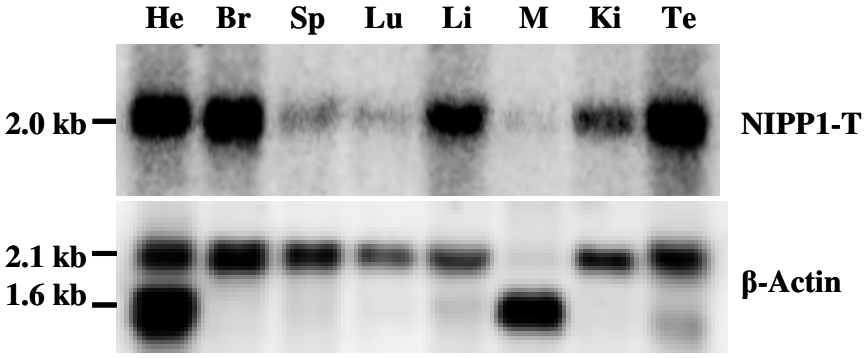


Figure III.16: *Northern analysis of different rat tissues using the cDNA of clone 273 as probe.The same blot was re-probed with β-actin as a control (lower panel). He, heart; Br, brain; Sp, spleen; Lu, lung; Li, liver; M, skeletal muscle; Ki, kidney; Te, testis.*

Table III.8: *Densitometric analysis of NIPP1 mRNA considering testis mRNA 100%.*

Tissue	Heart	Brain	Spleen	Lung	Liver	Kidney	Testis
%NIPP1 mRNA	125	93	22	18	86	50	100

Another Northern blot was also prepared with human testis RNA and several rat tissues mRNAs. The results demonstrated the existence of two mRNA bands in the human testis lane (Fig. III.17) that might correspond to NIPP1β and the novel NIPP1-T.

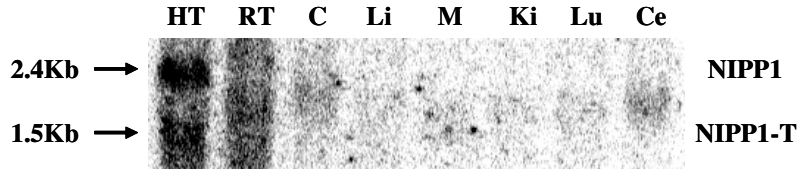


Figure III.17: *Northern analysis of human testis and several rat tissues using the cDNA of clone 273 as probe.HT, human testis; RT, rat testis; C, cortex; Li, liver; M,skeletal muscle; Ki, kidney; Lu, lung; Ce, cerebellum.*

These results, together with the RT-PCR analysis (Fig. III.15) and the existence of a human EST corresponding to this splice strongly support the existence of a novel NIPP1 splice variant, that we have named NIPP1-T.

Additionally, sequential transformation of yeast was performed with the bait (PP1γ1 or PP1γ2 or PP1γ2end) and the prey (NIPP1-T) in order to confirm the YTH interaction (Fig. III.18).

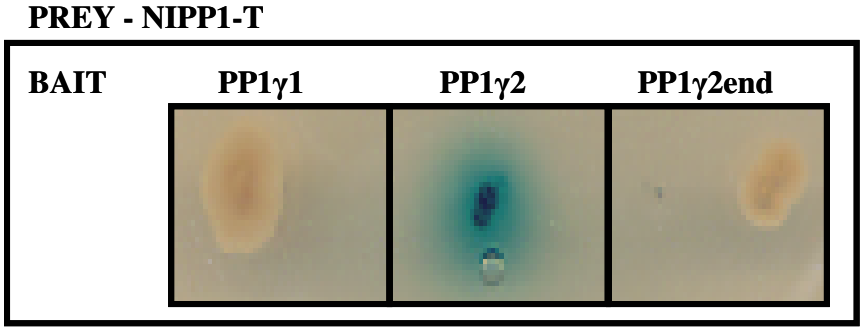


Figure III.18: *Sequential transformation of the yeast AH109 first with the bait plasmid (pAS2PP1γ1 or pAS2PP1γ2 or pAS2PP1γ2end) and then the prey plasmid (pACT2-273), followed by plating in selective medium containing X-α-Gal.*

The results obtained indicate that NIPP1-T interacts more strongly with PP1γ2 than with its C-terminal domain or with PP1γ1, since the blue colour due to X-α-Gal cleavage takes longer to develop with the latter two.

III.3.6 PPPR15B (Phosphoprotein Phosphatase Regulatory Subunit 15B)

Data from multiple eukaryotic genomes was used to trace the evolution of well established vertebrate PP1 regulators (Ceulemans *et al.*, 2002). That study also identified nine poorly characterized proteins as putative PP1 regulators. Among them was a protein named PPP1R15B (NM_032833; FLJ14744). This gene is predicted to encode a homologue of GADD34 (growth arrest and DNA damage-inducible protein) also known as PPP1R15A. GADD34 is a member of a group of genes whose mRNA levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents. GADD34 was identified in a yeast two hybrid screen of a human brain cDNA library by its interaction with I-1 (inhibitor-1). It was also shown that PP1 bound GADD34 near the C-terminus, while I-1 bound the central domain of GADD34. Modifications of the I-1/GADD34/PP1 signaling complex regulate initiation of protein translation in mammalian tissues in response to cell stress, by dephosphorylation of the eukaryotic initiation factor eIF-2α (Connor *et al.*, 2001). Recently, a yeast two hybrid screen using GADD34 as bait identified the human cochaperone protein BAG-1 (Hung *et al.*, 2003). Also, Hsp70 and PP1 were shown to associate reversibly with the GADD34/BAG-1 complex. It was

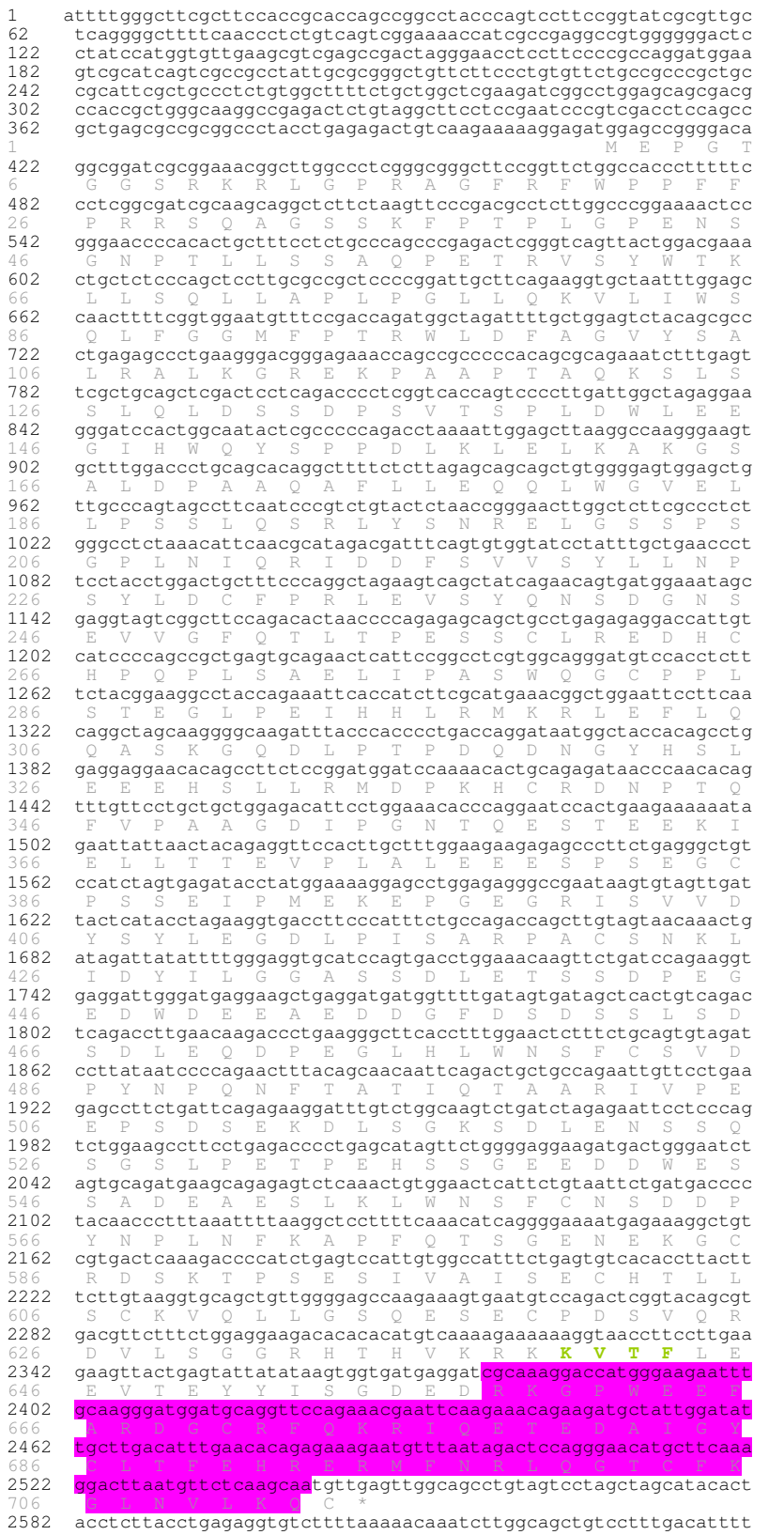
suggested that BAG-1 might function to suppress the GADD34-mediated cellular stress response and have a role in the survival of cells undergoing stress.

In our YTH screens we identified several independent PPP1R15B clones (Table III.9):

Table III.9: *Independent PPP1R15B clones identified in YTH1 and YTH2.*

Initial Sequence	first nt	YTH1	YTH2
CTTGGCTCTTCGC	1004	48QA	229,245,260,266,275,276,2110,2138,2142C, 2147A, 2192,2226,2271,2272,2308
GCTTGTAGTAACA	1664		214
GATGACTGGGAAT	2027	39Q	2227

We chose clone 275 for further studies, starting at nt 1004 and finishing after nt 2942 relatively to the database sequence NM_032833 (Fig. III.19). We have not get sequenced the remaining sequence but we have verified the existence of the poly-A tail. The total size of the 275 cDNA insert is approximately 2.8kb.



```
2642  ttttttagaggaaatgtaacttggatctagtttaatttttttttttgcaacatatccca
2702  ctcagaaacattcaggtttgaagccagccctgataatgaaggatgaactagtgtgatttc
2762  taatcctccctttttgatttagttggatgtgcttttaaatgtcctttgcctgcatgagg
2822  tggaaaggggacctttttgagttgtcattttgcactttcaaaacttattttcttggaaaa
2882  caatacttatagggcttaaagcccattttcatttctaatctaaattatgtgtgcctatct
2942  g
```

Figure III.19: *Sequence of PPP1R15B (NM_032833). Clone 275 starts at nt 1004. The PP1 binding motif is indicated in green. The pink area corresponds to the small C-terminal module that characterizes the GADD34 family.*

Aligning of the PPP1R15B sequence with human genome contig, lead us to map it to two exons on chromosome 1 (Fig. III.20). Interestingly, the exon boundaries occur within the PP1 binding motif.

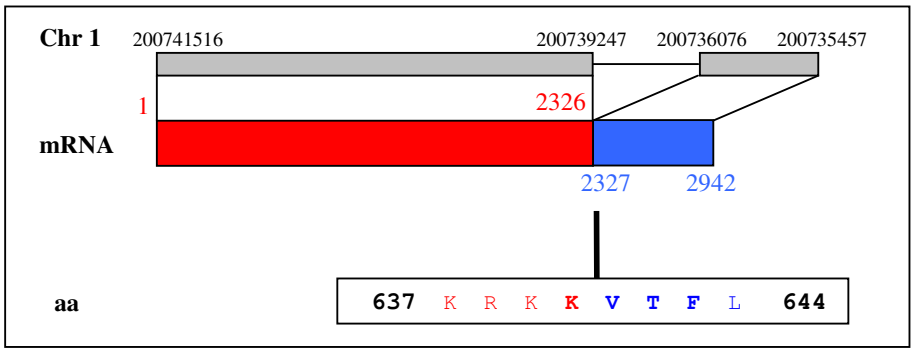
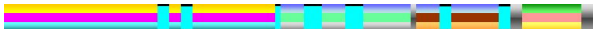
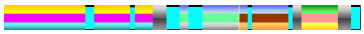


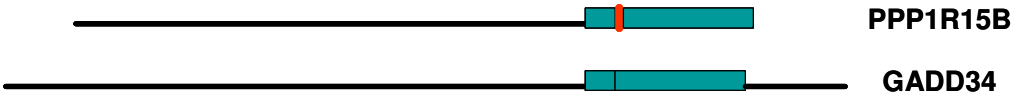
Figure III.20: *Genomic structure of PPP1R15B.*

By searching Pfam (Bateman *et al.*, 2002) with the PPP1R15B sequence, some special features could be identified. Notably, the existence of a Pfam-B domain (Pfam_9893) that characterizes the GADD34 family of proteins (Table III.10 and Fig. III.21), and is present in the C-terminus of all members. This family includes members from very different organisms like human, mouse, virus and fly.

Table III.10: *Members of the PPP1R15B family.*

Identification	Diagram of the protein domains	residues
MY16_MOUSE [Mus musculus (mouse)] myeloid differentiation primary response protein myd116		657
Q60465 [Cricetulus longicaudatus (long tailed hamster) (chinese hamster)] GADD34 protein		590
O75807 - GADD34 [Homo sapiens (human)] apoptosis associated protein (growth arrest and DNA-damage -inducible34)		674
Q9EML3 [Amsacta moorei entomo poxvirus (amepv)] amv193		150
Q9W1E4 [Drosophila melanogaster (fruit fly)] cg3825 protein (gh11727p)		317
Q91CH8 [Macropodid herpesvirus 1] icp34.5		170
Q96SN1 - PPP1R15B [Homo sapiens (human)] hypothetical protein flj14744		713

Transmembrane:  *; low complexity:*  *; pfamB:*  *(different colours indicate different Pfam-B families) ; GADD34 family domain:*  *. Pfam B domains are regions of proteins that belong to a Pfam-B family; Pfam-B is an automatically generated supplement to Pfam that is generated from the PRODOM database. Matches to Pfam-B are likely to indicate true relationships.*



A

```
PPP1R15B     634 THVKRKKVTFLEEVTEYYIS-----GDEDRKGPWEEFARDGCRFQKRIQETEDAIGYCLT
                   +  +       +   ++++          +    +          +   + ++ +
GADD34       549 TPLKARKVRFSEKVTVHFLAVWAGPAQAARQGPWEQLARDRSRFARRIAQAQEELSPCLT
                 *  *  ** * * **               * ****  ***  **  **         ***

PPP1R15B     689 FEHRERMFNRLQGTCFKGLNVLKQ
                       +   +       +
GADD34       609 PAARARAWARLRNPPLAPIPALTQ
                   * *   **           * *
Score = 53.1 bits (126), Expect = 3e-05
Identities = 29/84 (34%), Positives = 46/84 (54%), Gaps = 5/84 (5%)
```

B

Figure III.21: *A, Diagram of the alignment of the aa sequences of GADD34 and PPP1R15B using BLAST algorithm (Altschul et al., 1997). B, Alignment of the boxed aa sequences.* indicates identities and + indicates homologies.*

Searching PROSITE (Sigrist *et al.*, 2002) several putative motifs were identified in PPP1R15B, including glycosylation sites, sulfation sites, cAMP and cGMP-dependent protein kinase phosphorylations site, PKC phosphorylation sites and CK2 phosphorylation sites.

Sequential transformation of yeast with bait and prey plasmids confirmed the YTH interaction (Fig. III.22).

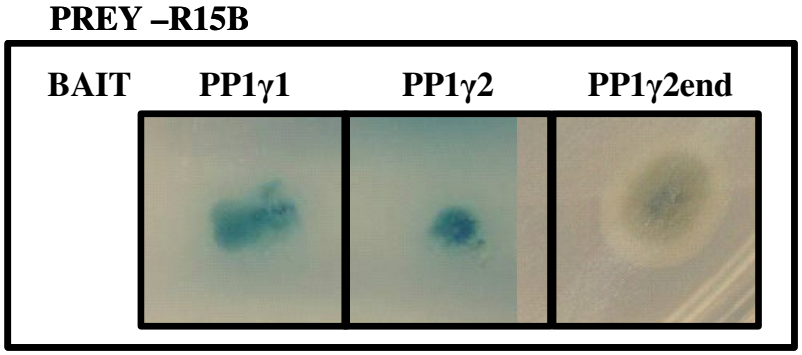


Figure III.22: *Sequential transformation of yeast AH109 first with, the bait plasmid (pAS2PP1γ1 or pAS2PP1γ2 or pAS2PP1γ2end) and then the prey plasmid (pACT2-275).*

The results indicated that R15B interacts both with PP1γ1 and PP1γ2. Although it also interacts with the PP1γ2 C-terminal domain, the interaction is likely weaker since the blue colour takes longer to develop.

III.3.7 KIAA1949

In this section a previously unpublished clone will be described. This cDNA termed KIAA1949 (XM_166376) in the database, seemed to be very interesting because it encodes for a protein sequence with some special features (Fig. III.23).

```
1      agacaaagcggtcgccgcccccgcccggccccctggtctctgtctccgtccctcctcctttg
63       ctgcctctttccctcctcctctccctccctcctccccctccctccagtctccggatctccc
123      tcggtccctctctcctcctcttcctctctctggacgcccggctcctccgcacccccctccc
183      ccgggggtcccgcggcctgtgagttgactgaggggctcagacttggggagtgggtgtctc
243      ctcgcccctgtccttgctcccgtccctggcccggaccttggctgtctcctctttgtgccg
303      agattgtcagtctgtgcggctacagcggggtggagacggccggctctgtcacggcttcat
363      gagagcggggacggggcgcaggacttgcaggcgccggggagaagagacatggagccggcc
423      cttggcactctggggtcgcgtggggcagtcggtgggggaggcaggcggtggtgacaggac
483      agggtgggggtggacgccagggttctgggaacgcgctggcagccctgacgcccgggttcc
543      gaaagtctcgggggtgggtatttcccccgacccgcctcgggggcggagtgcggggcagag
603      gggtgggggctggggagaggcgtggcccgagcggtgctggaagcggagccgggacctttg
663      gggcccgcgctgagacgcgcccggctgctgccgccgccctccttcccctcttccctggt
723      ttcccttctcctctagacctgttcgctctccgcccctccttgcctccccaacacccccctc
783      aggtcccgttgcctcctggtcctttcagggattcctggtccttccttcccacactagcct
843      ccctggggtatcgctgaggcagcctggcctgcacccaggttcccctcacccctgccacat
903      ttctctcttctccctcacgccaacttttccttttcgcccttctctctctttctcacatcct
963      agagacggtctttaatacgcattaaccctgtgctgccacatctggctcctgccctcattg
1023     cctccaatccggactcttcctctcacatcacccccaccacccccaacttgggctcacaac
1083     ttctcttcacttttccatttccccagttctctgccttccgtctttccctctgtcctcat
1143     ccttagcccctctgccctgctttgtgtcccacctctccccctccacttcctctcctccca
1203     ccctcagtctcacccccgggctgtctcactctctggagcctctccttcctgttctctgtc
1263     cccagtgctccctaccctcacctcaagacgaccatggccaccatcccagactggaagcta
1                                          M  A  T  I  P  D  W  K  L
1323     cagctgctagcccggcgccggcaggaggaggcgtccgttcgaggccgagagaaagcagaa
10        Q  L  L  A  R  R  R  Q  E  E  A  S  V  R  G  R  E  K  A  E
1383     cgggagcgcctgtcccagatgccagcctggaaacgagggctcctggagcgccgccgggcc
30        R  E  R  L  S  Q  M  P  A  W  K  R  G  L  L  E  R  R  R  A
1443     aagcttgggctgtcccctggggagcctagccctgtgctagggactgtagaggctggacct
50        K  L  G  L  S  P  G  E  P  S  P  V  L  G  T  V  E  A  G  P
1503     ccagacccggatgagtctgcggtccttctggaggccatcgggccagtgcaccagaaccga
70        P  D  P  D  E  S  A  V  L  L  E  A  I  G  P  V  H  Q  N  R
1563     ttcatccggcaggagcggcagcagcagcagcaacaacaacggagtgaagagctgcta
90        F  I  R  Q  E  R  Q  Q  Q  Q  Q  Q  Q  Q  R  S  E  E  L  L
1623     gcagagagaaagcctgggcctctggaggcccgggagcggagacccagccctggggagatg
110       A  E  R  K  P  G  P  L  E  A  R  E  R  R  P  S  P  G  E  M
1683     cgggatcagagcccaagggaagagagtcaagagaagagagactaagtccgagggagacc
130       R  D  Q  S  P  K  G  R  E  S  R  E  E  R  L  S  P  R  E  T
1743     agagagaggaggctggggatagggggagcccaagagttgagcctgaggcctctggaggct
150       R  E  R  R  L  G  I  G  G  A  Q  E  L  S  L  R  P  L  E  A
1803     cgggactggaggcaaagcccaggagaggtgggagacaggagctcccgactgtcagaggca
170       R  D  W  R  Q  S  P  G  E  V  G  D  R  S  S  R  L  S  E  A
1863     tggaaatggaggctgagtcctggagaaactccagagcggagtctgagactagcagagtct
190       W  K  W  R  L  S  P  G  E  T  P  E  R  S  L  R  L  A  E  S
1923     cgagagcaaagccccaggagaaaagaggtggaaagtagactgagcccaggggaatctgcc
210       R  E  Q  S  P  R  R  K  E  V  E  S  R  L  S  P  G  E  S  A
1983     taccagaagttgggcctgacagaggcccataaatggagacctgactccagagagtctcag
230       Y  Q  K  L  G  L  T  E  A  H  K  W  R  P  D  S  R  E  S  Q
2043     gaacagagtttggtacaactggaggcaacagagtggaggctgaggtcaggagaagaaaga
250       E  Q  S  L  V  Q  L  E  A  T  E  W  R  L  R  S  G  E  E  R
2103     caagactactcggaagaatgtgggagaaaagaagagtggccagttccaggggtagctcca
270       Q  D  Y  S  E  E  C  G  R  K  E  E  W  P  V  P  G  V  A  P
2163     aaagagactgcagagctgtccgagaccctgacaagggaggcccaaggcaacagttccgca
290       K  E  T  A  E  L  S  E  T  L  T  R  E  A  Q  G  N  S  S  A
2223     ggagtggaggcagcagagcagaggcctgtggaagatggcgagaggggcatgaagccaaca
310       G  V  E  A  A  E  Q  R  P  V  E  D  G  E  R  G  M  K  P  T
2283     gaagggtggaaatggaccctgaactccgggaaggctcgagaatggacacccagggacata
330       E  G  W  K  W  T  L  N  S  G  K  A  R  E  W  T  P  R  D  I
2343     gaggctcaaactcagaaactagaacctccagagtcagcagagaagcttctggaatctccc
```

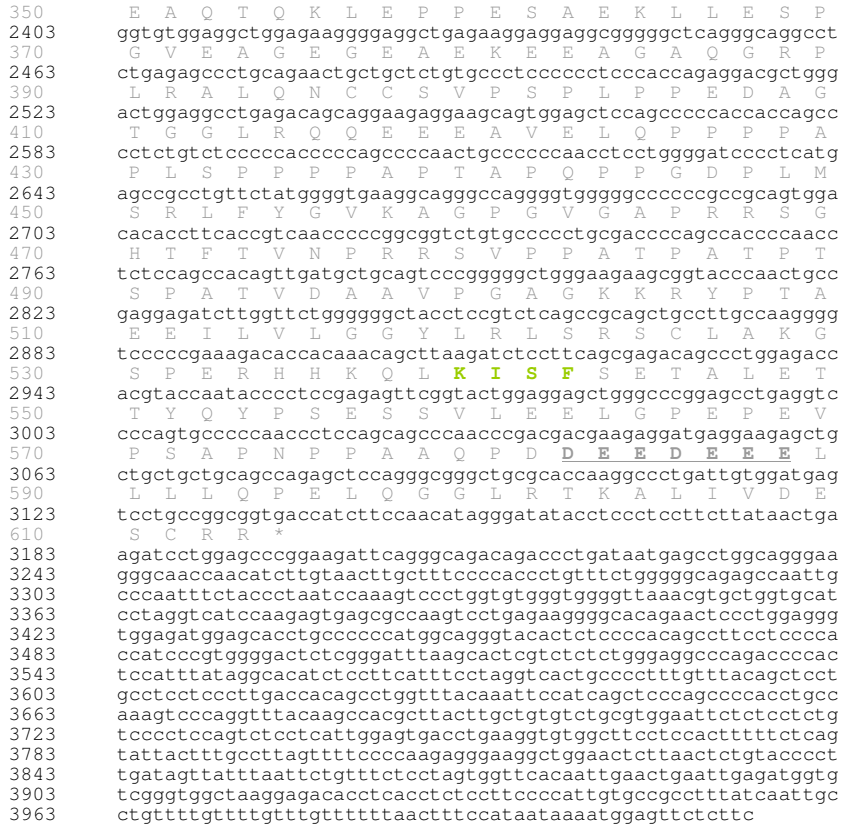


Figure III.23: N*ucleotide and deduced amino acid sequence of clone KIAA1949. Clone 4Q starts at nt 2763. The PP1 binding motif is indicated in green; A PEST sequence is underlined.*

Several KIAA1949 cDNAs were isolated in both YTH screens (Table III.11). Clone 4Q was selected for further examination. This clone starts at nt 2763 relatively to the XM_166376 sequence in the database.

Table III.11: *Independent KIAA1949 clones obtained in the YTH1 and YTH2.*

Sequence	first nt	YTH1	YTH2
TCTCCAGCCACAG	2763	4Q,29Q,33Q,34Q,35Q, 22T,27T,32T,141T	
GAAGCGGTACCC	2804		2238C

The protein encoded by KIAA1949 contains a putative PP1 binding motif, KISF, and a putative PEST sequence (responsible for targeting proteins to degradation). Other putative domains were also identified by searching Prosite: a N-glycosylation site (aa 306-309), a Tyr sulfation site (aa 265-279), a cAMP- and cGMP-dependent protein kinase phosphorylation site (aa 122-125), and several protein kinase C phosphorylation sites. Additionally, it has casein kinase II phosphorylation sites, a Tyr kinase phosphorylation site (264-272), several N-myristoylation sites, two amidation sites, a Arg rich region (aa 120-216), a Gln rich region (aa 87-103), a Glu rich region (aa 247-382) and a Pro rich region (aa 425-491).

By aligning the XM_166376 database sequence with the human genome, the corresponding gene sequence was determined to spread over 3 exons on chromosome 6 (TableIII.12).

Table III.12: *Exon-intron sizes of KIAA1949.*

Exon No.	Nt No.*	Exon size (bp)	Intron No.	Nt No.$^{+}$	Intron size (kb)
1	1-2906	2906			
2	2907-3116	209	1	30712912-30707910	5002
3	3117-4015	898	2	30707683-30705795	1888

*Clone 4Q starts near the end of exon 1. *, corresponding to sequence XM_166376.*$^{+}$*, corresponding to the human chromosome 6 sequence (NC_000006).*

By searching EST database, several ESTs were found to be present in the database. There are ESTs from brain, primary B-cells, spleen, thymus, brain and leukocytes. The existence of such ESTs supports the importance of this protein in several tissues.

