Universidade de Aveiro Departamento de Biologia
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CHARACTERIZATION OF TWO NOVEL PROTEIN PHOSPHATASE 1 REGULATORS: I2L AND NEK2C

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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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agradecimentos

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palavras-chave I2L, Nek2C, ‘splicing’ alternativo, mobilidade dos espermatozóides.

resumo

A infertilidade masculina tem vindo a assumir proporções preocupantes na nossa sociedade. Há inúmeros factores que contribuem para esta condição, incluindo problemas associados à motilidade dos espermatozóides. As bases moleculares da motilidade dos espermatozóides ainda não foi completamente desvendada, contudo a incubação de espermatozóides imaturos imóveis com inibidores de proteínas fosfatases induz a sua motilidade. A fosforilação de proteínas é, então, fundamental na regulação da motilidade dos espermatozóides. A fosforilação de proteínas é um dos principais mecanismos reguladores de cascatas de transdução de sinais em organismos eucariotas. Os mecanismos dinâmicos e reversíveis de fosforilação/desfosforilação são catalizados pelas proteínas cinases e pelas proteínas fosfatases, respectivamente. A PP1, uma fosfatase específica para serina/treonina, está envolvida no controlo da motilidade dos espermatozóides, e noutras funções nos testículos. Nas células somáticas, a PP1 está envolvida em diversos mecanismos, que incluem o controlo do ciclo celular, a contracção muscular, a expressão de genes, a actividade neuronal e o metabolismo do glicogénio. A especificidade da função da PP1 depende das proteínas reguladoras que interagem com a sua subunidade catalítica, direccionando-a para um dado substrato ou para uma determinada localização subcelular, e/ou modificando a sua actividade em relação ao substratos. Actualmente conhecem-se mais de cinquenta subunidades reguladoras da PP1, não relacionadas bioquimicamente.

A diversidade da PP1 também se deve à expressão de várias isoformas: existem três genes que codificam a PP1 no genoma humano, denominados PP1α, PP1β e PP1γ. Maior complexidade advem ainda do ‘splicing’ alternativo que é conhecido para a PP1α e PP1γ. O gene da PP1γ origina uma variante ubíqua, a PP1γ1, e uma variante enriquecida em testículo, a PP1γ2, que é também a isoforma da PP1 mais abundante em espermatozóides, e que pode ser alvo de uma terapêutica para a infertilidade masculina ou contracepção. Rastreios de uma biblioteca de cDNA de testículo humano utilizando o sistema dois-híbrido de levedura foram realizados usando como iscos a PP1γ1 ou a PP1γ2. Várias novas proteínas que se ligam à PP1 foram identificadas e as respectivas interacções validadas usando uma diversidade de métodos, quer *in vivo* quer *in vitro* (utilizando PP1γ1 e PP1γ2 recombinantes produzidas em sistemas de expressão

bacterianos). Nas condições testadas, ambas as variantes recombinantes da PP1γ apresentaram propriedades enzimáticas semelhantes, o que confirma que a sua especificidade funcional é provavelmente adquirida pela ligação de proteínas reguladoras. Neste trabalho iremos focar