III.3.8 I2-L (Protein Phosphatase 1 Inhibitor 2 Like)

Inhibitor 2 (I2) is a heat stable protein that inhibits PP1 activity *in vitro* with nanomolar affinity and was first purified from rabbit skeletal muscle (F. L. Huang and W. Glinsmann, 1976). It is a 23 KDa phosphoprotein well conserved among eukaryotes. PP1 is inactivated through binding to I2 and its activity is reestablished when I2 is phosphorylated at Thr72 by glycogen synthase kinase IIIβ (GSK-3β) (Hemmings *et al.*, 1982; Aitken *et al.*, 1984; DePaoli-Roach, 1984) and Cdk5 (Agarwal-Mawal and Paudel, 2001). PP1 can then dephosphorylate I2. I2 is also phosphorylated by casein kinase II (CK2) at Ser86, Ser120 and Ser121 (Holmes *et al.*, 1986b). Phosphorylation by CK2 does not alter the inhibitory activity of I2. Phosphorylation of Ser86 enhances the subsequent phosphorylation at Thr72 by GSK-3β (Park and DePaoli-Roach, 1994; Park *et al.*, 1994). A recent study showed that Ser120 and Ser121 phosphorylation, of previously unknown function, is also important for the binding of GSK-3β (Sakashita *et al.*, 2003). Other sequences are thought to be important in the formation of the PP1/I2 complex, such as the N-terminal IKGI sequence, Trp46 and the C-terminal FEMKRKLHY sequence (Helps *et al.*, 1998; H. B. Huang *et al.*, 1999; Yang *et al.*, 2000).

To date, two genes have been identified that code for I2, termed I2α and I2β (Osawa *et al.*, 1996). All the phosphorylation sites were found to be conserved in these I2 isoforms. Here, we report the finding of a new protein, termed I2-L (Inhibitor 2 Like) that is 96% identical at the nucleotide level and 95.6% identical at the aa level to I2 (Fig. III.24 and 25). Clone 48, shown in red in Figure III.26, starts at nt 57 and ends at nt 901 of the sequence XM_018216 in the database.

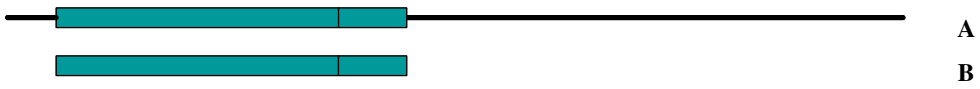


Figure III.24: *Diagram of the alignment of the nt sequences of **A)** I2 (NM_006241) and **B)** I2-L (XM_018216)).*

```
I2-L     1 MAASTASHRPIKGILKNKTSTTSSMVASAEQPRRSVDEELSKKSQKWDEINILATYHPAD
I2       1 MAASTASHRPIKGILKNKTSTTSSMVASAEQPRGNVDEELSKKSQKWDEMNILATYHPAD
             ********************************  ************** **********

I2-L    61 KGYGLMKIDEPSEPYHSMMGDDEDACRDTETTEAMAPDILAKKLAAAEGLEPKYRIQEQE
I2      61 KDYGLMKIDEPSTPYHSMMGDDEDACSDTEATEAMAPDILARKLAAAEGLEPKYRIQEQE
           * ********** ************* *** ********** ******************

I2-L   121 SSGEEDSDLSPEEREKKRQFEMRRKLHYNEGLNIKLARQLISKDLHDDDEDEEMLETADG
I2     121 SSGEEDSDLSPEEREKKRQFEMKRKLHYNEGLNIKLARQLISKDLHDDDEDEEMLETADG
           ********************** ************************************

I2-L   181 ESMNTEESNQGSTPSDQQQNKLRSS
I2     181 ESMNTEESNQGSTPSDQQQNKLRSS
           *************************
95.6% identity in 205 residues overlap; Score: 1006.0; Gap frequency:0.0%
```

Figure III.25: *Alignment of amino acid sequences of I2-L and I2. Highlighted residues represent important regulatory site; underlined region represents the most highly conserved region of the protein.*

```
1     gaggcagcaggtgcggccgctttagccctgagcgggctctgcggctgcctgcgagtctctg
62     ctgtgccgacccttctcttcgcggaccccacgccaagcagcgaccctgaggcgacagccg
122    gagcgcccggcaatggcggcctcgacggcctcccaccggcccatcaaggggatcttgaag
1                M  A  A  S  T  A  S  H  R  P  I  K  G  I  L  K
182    aacaagacctctacgacttcctctatggtggcgtcggccgaacagccccgcaggagtgtc
17      N  K  T  S  T  T  S  S  M  V  A  S  A  E  Q  P  R  R  S  V
242    gacgaggagctgagcaaaaaatcccagaagtgggatgaaattaacatcttggcgacctat
37      D  E  E  L  S  K  K  S  Q  K  W  D  E  I  N  I  L  A  T  Y
302    catccagcagacaaaggctatggtttaatgaaaatagatgaaccaagccctccttaccat
57      H  P  A  D  K  G  Y  G  L  M  K  I  D  E  P  S  P  P  Y  H
362    agtatgatgggtgatgatgaagatgcgtgtagggacaccgagaccactgaagccatggcg
77      S  M  M  G  D  D  E  D  A  C  R  D  T  E  T  T  E  A  M  A
422    ccagacatcctagccaagaaattagctgctgctgaaggcttggagccaaagtaccggatt
97      P  D  I  L  A  K  K  L  A  A  A  E  G  L  E  P  K  Y  R  I
482    caggaacaagaaagcagtggagaggaggatagtgacctctcacctgaagaacgagaaaaa
117     Q  E  Q  E  S  S  G  E  E  D  S  D  L  S  P  E  E  R  E  K
542    aagcgacaatttgaaatgagaaggaagcttcactacaatgaaggactcaatatcaaacta
137     K  R  Q  F  E  M  R  R  K  L  H  Y  N  E  G  L  N  I  K  L
602    gccagacaattaatttcaaaagacctacatgatgatgatgaagatgaagaaatgttagag
157     A  R  Q  L  I  S  K  D  L  H  D  D  D  E  D  E  E  M  L  E
662    actgcagatggagaaagcatgaatacggaagaatcaaatcaaggatctactccaagtgac
177     T  A  D  G  E  S  M  N  T  E  E  S  N  Q  G  S  T  P  S  D
722    caacagcaaaacaaattacgaagttcatagaagagatttgttcaacactgcaattgtttg
197     Q  Q  Q  N  K  L  R  S  S  *
782    ttagatataaaccctgtgactataatacattgcttcttgttctccacaattcatgactta
842    agtaccaaaatgcataccagttattatatattgccaagaattaaatgataaacttagaga
902    ctaattagactgaaaatgcctaattgatatatatattcttatgcctagtactttaccaca
962    aatacagtgtaatatcatcagtccaaaactgcattactttcataagaacactggttaatt
1022   tgtataagatattatagagctttttatgctttagaagttaagcaatatctttgggggga
1082   actaatttattttcatcactcgaaatgtggtagctcttacaaagtttgttgatttgtttt
1142   tttaaaaatcaaaagccagttgaacaacaggatatatagacttataaatattcaagctga
1202   atcgtattttaacacttctcttcaacttgatttgtctgtttaattgaaaagaattgtaag
1262   agttactgttgcattttctgacctactaccttaaaattcctgttgagtttctttgtgtt
1322   tacaaggaaaggactgaacttttttctcatcaaaactagctttttttccccacaaataaatt
1382   atcaggttaaactttc
```

Figure III.26: *Nucleotidic and amino acidic sequences of I2-L (XM_018216). In red is the sequence of the clone 48 (it starts at nt 57 and ends at nt 901). Underlined are the important aa sequences involved in PP1 binding. The blue adenine is absent in clone 48.*

The alignment of I2 and I2-L highlights the differences between the two proteins. The main difference is the Thr for Pro substitution at position 72. This Thr72 is the most important residue for the regulation of the PP1/I2 complex. Thr72 can be phosphorylated

by CDK5/p35, GSK3, MAPK and CDK2/cyclinB. The absence of Thr72 in I2-L raises an interesting question. Is I2-L an alternative constitutive inhibitor of PP1, not controlled by phosphorylation by GSK-3β but by an alternative mechanism? In Chapter V this question will be addressed in more detail. Another difference is the substitution of Ser86 by Arg. This fact also alters I2-L regulation by CK2 and subsequently the regulation of PP1 and other regulatory proteins, since it was recently shown that the PP1/I2 complex can bind a third protein, for instance, Nek2 (Eto *et al.*, 2002). It was shown that Ser86 phosphorylation by CK2 does not alter the inhibitory activity of I-2 but accelerates the subsequent phosphorylation at Thr72 by GSK-3 (Park and DePaoli-Roach, 1994).

By searching Prosite for motifs, several CK2 phospho sites (35-38 SvdE, 121-124 SsgE, 122-125 SgeE, 130-133 SpeE, 193-196 TpsD) were found, as expected. The 35-38 SvdE is a new putative CK2 phosphorylation site in I2-L that does not exist in I2. There are also other possible motifs: protein kinase C phosphorylation sites (7-9 ShR, 41-43 SkK, 44-46 SqK) and a putative ubiquitin interacting motif (170-189 EDEEMLETADGESMNTEESN).

Using the BLAST2 algorithm we aligned the XM_018216 sequence with the human genome and concluded that this sequence is present in chromosome 5, while the I2 gene is located in chromosome 3. The I2-L gene has an unique exon in contrast to the 5 exons of I2.

Curiously, *in vivo*, both negative and positive effects of I2 on PP1 activity were observed (Tung *et al.*, 1995; Nigavekar *et al.*, 2002). Overexpression of I2 induces a decrease in PP1 activity due to PP1 inhibition (Tung *et al.*, 1995). On the other hand, at physiological concentrations, I2 was found to lead to activation of PP1 (Tung *et al.*, 1995; Nigavekar *et al.*, 2002). This experiment suggests that I2 may not act simply as a direct inhibitor of PP1 activity *in vivo*. This idea is also supported by the fact that the *in vitro* inactivation of PP1 by I2 is blocked by salt at physiological concentrations (Bollen *et al.*, 1994). So the current notion is that I2 function may be to promote a PP1 conformational change that converts the enzyme to its active form. Supporting this view is the fact that the I2 phosphorylation mechanism is conserved: in yeast the major I2 kinase is Pho85, a cyclin-dependent kinase, which also phosphorylates glycogen synthase; in mammals I2 is phosphorylated, as pointed out above, by GSK-3β (Woodgett and Cohen, 1984) and also by Cdk5, the Pho85 orthologue (Agarwal-Mawal and Paudel, 2001).

In what concerns I2-L, the subtle differences in amino acid sequence with respect to I2 might induce a different behavior of the sperm PP1 holoenzyme, thus leading to a different mode of PP1 activity and sperm function.

III.4 DISCUSSION

PP1 involvement in diverse cellular processes is associated with its binding to a diverse set of regulatory subunits. Using the Yeast Two Hybrid System, new PP1 interactors from human testis were identified in order to gain insight into the various roles of PP1 in sperm motility. The results obtained validated the method as a promising approach to understand the multiple functions of PP1 catalytic subunit, being a method of choice to screen a large number of proteins for putative interactions. We set out to identify all the positive clones obtained in our screens. This strategy turned out to be very fruitful because we could identify several new very interesting clones that would never be found if we decided only to study the more abundant clones. Some bonafide, previously characterized, PP1 binding proteins were identified in both screens (YTH1 and YTH2) like NIPP1 and Nek2. These findings validated our approach by confirming that expression of reporter genes was due to a specific interaction between the bait protein (PP1γ2/ PP1γ1) and the identified library protein.

Nek2 was by far the most frequent interaction in both the YTH1 (47 out of 120) and the YTH2 (71 out of 155) screens. There is a panoply of data supporting the involvement of PP1 in mitosis. Specifically, PP1 was shown to be targeted to multiple mitotic structures such as chromosomes, spindle and centrosomes. Centrosomes duplicate during S-phase and remain paired during G2. Before the onset of mitosis the centrosomes split to form the poles of the mitotic spindle. Many of the changes that occur during the centrosome cycle are due to protein phosphorylation (Nigg, 2001). Two kinases have been shown to be involved in centrosome splitting and to be inactivated by PP1, indicating that PP1 prevents precocious separation of centrosomes. One of the kinases is Aurora-A, a homologue of Aurora-B (Katayama *et al.*, 2001), that interacts with PP1 strongly at mitosis. PP1 dephosphorylates and inactivates Aurora-A and is also an *in vitro* substrate for Aurora-A, being inactivated by its phosphorylation.

The other kinase related to centrosome separation is Nek2. Nek2 is tethered to centrioles via binding to a specific substrate called C-Nap1 (Nek-2 associated protein 1)

(Fry *et al.*, 1998a). Phosphorylation of C-Nap1 correlates to loss of centrosome cohesion and C-Nap1 is likely to be the substrate and effector of Nek2-PP1 complex. It has also been shown that I2, a PP1 inhibitor, can enhance the kinase activity of the Nek2-PP1 complex via inhibition of PP1 to initiate centrosome splitting (Eto *et al.*, 2002). This complex is auto-regulated since Nek2 phosporylates PP1 at Thr307 and Thr318. PP1, in turn, dephosphorylates Nek2 (Helps *et al.*, 2000). The kinase/phosphatase complex of Nek2-PP1 functions as a bistable switch, as first described by Sohaskey and Ferrell (2002).

Besides the two splice variants Nek2A and Nek2B, identified in primary blood lymphocytes and adult transformed cells (Hames and Fry, 2002), no other Nek2 isoforms have been described to date. Here we identified a new alternatively spliced isoform of Nek2 in human testis called Nek2A-T. Murine Nek2 was shown to be highly expressed in adult testis. It was also shown that Nek2 was expressed in a stage specific pattern during spermatogenesis and intense signals were also observed in oocytes (Tanaka *et al.*, 1997). The new Nek2A-T isoform misses a phosphorylatable domain of 8 amino acids (ELLNLPSS) which includes two serines (Fig. III.7.). These serines could be important for regulation of PP1 in two ways. First, PP1 may dephosphorylate these serine residues in Nek2A but since they are missing from Nek2A-T, the latter would not be subject to such regulation, ultimately leading to an increase in centrosome splitting, in agreement with testis being a tissue with high levels of cell division. Second, the absence of this 8 amino acids stretch could alter Nek2A-T conformation resulting in a different affinity for PP1. In conclusion, this may be a very important isoform of Nek2 in testis and further experimentation is required to address the significance of this splicing event. Nek2 was shown to be phosphorylated on the two serine residues that occur N-terminally to the PP1 binding domain, thus that are present in the phosphorylatable domain. It is interesting to note that it is precisely the phosphorylatable 8 amino acid domain of Nek2A that is missing from Nek2A-T. Thus, although Nek2A and Nek2A-T are likely to exhibit distinct properties, it may be difficult to specifically target Nek2A-T for therapeutics since it lacks the phosphorylatable, regulatory 8 amino acid domain. However, the effectiveness of siRNA directed against Nek2A-T can still be explored.

Another new alternatively spliced variant found in the YTH2 was designated NIPP1-T. While Nek2A-T derives from an alternative 3'splice-site of the pre-mRNA, the NIPP1-T results from excision of part of exon 7 (Fig. III.7 and III.14). NIPP1 was

originally identified in the particulate fraction of bovine thymus nuclei. Subsequent studies showed that the full length NIPP1 cDNA encoded a protein of 38.5 kDa that was extremely sensitive to proteolysis. NIPP1 is perhaps the most potent inhibitor of PP1 thus far identified, with a Ki in the picomolar range. The protein is largely nuclear in its localization and is ubiquitously expressed in mammalian tissues (Van Eynde *et al.*, 1995). Although NIPP1, like I-1 and DARPP-32, possesses the consensus PP1-binding motif, it is more like I-2 in its action, being a more potent phosphatase inhibitor in the dephosphorylated state. Moreover, NIPP-1, like I-2, forms a stable, inactive complex with PP1. Following phosphorylation of NIPP1 by PKA on a site within the PP1-binding motif, its activity as a PP1 inhibitor is dramatically reduced. NIPP1 is phosphorylated at Ser178 and Ser199 by PKA and on Thr161 and Ser204 by CK2 (Vulsteke *et al.*, 1997). Both kinases reduce the affinity of NIPP1 for PP1 and increase phosphatase activity. The effects of the two kinases in activating the NIPP1/PP1 complex are synergistic but do not cause dissociation of the complex. NIPP1 phosphorylation may prevent its re-association with PP1. NIPP1 contains a RNA-binding motif also found in the bacterial RNA processing enzyme, Ard-1. The extensive sequence homology between mammalian Ard-1 and the C-terminus of NIPP1 suggests that the two proteins are derived from the same gene through alternative splicing (Vulsteke *et al.*, 1997). Recombinant NIPP1 was shown to bind to RNA and its C-terminal fragment was shown to possess endonuclease activity. While RNA-binding was not affected by the presence of PP1, full length NIPP1 did not degrade RNA (Jagiello *et al.*, 1997).

Although several alternatively spliced variants of NIPP1 are known (Van Eynde *et al.*, 1999), NIPP1-T appears to be a novel testis-specific variant that uses the same polyadenylation site as NIPP1β. At the protein level, it differs from the latter in that it has a longer C-terminal extension. It is interesting to note that whereas NIPP1β possesses the sequence –KKPTP- in the spliced domain (a proline directed kinase consensus acceptor site), a related sequence also occurs in NIPP1-T (-NAPSP-). Both motifs are located exactly 4 amino acids from the C-terminus of the two proteins. In this case, the distinct C-terminal sequence of NIPP1-T could constitute a specific therapeutic target.

The specificity of the interactions between PP1γ1 or PP1γ2 and the identified positives was confirmed by sequential transformation of yeast strain AH109 with the putative interacting proteins (Fig. III.12 and III.18). To this end the longest cDNAs

obtained for Nek2A (clone 25), Nek2A-T (clone 3Q) and NIPP1-T (273) were used. All interactions described in this chapter were confirmed by this method. Furthermore, the binding of PP1γ2 to Nek2A and Nek2A-T was also verified by the blot overlay technique. Both proteins were expressed in *E. coli* and found to bind purified PP1γ2 (Fig. III.11). Thus, both the previously known Nek2A isoform and the novel Nek2A-T and NIPP1-T variants appear to bind PP1γ2 *in vitro* (blot overlays) and *in vivo* (yeast co-expression). In order to evaluate whether the novel protein variants detected were truly testis-specific and produced by alternative splicing, Northern blot and RT-PCR analyses were also performed. The results obtained indicate that Nek2A-T expression is largely restricted to the testis (Fig. III.10). However, both Nek2A and Nek2A-T appear to be expressed in testis, since the RT-PCR performed using the indicated primers detected fragments of the expected length for both (Fig. III.9 and III.15). The probe used for NIPP1-T was also expected to hybridize with NIPP1. Thus, only a single message was detected in rat tissues, being particularly abundant in testis, heart and brain. As expected, two mRNAs were detected in human testis, corresponding to NIPP1 and NIPP1-T (Fig. III.16 and III.17). Therefore, it appears that both Nek2A-T and NIPP1-T are new and testis-specific alternatively spliced variants. Although Nek2A-T and NIPP1-T were shown to interact with both PP1γ isoforms, the significance of these observations remains to be fully clarified. Since sperm were found to express almost exclusively the PP1γ2 isoform, the possible interaction of Nek2A-T and NIPP1-T with PP1γ1 would only be expected to have physiological relevance in other testis cell types.

Some of the positive clones encoded known PP1 binding proteins joined out of frame to GAL4-BD (Table III.3, WRF). We would therefore expect a totally different reading frame to code for an artificial protein that might not bind PP1. This was the case, for example, with well known PP1 binding proteins PPP1R13A and PP1R13B. Thus, the fact that several bonafide PP1 regulators were fused to the GAL4-BD in a wrong reading frame strongly suggested that the correct protein might still be produced by a well established mechanism in yeast, called programmed translational frameshift or translational recoding (Shah *et al.*, 2002). Some specific sequences, 7 nucleotides long, are known to induce ribosomal frameshifting. This is a directional and reading frame specific event. Several programmed frameshift heptanucleotides have been identified: CUU-AGG-C, CUU-AGU-U and GGU-CAG-A (Shah *et al.*, 2002).We concluded that translational

recoding may be a feasible explanation for these observations. Moreover, this mechanism could be enhanced because of the use of selective media without some amino acids. So, the yeast translation machinery might be using alternative codons favouring the existing amino acids.

The majority of the recovered clones encoded partial cDNA sequences (Table III.3) but each shows specific interaction with PP1 in the yeast two hybrid system. The large number of totally new putative PP1 binding proteins opens new fields of study. All the uncharacterized proteins need to be further analysed in order for their functions to be identified, allowing new roles to be attributed to PP1, not only in sperm motility but in other cellular functions. In what concerns PP1γ2 and sperm motility, this screen provided a large amount of data that will be useful in our search for testis-specific PP1 binding proteins that may be used as targets for therapeutic intervention at the level of male infertility or contraception.

IV SEARP – a novel PP1 interacting protein

IV.1 INTRODUCTION

It is now well estabilished that PP1 occurs in cells in multi-component complexes with regulatory/targeting subunits that are responsible for controlling its activity and fulfilling its cellular potential. Some PP1 regulatory subunits are known to direct the catalytic subunit to specific cellular loci (Ouimet *et al.*, 1995; Allen *et al.*, 1997), restricting the action of the catalytic subunit to the substrates expressed there. Thus, such regulatory subunits can be highly specific to particular subcellular compartments (Ouimet *et al.*, 1995) and control a much more restricted number of cellular events than the phosphatase catalytic subunit itself.

Until now more than 50 different PP1 regulatory subunits have been identified and were shown to be responsible for the involvement of PP1 in a number of diverse cellular functions from protein synthesis to regulation of the cell cycle, muscle contraction, glycogen metabolism, synaptic plasticity, gene transcription, etc (Cohen, 2002). The overall picture is summarized in Table IV.1, listing the classification of PP1 R-subunits.

Table IV.1: *Classification of the R subunits of PP1.*

Regulatory subunit		General Function	Reference
I-1	PPP1R1A	PP1 inhibitors	(F. L. Huang and W. Glinsmann, 1976)
DARPP-32	PPP1R1B		(Hemmings *et al.*, 1984c)
I-2	PPP1R2		(F. L. Huang and W. Glinsmann, 1976)
GM (RGL, R3)	PPP1R3A	Glycogen metabolism	(Stralfors *et al.*, 1985)
GL (R4)	PPP1R3B		(Doherty *et al.*, 1995; Moorhead *et al.*, 1995)
R5(PTG)	PPP1R3C		(Doherty *et al.*, 1996)
R6	PPP1R3D		(Armstrong *et al.*, 1997)
Sds22	PPP1R7	Mitosis/Meiosis	(Dinischiotu *et al.*, 1997)
NIPP-1 (Ard-1)	PPP1R8	RNA splicing	(Van Eynde *et al.*, 1995)
SIPP-1			(Llorian *et al.*, 2004)
PSF1			(Hirano *et al.*, 1996)
Neurabin I	PPP1R9A	Neurite outgrowth, synapse morphology	(MacMillan *et al.*, 1999)
Spinophilin (neurabin II)	PPP1R9B	Glutamatergic synaptic transmission, dendritic morphology	(Allen *et al.*, 1997)
p99 (R111, PNUTS)	PPP1R10	RNA processing or transport?	(Allen *et al.*, 1998)

Hox11		Cell cycle checkpoint	(Kawabe *et al.*, 1997)
Inhibitor-3 (HCG-V)	PPP1R11	Inhibits PP1	(Zhang *et al.*, 1998)
HCF		Transcription, cell cycle	(Ajuh *et al.*, 2000)
MYPT1 (M110, MBS, M130)	PPP1R12A	Myosin/actin targeting	(Alessi *et al.*, 1992)
MYPT2 (PP1bp55, M20-spliced form)	PPP1R12B		(Moorhead *et al.*, 1998)
p85	PPP1R12C		(Tan *et al.*, 2001)
Phactr1			(Allen *et al.*, 2004)
L5 ribossomal protein		Protein synthesis?	(Hirano *et al.*, 1995)
RIPP1			(Beullens *et al.*, 1996)
53BP2 (TP53BP2, p53-binding protein 2)	PPP1R13A	Cell cycle checkpoint?	(Helps *et al.*, 1995)
CPI-17	PPP1R14A	Inhibits the myosin bound PP1 complex	(Eto *et al.*, 1997)
PHI-2	PPP1R14B	Inhibits PP1	(Eto *et al.*, 1999)
PHI-1			(Eto *et al.*, 1999)
KEPI			(Liu *et al.*, 2002)
GBPI-1		Inhibits PP1 when phosphorylated (Brain/Stomach)	(Liu *et al.*, 2004)
GBPI-2		Inhibits PP1 when phosphorylated (Testis)	(Liu *et al.*, 2004)
I-4			(Shirato *et al.*, 2000)
GADD34	PPP1R15A	Protein synthesis	(Connor *et al.*, 2001)
CReP	PPP1R15B	Protein synthesis	(Jousse *et al.*, 2003)
AKAP 149		Nuclear envelope reassembly (dephosphorylation of B-type lamins)	(Steen *et al.*, 2000)
NF-L		Synaptic transmission?	(Terry-Lorenzo *et al.*, 2000)
AKAP-220		Coordination of PKA/PP1 signaling	(Schillace *et al.*, 2001)
Yotiao		Synaptic transmission (NMDA receptor ion channel activity)	(Westphal *et al.*, 1999b; Zhao *et al.*, 1999)
BH-protochaderin c		Neuronal cell-cell interaction	(Yoshida *et al.*, 1999)
Rynanodyne receptor		Calcium ion channel activity?	(Zhao *et al.*, 1998)
NKCCl		Cl transport	(Darman *et al.*, 2001)
AKAP 350 (CG-NAP, AKAP450)		Centrosomal function	(Takahashi *et al.*, 1999)
Nek2			(Helps *et al.*, 2000)
Tau		Microtubule stability?	(Liao *et al.*, 1998)
Bcl2		Apoptosis	(Ayllon *et al.*, 2001)
RB		Cell cycle progression	(Durfee *et al.*, 1993)
PRIP-1 (p130, PLC-L1)		Calcium signaling?	(Yoshimura *et al.*, 2001)
PFK		Glycolisis?	(Zhao and Lee, 1997b)
PP1bp80		Myosin targeting	(Damer *et al.*, 1998)
MYPT3	PPP1R16A		(Skinner and Saltiel, 2001)
$I1^{PP2A}$(PHAPI)		Stimulation of PP1 and Inhibition of PP2A	(Katayose *et al.*, 2000)
$I2^{PP2A}$(SET, PHAPII, TAF1β)			(Katayose *et al.*, 2000)
G-substrate		Inhibition of PP1	(Aitken *et al.*, 1981)

Grp78		Unknown	(Chun *et al.*, 1994)
NCLK		Activates PP1	(Agarwal-Mawal and Paudel, 2001)
Myr8		Brain development	(Patel *et al.*, 2001)
FAK		PP1 dephosphorylates FAK in cells released from mitosis	(Fresu *et al.*, 2001)
Herpes vírus γ1 34.5 protein		Inhibits protein synthesis	(He *et al.*, 1997)
mGluR7b		Unknown	(Enz, 2002)
Histone H3		Mitosis	(Hsu *et al.*, 2000)
Scapinin		Associated with the nuclear nonchromatin structure	(Sagara *et al.*, 2003)
14-3-3		sperm	(Huang *et al.*, 2004)

Abbreviations: PTG, Protein targeting to glycogen; MYPT, myosin phosphatase targeting subunit; MBS, myosin binding subunit; NIPP, Nuclear inhibitor of PP1; PSF, polypyrimidine tract-binding protein associated splicing factor; PNUTS, phosphatase 1 nuclear targeting subunit; Hox11, Homeodomain transcription factor; HCF, host cell factor or human factor C1; RIPP1, ribosomal inhibitor of PP1; PHI, phosphatase holoenzyme inhibitor; KEPI, kinase-enhanced protein phosphatase type 1 inhibitor; GADD34; growth arrest and DNA damage protein; AKAP, A-kinase anchoring protein; NKCCl, Na-K-Cl cotransporter; Nek2; NIMA-related protein kinase; Rb, retinoblatoma protein; PRIP-1; phospholipase C-related inactive protein; PFK, Phosphofrutokinase; G-substrate, cGMP-dependent protein kinase substrate; Grp-78, glucose-regulated protein, member of the HSP-70 family; NCLK, neuronal cdc2-like kinase; FAK; Focal adhesion kinase. The human genome nomenclature for the regulatory subunits that are only classified as PP1 regulators is indicated in column 2. PPP1R4, PPP1R5 and PPP1R6 have recently been re-classified as PPP1R3B, PPP1R3C and PPP1R3D.

The cellular functions of several PP1 complexes are now well established although there are a number of interactions that have not yet been fully characterized. Most PP1 regulatory subunits have a consensus binding motif but they do not exhibit any other sequence similarities that would allow their identification them by bioinformatic means. The methods that are used to identify PP1 regulators include the Yeast Tho Hybrid System, immunoprecipitation of PP1 complexes and the screening of expression libraries with PP1 as probe. The known PP1 regulators can be classified into targeting or modulator subunits. Several PP1 targeting subunits can target PP1 to glycogen. These include GM, GL, R5 and R6 that possess a glycogen targeting domain (Doherty *et al.*, 1996; Wu *et al.*, 1996; Armstrong *et al.*, 1997; Armstrong *et al.*, 1998; Wu *et al.*, 1998). Three novel proteins encoded in the human genome also have a similar domain (Ceulemans *et al.*, 2002). GM

(124 kDa) is a G subunit that directs PP1 to glycogen particles and membranes of the sarcoplasmic reticulum in muscle (Hubbard and Cohen, 1993), GL (33kDa) directs PP1 to glycogen in liver (Cohen, 1989) and R5 (36kDa) is related to GL but is ubiquitously expressed (Doherty *et al.*, 1995).

MYPT1, MYPT2, MYPT3, p85 and phactr1 target PP1 to myosin/actin (Hirano *et al.*, 1997; Johnson *et al.*, 1997; Skinner and Saltiel, 2001; Tan *et al.*, 2001; Allen *et al.*, 2004). Smooth muscle myosin phosphatase is composed of three subunits: PP1c and large (110kDa) and small (20kDa) noncatalytic subunits. The large subunit binds to myosin and to PP1. This subunit was called MYPT1 (myosin phosphatase target subunit 1) because it binds to myosin and to PP1cβ activating it (Tanaka *et al.*, 1998). The N-terminal sequence and the ankyrin repeats of MYPT1 are involved in PP1 binding. PKC phosphorylates Thr34, the residue that precedes the consensus PP1-binding motif, KVKF, in MYPT1 but this did not affect binding of the peptide to PP1c. Another phosphorylation site, within the ankyrin repeat, diminished the stimulatory effect of MYPT1 on the phosphorylated 20kDa myosin light chain phosphatase activity of PP1c (Toth *et al.*, 2000a). In a yeast two hybrid screen using PP1 as bait a new homolog of the MYPT family, MYPT3 (Skinner and Saltiel, 2001), was found to interact with PP1. The N-terminal region of MYPT3 consists of a consensus PP1-binding motif and multiple ankyrin repeats. MYPT3 is distinguished from the other members of the MYPT family by its molecular mass of 58kDa and a unique C-terminal region that contains several potential signaling motifs and a CaaX prenylation site. Endogenous PP1 from 3T3-L1 lysates specifically interacts with MYPT3. Additionally, purified PP1 was inhibited by recombinant GST-MYPT3 using phosphorylase a, myosin light chain and myosin substrates *in vitro* (Skinner and Saltiel, 2001).

PP1 can also be targeted to particular subcellular locations by interacting with A-kinase-anchoring proteins (AKAPs) that keep PKA and PP1 in close proximity (Schillace and Scott, 1999). These include AKAP-149, AKAP-220, Yotiao and AKAP-350 (Takahashi *et al.*, 1999; Westphal *et al.*, 1999b; Steen *et al.*, 2000; Schillace *et al.*, 2001). For example, AKAP350 (Takahashi *et al.*, 1999) is a scaffold protein that assembles several protein kinases and protein phosphatases, including PP1, at the centrosomes throughout the cell cycle and at the Golgi during interphase.

There are several PP1 regulators that are involved in RNA processing. L5 (Hirano *et al.*, 1995) is part of the 60S ribosomal subunit and localizes in both the cytoplasm and the nucleus of eukaryotic cells, accumulating particularly in the nucleoli. L5 is known to bind specifically to 5S rRNA and is involved in nucleocytoplasmic transport of this rRNA (Rosorius *et al.*, 2000). L5 was first isolated from rat liver ribosomes and activated phosphatase activity of a myosin-bound phosphatase and the isolated type 1 catalytic subunit using phosphorylated myosin light chains and phosphorylase a as substrates. In addition, it was shown that phosphatase sedimented with ribosomal subunits containing L5 but did not sedimented with those deficient in L5. These data indicated that L5 binds to PP1 and may act as a target molecule for PP1 in ribosomal function or other cell mechanisms. PSF (polypirimidine tract-binding protein associated splicing factor) (Hirano *et al.*, 1996) was found to interact with and inhibit PP1β. PSF contains the consensus PP1-binding motif and may therefore act as a PP1 target molecule in the spliceosome (Hirano *et al.*, 1996). RIPP-1 (ribosomal inhibitor of PP1) (Beullens *et al.*, 1996) is a 23 kDa basic polypeptide that is complexed with PP1 in rat liver ribosomes. *In vitro*, RIPP-1 is a potent inhibitor of PP1 (Ki=20nM) with some substrates, (phosphorylase a and myelin basic protein) but a much poorer inhibitor (Ki=400nM) with other substrates (histone IIA and casein). RIPP-1 inhibits PP1-mediated dephosphorylation of ribosomal S6, a component of the 40S ribosomal subunit and suggests a role in the control of protein synthesis (Beullens *et al.*, 1996). PNUTS (Kreivi *et al.*, 1997; Allen *et al.*, 1998) was found to bind PP1 in a two hybrid assay. PNUTS is widely expressed in human tissues and shows a punctate nucleoplasmic staining with additional accumulation within the nucleolus. The C-terminus of PNUTS contains seven RGG RNA-binding motifs, and a putative zinc-finger domain. Recombinant PNUTS suppresses the phosphorylase phosphatase activity of PP1 and the canonical PP1-binding motif (residues 396-401) is unusual in that the phenylalanine is replaced by a tryptophan. Recently, PNUTS was shown to inhibit PP1 activity towards the retinoblastoma protein (Rb) (Udho *et al.*, 2002). Also NIPP1 targets PP1 to the splicing machinery (Jagiello *et al.*, 2000). This regulator was discussed in the previous chapter.

Some PP1 binding proteins are brain specific. Cyclic GMP and cGMP-dependent protein kinase (PKG) mediate several of the actions of nitric oxide in the brain (Wang and Robinson, 1997). A specific substrate for PKG, termed G substrate (Hall *et al.*, 1999), is highly expressed in Purkinje cells of the cerebellum. Purified recombinant G substrate has

recently been shown to be an inhibitor of PP1. The inhibition was dependent on phosphorylation by PKG (Hall *et al.*, 1999). Despite its structural similarity to DARPP-32, which is highly specific for PP1, recombinant G substrate inhibits PP2A more effectively than PP1. Phosphorylation of G substrate may represent a novel pathway for regulation of neural PP1 and PP2A in response to nitric oxide production. Myr8 myosins comprise a new class of myosins that have been designated class XVI. The head domain contains a large N-terminal extension composed of multiple ankyrin repeats implicated in mediating an association with PP1α and γ1. The structural features and restricted expression patterns suggest that members of this novel class of unconventional myosins comprise a mechanism to target selectively the PP1 in developing brain (Patel *et al.*, 2001). Neurofilament-L (NF-L), a 70kDa membrane-bound protein, was identified as a PP1 binding protein in bovine brain cortex plasma membranes. Bovine NF-L, at nanomolar concentration, inhibited the phosphorylase phosphatase activity of rabbit skeletal muscle PP1c but not the activity of PP2A. PP1 binding to the bovine NF-L was mapped to the head region. NF-L may target the functions of PP1 in membranes and cytoskeleton of mammalian neurons (Terry-Lorenzo *et al.*, 2000). NCLK (neuronal Cdc2-like protein kinase) a heterodimer of Cdk5 and the regulatory p25 subunit binds PP1 through Cdk5. In brain extracts PP1/I-2 and NCLK are associated within a 450kDa complex suggesting that NCLK is one of the PP1/I-2 activating kinases in mammalian brain. *In vitro* NCLK phosphorylates I-2 on Thr72 and activates PP1/I-2 in an ATP/Mg-dependent manner, just like GSK3 (Agarwal-Mawal and Paudel, 2001). Neurabin I is a brain-specific actin-binding protein that binds PP1 and inhibits its activity. Phosphorylation of recombinant GST-neurabin I (residues 318-661) by PKA significantly reduced its binding to PP1. These findings identify a signaling mechanism involving the regulation of PP1 activity and localization mediated by the cAMP pathway (McAvoy *et al.*, 1999). Dendritic spines receive the vast majority of excitatory synaptic contacts in the mammalian brain and are presumed to contain machinery for the integration of various signal transduction pathways. PP1 is greatly enriched in dendritic spines and has been implicated in both the regulation of ionic conductance and long-term synaptic plasticity. A novel PP1 binding protein, spinophilin (neurabin II), was identified by the yeast two hybrid approach that is a potent modulator of PP1 enzymatic activity *in vitro*. Spinophilin has the properties expected of a scaffold protein localized to the cell membrane. Spinophilin represents a novel targeting subunit for

PP1 which directs the enzyme to those substrates in the dendritic spine compartment, e. g., neurotransmitter receptors, which mediate the regulation of synaptic function by PP1 (Allen *et al.*, 1997). PP1 is distributed in many regions of neurons, in addition to dendritic spines where it is target by spinophilin, suggesting the existence of multiple targeting proteins (Ouimet *et al.*, 1995). PP1 is associated with neurofilaments. As phosphorylation of neurofilament proteins is associated with depolymerization, neurofilament-associated PP1 may regulate neurofilament stability. PP1c also associates with brain microtubules where it appears to be tethered by the microtubule-associated protein tau. Tau acts as targeting protein that bridges PP1 to microtubules and can also be a substrate for the phosphatase (Liao *et al.*, 1998).

The protein kinase Nek2 (Fry *et al.*, 1998b) targets PP1 to the centrosomes and it was extensively discussed in Chapter III. Regulatory subunits that do not target PP1 to any particular location may instead act on its specificity to certain substrates. For instance, RB (retinoblastoma) interacts with many cellular proteins in complexes potentially important for its growth-suppressing function (Durfee *et al.*, 1993). A yeast two hybrid screen using RB as bait was performed and PP1α2 was found to interact with it. PP1α2 differs from the originally defined PP1α isoform by an amino-terminal 11 amino acid insert. PP1α isoforms preferentially bind the hyperphosphorylated form of RB. Cell cycle synchrony experiments revealed that this association occurs from mitosis to early G1. More recent studies have shown that PP1 is associated with the direct dephosphorylation of RB while PP2A is involved in pathways regulating G1 cyclin-dependent kinase activity (Yan and Mumby, 1999). The p53 binding protein (p53BP2) (Helps *et al.*, 1995) was identified as a protein interacting with PP1 also in a yeast two hybrid screen. The p53BP2-PP1 complex was stable at NaCl concentrations that dissociate the p53-p53BP2 complex, and the binding of PP1 and p53 to p53BP2 was found to be mutually exclusive. The region required for interaction with PP1 was shown to be contained within amino acids 297-431 of p53BP2, which includes two ankyrin repeats. The phosphorylase phosphatase activity of PP1 was inhibited by p53BP2 at nanomolar concentrations. These results suggest that PP1 may be involved in dephosphorylation and regulation of p53 through interaction with p53BP2 (Helps *et al.*, 1995). Bcl-2 was also found to interact with PP1α and Bad. Bcl-2 depletion decreased phosphatase activity and association of PP1α and Bad. Bcl-2 contains

the PP1-binding motif RIVAF. So Bcl-2 seems to target PP1 to Bad, directly involving PP1 in apoptosis (Ayllon *et al.*, 2001).

Most PP1 inhibitor proteins are thermostable inhibitors of low molecular mass. The existence of such a high number of inhibitor proteins (I-1, DARPP-32, I-2, I-3, CPI-17, PHI-2, PHI-1, I-4, $I1^{PP2A}$, $I2^{PP2A}$) indicates that the activity of free PP1 catalytic subunit must be kept under strict control.

The role of I-1 in regulating PP1 function has been investigated in many different physiological settings: hormonal control of glycogen metabolism, synaptic plasticity controlled by neurotransmitters, growth of pituitary tumor cells and the control of muscle contraction (F. L. Huang and W. H. Glinsmann, 1976). Some but not all hormones that elevate intracellular cAMP activate I-1. Hormones that activate I-1 induce much larger and more rapid changes in signal transduction pathways transduced by PP1 substrates. Hormones, like adrenalin, which activate PKA result in increased phosphorylation and inactivation of glycogen synthase in mammalian skeletal muscle. This activation is not due to the direct action of PKA on glycogen synthase. Although PKA can phosphorylate and inactivate glycogen synthase *in vitro*, adrenalin promotes the phosphorylation of glycogen synthase at serine residues that are not PKA targets (Poulter *et al.*, 1988). Moreover, the protein kinase, GSK3, that phosphorylates these serines is itself not activated by PKA. So, it was determined that the mechanism for enhanced glycogen synthase phosphorylation was the PKA-mediated activation of I-1 and the consequent inhibition of PP1 (Nakielny *et al.*, 1991). This revealed a new mechanism for hormone action in that PP1 modulation via I-1 mediated cAMP regulation of proteins that are substrates for kinases other than PKA. Other hormones, such as insulin, result in the dephosphorylation and activation of glycogen synthase. Numerous studies have reported that insulin activates PP1 in insulin-sensitive cells (Chan *et al.*, 1988). In contrast to GM, some of the G-subunits present in other tissues are not subject to regulation by insulin (Brady *et al.*, 1997). This makes the role of I-1 in hormonal control of glycogen metabolism even more important in these tissues.

Changes in synaptic transmission elicited by prior neuronal activity have been extensively studied in the hippocampus as a potential model for learning and memory. Activity-dependent enhancement of synaptic transmission is seen as long term potentiation or LTP. Other stimuli depress the functions of hippocampal synapses leading to long term

depression or LTD. Several protein kinases, including CaM-kinase II, PKC (Malinow *et al.*, 1989) and fyn (Grant *et al.*, 1992), have been implicated in LTP. Recent studies have established that two protein phosphatases, PP1 and PP2B, act in tandem to regulate LTD (Mulkey *et al.*, 1994). I-1 is highly expressed in the hippocampus and functions as the link between PP2B and PP1. LTP and LTD are both activated by the excitatory neurotransmitter glutamate, acting via the second messenger calcium. A single second messenger mediates such opposing physiological effects because of the different calcium sensitivities of PP2B and CaM-kinase II in neurons. Low calcium levels activate PP2B and induce LTD, while higher concentrations of calcium activate CaM-kinase II which is required to trigger LTP. Thus, the current model is that LTP-generating stimuli result in the activation of both CaM-kinase II and calcium/calmodulin-stimulated adenylyl cyclase. The role of cAMP in the LTP pathway is to activate I-1 and suppress PP1 activity which reverses the actions of CaM-kinase II. Hence, I-1 functions as the gatekeeper which determines whether the neuron will transition from early to intermediate or late phases of LTP. In contrast to LTP, LTD results from the small influx of calcium which is unable to activate either CaMKII or adenyl cyclase, and the predominant signal transducer for LTD is PP2B.

The β-adrenergic agonist isoproterenol has a positive ionotropic effect in the heart. Isoproterenol increases I-1 phosphorylation and results in the inhibition of PP1 activity in the myocardium (Neumann *et al.*, 1991). This decrease in PP1 activity enhances cardiac contractility by preventing the dephosphorylation of proteins such as the Na/K-ATPase, phospholamban, troponin I and voltage sensitive calcium channels which are all involved in maintaining the contractile state of heart muscle (Lindmann and Watanabe, 1989). On the other hand, smooth muscle PP1 is tightly bound to myosin and is the primary myosin phosphatase (Chisholm and Cohen, 1988). Dephosphorylation of myosin light chain results in the relaxation of smooth muscle. Thus, hormones such as epinephrine that increase intracellular cAMP and lead to I-1 phosphorylation promote the relaxation of smooth muscle. This paradox was resolved by the finding that smooth muscle myosin light chain kinase (MLCK) is phosphorylated at an inhibitory site in response to cAMP. The dephosphorylation of MLCK at this site is also mediated by PP1. Thus, it has been proposed that hormonal activation of I-1 leads to the predicted inhibition of PP1 that acts on MLCK. MLCK is inactivated and smooth muscle relaxation occurs. Unlike CaM-kinase

II which is inactivated by PP1, MLCK requires PP1 activity to remain active. So, I-1 functions in opposing ways to control protein kinases in neurons and smooth muscle. This model also predicts that the myosin bound PP1 is not regulated by I-1. This may be consistent with the presence of the common PP1-binding motif in I-1 and the 110kDa myosin-targeting subunit of PP1 (MYPT1), see below. Alternatively, the interplay between I-1, the myosin and the MLCK phosphatases may modulate the rate of protein phosphorylation and dephosphorylation that set the contractile tone of the smooth muscle.

DARPP-32 (dopamine and cAMP-regulated phosphoprotein) (Hemmings *et al.*, 1984c) although originally identified by its enhanced phosphorylation in response to dopamine, is activated by many hormones and neurotransmitters that modulate cAMP levels (Snyder *et al.*, 1992). DARPP-32 shows a more complex mode of regulation than I-1. For instance, DARPP-32 is phosphorylated *in vivo* by both PKA and casein kinase I. Phosphorylation by casein kinase I impairs the turnover of phosphate at the activating Thr-34 site (Desdouits *et al.*, 1995b). Indeed, two different phosphatases, PP2B acting on Thr34 and PP2C dephosphorylating the casein kinase I sites, may regulate DARPP-32 function. In brain, dopamine and glutamate have antagonic effects on the excitability of neurons, possibly mediated by their opposing effects on DARPP-32. Dopamine acting via D1 receptors activates adenyl cyclase, and through PKA, activates DARPP-32 (Walaas *et al.*, 1983). Glutamate on the other hand, working through NMDA receptors, increases intracellular calcium that activates PP2B and reverses DARPP-32 activity. DARPP-32 also appears to be a critical component of the control of salt balance in the mammalian kidney. Use of DARPP-32 phosphopeptides showed that PP1 inhibition increased the phosphorylation of Na/K-ATPase. This resulted in increased sodium excretion as the ATPase no longer pumped sodium from the urine back into the blood stream. Vasodilation occurs as water flows into the kidney to maintain the ion gradient (Aperia *et al.*, 1991). DARPP-32, but not I-1, is present in 3T3-L1 adipocytes and in these cells it is required for adipogenesis. Active DARPP-32 maintains low PP1 activity in differentiated fat cells and is essential for insulin to stimulate PP1 activity and facilitate triglyceride biosynthesis (Brady *et al.*, 1997). cAMP- mediated activation of DARPP-32 may be critical for amplifying the phosphorylation events that lead to activation of hormone-sensitive lipase and enhanced lipolysis.

I-2 (F. L. Huang and W. H. Glinsmann, 1976) forms a stable and inactive complex with PP1c. This inactive complex was isolated as an ATP-Mg-dependent phosphatase activity which remained inactive until incubated with ATP-Mg. Activation of the latent complex is accompanied by the phosphorylation of I-2 on Thr-72 by GSK3. I-2 is also phosphorylated on three serines by casein kinase II (CK2). Phosphorylation by CK2 does not alter I-2 activity but greatly facilitates the subsequent phosphorylation by GSK3 (DePaoli-Roach, 1984; Holmes *et al.*, 1986b). The activation cycle for the ATP-Mg-dependent phosphatase is complicated and controversial. The fundamental aspect of this cycle is that I-2 phosphorylation by GSK3 promotes a conformational change in the PP1/I-2 complex which does not by itself activate the enzyme. The slow auto-dephosphorylation of I-2 correlates best with the increase in PP1 activity. Throughout this process I-2 remains bound to PP1 and in a longer time frame the complex relaxes back to its original inactive conformation. Recent studies show that multiple domains mediate the rapid and reversible inhibition of PP1 and the slower inactivation to the stable complex that can be reactivated by GSK3 (Park and DePaoli-Roach, 1994). The active PP1/I-2 complex can itself be inhibited by addition of exogenous I-2. *In vitro* studies suggest that I-2 interacts with denaturated PP1 to promote a rapid and effective refolding of this protein and yield an active enzyme. According to this idea recombinant PP1 behaves much more like the native enzyme after incubation with I-2 and reactivation by GSK3 (Alessi *et al.*, 1993; Tung *et al.*, 1995; MacKintosh *et al.*, 1996). This led to the proposal that I-2 might be acting as a chaperone for PP1. This hypothesis may also be consistent with preliminary reports that overexpression of I-2 in mammalian cells does not inhibit cellular PP1 activity but in fact elevates the cellular content of PP1c. Studies with Glc8, the yeast homologue of I-2, came to a more complex conclusion, suggesting that under different circumstances, Glc8 was either an inhibitor or an activator of yeast PP1 (Tung *et al.*, 1995). So, despite extensive studies, the functions of I-2 remain elusive. Perhaps the most intriguing property of I-2 is that its protein and mRNA levels fluctuate during the cell cycle, peaking twice, at S phase and mitosis (Brautigan *et al.*, 1990). More recent studies used I-2 fused to the green fluorescent protein to show that I-2 was cytosolic during G1 and translocated to the nucleus in S phase (Kakinoki *et al.*, 1997). As I-2 localization failed to correlate with PP1 distribution in cells, this has raised questions about I-2's role as a PP1 regulator, but no other functions have been described for I-2. However, Brautigan and coworkers,

discovered that I-2 is concentrated in the nucleus of cells cultured at low densities, while cells growing at high density excluded I-2 from the nucleus (Leach *et al.*, 2003). This change in I-2 localization may direct I-2 to different forms of PP1 or change the localization of PP1 in response to cell-cell contacts at high density.

In testis and sperm a few PP1 regulators have been identified. Indeed, the only physiological role proposed for I-2 is in the control of sperm motility. The testis-specific isoform PP1γ2 forms an inactive complex with an I2-like activity. The increased PP1 activity seen in nonmotile immature sperm was accredited to an elevation in GSK3 activity which activates the PP1/I-2 complex. Incubation of immature sperm with phosphatase inhibitors, okadaic acid and calyculin A, induced motility (Vijayaraghavan *et al.*, 1996), suggesting that I-2 inhibits PP1 activity in mature mammalian sperm to facilitate their motility. In this work we may have identified this I2-like activity, the I2-like protein, discussed in the previous chapter.

Sds22 (Dinischiotu *et al.*, 1997) is a regulatory polypeptide of PP1 that is required for the completion of mitosis in both fission and budding yeast. Renouf and coworkers (Renouf *et al.*, 1995) reported the cDNA cloning of a human polypeptide that is 46% identical to yeast sds22. In *S. cerevisiae*, sds22 is largely a nuclear protein mostly present as a stable 1:1 complex with yeast PP1 (Glc7p). It was suggested recently (Peggie *et al.*, 2002) that sds22 functions positively with Glc7p to promote dephosphorylation of nuclear substrates required for faithful transmission of chromosomes during mitosis and that this role is at least partly mediated by effects of sds22 on the nuclear distribution of Glc7p. Interestingly, a new splice variant of sds22 is associated with PP1γ2 in rat testis (Chun *et al.*, 2000). Bovine PP1γ2 was also found to bind sds22 (Huang *et al.*, 2002) and both proteins were shown to be present in the spermatozoid tail and head. Therefore, mechanisms regulating sds22 binding to PP1γ2 are likely to be important in understanding the biochemical basis underlying development and regulation of sperm function.

Recently, a new PP1 testis specific regulator, GBPI-2 (gut and brain phosphatase inhibitor) was identified (Liu *et al.*, 2004) that belongs to the PKC-potentiated PP1 inhibitors (such as CPI, KEPI and PHI). A shorter mRNA encodes another isoform, GBPI-1 that is expressed in brain, stomach, small intestine, colon and kidney. GBPI-1 inhibits PP1 only after phosphorylation by PKC. Interestingly when PKC-phosphorylated GBPI-1 is phosphorylated by PKA it is unable to inhibit PP1. This new protein may be involved in

the integration of the PKC and PKA signal transduction mechanisms. It would be interesting to study the effect of GBPI-2 on PP1γ2 activity in testis and its relation to sperm motility.

For several targeting subunits there is a strong association with PP1 through the canonical PP1 binding motif allowing the formation of a weaker interaction with other regulator motifs that enhance binding or modulate PP1 activity and specificity. One of these other motifs are the ankyrin repeats that acts as additional contacts for the interaction with PP1. PP1 regulators that possess these domains include Myr8, MYPT1, MYPT3 and p53BP2. MYPT1 has seven ankyrin repeats and p53BP2 has four of these domains. Ankyrin repeats are motifs usually involved in protein/protein interactions. In the PP1 binding partners this motifs are usually preceded by the RVxF PP1 binding motif.

Interestingly, in the YTH1 screen a unique clone was identified encoding a previously unknown protein that possesses eight ankyrin repeats and other interesting features that attracted us for further studies. We named this protein SEARP (Six to Eight Ankyrin Repeat Protein). SEARP, although having been identified as a PP1γ1 binding protein, also binds to PP1γ2. Several studies were performed in order to describe SEARP'S localization, tissue distribution and possible function in the human testis and sperm motility. SEARP is present in a variety of tissues, mainly in testis. There are two splice variants of SEARP, one in testis and another in other tissues. The levels of SEARP mRNA are amazingly high in testis when compared to other tissues. Immunolocalization studies revealed that SEARP in present in the cell cytoplasm and is present throughout spermatogenesis and spermiogenesis.

IV.2 MATERIALS AND METHODS

For the complete composition of all reagents, media and solutions used, see the list presented in Appendix I. All reagents were cell culture grade or ultrapure.

IV.2.1 Tissue preparation

Rat tissues were obtained from sacrificed animals and immediately frozen on dry ice. Frozen rat tissues were resuspended in boiling 1%SDS and sonicated for 30sec. Human testis samples were obtained via the kind efforts of Dr. Jorge Oliveira (IPO, Porto)

and processed in the same way as rat tissues. Testes from mature bulls with intact tunica were obtained from a local slaughter house and spermatozoa were isolated from caput and caudal epididymis (Fig. IV.1). Bovine testes were treated as previously described for rat testis for immunoblot analysis. Human semen samples were collected from healthy volunteer adult men and had an average sperm concentration of $2.2x10^8$cells/ml (range: 12-440x10^6), and an average 62% motility (range 31-85%). The average volume was 2.7ml (range 1.5-3.5ml). Samples were washed and processed for preparation of sperm sonicates (in boiling 1%SDS), immunoprecipitation (see below), immunocytochemistry (see below), or directly mounted on glass slides for motility video capture.

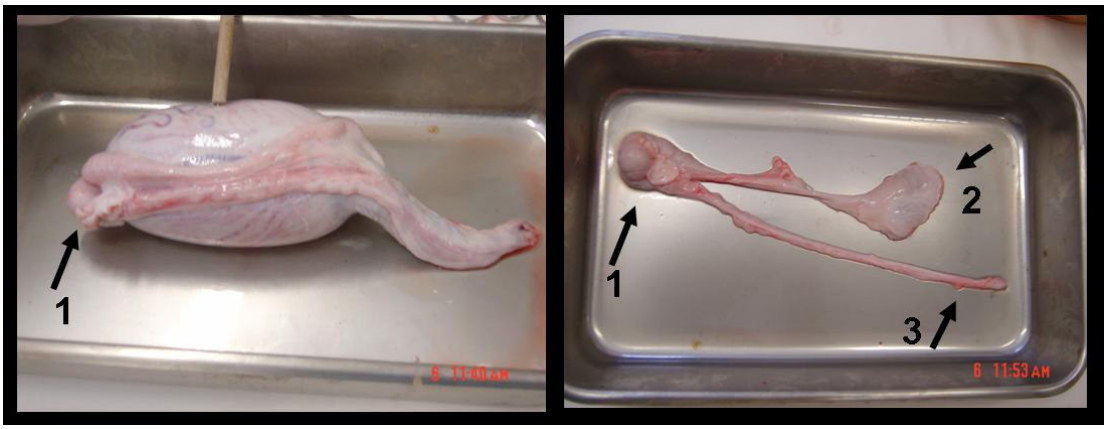


Figure IV.1: Adult b*ovine testis (left) and epididymis (right). Corpus (1), caput (2) and caudal (3) sections of the epididymis are indicated.*

IV.2.2 Immunoprecipitation

Fresh rat testis (1-10mg) were lysed in 100µl of lysis buffer (see Appendix) for 15min on ice and sonicated for 30sec. The lysates were precleared with 25µl of Protein A Sepharose slurry (Pharmacia) for 1h at 4ºC with agitation. After centrifuging the sample for 1min at 4ºC at 10,000g, the supernatant was transferred to a new tube and 25µl of Protein A Sepharose slurry and the primary antibody were added. The mixture was incubated overnight at 4ºC with agitation. For the anti-PP1γ2 antibody 25µl were used for 10mg of rat testis and 5µl of anti-PP1γ or 5µl of anti-PP1α were used for 1mg of rat testis; 25µl of pre-immune serum were used with 10mg of rat testis homogenate as a negative control. The mixture was then centrifuged for 1min at 4ºC at 10,000g and the pellet washed 4 times for 15min with 500µl of 50mM Tris-HCl (pH8.0), 120mM NaCl. Finally, 2 washes

were performed with 50mM Tris-HCl (pH8.0). The pellet was ressuspended in 100µl of electrophoresis buffer (loading buffer for SDS-PAGE of the immunoprecipitation with 1mg of testis or re-hydratation solution for 2D electrophoresis of the immunoprecipitation with 10mg of testis). Before loading, the samples for SDS-PAGE were boiled for 3min and briefly centrifuged to pellet the Sepharose beads; for the 2D gel analysis, the samples were only centrifuged, but not boiled.

For the immunoprecipitation of human sperm, the samples were centrifuged at 350g for 7min at RT and the pellet resuspended in 1ml of lysis buffer. 250µl of sperm homogenate (corresponding to 500mg of protein) were used for immunoprecipitation with 2µl anti-PP1γ2 or 2µl of anti-PP1γ1 or 2µl of pre-immune serum or 2µl anti-SEARP (gift from P. T. Cohen), using the procedure described above.

IV.2.3 2D Gel Electrophoretic Analysis

Two dimensional gel electrophoresis combines protein separation by isoelectric focusing (IEF), in the first dimension, with SDS-PAGE in the second dimension. In a pH gradient, under the influence of an electric field, proteins migrate to the position in the gradient where their net charge is zero; proteins will separate according to their isoelectric point. The original method for the first dimension separation depended on carrier ampholyte-generated pH gradients in polyacrylamide tube gels. Because of the limitations of this method, an alternative technique for pH gradient formation was developed more recently: immobilized pH gradients (IPG) (Bjellqvist *et al.*, 1982; Gorg *et al.*, 1988). This type of gradient is created by covalently incorporating a gradient of acidic and basic buffering groups into a polyacrylamide gel at the time it is cast.

The pelleted samples from the immunoprecipitation experiments were resuspended and properly solubilized in 100µl re-hydration buffer [lysis buffer supplemented with 0.5% of IPG buffer (in the 3-10 pH range), 0.7mg of DTT per strip and 0.002% of bromophenol blue]. Each sample thus prepared was pipeted into a strip holder and overlaid with dry strip cover fluid (Amersham Pharmacia). The strips were placed in the IPGphor electrophoresis equipment and the electrophoresis was started. The IEF program used was as follows:

Step	Voltage (V)	Duration (h)
Rehydration of the strip under voltage	30	12
Ionic impurities removal	150	2
Sample entry into the strip	500	1
Protein movement	1000	1
High definition isoelectric focusing	8000	2

For the second dimension (SDS-PAGE), 11% gels were cast using a 14x16cm gel apparatus (Hoefer SE 600 system) and the strips from the first dimension were placed on top of the casting gel. Electrophoretic separation was performed at 90mA until the tracking dye reached the bottom of the gel. Proteins were later visualized by silver staining or transferred to nitrocellulose membranes for immunoblot with anti-SEARP and anti-PP1γ2 antibodies.

IV.2.4 Silver Staining

For increased sensitivity in protein detection the reversible silver staining procedure was used. In this technique the gel is impregnated with soluble silver ions and developed by treatment with formaldehyde, which reduces silver ions to form an insoluble brown precipitate. Proteins promote this reduction. Gels from the 2D electrophoresis were incubated overnight in fixing solution and then transferred into sensitizing solution for 30 min. The gels were washed three times with distilled water and incubated for 20 min in silver solution, in the dark. Following two washes with distilled water to remove excess silver nitrate, the gels were placed in developing solution until protein bands appeared. The reaction was stopped with stop solution. Finally, the gels were washed with distilled water and scanned in a GS-710 calibrated imaging densitometer (Amersham-Pharmacia) and analyzed with Quantity One software (version 4.2.1). Alternatively, gels were destained with 100mM potassium ferricyanide for a few minutes, rinsed several times in water and then transferred to nitrocellulose membranes for immunoblot analysis.

IV.2.5 Cell Culture

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen). Cells were grown in 10cm plates or 6-well plates in a 5%CO_2 humidified incubator at 37ºC and subcultured every 2-3 days.

IV.2.6 Cell transfection

For expression in COS-7 cells, the cDNA insert from clone 40Q was PCR-amplified using high fidelity Pfu polymerase (Stratagene) with two different set of primers (40Q3P1/40Q3P2 and 40Q3P2/40Q3P3) and subcloned between the *Eco*RI and *BamH*I sites of the pEGFP vector, upstream of the enhanced green fluorescent protein (EGFP) cassette. The cDNA for PP1γ2 was also subcloned into the *Eco*RI site of the pcDNA3.1 expression vector (Invitrogen). Lipofectamine (Invitrogen) was used for transfection of COS-7 cells using the pcDNA3.1-PP1γ2 expression vector, the pEGFP expression vectors encoding GFP-40Q or GFP-32240Q (starting at amino acid 322, Fig. IV.II), or GFP alone, as a control. One day before transfection, the cells were plated in DMEM with 10% fetal fetal calf serum so they were 90% confluent at the time of transfection. For each transfection sample in 6-well plates the DNA/Lipofectamine complexes were prepared as follows: 10µl of Lipofectamine were diluted in 500µl DMEM without serum or antibiotics and added to 1µg of plasmid DNA previously diluted in 500µl of the same medium. The mixture was allowed to complex for 20min at RT and then the 1ml sample was added to each well and gently mixed by rocking the plate back and forth. 20h after transfection, cells were harvested and replated in poly-L-ornithine coated cover slips placed in 6-well plates. After 4h, cells were washed 3 times with DMEM without serum and fixed for 25min with 4% paraformaldheyde. After 3 washes with 1XPBS the cover slips were mounted on a slide with one drop of Fluoroguard. Cells were then visualized in an epifluorescence Olympus microscope.

IV.2.7 Immunocytochemistry

COS-7 cells were fixed with 4% paraformaldehyde and washed with PBS as described above. Then were permeabilized with methanol for 2min and immediately washed with 1XPBS. After, cells were incubated with primary antibody diluted in 3%BSA in PBS (50µl) for 1h at RT (diluted 1:1000 for anti-PP1γ2, or 1:250 for anti-SEARP). The primary antibody was removed by washing 3 times with 1XPBS and the secondary antibody placed over the cells for 1h at RT (the secondary antibody, Texas Red conjugated Goat Anti-Rabbit was diluted 1:300 according to the manufacturer's instructions). After washing 3 times with 1XPBS the cover slips were mounted in one drop of Fluoroguard in a glass slide. Cells were visualized in an Olympus epifluorescence microscope.

The immunocytochemistry protocol used for spermatozoa was very similar to that described above. Briefly, bovine or human spermatozoa were resuspended in 1XPBS after centrifugation at 350g for 7min. Samples (500µl) were then plated in poly-L-ornithine coated cover slips placed in 6-well plates for 15min. The spermatozoa were then fixed with 500µl of 4% paraformaldehyde, washed with 1XPBS and permeabilized with methanol for 2min or 0.2%Triton for 7min. The spermatozoa were then treated for immunocytochemistry as described above.

IV.2.8 Immunohistochemistry

Adult mice were perfused with formaldehyde to fix all tissues. Testis were removed and submersed in formaldehyde overnight with agitation. The fixed testes were cut in slices and put in minicassettes. The tissue was then washed 6 times for 15min with 30%ethanol and left overnight in 30%ethanol. Afterwards, the samples were processed according to the manufacturer's instructions in a sample processor (Table IV.2).

Table IV.2: *Paraffin embedding of fixed rat testes.*

Step	Solution	Time/h
1	70%ethanol	1
2	70%ethanol	1
3	90%ethanol	1
4	96%ethanol	1
5	96%ethanol	1
6	Toluene	1
7	Toluene	1
8	Toluene	1
9	Paraffin	10
10	Paraffin	4

The processed tissue was mounted on paraffin and stored at -20ºC. The tissue was sliced into 3µm sections using a microtome (Leica). Sections were then stretched on a histological water bath with gelatine (Sigma) at 37ºC, put on a glass slide and dried for 30min in a 60ºC incubator. To check tissue integrity, samples were stained with haematoxylin and eosin as illustrated in Table IV.3.

Table IV.3: *Haematoxylin and eosin staining procedure.*

Step	Solution	Time/min
1	Xylene	15
2	Xylene	10
3	96%ethanol	10
4	96%ethanol	10
5	70%ethanol	10
6	H_2O	10
7	Haematoxylin	2-10
8	H_2O	5
9	Ethanol-HCl	5-30sec
10	96%ethanol	10
11	H_2O	10
12	Eosin	2-6
13	70%ethanol	5
14	90%ethanol	5
15	96%ethanol	10
16	96%ethanol	10
17	Xylene	5
18	Xylene	5

After this procedure the tissue was mounted in DPX and visualized in a phase contrast microscope. After verification of tissue integrity, immunohistochemistry was performed (Table IV.4):

Table IV.4: *Immunohistochemistry method.*

Step	Solution	Time/min
1	Xylene	10
2	Xylene	10
3	96%ethanol	5
4	90%ethanol	5
5	70%ethanol	5
6	H_2O	5
7	0.04%pepsine in 0.1M HCl pH2.0, 37ºC	30
8	1XPBS	5

The samples were then treated for 5min with a solution to block endogenous peroxidase activity (160ml 1XPBS, 20ml methanol, 20µl 30%H_2O_2). This step was omitted for immunofluorescence. After three 5min washes with 1XPBS, samples were incubated for 1h with serum of the animal of the secondary antibody diluted 1:30 in 1XPBS, to block non-specific background. Afterwards, the samples were incubated with the primary antibody diluted in 1XPBS for 1h at RT, or overnight at 4ºC. The primary antibodies used and their working dilutions are summarized below (Table IV.5).

Table IV.5: *Primary antibody dilution.*

Antibody	Dilution
Preimmune serum	1:1000
Anti-PP1γ2	1:500
Anti-PP1α	1:500
Anti-SEARP	1:250

Following three 5min washes with 1XPBS, the samples were incubated for 1h with secondary antibody diluted in 1XPBS, and washed again as before. If the secondary antibody was conjugated with peroxidase, a developing solution [198ml 1XPBS, 2ml DAB (diaminobenzine), 200ml 30%H_2O_2 solution, previously filtered with a 0.22µm filter], was left over the sample until a brown colour appeared (~10min). Samples were finally stained with haematoxylin (as described above) and mounted in DPX. If the secondary antibody

was conjugated with a fluorocrome (Cy3, cyanide dye), the sample was directly mounted with Fluoroguard and kept in the dark until visualization in the microscope.

IV.3 RESULTS

IV.3.1 SEARP sequence analysis

Clone number 40Q, identified in YTH1, encoded a protein displaying interesting characteristics that warranted its further study. First, the clone was completely sequenced with the primers GAL4-AD, OLIGO40Q3, OLIGO40Q3/2, OLIGO40Q3/3 and OLIGO40Q3/4 (see Appendix). After complete insert sequencing and database searching it was concluded that the encoded protein was a completely new protein. When we first identified this clone it only existed in the database at the genomic level, mapping to chromosome 11. After a few months, a putative coding sequence (Gi: 21754395) was submitted containing part of our clone. This cDNA starts at position 964 below (Fig. IV.2), whereas the 40Q sequence starts at position 169 and is 2415 nt long:

```
   1  atgcccctggagggcaggcgacggccctgctgcctctccagccaagtggctggagtcggg
   1   M  P  L  E  G  R  R  R  P  C  C  L  S  S  Q  V  A  G  V  G
  61  aggctggaaagagactccgagaaagtaccagcggaaggcggccgccgctacggcgattcg
  21   R  L  E  R  D  S  E  K  V  P  A  E  G  G  R  R  Y  G  D  S
 121  cagggagtagcagacgaagacggtggccgccgcactagccaccacgtgtggaggataaac
  41   Q  G  V  A  D  E  D  G  G  R  R  T  S  H  H  V  W  R  I  N
 181  ggtctacacggccattccggcgccgagtctagggaaagagttagcgacgacgggggaaaga
  61   G  L  H  G  H  S  G  A  E  S  R  E  R  V  S  D  D  G  E  R
 241  aaatgtgaagagagcgaccgccgctccagggtcgctgcaggaagcctaagtgcagacgcc
  81   K  C  E  E  S  D  R  R  S  R  V  A  A  G  S  L  S  A  D  A
 301  ggcttctcccgcagtgacttgagaagggtcagtgaaaacctcggccactgccgcagcgtc
 101   G  F  S  R  S  D  L  R  R  V  S  E  N  L  G  H  C  R  S  V
 361  tctagggagagagttaggggagatagtggccacagtcacagctgctcttgggagagagtt
 121   S  R  E  R  V  R  G  D  S  G  H  S  H  S  C  S  W  E  R  V
 421  aggggagacagcaccttctgcagcagcgacgtgaattttagtgaagttggaggccaccaa
 141   R  G  D  S  T  F  C  S  S  D  V  N  F  S  E  V  G  G  H  Q
 481  actaccgactccaggggaacagccagagaagaccgaggcctccgcctcagtggtccttgg
 161   T  T  D  S  R  G  T  A  R  E  D  R  G  L  R  L  S  G  P  W
 541  gagggagtcagtgacattcgggacccgcgaactagtgacttcggggatagagtcagtgac
 181   E  G  V  S  D  I  R  D  P  R  T  S  D  F  G  D  R  V  S  D
 601  gatcgcagtcgccgcttcagtggctcctgggagggagggagtgtcgaaggcggccacagc
 201   D  R  S  R  R  F  S  G  S  W  E  G  G  S  V  E  G  G  H  S
 661  gttggtagttcttgggaggaagtaagtggagaccgcggctacgcagccagcgactcctct
 221   V  G  S  S  W  E  E  V  S  G  D  R  G  Y  A  A  S  D  S  S
 721  ggtgtgagcggcagtgaagacgccagctaccgcttcagtggcttttgggagagagaaagt
 241   G  V  S  G  S  E  D  A  S  Y  R  F  S  G  F  W  E  R  E  S
 781  gaagacgaaggtttccgctgcagcttctgggagagagcaagagaggaccttgggccccgt
 261   E  D  E  G  F  R  C  S  F  W  E  R  A  R  E  D  L  G  P  R
 841  cctagtgacgacggagaagagggccgctgccgctgcagtggctcgtgggtgagagcaagt
 281   P  S  D  D  G  E  E  G  R  C  R  C  S  G  S  W  V  R  A  S
 901  gaagaccgccgcagcatcaggggcctggactcaactcctccccagagtcggaggtgttgc
 301   E  D  R  R  S  I  R  G  L  D  S  T  P  P  Q  S  R  R  C  C
 961  gccatgcccgggggtggccaattcaggcccctccacttcctctagggagactgcaaacccc
 321   A  M  P  G  V  A  N  S  G  P  S  T  S  S  R  E  T  A  N  P
1021  tgttccaggaagaaggtgcattttggcagcatacatgatgcagtacgagctggagatgta
```

```
 341   C  S  R  K  K  V  H  F  G  S  I  H  D  A  V  R  A  G  D  V
1081  aagcagctttcagaaatagtggtacgtggagccagcattaatgaacttgatgttctccat
 361   K  Q  L  S  E  I  V  V  R  G  A  S  I  N  E  L  D  V  L  H
1141  aagtttacccctttacattgggcagcacattctggaagtttggagtgtcttcattggctg
 381   K  F  T  P  L  H  W  A  A  H  S  G  S  L  E  C  L  H  W  L
1201  ctctggcatggagctgatatcacacacgtaacaacgagaggttggacagcatctcacata
 401   L  W  H  G  A  D  I  T  H  V  T  T  R  G  W  T  A  S  H  I
1261  gctgcaatcaggggtcaggatgcttgtgtacaggctcttataatgaatggagcaaatctg
 421   A  A  I  R  G  Q  D  A  C  V  Q  A  L  I  M  N  G  A  N  L
1321  acagcccaggatgaccggggatgcactcctttacatcttgctgcaactcatggacattct
 441   T  A  Q  D  D  R  G  C  T  P  L  H  L  A  A  T  H  G  H  S
1381  ttcactttacaaataatgctccgaagtggagatcccagtgtgactgataagagagaatgg
 461   F  T  L  Q  I  M  L  R  S  G  D  P  S  V  T  D  K  R  E  W
1441  agacctgtgcattatgcagcttttcatgggcggcttggctgcttgcaacttcttgttaaa
 481   R  P  V  H  Y  A  A  F  H  G  R  L  G  C  L  Q  L  L  V  K
1501  tggggttgtagcatagaagatgtggactacaatggaaaccttccagttcacttagcagcc
 501   W  G  C  S  I  E  D  V  D  Y  N  G  N  L  P  V  H  L  A  A
1561  atggaaggccaccttcactgtttcaaattcctagtcagtagaatgagcagtgcgacgcaa
 521   M  E  G  H  L  H  C  F  K  F  L  V  S  R  M  S  S  A  T  Q
1621  gttttaaaagctttcaatgataatggagaaaatgtactggatttggcccagaggttcttc
 541   V  L  K  A  F  N  D  N  G  E  N  V  L  D  L  A  Q  R  F  F
1681  aagcagaacattttacagtttatccaggggggctgagtatgaaggaaaagacctagaggat
 561   K  Q  N  I  L  Q  F  I  Q  G  A  E  Y  E  G  K  D  L  E  D
1741  caggaaactttagcatttccaggtcatgtggctgcctttaagggtgatttggggatgctt
 581   Q  E  T  L  A  F  P  G  H  V  A  A  F  K  G  D  L  G  M  L
1801  aagaaattagtggaagatggagtaatcaatattaatgagcgtgctgataatggatcaact
 601   K  K  L  V  E  D  G  V  I  N  I  N  E  R  A  D  N  G  S  T
1861  cctatgcataaagctgctggacaaggccacatagagtgtttgcagtggttaattaaaatg
 621   P  M  H  K  A  A  G  Q  G  H  I  E  C  L  Q  W  L  I  K  M
1921  ggagcagacagtaatattaccaacaaagcaggggagagacccagtgatgtggcaaagagg
 641   G  A  D  S  N  I  T  N  K  A  G  E  R  P  S  D  V  A  K  R
1981  tttgcccatttggcagcagtgaagctgttagaggagctacagaaatatgatatagatgac
 661   F  A  H  L  A  A  V  K  L  L  E  E  L  Q  K  Y  D  I  D  D
2041  gaaaatgaaattgatgaaaatgatgtgaaatattttataagacatggtgttgagggaagc
 681   E  N  E  I  D  E  N  D  V  K  Y  F  I  R  H  G  V  E  G  S
2101  actgatgccaaggatgatttatgtctgagtgacttggataaaacagatgccagaatgaga
 701   T  D  A  K  D  D  L  C  L  S  D  L  D  K  T  D  A  R  M  R
2161  gcttacaagaaaattgtagaattgagacacctcctggaaattgccgagagcaactataaa
 721   A  Y  K  K  I  V  E  L  R  H  L  L  E  I  A  E  S  N  Y  K
2221  cacttgggaggcataacagaagaagatttaaagcagaagaaagaacagcttgagtctgaa
 741   H  L  G  G  I  T  E  E  D  L  K  Q  K  K  E  Q  L  E  S  E
2281  aagaccatcaaagaactgcagggccagctggagtatgaacgactacgtagagaaaaatta
 761   K  T  I  K  E  L  Q  G  Q  L  E  Y  E  R  L  R  R  E  K  L
2341  gaatgtcagcttgatgaatatcgagcagaagttgatcaactcagggaaacactggaaaaa
 781   E  C  Q  L  D  E  Y  R  A  E  V  D  Q  L  R  E  T  L  E  K
2401  attcaagtcccaaactttgtggctatggaagacagcgcttcttgtgagtcaaacaaagag
 801   I  Q  V  P  N  F  V  A  M  E  D  S  A  S  C  E  S  N  K  E
2461  aagaggcgagtaaaaaaaaaggtttcttctggaggggtgtttgtgagaaggtactaatc
 821   K  R  R  V  K  K  K  G  F  F  W  R  G  V  C  E  K  V  L  I
2521  agtgaaataactaaattgacctgctagatttttctctttcattaaaaaaattgatataaa
 841   S  E  I  T  K  L  T  C  *
2581  tgtg
```

Figure IV.2: *Nucleotide and amino acid sequence of SEARP. Clone 40Q starts at nt 169 while the database clone (Gi: 21754395) starts at nt 964 and exhibits a different splicing pattern. The PP1 binding motif is in green. The red and blue colours identify different exons.*

Examination of the amino acid sequence for signaling motif (Sigrist *et al.*, 2002) identified the existence of eight ankyrin repeats. Arround the same time, a lymphocyte specific isoform was also reported (Cohen P. T., personal communication), but with a different C-terminal sequence (Fig. IV.3). The protein encoded by the 40Q cDNA was termed SEARP-T, whereas the lymphocyte protein was termed SEARP-L.

```
SEARP-L          MPLEGRRRPCCLSSQVAGVGRLERDSEKVPAEGGRRYGDSQGVADEDGGRRTSHHVWRIN
SEARP-T          ------------------------------------------------------WRIN
                                                                       ****

SEARP-L          GLHGHSGAESRERVSDDGERKCEESDRRSRVAAGSLSADAGFSRSDLRRVSENLGHCRSV
SEARP-T          GLHGHSGAESRERVSDDGERKCEESDRRSRVAAGSLSADAGFSRSDLRRVSENLGHCRSV
                 ************************************************************


SEARP-L          SRERVRGDSGHSHSCSWERVRGDSTFCSSDVNFSEVGGHQTTDSRGTAREDRGLHLSGPW
SEARP-T          SRERVRGDSGHSHSCSWERVRGDSTFCSSDVNFSEVGGHQTTDSRGTAREDRGLRLSGPW
                 ************************************************:***********

SEARP-L          EGVSDIRDPRTSDFGDRVSDDRSRRFSGSWEGGSVEGGHSVGSSWEEVSGDRGYAASDSS
SEARP-T          EGVSDIRDPRTSDFGDRVSDDRSRRFSGSWEGGSVEGGHSVGSSWEEVSGDRGYAASDSS
                 ************************************************************

SEARP-L          GVSGSEDASYRFSGFWERESEDEGFRCSFWERAREDLGPRPSDDGEEGRCRCSGSWVRAS
SEARP-T          GVSGSEDASYRFSGFWERESEDEGFRCSFWERAREDLGPRPSDDGEEGRCRCSGSWVRAS
                 ************************************************************

SEARP-L          EDRRSIRGLDSTPPQSRRCCAMPGVANSGPSTSSRETANPCSRKKVHFGSIHDAVRAGDV
SEARP-T          EDRRSIRGLDSTPPQSRRCCAMPGVANSGPSTSSRETANPCSRKKVHFGSIHDAVRAGDV
                 ************************************************************

SEARP-L          KQLSEIVVRGASINELDVLHKFTPLHWAAHSGSLECLHWLLWHGADITHVTTRGWTASHI
SEARP-T          KQLSEIVVRGASINELDVLHKFTPLHWAAHSGSLECLHWLLWHGADITHVTTRGWTASHI
                 ************************************************************

SEARP-L          AAIRGQDACVQALIMNGANLTAQDDRGCTPLHLAATHGHSFTLQIMLRSGVDPSVTDKRE
SEARP-T          AAIRGQDACVQALIMNGANLTAQDDRGCTPLHLAATHGHSFTLQIMLRSG-DPSVTDKRE
                 ************************************************ *********

SEARP-L          WRPVHYAAFHGRLGCLQLLVKWGCSIEDVDYNGNLPVHLAAMEGHLHCFKFLVSRMSSAT
SEARP-T          WRPVHYAAFHGRLGCLQLLVKWGCSIEDVDYNGNLPVHLAAMEGHLHCFKFLVSRMSSAT
                 ************************************************************

SEARP-L          QVLKAFNDNGENVLDLAQRFFKQNILQFIQGAEYEGKDLEDQETLAFPGHVAAFKGDLGM
SEARP-T          QVLKAFNDNGENVLDLAQRFFKQNILQFIQGAEYEGKDLEDQETLAFPGHVAAFKGDLGM
                 ************************************************************

SEARP-L          LKKLVEDGVININERADNGSTPMHKAAGQGHIECLQWLIKMGADSNITNKAGERPSDVAK
SEARP-T          LKKLVEDGVININERADNGSTPMHKAAGQGHIECLQWLIKMGADSNITNKAGERPSDVAK
                 ************************************************************

SEARP-L          RFAHLAAVKLLEELQKYDIDDENEIDENDVKYFIRHGVEGSTDAKDDLCLSDLDKTDARM
SEARP-T          RFAHLAAVKLLEELQKYDIDDENEIDENDVKYFIRHGVEGSTDAKDDLCLSDLDKTDARM
                 ************************************************************

SEARP-L          RAYKKIVELRHLLEIAESNYKHLGGITEEDLKQKKEQLESEKTIKELQGQLEYERLRREK
SEARP-T          RAYKKIVELRHLLEIAESNYKHLGGITEEDLKQKKEQLESEKTIKELQGQLEYERLRREK
                 ************************************************************

SEARP-L          LECQLDEYRAEVDQLRETLEKIQVPNFVAMTALLVSQTKRRGE-----------------
SEARP-T          LECQLDEYRAEVDQLRETLEKIQVPNFVAMEDSASCESNKEKRRVKKKGFFWRGVCEKVL
                 ******************************     .::::. .

SEARP-L          ---------
SEARP-T          ISEITKLTC
```

Figure IV.3: *Comparison of the amino acid sequences of SEARP-L and SEARP-T.*

Other motifs present include a EF-calcium binding domain (aa 677-689), a BZip-Maf transcription factor domain (aa 721-618) and the PP1 binding domain (aa 345-348) upstream of the eight ankyrin repeats (aa 346-375, 380-409, 413-442, 446-476, 478-507, 511-540, 583-613, 617-646). The ankyrin repeats mediate interaction between a wide spectrum of proteins. Ankyrin repeats are tandemly repeated modules of about 33 amino acids that occur in a large number of functionally diverse proteins. This domain has been described as an L-shaped structure consisting of a beta-hairpin and two alpha helices (Gorina and Pavletich, 1996). The appearance of the PP1 binding motif just before the

eight ankyrin repeats also occurs in another well known PP1 binding protein, the p53BP2 or PPP1R13A (Helps *et al.*, 1995). SEARP also possesses consensus sites for phosphorylation by Protein kinase C, Casein kinase II, cAMP- and cGMP-dependent protein kinases and tyrosine kinases. Thus, SEARP itself may be regulated by phosphorylation.

The exon-intron organization of SEARP was determined by aligning SEARP cDNA sequence with the nucleotide sequence of chromosome 11 (Table IV.6). The SEARP-T transcript spreads over 63Kb on chromosome 11 and is 2604bp long.

Table IV.6: *Exon-intron organization of SEARP-T.*

Exon nº	Exon seq	Intron sequence	Exon seq	Exon nº
1	gagggcag[17]	[63058]**gt**aattct….tttcca**ag**[63812]	[18]gcgacggc	2
2	aaacccct[1041]	[64817]**gt**gagtca….ttcaat**ag**[68566]	[1042]gttccagg	3
3	gtttggag[1206]	[68731]**gt**aagaaa….tttctt**ag**[76145]	[1207]tgtcttca	4
4	gtgtacag[1314]	[76254]**gt**aataat….atttt**ag**[80387]	[1315]gctcttat	5
5	gaagtgga[1431]	[80505]**gt**ggtgag….acctct**ag**[81382]	[1432]gatcccag	6
6	ccttccag[1567]	[81519]**gt**atttta….tcctct**ag**[94942]	[1568]ttcactta	7
7	tcaggaaa[1767]	[95144]**gt**aagtaa….attcat**ag**[97834]	[1768]ctttagca	8
8	gcataaag[1893]	[97961]**gt**gagtta….ttgaac**ag**[106491]	[1894]ctgctgga	9
9	gcaaagag[1999]	[106598]**gt**ataaat…ttctac**ag**[110846]	[2000]gtttgccc	10
10	tgccagaa[2175]	[111023]**gt**aagtat….tcttgc**ag**[115585]	[2176]tgagagct	11
11	tctgaaaa[2302]	[115613]**gt**aatgtc….tttgat**ag**[118030]	[2303]gaccatca	12
12	tggctatg[2448]	[118176]**gt**tggtgt….gcttat**ag**[125932]	[2449]gaagacag	13

Nucleotide sequences around the identified splice junctions of the human testis specific SEARP-T transcript. The conserved gt-ag of splice donor and acceptor sites are shown in bold. The exon nucleotide numbers related to Figure IV.2 and the introns nucleotides correspond to the sequence of chromosome 11 (Gi:13094222).

IV.3.2 Tissue distribution of SEARP

Table IV.7 compares the general properties of SEARP-T and SEARP-L. The two proteins arise by alternative splicing but are very similar, as already pointed out above (Fig. IV.3).

Table IV.7: *Features of SEARP-testis specific and SEARP-lymphocyte specific.*

Characteristic	SEARP-T	SEARP-L
No. of amino acids	848	823
Molecular weight (KDa)	94.5	91.5
pI	5.99	5.89

The tissue distribution of SEARP was evaluated by immunoblot analysis of different rat tissues, human testis (Fig. IV.4), bovine testis (Fig IV.5) and human sperm

(Fig. IV.5). From Figure IV.4 it is evident that SEARP is present in human and rat testis and also in ovary. Besides these reproductive tissues, SEARP is also expressed in lung and liver, and is apparently also present in the other tissues examined but in much lower abundance. The observed slight difference in mobility in rat testis (RT) and ovary (Ov) is in agreement with the predicted higher molecular mass of SEARP-T (TableIV.7). In the brain, SEARP was only detected in cerebellum and olfactory bulb although at much lower levels. Since high levels of PP1 activity were co-immunoprecipitated with SEARP from brain (P. T. Cohen, personal communication), the possibility exists that another, different 796alternatively spliced SEARP isoform may occur in brain. The tissue distribution of PP1γ2 is also shown in Fig. IV.4 for comparison. PP1γ2 is most abundant in testis as reported before (Vijayaraghavan *et al.*, 1996) but is also found in other tissues. As for SEARP, high levels of PP1γ2 were also detected in testis, ovary and lung. Additionally, PP1γ2 seems to be also highly expressed in striatum, olfactory bulb and hippocampus.

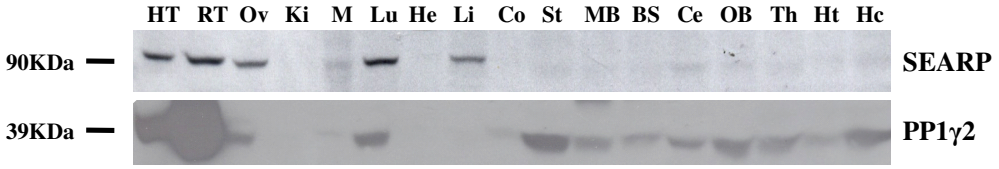
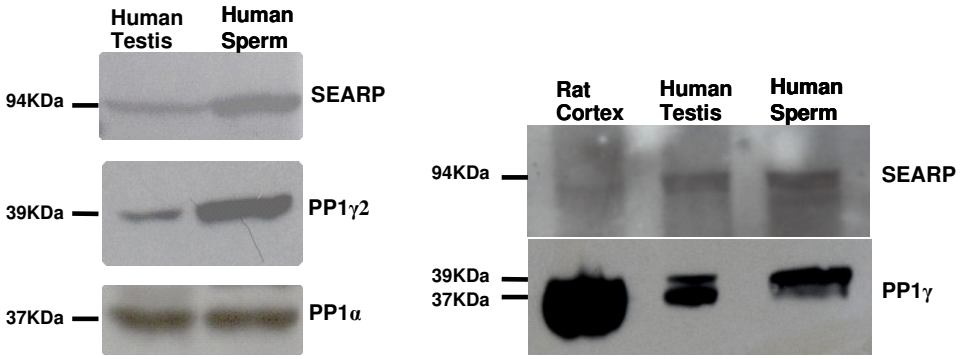


Figure IV.4: *Immunoblot analysis using anti-SEARP and anti-PP1γ2 antibodies with different rat tissues and human testis (100μg). HT, human testis; RT, rat testis; Ov, rat ovary; Ki, rat kidney; M, rat skeletal muscle; Lu, rat lung; He, rat heart; Li, rat liver; Co, rat cortex; St, rat striatum; MB, rat mid brain; BS, rat brain stem; Ce, rat cerebellum; OB, rat olfactory bulb; Th, rat thalamus; Ht, rat hypothalamus; Hc, rat hippocampus.*

It is also apparent by analyzing Fig. IV.4 that there is a huge difference in the amount of PP1γ2 in rat testis compared with human testis. For that reason, the abundance of PP1γ2 in bovine testis was also verified (Fig. IV.5).

Figure IV.5: *Immunoblot analysis of PP1γ2 in human (100µg), rat (10µg) and bovine (10µg) testis.*

Whereas bovine and rat testis express approximately the same amounts of PP1γ2, human testis contains more than 10-fold less PP1γ2, as can be seen in Fig. IV.5. However, both SEARP-T and PP1γ2 appear to be relatively enriched in sperm (Fig. IV.6), in contrast to PP1α, which is equally expressed in both samples. Using an anti-PP1γ antibody recognizing both PP1γ1 (37kDa) and PP1γ2 (39kDa), it is apparent that while human testes express both PP1γ1 and PP1γ2, human sperm has only PP1γ2 (Fig. IV.6B). In comparison, very little PP1γ2 is detected in rat cortex.



A) B)

Figure IV.6*: **A)** Immunoblot analysis of SEARP, PP1γ2 and PP1α expression in human testis and human sperm (50µg). **B)** Immunoblot comparison of SEARP (100µg each tissue), and PP1γ (25µg of rat cortex, 200µg human testis and 100µg human sperm) expression in rat cortex and human testis and sperm.*

In order to verify the expression of 40Q mRNA in different tissues, Northern blot analysis was performed using a Rat Multiple Tissue Northern (MTN) Blot. Using the 40Q cDNA as probe, SEARP transcript of 3.6kb was detected (Fig. IV.7). The expression of SEARP in testis is amazingly high when compared to the expression of SEARP in the

other tissues analyzed. Brain and liver also expressed detectable levels of SEARP mRNA. As control we used a β-actin probe that detected a transcript of 2.1kb in non-muscle tissues and another transcript of 1.6kb in both cardiac and skeletal muscle.

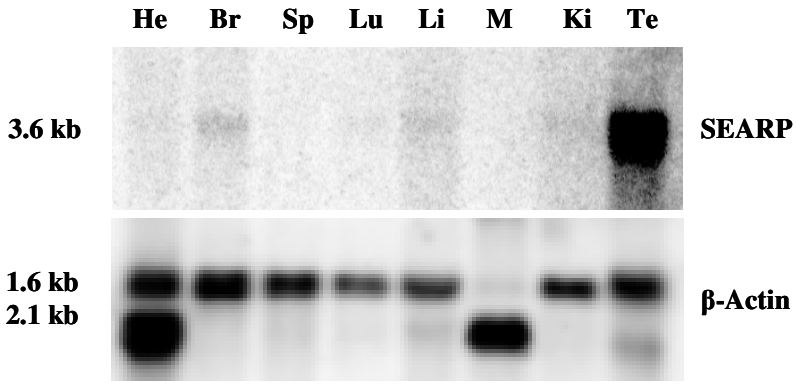


Figure IV.7: *MTN blot analysis of different rat tissues using the cDNA of clone 40Q as probe.He, heart; Br, brain; Sp, spleen; Lu, lung; Li, liver; M, muscle; Ki, kidney; Te, testis.*

Densitometric analysis of the SEARP mRNA band, corrected against the 2.1kb actin control yielded the results shown in Table IV.8. Skeletal muscle was excluded from this analysis since it expresses little or none of this actin transcript. Clearly, SEARP mRNA is more abundant in testis than in all the other tissues analysed.

Table IV.8: Relative abundance of SEARP mRNA.

Tissue	Heart	Brain	Spleen	Lung	Liver	Kidney	Testis
%SEARP mRNA	2.3	5.6	0.24	2.4	3.9	1.9	100

The value for testis was set at 100% and all other tissues were expressed as a percentage of this value.

IV.3.3 Confirmation of SEARP/PP1γ2 interaction

Sequential transformation of yeast with the bait and the prey vectors performed in order to confirm the YTH interaction (Fig. IV.8).

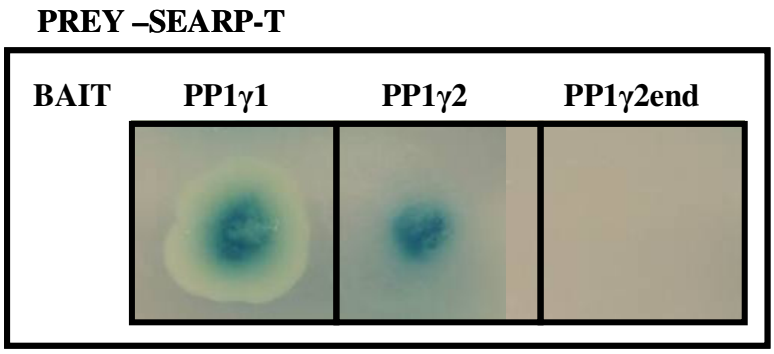


Figure IV.8: *Sequential transformation of yeast AH109 (see Appendix) first with the bait plasmid (pAS2PP1γ1 or pAS2PP1γ2 or pAS2PP1γ2end) and then the prey plasmid (pACT-2-40Q).*

The results obtained (Fig. IV.8) indicate that SEARP-T interacts with PP1γ1 and PP1γ2 but not with the unique C-terminal domain of PP1γ2. The interaction of SEARP-T with PP1γ2 appear to be stronger than with PP1γ1, since with the latter the colony grew white and only turned blue after several days.

Since it was developed, 2D gel electrophoresis has been the technique of choice to study the protein content of a certain tissue or to compare protein expression profiles of sample pairs, for instance, healthy versus disease conditions. 2-D technology can provide a huge amount of putative protein targets for drug development strategies. Protein spots can be further analyzed by mass spectrometry, peptide sequencing and 2D immunoblotting. Information taken from 2-D gel analysis can be stored in databases like http://biobase.dk/cgi-bin/celis and http://expasy.hcuge.ch/ch2d/2d-index.html.

Here, we made use of this technique to further characterize the PP1γ2 interactome from rat testis and human sperm. Thus, to obtain a global picture of the proteins from rat testis or human sperm that interact with PP1γ2 we performed immunoprecipitation of protein extracts from these tissues with the highly specific anti-PP1γ2 antibody. Furthermore, this experiment was also important to provide biochemical evidence of the interaction of SEARP with PP1γ2. After immunoprecipitation, the samples were subjected

to 2D gel electrophoresis and the gel was silver stained to visualize the protein spots (Fig. IV.9). Many protein spots were obtained, consistent with the multiple interactions postulated for PP1 isoforms. The pattern obtained from the immunoprecipitation of human sperm was very similar (data not shown). To confirm the interaction of PP1γ2 with SEARP, the destained 2D gel from human sperm was transferred to a nitrocellulose membrane and immunoblotted with anti-SEARP antibody (Fig. IV.10A). A similar analysis was performed with the rat testis immunoprecipitated sample (Fig. IV.10B). In both cases a single immunoreactive spot was detected with the anti-SEARP antibody. Afterwards both blots were stripped and immunoblotted with anti-PP1γ2 antibody.

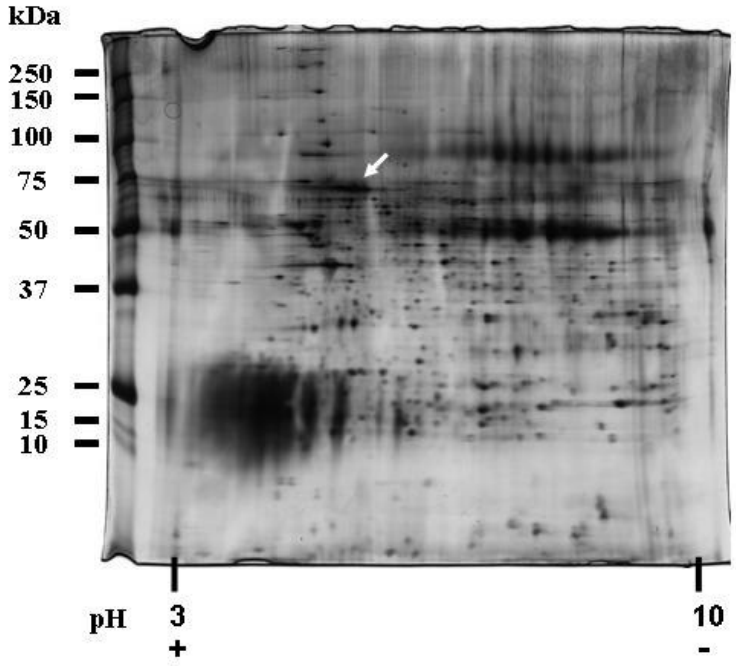


Figure IV.9: *Silver stained 2D gel of the immunoprecipitation of 10mg of rat testis homogenate with anti-PP1γ2. The arrow points to the anti-SEARP immunoreactive spot (see Fig. IV.10).*

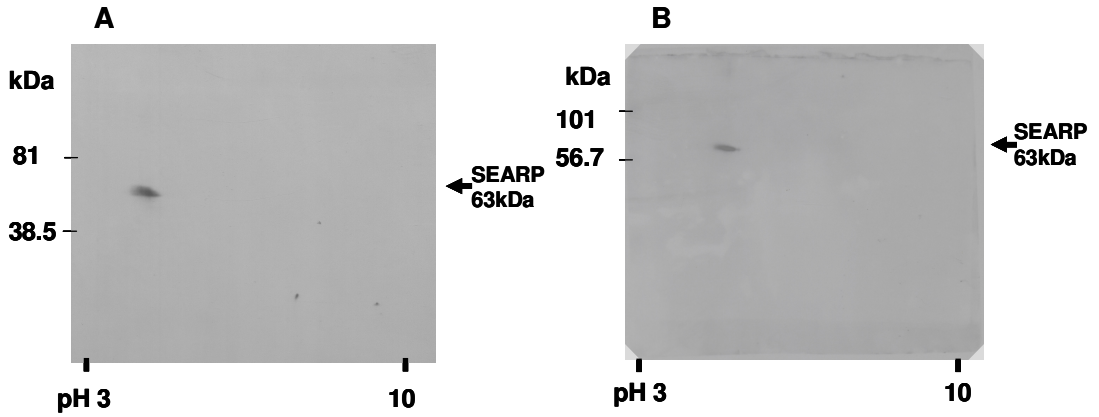
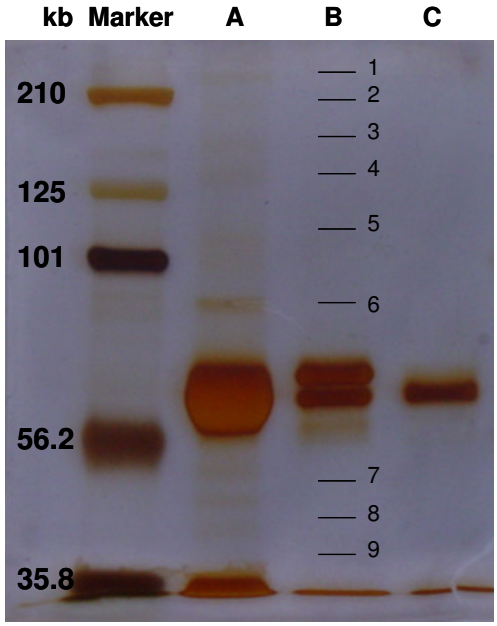


Figure IV.10: Anti-SEARP i*mmunoblot of the anti-PP1γ2 immunoprecipitates from human sperm (A) and rat testis (B).*

By analyzing Figure IV.10 it is clear that SEARP co-immunoprecipitates with PP1γ2 from human sperm and rat testis. Although the predicted molecular mass for SEARP was 94.5kDa (Table IV.7) the spot detected was approximately 63kDa. Since proteolysis is an unlikely explanation, given the use of protease inhibitors, this observation leads us to believe that the complete protein may be cleaved before it interacts with PP1γ2. Alternatively, the detected protein may result from translation initiation from an internal initiation codon. Indeed, if Met322 is used as the initiator codon, then a protein of approximately 60kDa would be expected. Further experimentation will be required in order to clarify this point.

This experiment clearly shows that SEARP interacts with PP1γ2 in rat testis and human sperm. Appropriate negative controls, including immunoprecipitating rat testis or human sperm with rabbit pre-immune serum, as expected, failed to produce protein spots, as visualized by silver staining (data not shown).

Immunoprecipitations of rat testis with anti-PP1γ and anti-PP1α were also performed. Samples were run on a SDS-PAGE gel and immunoprecipitated proteins detected by silver staining (Fig. IV.11) to compare with the anti-PP1γ2 immunoprecipitate.



I

Arrow No.	MW (kDa)
1	282
2	211
3	164
4	130
5	110
6	87
7	50
8	45
9	40

II

Figure IV.11: ***I****, SDS-PAGE gel showing rat testis immunoprecipitates with anti-PP1γ2 (A), anti-PP1α (B) and anti-PP1γ (C).* ***II****, Corresponding table of the molecular masses of the protein bands indicated with arrows.*

From the analysis of the results obtained (Fig. IV.11) it was concluded that several proteins co-immunoprecipitated with PP1γ2, when compared with the proteins that are immunoprecipitated with the other two antibodies (anti-PP1γ and anti-PP1α). The strongest bands in the gel of approximately equal molecular mass correspond to the antibodies used to immunoprecipitate the proteins.

In the future, we will subject the SEARP immunoreactive spot to mass spectrometry analysis to confirm its identity, and other spots will also be analysed to obtain their identification. Thus, a comparative picture will be obtained of the PP1γ2 interactome from human sperm, in relation to the interactome defined by the YTH analysis. The 2D electrophoresis turned out to be a valuable complementary technique to the Yeast Two Hybrid System since it confirmed the SEARP/PP1γ2 interaction *in vivo*. Without an antibody against the putative interactor, the only way to confirm an interaction would be by mass spectrometry analysis of the spots in the gel. Further analysis of the immunoprecipitated protein spots will continue in the future.

IV.3.4 Intracellular localization of SEARP and PP1γ2 in COS-7 cells

The cDNA corresponding to clone 40Q was subcloned into the pEGFP vector in frame with the GFP (Green Fluorescent Protein) protein. GFP was first described by Davenport and Nicol (1955) who reported that the jellyfish *Aequorea victoria* fluoresced green when irradiated with UV light. However, interest on this protein has grown enormously since its cloning (Prasher *et al.*, 1992; Chalfie *et al.*, 1994). The usefulness of GFP derives from the finding that the protein's fluorescence is due to the cyclization of the peptide backbone, and no other cofactor is needed in order to have fluorescence. Another advantage of GFP is that it can be examined in living cells without fixation and permeabilization. In our study, GFP was used as a biological marker, overcoming the need to have an antibody against SEARP. It allowed us to evaluate the subcellular localization of SEARP in cultured cells by expressing a SEARP-GFP fusion protein and monitoring the green fluorescence within the cells. COS-7 cells were transfected with the recombinant construct (pEGFP-SEARP) and the cells were immunostained with the anti-PP1γ2 antibody (Fig. IV.12 and Fig. IV.13). The results demonstrate that PP1γ2 is present throughout the cell (Fig. IV.12, C and IV.13, C), but may be particularly enriched in the nucleus. In contrast, SEARP is mostly absent from the nucleus, but in some cells it was observed in cytoplasmic structures surrounding the nuclear membrane (Fig. IV.12, D). This unusual distribution warrants further studies. In other cells, SEARP appears to be enriched in the Golgi apparatus (Fig. IV.13, D) and may also be associated with the cytoskeleton. In this case, SEARP co-localizes with PP1γ2 in some regions (Fig. IV.13, E and F).

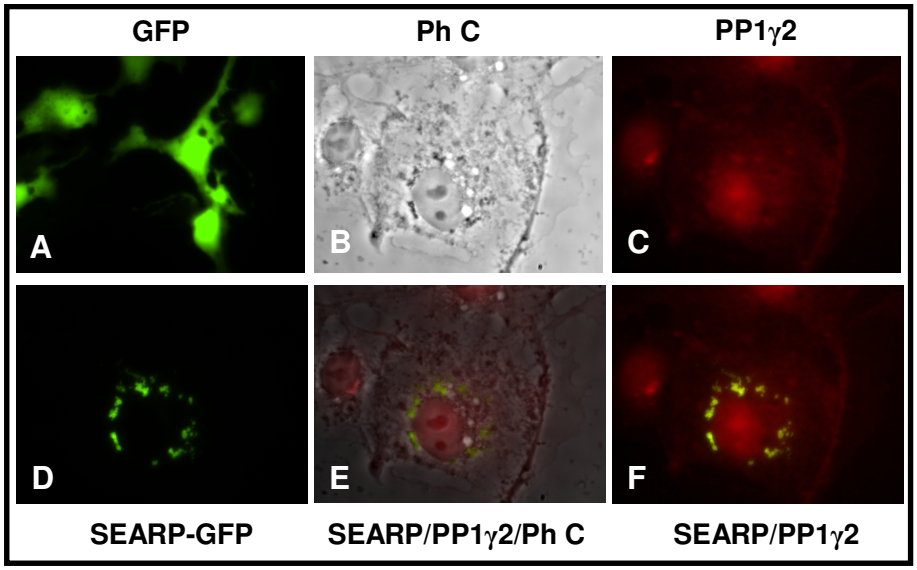


Figure IV.12: *Intracellular localization of SEARP-GFP and PP1γ2 in COS-7 cells (60X magnification).* ***A****, Control COS-7 cells transfected with the pEGFP vector;* ***B****, phase contrast;* ***C****, COS-7 cells immunostained with anti-PP1γ2 antibody;* ***D****, COS-7 cells transfected with pEGFP-SEARP;* ***E****, composite image of the immunostaining with anti-PP1γ2 antibody and GFP-SEARP fluorescence overlaid onto the phase contrast image;* ***F****, same as* ***E****, but without phase contrast.*

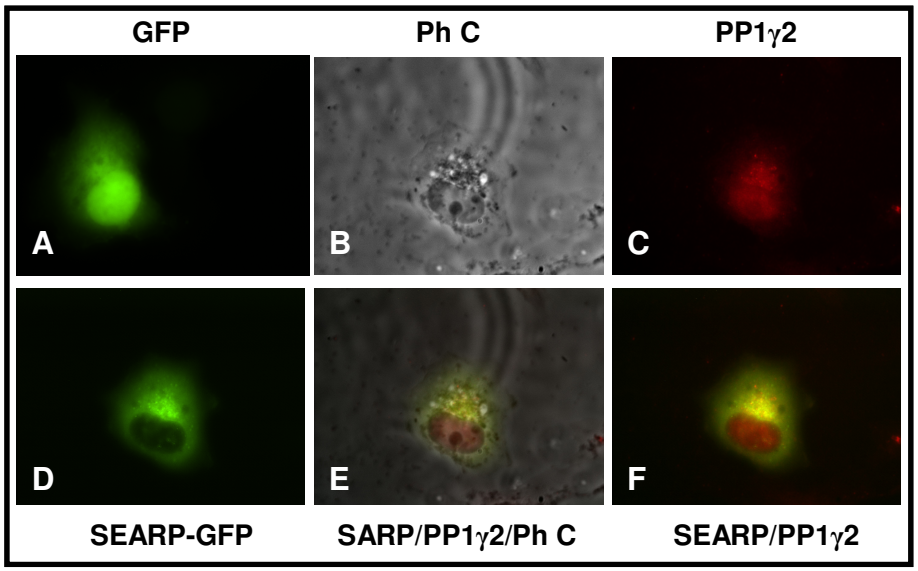
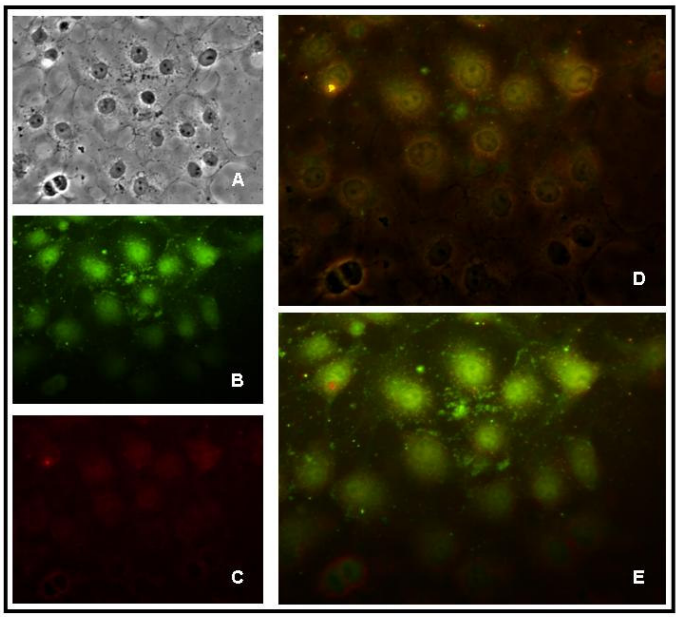


Figure IV.13: *Intracellular localization of SEARP-GFP and PP1γ2 in COS-7 cells (60X magnification).* ***A****, Control COS-7 cells transfected with the pEGFP vector; B, phase contrast of COS-7 cell ;* ***C****, COS-7 cells immunostained with anti-PP1γ2 antibody;* ***D****, COS-7 cells transfected with pEGFP-SEARP;* ***E****, composite image of the immunostaining with anti-PP1γ2 antibody and GFP-SEARP fluorescence overlaid onto the phase contrat image;* ***F****, same as* ***E****, but without phase contrast.*

Nevertheless, some interesting differences were observed in the distribution of COS-7 cells endogenous SEARP using specific antibodies to detect endogenous SEARP and PP1γ (Fig. IV.14).

1



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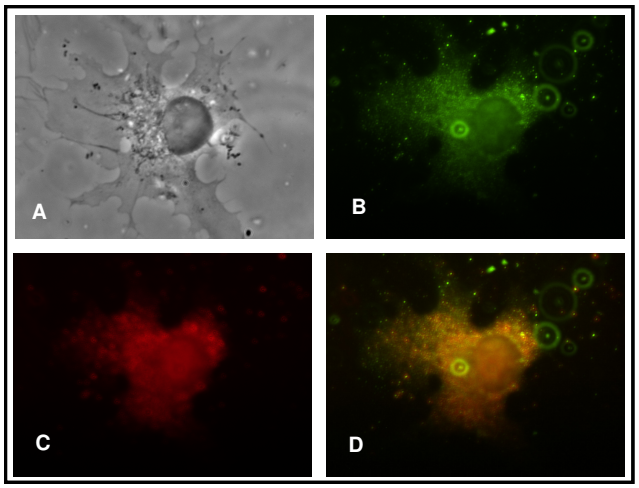


Figure IV.14: *Co-localization of SEARP and PP1γ2 in COS-7 cells by immunocytochemistry using anti-SEARP and anti-PP1γ2 antibodies, and specific secondary antibodies conjugated with FITC and Texas Red, respectively. 1: A, phase contrast micrograph of high density COS-7 cells; B, immunostaining for SEARP; C, immunostaining for PP1γ2; D; combined phase contrast and fluorescent micrograph of the co-localization of SEARP and PP1γ2; E, overlay of the co-localization of SEARP and PP1γ2 (20X magnification). 2: A, phase contrast micrograph of a low density COS-7 cell; B, immunostaining for SEARP; C, immunostaining for PP1γ2; D; overlay of the co-localization of SEARP and PP1γ2 (60X magnification).*

PP1γ2 is present mainly in the nucleus, although it is also found all over the cytoplasm. Some regions in the nucleus are not stained, indicating that there may be selective PP1γ2 enrichement in specific nuclear loci. In what concerns SEARP, it is also found in the nucleus and albeit with lower intensity, in some areas of the cytoplasm. The different distributions of SEARP fused to GFP compared to endogenous SEARP, may be due to the absence of the complete N-terminal sequence from the former. This N-terminal sequence might be essential to target SEARP to the nucleus, or for its interaction with some other protein responsible for taking SEARP into the nucleus.

Recent studies point to the importance of PP1 regulators as targeting subunits in response to cell-cell contact. For instance, I-2 is concentrated in the nucleus of cells cultured at low densities, whereas high densities cells excluded I-2 from the nucleus. The change in I-2 localization may direct I-2 to different forms of PP1 or directly change PP1 localization in response to different cell confluency signals (Leach *et al.*, 2002). Similar results were observed with SEARP. At low density, in the absence of intercellular contacts, SEARP is found throughout the cell but mainly in the cytoplasm (Fig. IV.14-2). However, when cells become confluent, SEARP becomes more highly enriched in the nucleus (Fig. IV.14-1) but can also be detected in the Golgi apparatus. Probably, the putative nuclear targeting sequence present in the N-terminal of full length SEARP is absent from the truncated SEARP-GFP fusion protein, (Fig. IV.12 and IV.13) leading to the retention of expressed recombinant SEARP in the cytoplasm. PP1 localizes mostly to the nucleus of low-density cells. Interestingly, in Fig. IV.14-1D a dividing cell is visible in the lower left corner and exhibits an interesting distribution of PP1γ2 and SEARP, suggesting their possible involvement in mitotic mechanisms. There is intense PP1γ2 staining around the nucleus, forming a ring structure, and SEARP staining is clearly visible in the nuclei of the daughter cells.

IV.3.5 Sperm maturation and SEARP expression

Immunohistochemistry was performed on rat testes sections in order to evaluate the presence of SEARP and its relationship with PP1γ2 and PP1α. First, rat testes sections were analyzed to verify the integrity of cellular structures by haematoxylin/eosin staining

(Fig. IV.15). Haematoxylin is a natural dye that after oxidation gives haematin, a basic dye that stains the nucleic acids in the nuclei blue. Eosin is an acidic dye that stains red the components of the cytoplasm.

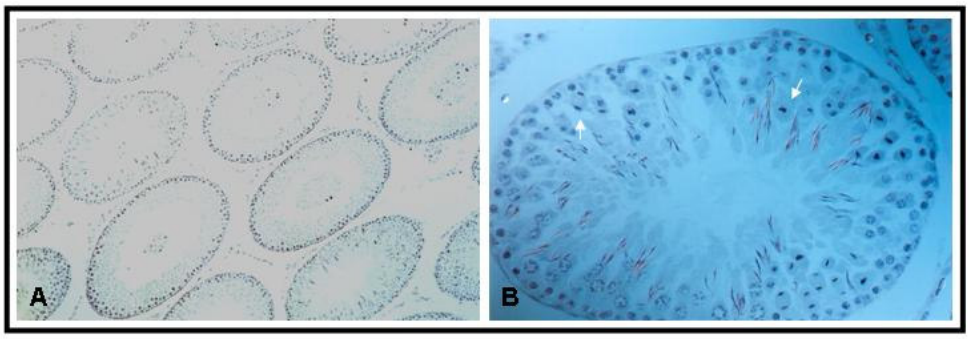


Figure IV.15: *Haematoxylin/eosin staining to confirm testes integrity.* ***A****, Several seminiferous tubules (10X magnification);* ***B****, Seminiferous tubule where the arrows mark meiotic figures (40X magnification).*

The seminiferous tubule is the structural unit of the testis, being approximately 180-300µm in diameter and 80cm long in men. The total length of all tubules in each human testis has been calculated to be around 300-900m. In a sexually mature adult, each seminiferous tubule has a central lumen surrounded by an actively replicating epithelium, mixed with supporting cells, the Sertoli cells (Fig. IV.16). Blood vessels and Leydig cells, that synthesize the hormone testosterone, are localized in the interstitials space between seminiferous tubules. The replicating epithelium (or germinal epithelium) lining the seminiferous tubules produces the haploid male gametes (spermatozoa), by a series of steps called spermatogenesis and spermiogenesis. Spermatogenesis begins with the development of spermatogonia, of which three types are recognized according to their nuclear appearance: A1, dark nuclei, A2, pale nuclei and B, coarsely clumped chromatin and pale central nucleolus. Spermatogonia type A1 undergo mitosis to give more spermatogonia type A1 or type A2. Type A2 mature giving rise to type B. Types A2 and B can still undergo mitosis to give more daughter cells. Diploid type B matures into primary spermatocytes, which duplicate their DNA in order to undergo the first meiotic division (lasting about 22 days). Primary spermatocytes are similar to type B spermatogonia but located further away from the basal lamina, and often the different stages of meiosis can be identified. Meiosis ends with the formation of diploid secondary spermatocytes which

rapidly (within a few hours) undergo the second meiotic division to produce haploid spermatids. The spermatids will subsequently undergo maturation, producing mature but immotile spermatozoa.

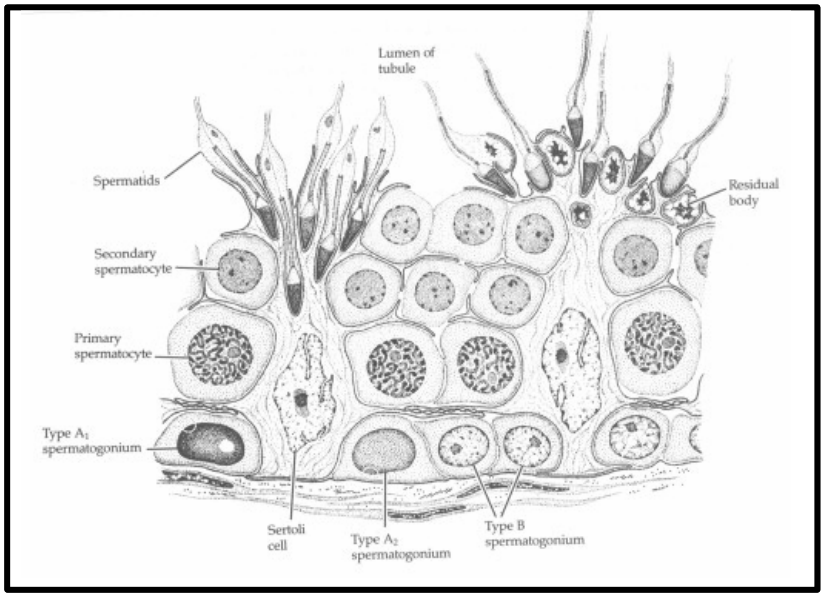


Figure IV.16: *Diagram of spermatogenesis and spermiogenesis stages in the seminiferous tubules (adapted from www.erin.utoronto.ca/~w3bio380/Lectsked/Lect06/Sperm.2htm).*

Only after going through the epididymis do the spermatozoa become motile (this process will be addressed later in this chapter).

In order to determine in which cell types SEARP, PP1γ2 and PP1α were expressed, immunohistochemistry analysis was performed on sections prepared from the testes of 8-week-old adult mice. These were incubated with the appropriate primary antibody and with peroxidase-conjugated secondary antibody (Fig. IV.17) or, for PP1γ2 and PP1α with a Cy3-conjugated secondary antibody, a red fluorescent dye (Fig. IV.18). As can be seen from the brown peroxidase staining in Fig. IV.17, all three proteins are present in rat testes. PP1γ2, PP1α and SEARP are found in the spermatozoa tails and possibly in the acrosome region of the head. Other cells present in the seminiferous tubule exhibit immunoreactive staining indicating the presence of PP1γ2, PPα1 and SEARP mainly in the cytoplasm of those cells and in the basal lamina.

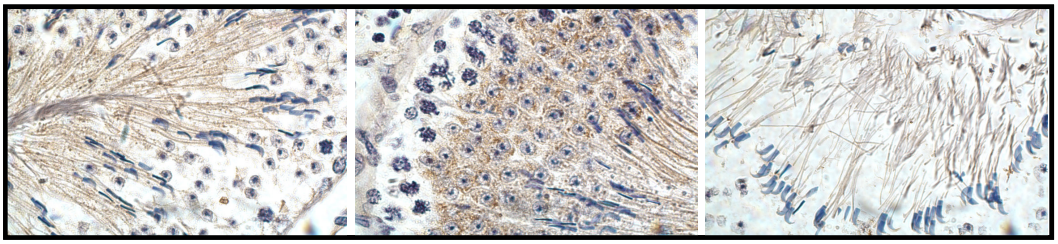


Figure IV.17: *Immunolocalization of SEARP, PP1γ2 and PP1α in the mouse testis using a peroxidase- conjugated secondary antibody (100X magnification).*

Using the Cys3-conjugated secondary antibody, red fluorescence was clearly detected in the spermatozoa tails (Fig. IV.18, 1). Especially for PP1γ2 red fluorescence was also detected in the basal lamina (Fig. IV.18, 3), in other cells (Fig. IV.18, 2), and in Leydig cells (Fig. IV.18, 4).

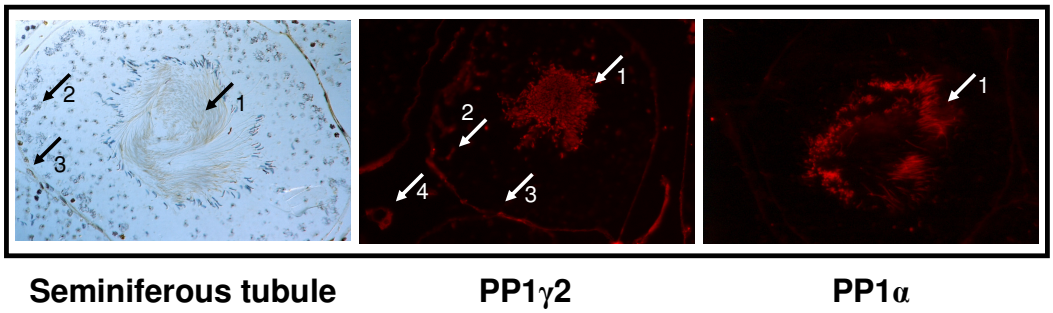


Figure IV.18: *Localization of PP1γ2 and PP1α in the mouse testis using a fluorescent secondary antibody (Cy3- conjugate Goat anti-rabbit). Arrow 1, spermatozoa tails; Arrow 2, other cells; arrow 3, basal lamina; Arrow 4, Leydig cells (40X magnification).*

Spermatogenesis occurs in waves along the seminiferous tubules and thus adjacent areas of the same tubule show different stages of spermatogenesis and spermiogenesis (Fig. IV.19). Each stage is characterized by a combination of certain cell types. The most frequently encountered is stage XIV, since it lasts approximately 22h.

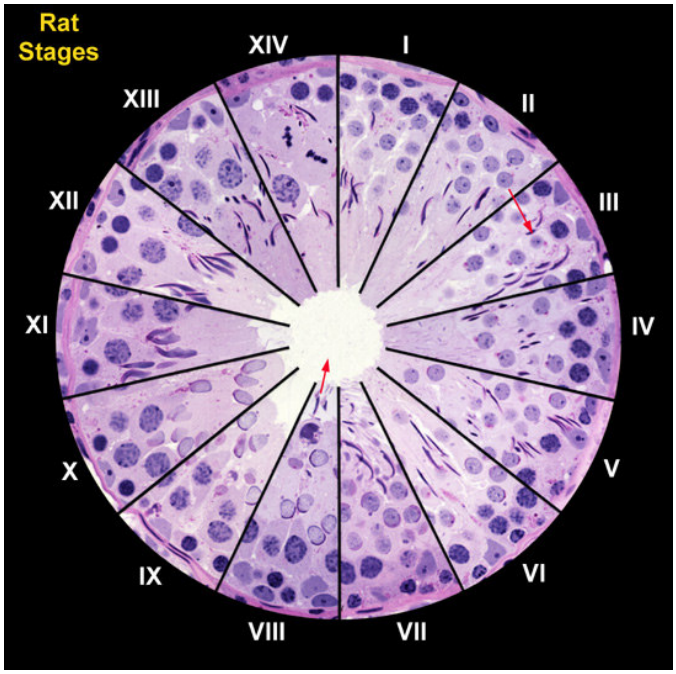


Figure IV.19: *Diagram of the different stages of the cycle of the seminiferous epithelium. Notice the different cell types present in each stage (adapted from www.users.von.uc.edu/hnaskor/plasmalemma_vesicle_protein.htm).*

SEARP, PP1γ2 and PP1α are all present during stage XIV in the tails of spermatozoa and in the cytoplasm of the different types of cells involved in spermatogenesis and spermiogenesis (Fig. IV.20). SEARP is present in spermatozoa tails, in the basal lamina and in Leydig cells. Also the cytoplasms of spermatids and the meiotic or mitotic fuse seem to be immunoreactive for SEARP. Clearly, PP1γ2 is present in the cytoplasm of secondary spermatocytes, in the basal membrane, in Leydig cells and in spermatids. Spermatozoa tails are also immunoreactive for PP1γ2. PP1α detected in spermatozoa flagella, basal membrane and in the cytoplasm of spermatids. The feature that is more relevant in Fig. IV.20, for PP1α, is the existence of very distinguishable meiotic figures that are likely to have PP1α somewhere around the condensed chromosomes, consistent with the postulated central role of PP1α in the control of cell division in other cell types.

Thus, it is obvious that all three proteins (SEARP, PP1γ2 and PP1α) are present in a variety of cells from the events of spermatogenesis and spermiogenesis. Their exact co-localization within the same subcellular structure still needs to be investigated.

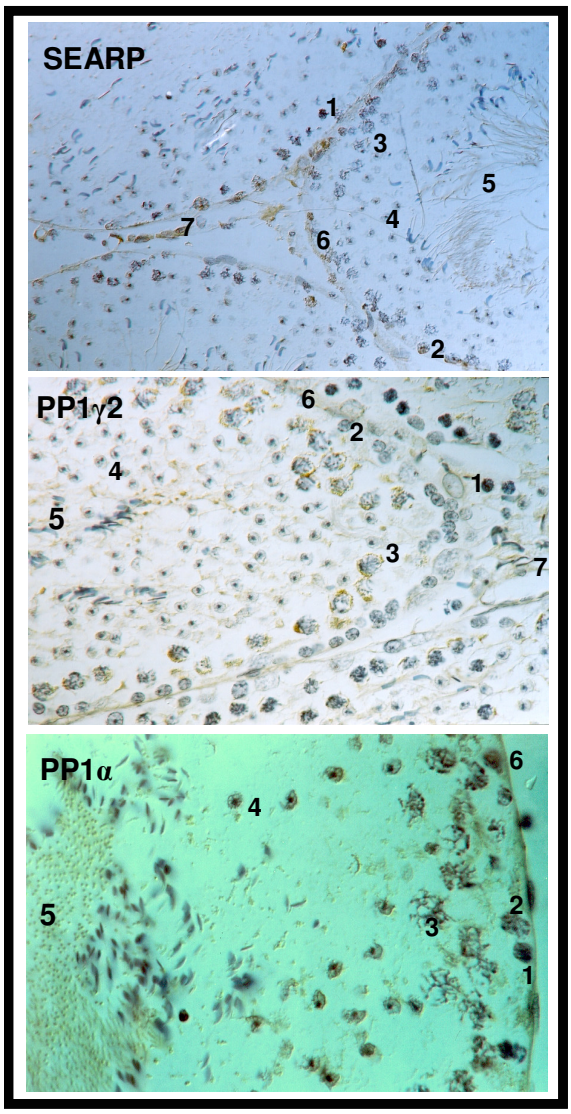


Figure IV.20: *Stage XIV of the cycle of the rat seminiferous tubules. Top panel: section of a rat seminiferuos tubule immunostained with anti-SEARP antibody; Middle panel:seminiferous tubule section immunostained with anti-PP1γ2 antibody; Lower panel: seminiferous tubule immunostained with Anti-PP1α antibody. All three micrographs were taken after incubation of the sections with a peroxidase-conjugated secondary antibody. Magnification used: 40X (top) and 100X (middle and lower oanels). 1, spermatogonia type A1; 2, Primary spermatocyte; 3, Meiotic secondary spermatocyte; 4, Spermatid; 5, Spermatozoa; 6; Basal membrane; 7; Leydig cell.*

Images obtained by immunohistochemistry with the fluorescent secondary antibodies were also analysed by confocal microscopy (Figure IV.21). The presence of PP1γ2 and PP1α in the rat spermatozoa tails was thus further confirmed. An interesting observation was that PP1α staining seemed to be restricted to the tail periphery. That is, the inner part of the tails is devoid of PP1α immunoreactive material. This raises interesting questions in relation to the structure of the spermatozoa tail (Fig. IV.22). Spermatozoa are among the smallest cells in the human body. They can be divided in head (5µm) and tail, and the tail comprises the neck, middle piece (7µm), principal piece (40µm) and end piece

(5-10µm). Along the tail is the axoneme that is responsible for spermatozoa motility. Essentially, it is a long, specialized cilium with nine outer doublet tubules organized around a central tubule pair. The proximal part of the tail is the neck, containing a pair of centrioles and a connecting piece which forms the nine fibrous rings surrounding the axoneme. The axoneme runs through the centre of the middle piece, and is surrounded by nine longitudinal fibers and elongated mitochondria. The principal piece is the longest part of the tail and comprises the axoneme, the nine longitudinal fibers surrounded by external fibers oriented circumferentially. The end piece is composed of axoneme only. So, it seems that PP1α is not present in the axoneme but might be in the fibrous sheath or, what might be more important, in the mitochondria that surround the fibrous sheath and the axoneme and are responsible for generating energy for sperm movement.

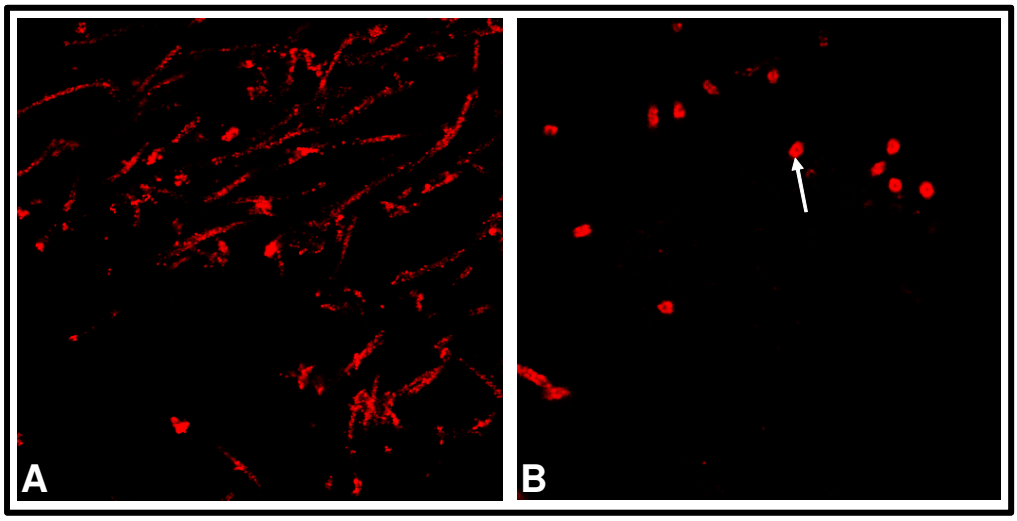


Figure IV.21: *Confocal microscopy of rat testis sections immunostained with Anti-PP1γ2* ***A)*** *and Anti-PP1α* ***B)****. The arrow marks a tail in a transverse section (100X magnification).*

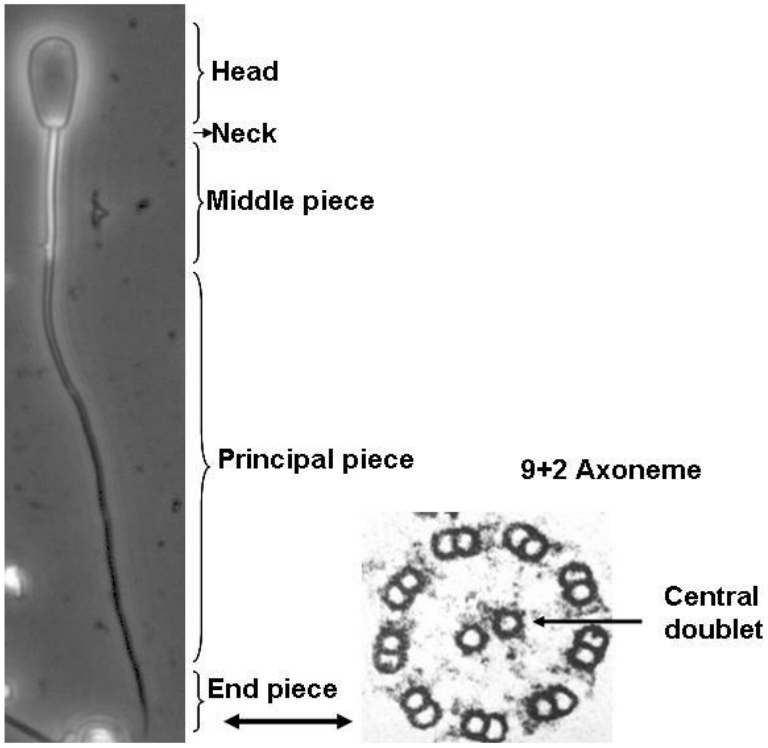


Figure IV.22: *Schematic representation of a mammalian spermatozoon. This is a phase contrast micrograph of a bovine caudal spermatozoon with a 60X magnification. A transverse section through the end piece is shown to illustrate the axonemal 9+2 structure.*

IV.3.6 Colocalization of SEARP and PP1γ2 in human spermatozoa

Human sperm has a corpuscular and a liquid component (Fig. IV.23). The corpuscular components are spermatozoa, immature germ cells, sloughed-off epithelial cells from the seminiferous tubules, spermatophages, cytoplasmic droplets and leukocytes. Cells of the testes, epididymis and accessory glands secrete the liquid components of the seminal plasma. The seminal plasma also contains proteolytic enzymes produced by the prostate gland. Normally, the volume of the human sperm ejaculate is approximately 3ml and contains 50-150 million spermatozoa per ml. In fertile men 25% of the spermatozoa are abnormal or degenerated. Ejaculated spermatozoa are motile but they need to undergo capacitation in the female genital tract before they can fertilize an oocyte.

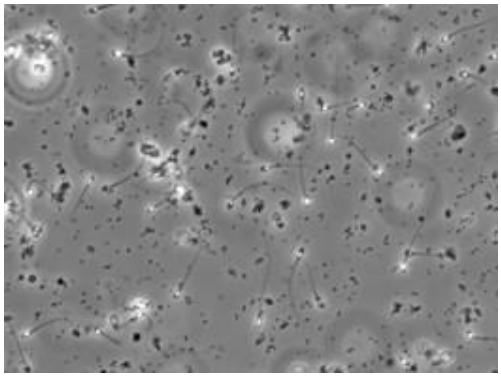


Figure IV.23: *Phase contrast micrograph of Human sperm (20X amplification).*

Immunofluorescence was used to localize PP1γ2 and SEARP within human spermatozoa. As seen in Fig. IV.24, PP1γ2 is present along the entire length of the flagellum including the middle piece. In the head staining is intense in the equatorial segment (because of the different flourescence intensities observed in the tail and in the equatorial region it is not possible to visualize both patterns in the same image). SEARP immunoreactivity was detected in the principal piece of the flagellum and the head-neck junction. Staining is relatively weak in the middle piece. In the head region, staining appears also in the equatorial region. Thus, there are regions within spermatozoa where PP1γ2 and SEARP co-localize (Fig. IV.25). The staining appears to be specific since there was no fluorescence observed when the primary antibodies were omitted (data not shown).

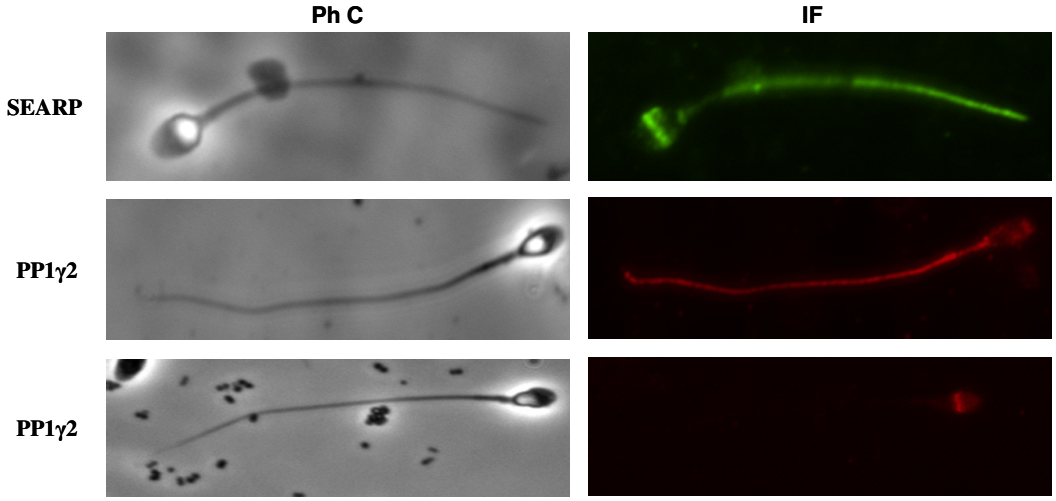


Figure IV.24: *Immunolocalization of PP1γ2 and SEARP in human spermatozoa. Human spermatozoa were labeled with anti-PP1γ2 and anti-SEARP antibodies.Ph C, phase contrast; IF, immunofluorescence (60X magnification).*

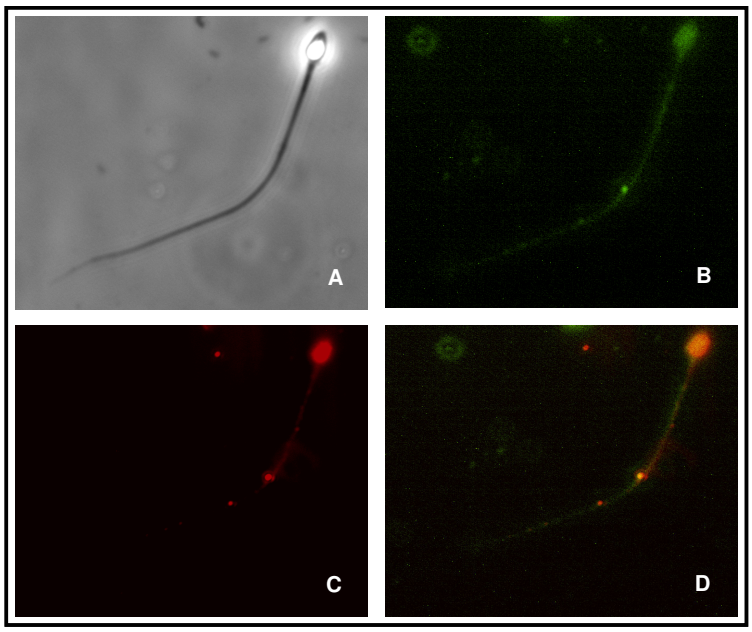
b 

Figure IV.25: Co-*Immunolocalization of PP1γ2 and SEARP in human spermatozoa. **A**, phase contrast micrograph of human spermatozoa; **B**, immunolocalization of SEARP; **C**, immunolocalization of PP1γ2; **D** ,* overlay *of PP1γ2 and SEARP immunoreactivity.*

IV.4 DISCUSSION

SEARP (Six to Eight Ankyrin Repeat Protein) was identified in a Yeast Two Hybrid screen using PP1γ1 as bait with a human testis cDNA library. Subsequently, it was shown by sequential transformation with the bait plasmid (pASPP1γ2) and the prey plasmid (pACT-2SEARP), that SEARP also interacts with PP1γ2.

This new protein was characterized in several ways. First, the complete nucleotide sequence was analyzed in what concerns, for instance, its genomic or exon-intron boundaries. Afterwards, bioinformatic analysis of the encoded protein revealed several interesting features of the protein. Eight ankyrin repeats were identified, as well as a PP1 consensus binding motif just upstream of these ankyrin repeats. Several putative phosphorylation sites were also identified, strongly suggesting that SEARP may be regulated itself by phosphorylation mechanisms.

Immunoblot analysis of several rat tissues, and bovine and human testis and sperm, showed that SEARP is present in residual amounts in most of the analyzed tissues (such as brain) but is expressed in testis, sperm, ovary, lung and liver in relatively large amounts.

The relatively slower electrophoretic mobility of testis SEARP compared to other tissues is in agreement with the existence of an alternatively spliced SEARP isoform, detected in a Yeast Two Hybrid screen of a human lymphocyte library (P. T. Cohen, personal communication). Thus, SEARP-L has a shorter C-terminus compared to SEARP-T. Northern blot analysis revealed that SEARP mRNA is strongly enriched in testis, compared with other tissues, and that the transcript is 3.6kb.

Analysis with anti-SEARP antibody of a 2D-gel immunoblot of immunoprecipitated samples of rat testis and human sperm with anti-PP1γ2 antibody clearly demonstrated the *in vivo* biochemical interaction between PP1γ2 and SEARP in both testis and sperm.

SEARP co-localizes with PP1γ2 in some regions of COS-7 cells and, more importantly, in some areas of spermatozoa, specially in the principal piece of the tail and the equatorial segment of the head (involved in the acrosome reaction). In COS-7 cells SEARP is mainly nuclear in confluent cells but is enriched in the cytoplasm in low density cells. However, when COS-7 are transfected with our truncated SEARP sequence (corresponding to clone 40Q) fused to GFP, the immunofluorescence is detected almost exclusively in the cytoplasm. This may be explained by the absence of the N-terminus. This region might be responsible for targeting SEARP to the nucleus or may interact with another protein that targets it to the nucleus.

Considering the above data, it seems reasonable to hypothesise two possible roles for SEARP in sperm function. First, being associated with PP1γ2 in the sperm tail, it might be involved in the control of sperm motility. Secondly, the COS7 confluency experiments might indicate that in sperm, SEARP is present in the cytoplasm (as confirmed by its localization in the equatorial head region) being thus essential for the acrossome reaction. After sperm-egg interaction, SEARP might translocate to the nucleus where it may alter gene expression. This hypothesis is supported by the presence, on the C-terminal domain of SEARP, of a putative transcription factor motif. Finally, SEARP is also present in cells involved in spermatogenesis and spermiogenesis. The mechanism by which SEARP may be involved in these events still needs to be investigated.

Sds22 (suppressor of the dis2-mutant) was originally identified in yeast as a PP1 regulatory subunit essential for the completion of mitosis (MacKelvie *et al.*, 1995). The mammalian counterpart, PPP1R7, was cloned by Renouf *et al.* (1995) and inhibits PP1 in rat liver nuclei (Dinischiotu *et al.*, 1997). Sds22 consists of a poorly conserved N-terminus

which appears to be essential for protein stability and a well conserved C-terminus that is probably involved in the nuclear localization of sds22. The central domain of sds22 has highly conserved tandem array of 11 leucine–rich repeats of 22 residues that mediate the binding to PP1. Sds22 is ubiquitously expressed in human tissues and up to six transcripts can be generated from PPP1R7 gene by alternative polyadenylation and skipping of exon 2 (Ceulemans *et al.*, 1999). It was shown that sds22 functions positively with GLC7 (yeast PP1) to promote dephosphorylation of nuclear substrates required for faithful transmission of chromosomes during mitosis (Peggie *et al.*, 2002), primarily by keeping PP1 in the nucleus. A sds22 homolog was identified in rat testis that associates with PP1γ2 (Chun *et al.*, 2000). The expression pattern of the rat sds22 homolog matches with that of PP1γ2, suggesting that it is involved in spermatogenesis by controlling PP1γ2 activity. Using immunoaffinity choromatography, sds22 was identified in motile caudal spermatozoa as a regulator of PP1γ2 catalytic activity (Huang *et al.*, 2002). In contrast, PP1γ2 and sds22 do not interact in caput bovine sperm (Mishra *et al.*, 2003). This same study showed that caput sds22 is bound to a p17kDa protein suggesting that binding to PP1γ2 requires sds22 dissociation of p17 or some other post-translational modification. This protein, like SEARP, is present in the principal piece of the spermatozoa flagellum and in the head-neck junction although in somatic cells they are mainly nuclear. Possibly, SEARP may also need to be cleaved to interact with PP1γ2, as seen in the 2D-gel immunoblot with anti-SEARP antibody (Fig. IV.10).

SEARP also has a putative calcium binding domain, indicating that SEARP could act as an intermediary between calcium signaling and PP1γ2 regulation of sperm motility.

Mechanisms regulating binding of SEARP to PP1γ2 should be important in understanding the biochemical events that underline sperm maturation and movement. Studies are underway to use recombinant PP1γ2 and recombinant SEARP to further analyze the nature of the interaction between these proteins. The complex set off signaling motifs detected in SEARP, its restricted expression in specific tissues, and its subcellular localization, coupled with the intriguing cell contact-dependent expression pattern, make SEARP an ideal target for signal transduction therapeutics. Future work will evaluate its usefulness as a target for the development of a male contraceptive or a target for the treatment of male infertility.

V DISCUSSION AND CONCLUSION

In somatic cells, PP1 isoforms are regulated by a large number of ubiquitous and cell-specific regulatory and targeting proteins (Oliver and Shenolikar, 1998). Germ cell-specific phosphatase PP1γ2 is the predominant serine/threonine phosphatase in spermatozoa (Smith *et al.*, 1996; Vijayaraghavan *et al.*, 1996). PP1γ2 activity is inversely correlated with sperm motility. Enzyme inhibition with okadaic acid or calyculin A induces motility initiation and stimulation. Thus, our goal was to understand how PP1γ2 activity is regulated during sperm maturation and to identify the sperm-specific partners in this event. Therefore, in order to identify the sperm proteins that interact with PP1, we performed 2 separate yeast two-hybrid screens of a human testis cDNA library using the baits PP1γ1 and PP1γ2. The validity of the results obtained was confirmed by a variety of criteria, including the isolation of bona fide PP1 binding proteins and the occurrence of a PP1 consensus binding motif in the newly identified proteins. We recovered 120 positive clones in the YTH1 and 155 positive clones in the YTH2. Among these were clones encoding previously known PP1 interactors, such as Nek2 and NIPP1, and also previously uncharacterized proteins. We performed a detailed study of one such novel protein of 93kDa that is expressed mainly in testis. We named this protein SEARP-T (**S**ix to **E**ight **A**nkyrin **R**epeat **P**rotein from **T**estis). Fluorescence immunocytochemistry was used to compare the intra sperm localization of PP1γ2 and SEARP-T. Both proteins co-localize in the tail and in the equatorial segment of the head.

These results provide new insights into PP1 function in human testis and sperm motility and validate the Yeast Two Hybrid System as a mean to understand the roles played by PP1 in diverse cellular regulatory events, thereby allowing possible targets to be investigated for pharmacological intervention, particularly regarding infertility and contraception.

V.1 THE YEAST TWO HYBRID SYSTEM

The availability of complete genome sequences from a variety of organisms initiated the development of new approaches to deal with a huge amount of genomic information (Table V.1). Thus, high-throughoutput methods for detecting protein interactions were

developed. Among them, the most used are the Yeast Two Hybrid System, Affinity Purification coupled to Mass Spectrometry and, on a smaller scale, proteome chips (Auerbach *et al.*, 2002), which have yielded vast amounts of data that can be exploited to infer protein function and regulation. These methods together with the availability of sophisticated databases help to decode complex interactions among proteins and to integrate interacting proteins in complex cellular pathways.

Table V.1: *Genome sequences of some organisms.*

Organism	Sequence published (year)	No. of Predicted ORFs	Reference
Haemophilus influenzae (bacteria)	1995	1720	(Fleischmann *et al.*, 1995)
Saccharomyces cerevisiae (Yeast)	1996	6234	(Goffeau *et al.*, 1996)
Caenorhabditis elegans (Worm)	1998	19099	(consortium, 1998)
Drosophila melanogaster (Fruit fly)	2000	13061	(Adams *et al.*, 2000)
Arabidopsis thaliana (Plant)	2000	~25000	(Initiative, 2000)
Homo sapiens (Human)	2001	~35000	(Lander *et al.*, 2001; Venter *et al.*, 2001)
Plasmodiumfalciparum (Parasite)	2002	5279	(Lander *et al.*, 2001; Hall *et al.*, 2002; Hyman *et al.*, 2002)
Mus musculus (Mouse)	2002	~30000	(Waterston *et al.*, 2002)

ORFs: open reading frames.

However, sequencing the genome of an organism does not mean decoding the functions of the proteins it encodes. Thus, the more challenging issue is to clarify the proteome of a given organism, and even more importantly, to characterize the interactions between its constituent proteins. Additionally, the goal of proteomics is also to determine when and where the proteome of an organism is expressed and to identify post-translational modifications that regulate protein function, ultimately elucidating how an organism or cell in that organism behaves. Proteomics tries to identify the interactions

between proteins in a normal versus a disease state, being of great biomedical value in order to identify putative protein targets to cure a certain disease and disease markers.

The Yeast Two Hybrid System has been rapidly adopted since it was first developed by Fields and Song (1989) with the number of papers citing "Two-Hybrid" growing markedly since its original introduction (Fig. V.1).

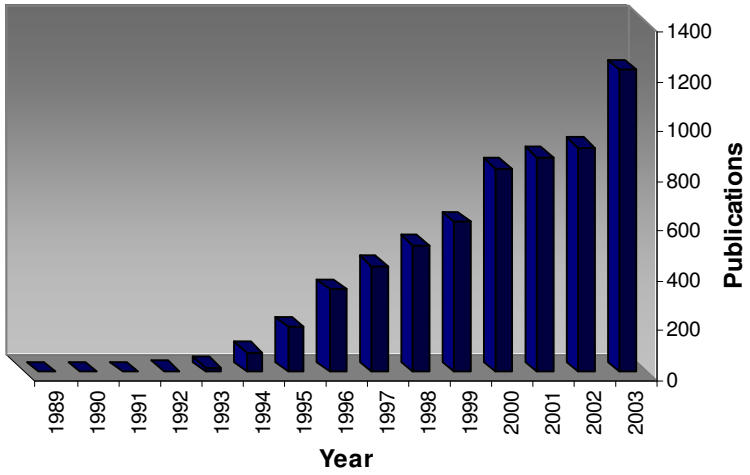


Figure V.1: *Number of publications obtained by searching PubMed (www.ncbi.nlm.nih.gov) with the words "Two-Hybrid" from 1989 to 2003.*

Only the Yeast Two Hybrid System will be discussed in detail here. The main advantages of this method are that the interactions are detected *in vivo*, there are no artificial lyses or washing steps and it has the potential to detect weak or transient interactions. Its drawbacks are that it mostly detects binary interactions and the forced nuclear localization of the interacting proteins can create false positives or false negatives. Also, artificial fusion may inhibit certain interactions and some interactions are preferred with regard to cellular compartments. The Yeast Two Hybrid System is a genetic method in which an interaction between a bait protein and its library expressed partner is detected via reconstitution of a transcription factor and subsequent activation of a set of reporter genes. Large scale two hybrid approaches have used the complementary methods called "matrix approach" and the "library screening approach" for screening large sets of proteins. In the matrix approach a yeast strain expressing the bait protein is mated with an array of yeast strains expressing many different prey proteins. The interactions are detected by growth on selective media. Here, a library screening method was used to screen our bait

proteins (PP1γ1 and PP1γ2) against a human testis cDNA library. Colonies expressing the interacting proteins are selected and the library plasmids are isolated and sequenced to determine the identity of the interacting prey proteins.

Overall, our results were very satisfying (Table III.3). Several previously known PP1 binding proteins were identified in our screens, thus validating the method used. Among them are Nek2 and NIPP1 (discussed in Chapter III). Additionally, proteins known in other contexts were also identified as putative PP1 regulators, for example, RANBPM (Nakamura *et al.*, 1998) and DAPPER1 (Katoh, 2003). Even more interesting was the fact that the vast majority of the proteins identified were completely new proteins. Chapter IV describes the characterization of a new protein that we named SEARP-T.

V.2 MECHANISMS INVOLVED IN SPERM MOTILITY

Several studies demonstrate the involvement of cyclic nucleotides, bicarbonate, cholesterol and protein phosphorylation in acquisition of progressive motility, capacitation, hyperactivation of motility, directed motility or induction of acrosome reaction (Garbers and Kopf, 1980; Smith *et al.*, 1996; Vijayaraghavan *et al.*, 1996; Vijayaraghavan *et al.*, 1997; Cross, 1998; Visconti and Kopf, 1998; Smith *et al.*, 1999; Huang *et al.*, 2002; Mishra *et al.*, 2003). Calcium ions are also primary determinants of sperm cell behavior being absolutely required for the processes described above. Immunostaining studies and transcript analysis revealed that spermatozoa possess several calcium channels.

Ren *et al.* (2001) discovered a new protein, crucial to sperm swimming in mice that could be a target for male contraceptives or fertility treatments. The protein forms a channel through the membrane of sperm tail and controls the inflow of calcium ions that triggers swimming. This protein was named CatSper and is located mainly in the principal piece of the sperm tail. This channel contains 6 transmembrane segments and maps to chromosome 15q13 in humans. This explains, at least in part, the role of calcium in sperm function, that despite having been known for a long time, the details have been always elusive. Mutant mice lacking this channel are sterile. Sperm motility is markedly decreased and sperm are unable to fertilize intact eggs. All humans have the gene that encodes the channel, but it is switched on only is sperm cells.

Another putative ion channel, CatSper2, was also found to contain 6 transmembrane segments and also mapped to human chromosome 15q13 (Quill *et al.*, 2001). CatSper represents a target for non-hormonal male contraceptives.

Worldwide, the market for oral contraceptives is worth billions of euros and the demand for a non-hormonal oral male contraceptive is likely to be phenomenal.

In our group we are especially interested in the involvement of protein phosphorylation on sperm motility. There are several evidences demonstrating that protein phosphatases are direct players in the acquisition of sperm movement. Indeed, PP1 inhibition of immotile caput sperm with specific inhibitors, results in them acquiring normal motility parameters. Intriguingly, these effects are completely independent of calcium.

V.2.1 PP1γ2/I2-L/GSK-3

Sperm contains a specific PP1 isoform (PP1γ2) as well as the kinase glycogen synthase kinase-3 (GSK-3) (Smith *et al.*, 1996; Vijayaraghavan *et al.*, 1996). PP1γ2 and GSK-3 activities are two- and six-fold higher, respectively, in immotile caput compared to motile caudal epididymal sperm. The PP1γ2 catalytic activity is regulated by its interaction with an inhibitor similar to somatic cells I-2. This inhibitor may correspond to a clone we identified in our screen, that we named I2-L (Inhibitor 2 Like, XM_018216). The inactive PP1γ2-inhibitor complex is activated by phosphorylation of this I2-L inhibitor by GSK-3 (Fig V.2). Interestingly, the I2-L protein has a Thr/Pro substitution at position 72. The absence of the most important regulatory residue of I-2 in I2-L must have consequences on the regulation of PP1 and ultimately on the acquisition of sperm motility. Apparently, I2-L represents a constitutive inhibitor of PP1 that is independent of GSK-3 phosphorylation. One hypothesis might be that I2-L is only expressed in caudal motile sperm, leading to constitutive inactivation of PP1γ2. Another hypothesis is that I2-L is bound to a protein that keeps it from binding PP1γ2 in immotile caput sperm, paralleling what happens with sds22. To test these hypotheses, two parallel sperm immunoprecipitation experiments need to be performed with caudal and caput epididymal sperm, followed by a 2D analysis, allowing the differences in the I2-L and I-2 spots to be detected. I2-L has a theoretical molecular mass of 23.1kDa and a pI of 4.77, whereas I-2 has a molecular mass of 23kDa and a pI of 4.64, although it migrates with an apparent molecular mass of 31kDa in SDS-

PAGE gels. The study of I2-L is difficult, since it is very similar to I-2. They are 96% similar at the nucleotide level and 96.5% similar at the protein level. It is therefore very difficult to produce I2 or I2-L specific molecular tools (antibodies, probes, etc) for further analysis. The protein complex PP1γ2/I2-L/GSK-3 may explain the observed sperm motility changes during epididymal transit.

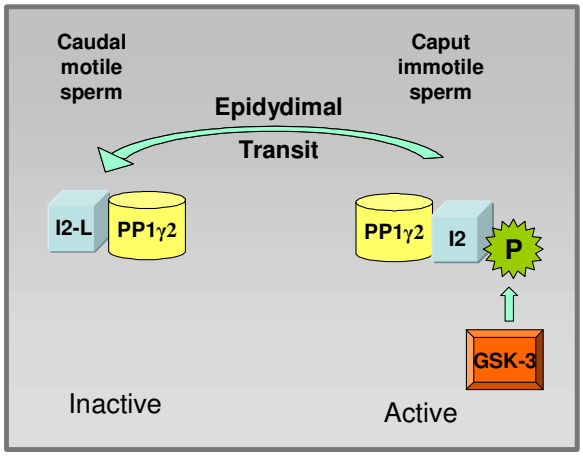


Figure V.2: *Possible mechanism of regulation of PP1γ2 by I2-L and GSK-3 in sperm.*

V.2.2 PP1γ2/sds22

A sds22 homologue was recently identified in sperm (Mishra *et al.*, 2003). The mammalian counterpart, PPP1R7 inhibits PP1 catalytic subunit in rat liver nuclei (Dinischiotu *et al.*, 1997). A sds22 homolog was identified in rat testis that associates with PP1γ2 (Chun *et al.*, 2000). The expression pattern of the rat sds22 homolog matches that of PP1γ2, suggesting that its involvement in spermatogenesis relates to the control of PP1γ2 activity. Sds22 was identified in motile caudal spermatozoa as a regulator of PP1γ2 catalytic activity (Huang *et al.*, 2002). In contrast, PP1γ2 and sds22 do not interact in caput bovine sperm (Mishra *et al.*, 2003). This same study showed that caput sds22 is bound to a 17kDa protein, suggesting that binding to PP1γ2 requires sds22 dissociation from p17 or some other post-translational modification. This protein is present in the principal piece of the spermatozoa flagellum and in the head-neck junction although in somatic cells it is mainly nuclear. Sds22 has consensus sites for phosphorylation by GSK3, PKA and CDKII (calmodulin dependent kinase II), all present in sperm (Fig. V.3).

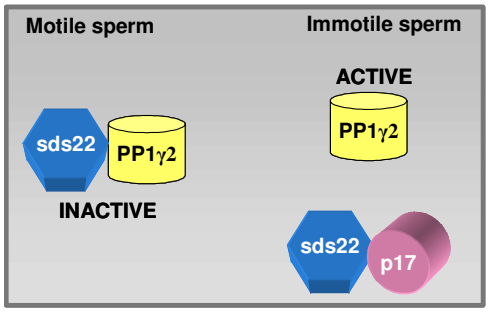


Figure V.3: *Possible mechanism of regulation of PP1γ2 by sds22 in sperm.*

V.2.3 PP1γ2/AKAP220/PKARII

The cyclic AMP (cAMP)-dependent protein kinase (PKA) and PP1 are broad specificity signaling enzymes with opposing actions that catalyse changes in the phosphorylation state of cellular proteins. PKA is a ubiquitous, multifunctional enzyme involved in the regulation of several cellular events. PKA holoenzyme consists of four subunits, two catalytic and two regulatory (RI and RII). Subcellular targeting to the vicinity of preferred substrates is a means of restricting the specificity of these enzymes (Hubbard and Cohen, 1993; Faux and Scott, 1996). Several PP1 targeting subunits direct PP1 to specific locations (Egloff *et al.*, 1997), while compartmentalization of PKA is mediated through association of the its regulatory subunits with A-kinase anchoring proteins (AKAPs) (Faux and Scott, 1996). Over 40 AKAPs have been identified to date, targeting PKA to the plasma membrane, cytoskeleton, endoplasmic reticulum, Golgi, mitochondria and nuclear matrix (Schillace *et al.*, 2001). This high level of molecular organization ensures selectivity in cAMP-responses. Several anchoring proteins have been identified that can simultaneously associate with kinases and phosphatases (Faux and Scott, 1996). Neuronal AKAP79 binds to PKA, PKC and PP2B, and AKAP149 recruits PKA and PP1 to the lamina of nuclear membranes (Steen *et al.*, 2000). Similarly, the NMDA receptor-associated protein Yotiao maintains an anchored PKA constitutively active PP1 to regulate the phosphorylation state of the NMDA receptor ion channel (Westphal *et al.*, 1999b). Another example is AKAP220 that binds PKA and PP1, being a competitive inhibitor of PP1 (Schillace *et al.*, 2001). AKAP220 also associates with PKA in testis, where it may target the kinase to peroxisomes. AKAP220 mRNA is expressed at high levels in human testis and in isolated human pachytene spermatocytes and round spermatids. AKAP220 is present in human male germ cells and mature sperm. In sperm,

AKAP220, as well as RIIα, is located in the middle-piece and is probably associated with cytoskeletal structures (Reinton *et al.*, 2000). AKAP220 binds the RIα, RIIα and RIIβ subunits of PKA in human testis. The centrosomal AKAP220/PKA complex may regulate spindle formation during meiosis. The middle-piece associated AKAP220 could serve to anchor PKA and /or PP1γ2, directly regulating the contractile machinery in the sperm axoneme (Fig. V.4). Furthermore, it has been shown that disruption of RII interaction with AKAPs, by membrane-permeable peptides, causes the arrest of sperm motility (Vijayaraghavan *et al.*, 1997). Together, this data suggest that the AKAP/PKA/PP1 complex is important for regulation of sperm motility.

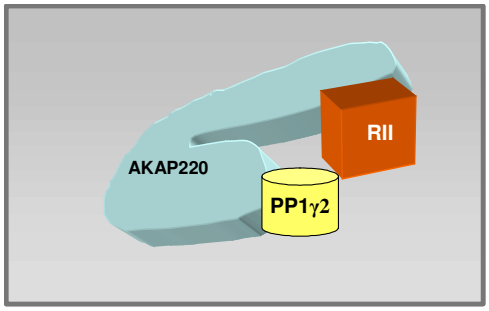


Figure V.4: *Putative AKAP220/PP1γ2/PKARII complex involved in the regulation of sperm motility.*

V.2.4 PP1γ2/AKAP110/PKA

Several AKAPs have been shown to be present in germ cells. Among them is S-AKAP84 that localizes in the middle-piece of mouse elongating spermatids (Lin *et al.*, 1995); its splice variants D-AKAP-1 (L. J. Huang *et al.*, 1999) and AKAP121 (Feliciello *et al.*, 1998) are also present in condensing spermatid middle-piece. AKAP82 (Turner *et al.*, 1998) and TAKAP80 (Mei *et al.*, 1997) are found in the sperm fibrous sheath. Finally, AKAP110, recently identified by Vijayaraghavan *et al.* (1999), is localized to the fibrous sheath and acrosomal region. There seems to be an abundance of dual specificity AKAPs, which bind RI and RII PKA regulatory subunits. RI subunit localizes primarily in the acrosomal region and RII exclusively to the tail.

Northern analysis and in situ hybridization detected AKAP110 only in testis, more specifically in round spermatids. In the spermatozoon AKAP110 localizes to the acrosome

and tail (Smith *et al.*, 1999). In sperm AKAP110 apparently binds RII on the mitochondria membrane. AKAP110 may also bind PP1γ2 that, as we demonstrated in Chapter IV, is also present in the tail (Fig. V.5).

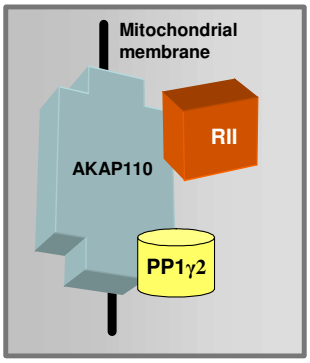


Figure V.5: *Putative AKAP110/PP1γ2/PKARII complex involved in the regulation of sperm motility.*

Sperm specific AKAPs seem to be a very directional target for the development of a male contraceptives or for infertility therapeutics. Although none of these AKAP proteins has been identified in the YTH screens performed, they might be present in the immunoprecipitate samples from human sperm and rat testis. Several putative spots corresponding to these proteins can be seen in the 2D-gel (Fig. IV.9). Mass spectrometric analysis of the identified spots should resolve this question.

V.3 SPLICING AS A FEASIBLE THERAPEUTICAL TARGET

Mammalian genes are organized on chromosomes in an exon-intron structure, with an average of 8.7 exons per gene (Waterston *et al.*, 2002). After transcription, the pre-mRNA undergoes splicing, the process of intron excision, performed by a huge RNA-protein complex, the spliceosome (Will and Luhrmann, 2001). This complex recognizes conserved sequences on exon-intron boundaries (the splice sites) and joins exons together removing the intermediate introns. Alternative splicing occurs when the introns from a pre-mRNA are spliced in more than one way resulting in more than one mature mRNA per gene. Therefore, one gene can yield several, sometimes functionally different, proteins (Graveley, 2001).

As was evident from the results of the YTH screens presented, a large number of the proteins identified as putative PP1 binding partners derive from an alternative splice of the pre-mRNA (Fardilha *et al.*, 2004a; Fardilha *et al.*, 2004b). For instance, Nek2A-T derives from an alternative 3'splice-site of the pre-mRNA; NIPP1-T mRNA suffers an excision of part of exon 7; SEARP-T and SEARP1 differ by alternative 3' splice-site (Browne *et al.*, 2004) (Fig. V.6).

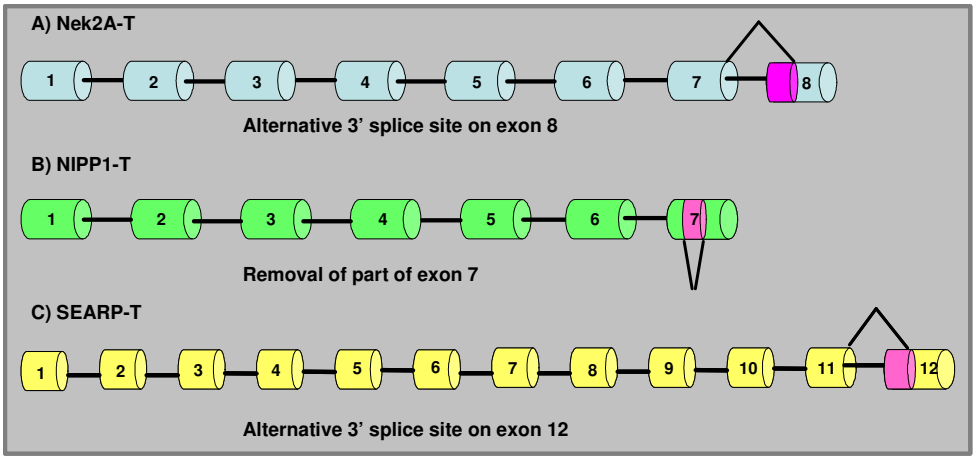


Figure V.6: *Spliced variants of Nek2A-T, NIPP1-T and SEARP-T. Pink cilinders are the alternative spliced parts of the respective exon. Exons and introns are not drawn to scale.*

Interestingly, for unknown reasons, several testis mRNAs are alternatively spliced. This is also true for PP1γ (da Cruz e Silva *et al.*, 1995b). The alternatively spliced PP1γ2 (Fig. V.7) protein appears to be the major PP1 isoform expressed in mammalian sperm (Vijayaraghavan *et al.*, 1996), although other PP1 isoforms can be detected in other cell types in testis.

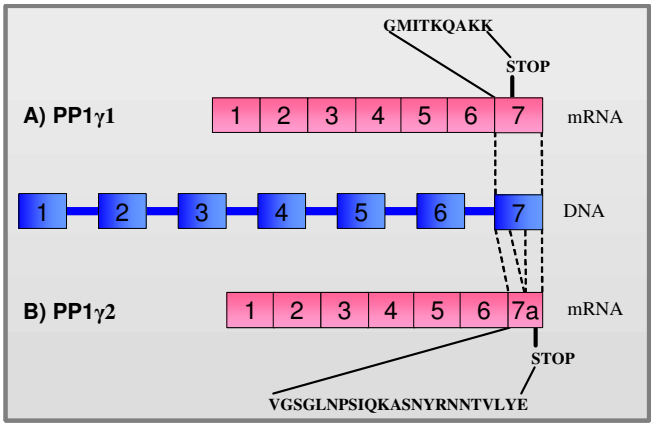


Figure V.7: *PP1γ1 and PP1γ2 splice variants. Exons and introns are not drawn to scale.*

Alternative splicing is a mechanism strictly regulated, being specific to a tissue, to a development stage, to a condition or to a pathological stage. Different splice variants of the same gene may also exist in the same place (cell or tissue), and act as regulators of each other. Errors that occur during splicing can lead to a pathological situation, and splice variants can be good diagnostic markers for disease conditions. In addition, different splice variants, being tissue specific, can function as ideal targets for the development of new drugs that will not affect similar mechanisms occurring in other tissues. For instance, if a drug is designed against the C-terminus of testis-enriched PP1γ2, it is unlikely to affect other organs except testis. However, an even more specific scenario would be to target a PP1γ2 binding protein that, presumably, would be involved in a much more restricted number of cellular events than the phosphatase catalytic subunit (Amador *et al.*, 2004). Our results indicate the existence of a large number of putative PP1γ2 binding proteins, some of which may ultimately be used as targets to develop new drugs to modulate sperm motility and other testis specific processes (Fardilha *et al.*, 2004a). The possibility of precise and specific modulation of sperm motility should prove inestimable for the treatment of male infertility and for developing efficient male contraceptives. Male infertility is a growing concern in the industrialized nations and defects in sperm motility appear to be one of the main underlying causes. In fact, this approach may also be used to identify novel therapeutic targets for other conditions, including neurodegenerative diseases (da Cruz e Silva and da Cruz e Silva, 2003; da Cruz e Silva *et al.*, 2004).

In conclusion, alternative splicing appears to have a significant dimension in functional genomics, possibly explaining the much lower number of human protein coding

genes that were revealed when the human genome was published than was previously estimated. Alternative splicing has thus acquired renewed importance as a mechanism for generating biological diversity, probably accounting for the observed discrepancy between the sizes of mammalian genome and proteome. For reasons that still need to be fully elucidated, alternatively spliced proteins predominate in mammalian testis. The identification of testis-specific variants of PP1 regulatory proteins may therefore constitute excellent candidates to develop new drugs to control testis-specific processes (including infertility/contraception), without affecting other PP1-dependent signal transduction pathways in other organs (Fig. V.8). This approach should be useful for designing rational intervention strategies both for the development of male contraceptives and for the treatment of male infertility.

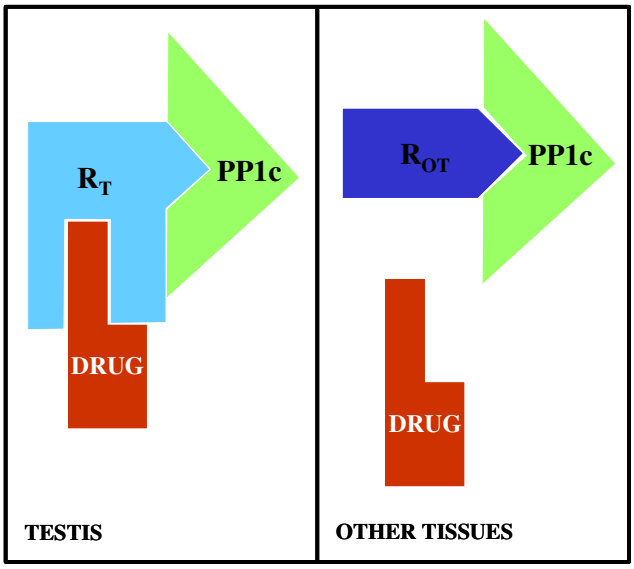


Figure V.8*: Signal transduction therapeutics for testis-specific processes based on the targeting of alternatively spliced protein domains expressed specifically in testis.* R_T*, testis-specific, alternatively spliced PP1 regulatory subunit;* R_{OT}*, alternative splice variant(s) of the regulatory protein expressed in other tissues.*

V.4 CONCLUDING REMARKS

Reversible protein phosphorylation is the major control mechanism of eukaryotic cells. Many diseases and dysfunctional states are associated with abnormal phosphorylation of key proteins (e.g. cancer, diabetes, Alzheimer's disease, etc.) (da Cruz

e Silva *et al.*, 2004). As a result, protein phosphorylation systems represent attractive targets for diagnostics and therapeutics. An increasing number of proteins have been identified in diverse cell types that regulate the catalytic activity of PP1. Indeed, the diversity of such PP1 regulatory subunits makes them attractive pharmacological targets. Besides, PP1 isoforms are highly mobile in cells and can dynamically relocalize through the direct interaction with targeting subunits (Trinkle-Mulcahy *et al.*, 2001). Clearly, a full understanding of the regulation of different cellular processes by PP1 requires the identification and characterization of the various PP1 regulatory proteins and holoenzyme complexes. The PP1γ gene is known to undergo alternative splicing to yield the ubiquitous PP1γ1 and the testis-specific PP1γ2 isoforms. Previous work has also shown that PP1γ2 is specifically expressed in sperm and that PP1 plays a key role in the control of sperm motility. Therefore, in order to identify the sperm proteins that interact with PP1 two separate yeast two-hybrid screens of a human testis library were performed using PP1γ1 and PP1γ2 as bait. Several new proteins were identified as PP1 regulatory subunits in human testis. These proteins represent potential specific targets to treat infertility and to develop new non-hormonal contraceptives. SEARP-T, is a new protein that interacts both with PP1γ1 and PP1γ2, and is a good candidate target to interfere with sperm motility. Mechanisms regulating binding of SEARP-T to PP1γ2 should be important in understanding the biochemical events that underlie sperm maturation and movement.

PP1γ1 and PP1γ2 interactomes described here constitute important tools for future studies, and represent a significant advance in our understanding of PP1 mediated signal transduction mechanisms.

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Appendix I - Culture media and solutions

- LB (Luria-Bertani) Medium

To 950ml of deionised H_2O add:

Bacto-tryptone 10g

Bacto-yeast extract 5g

NaCl 10g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5N NaOH. Adjust the volume of the solution to 1 liter with deionised H_2O. Sterilize by autoclaving.

- SOB Medium

To 950ml of deionised H_2O add:

Bacto-tryptone 20g

Bacto-yeast extract 5g

NaCl 0.5g

Shake until the solutes have dissolved. Add 10ml of a 250mM KCl (prepared by dissolving 1.86g of KCl in 100ml of deionised H_2O). Adjust the pH to 7.0 with 5N NaOH. Adjust the volume of the solution to 1 liter with deionised H_2O. Sterilize by autoclaving. Just prior to use add 5ml of a sterile solution of 2M $MgCl_2$ (prepared by dissolving 19g of $MgCl_2$ in 90ml of deionised H_2O; adjust the volume of the solution to 100ml with deionised H_2O and sterilize by autoclaving).

- SOC Medium

SOC is identical to SOB except that it contains 20mM glucose. After the SOB medium has been autoclaved, allow it to cool to 60ºC and add 20ml of a sterile 1M glucose (this solution is made by dissolving 18g of glucose in 90ml of deionised H_2O; after the sugar has dissolved, adjust the volume of the solution to 100ml with deionised H_2O and sterilize by filtration through a 0.22-micron filter).

- 50X TAE Buffer

242g Tris base

57.1ml glacial acetic acid

100ml 0.5M EDTA (pH 8.0)

- TE buffer (pH 7.5)

10mM Tris-HCl (pH 7.5)

1mM EDTA, pH 8.0

- Competent cell solutions:

Solution I (1L): 9.9g $MnCl_2.4H_2O$, 1.5g $CaCl_2.2H_2O$, 150g glycerol, 30ml KHAc 1M; adjust pH to 5.8 with HAc, filter through a 0.2µm filter and store at 4ºC.

Solution II (1L): 20ml 0.5M MOPS (pH 6.8), 1.2g RbCl, 11g $CaCl_2.2H_2O$, 150g glycerol; filter through a 0.2µm filter and store at 4ºC.

- RNAase stock solution

10mg/ml (ddH_2O)

SDS-PAGE Solutions

- Loading Gel Buffer

250mM Tris-HCl (pH 6.8)

8% SDS

40% Glycerol

2% 2-mercaptoethanol

0.01% Bromophenol blue

- LGB (Low Gel Buffer)

To 900ml of deionised H_2O add:

181.65g Tris

4g SDS

Shake until the solutes have dissolved. Adjust the pH to 8.9 and adjust the volume to 1L with deionised H_2O.

- UGB (Upper Gel Buffer)

To 900ml of deionised H_2O add:

75.69g Tris

Shake until the solute has dissolved. Adjust the pH to 6.8 and adjust the volume to 1L with deionised H_2O.

- 30%Acrylamide/0.8% Bisacrylamide

To 70ml of deionised H_2O add:

29.2g Acrylamide

0.8g Bisacrylamide

Shake until the solutes have dissolved. Adjust the volume to 100ml with deionised H_2O. Store at 4ºC.

- Coomassie blue staining solutions:

Staining solution

0.1% Coomassie Brilliant Blue R250

25% Methanol

10% Acetic Acid

Fixing solution

25% Methanol

10% Acetic Acid

-1X Running Buffer

25mM Tris-HCl (pH8.3)

250mM Glycine

0.1% SDS

-1X Transfer buffer

25mM Tris-HCl (pH8.3)

192mM Glycine

20% Methanol

-10X TBS

10mM Tris-HCl (pH 8.0)

150mM NaCl

-10X TBST

10mM Tris-HCl (pH 8.0)

150mM NaCl

0.05% Tween

- Stripping solution

100mM β-mercaptoethanol

2% SDS

62.5mM Tris-HCl (pH 6.7)

Yeast Two-Hybrid Solutions

- YPD medium

To 950ml of deionised H_2O add:

20g Difco peptone

10g Yeast extract

20g Agar (for plates only)

Shake until the solutes have dissolved. Adjust the volume to 1L with deionised H_2O and sterilize by autoclaving. Allow medium to cool to 60ºC and add glucose to 2% (50ml of a sterile 40% stock solution).

- SD synthetic medium

To 800ml of deionised H_2O add:

6.7g Yeast nitrogen base without amino acids (DIFCO)

20g Agar (for plates only)

Shake until the solutes have dissolved. Adjust the volume to 850ml with deionised H_2O and sterilize by autoclaving. Allow medium to cool to 60ºC and add glucose to 2% (50 ml of a sterile 40% stock solution) and 100ml of the appropriate 10X dropout solution .

- 10X dropout solution (DO)

This solution contains all but one or more of the following components:

	10X concentration (mg/L)	SIGMA #
L-Isoleucine	300	I-7383
L-Valine	1500	V-0500
L-Adenine hemisulfate salt	200	A-9126
L-Arginine HCl	200	A-5131
L-Histidine HCl monohydrate	200	H-9511
L-Leucine	1000	L-1512
L-Lysine HCl	300	L-1262
L-Methionine	200	M-9625
L-Phenylalanine	500	P-5030
L-Threonine	2000	T-8625
L-Tryptophan	200	T-0254
L-Tyrosine	300	T-3754
L-Uracil	200	U-0750

10X dropout supplements may be autoclaved and stored for up to 1 year.

- 2X YPDA

Prepare YPD as above. After the autoclaved medium has cooled to 55ºC add 15ml of a 0.2% adenine hemisulfate solution per liter of medium (final concentration is 0.003%).

- Solutions for preparation of yeast protein extracts

a) Protease inhibitor solution

Always prepare solution fresh just before using. Place on ice to prechill. To prepare 688µl add in a microfuge tube:

66µl Pepstatin A (1mg/ml stock solution in DMSO)

2µl Leupeptin (10.5mM stock solution)

500µl Benzamidine (200mM stock solution)

120µl Aprotinin (2.1mg/ml stock solution)

b) PMSF (phenylmethyl-sulfonyl fluoride) stock solution (100X)

Dissolve 0.1742g of PMSF (SIGMA) in 10ml isopropanol. Wrap tube in foil and store at RT.

c) Cracking buffer stock solution

To 80ml of deionised H_2O add:

48g Urea

5g SDS

4ml 1M Tris-HCl (pH6.8)

20µl 0.5M EDTA

40mg Bromophenol blue

Shake until the solutes have dissolved. Adjust the volume to 100ml with deionised H_2O.

d) Cracking buffer

To prepare 1.13ml add in a microfuge tube:

1ml Cracking buffer stock solution (recipe above)

10µl β-mercaptoethanol

70µl Protease inhibitor solution (recipe above)

50µl 100X PMSF stock solution

Solutions for yeast colony hybridization experiments

a) SCE solution

1M sorbitol

100mM sodium citrate

60mM EDTA

b) SCE/DTT/Lyticase

40ml SCE

300µl 2M DTT

5600 units Lyticase

c) 20X SSC

87.65g NaCl

44.1g Sodium citrate

Dissolve in 400ml dH_2O, adjust pH to 7.0 with NaOH, adjust the volume to 500ml and sterilize by autoclaving

d) 200mM Tris/2X SSC

200ml 1M Tris-HCl (pH7.5)

100ml 20X SSC

700ml dH_2O

e) 3X SSC/ 0.1%SDS/ 1mM EDTA

7.5ml 20XSSC

0.5g SDS

1ml EDTA 0.5M

Adjust the volume to 50ml with dH_2O

f) 60mM Tris-HCl (pH 7.8)/ 10mM $MgCl_2$ / 14mM 2-mercaptoethanol

3ml 1M Tris-HCl (pH7.8)

0.5ml 1M $MgCl_2$

48.5µl 2-mercaptoethanol

Adjust the volume to 50ml with dH_2O

g) Hybridization solution

0.009M Tris-HCl (pH7.4)

0.9M NaCl

6mM EDTA

0.5% Nonidet P40

2X Denhart's solution

0.2%SDS

0.05% sodium pyrophosphate

(70µg/ml) *E. coli* tRNA

(100µg/ml) *E. coli* DNA

h) 50X Denhart's solution

1% Ficoll 400

1% polyvinylpyrrolidone

1% BSA (Pentax fraction V)

Solutions for Northern Analysis

a) 5X formaldehyde gel-running buffer

0.1M MOPS (pH7.0)

40mM sodium acetate

5mM EDTA (pH8.0)

b) Formaldehyde gel-loading buffer

50% glycerol

1mM EDTA (pH8.0)

0.25% bromophenol blue

0.25% xylene cyanol FF

Solutions for the 2D gel electrophoresis

a) Lysis buffer

9M Urea

4% CHAPS

b) Equilibration buffer

50mM Tris-HCl (pH8.8)

6M Urea

30% Glycerol

2% SDS

0.002% Bromophenol blue

-Silver Staining

a) Fixing solution

40%Methanol

10%Acetic Acid

b) Sensitizing solution

30% Methanol

0.2% Sodium Thiossulfate

6.8% Sodium Acetate

c) Silver solution

2.5% $AgNO_3$

d) Developing solution

2.5% Sodium carbonate

0.04% Formalin

e) Stop solution

1.46% EDTA

Immnunoprecipitation Solutions

-Lysis buffer

50mM Tris-HCl (pH8.0)

120mM NaCl

4% CHAPS

0.1mg/ml Pepstatin A

0.03mM Leupeptin

145mM Benzamidine

0.37mg/ml Aprotinin

4.4mM PMSF in isopropanol

- 1X PBS

8mM Sodium Phosphate

2mM Potassium Phosphate

140mM NaCl

10mM KCl

Appendix II - Primers

PRIMER	SEQUENCE (5':::: 3')	Nt No.	MT (ºC)
GAL4 AD	TACCACTACAATGGATG	17	48
GAL4 BD	TCATCGGAAGAGAGTAG	17	50
OLIGONEK	CAGCCCTGTATTGAGTGAGC	20	62
OLIGONEK2	TCTTCTTAATTACTGGATTACTTG	24	62
OLIGONEK3	CTGAGGATGGAAGATTAAGAAG	22	62
OLIGONEK4	GAATACTTTCTTGGTTGGGC	20	58
OLIGONEK5	GGCTTCACGCTGCCCAGCTG	20	68
OLIGONIPP	GACTCCTGTTGTGCCGTCAG	20	64
OLIGONIPP2	TGGAGCATTGCCTCCCAAGC	20	64
OLIGO40Q3	GAGTCAGTGACATTCGGGAC	20	62
OLIGO40Q3/2	TCTGGCATGGAGCTGATATC	20	60
OLIGO40Q3/3	CCAGGTCATGTGGCTGCCTT	20	64
OLIGO40Q3/4	GTAGAATTGAGACACCTCCTG	21	62
40Q3P1	CGGAATTCGCCATGTGGAGGATAAACGG	28	86
40Q3P2	CGGAATTCGCCATGCCCGGGGTG	23	78
40Q3P3 (reverse)	CGGGATCCGCAGGTCAATTTAGTTA	25	74
Amplimer 3' (reverse)	ATCGTAGATACTGAAAAACCCCGCAAGTTCAC	32	84
Amplimer 5'	CTATTCGATGATGAAGATACCCCACCAAACCC	32	94
OLIGO4Q1	CTGAAGATCCTGGAGCCCGG	20	58
OLIGO4Q1/2	TTGACCACAGCCTGGTTTAC	20	60
OLIGO4Q1/3	GCACTCGTCTCTCTGGGAGG	20	66
OLIGO275.2	CCTGGAGAGGGCCGAATAA	19	60
OLIGO275.2/2	GACGTTCTTTCTGGAGGAAG	20	60

Appendix III - Bacteria and yeast strains

- E. coli XL1- blue: *recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F' proAB lacZΔM15 Tn10(Tet^r)]*

- S. cerevisiae AH109: MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal 80Δ, LYS2:: $GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3::$MEL1_{UAS}$-$MEL1_{TATA}$-lacZ, MEL1

- S. cerevisiae Y187: MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met-, gal 80Δ, URA3:: $GAL1_{UAS}$-$GAL1_{TATA}$-lacZ, MEL1

Appendix IV - Plasmids

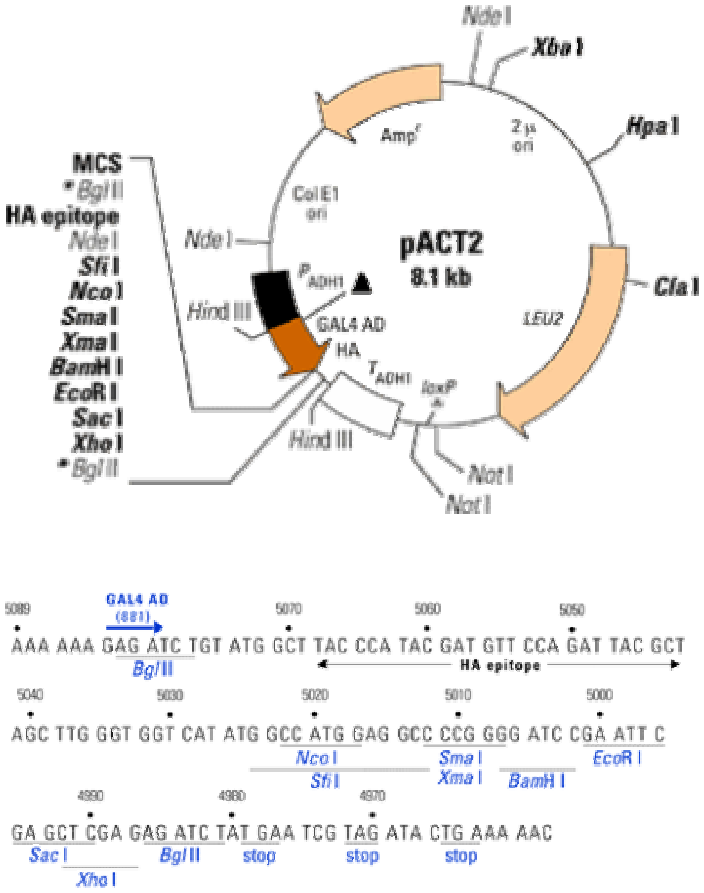

Figure 1: pACT2 (CLONTECH) map and MCS. Unique sites are coloured blue. pACT2 is used to generate a hybrid containing the GAL4 AD, an epitope tag and a protein encoded by a cDNA in a fusion library. The hybrid protein is expressed at medium levels in yeast host cells from an enhanced, truncated ADH1 promoter and is target to the nucleus by the SV40 T-antigen nuclear localization sequence. pACT2 contains the LEU gene for selection in Leu$^-$ auxotrophic yeast strains.

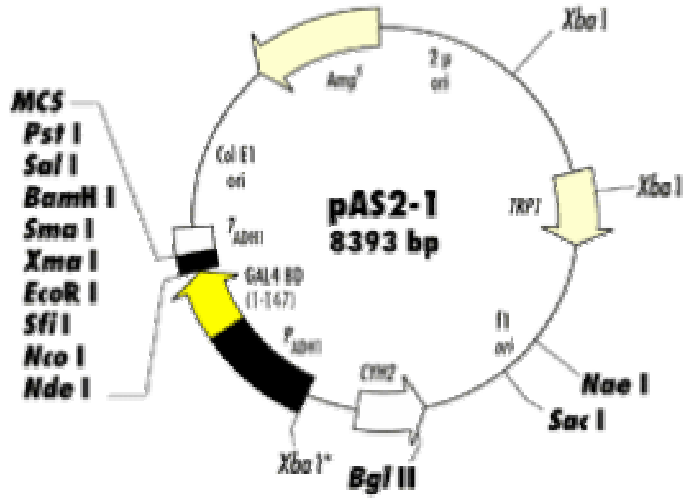

Figure 2: *pAS2-1* (CLONTECH) *map and MCS. Unique sites are coloured blue. pAS2-1 is a cloning vector used to generate fusions of a bait protein with the GAL4 DNA-BD. The hybrid protein is expressed at high levels in yeast host cells from the full-length ADH1 promoter. The hybrid protein is target to the yeast nucleus by nuclear localization sequences. pAS2-1 contains the TRP1 gene for selection in Trp$^-$ auxotrophic yeast strains.*

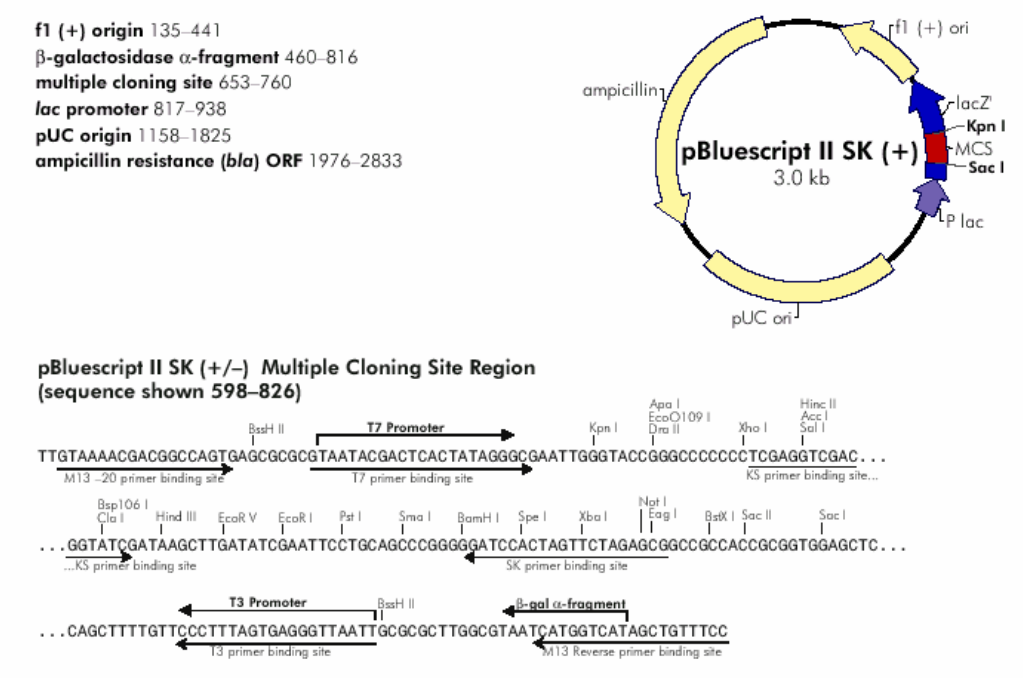

Figure 3: *pBluescript II SK map and MCS. This vector is a 2961 bp phagemid derived from pUC19. The SK designation indicates that the polylinker is oriented such that lacZ transcription proceeds from SacI to KpnI.*

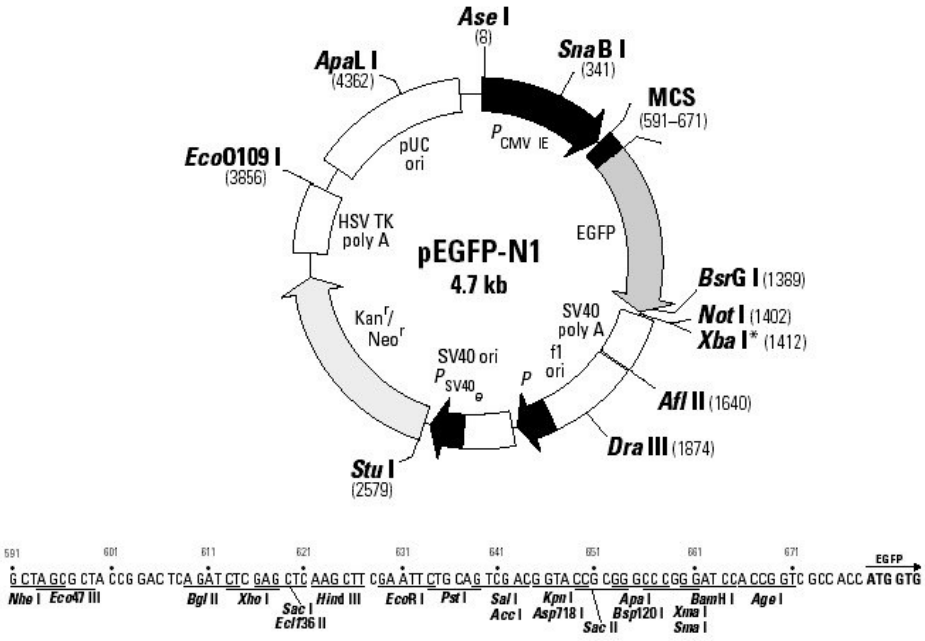

Figure 4: *pEGFP vector map (Clontech). This eukaryotic exppression vector was used to produce GFP fusion proteins*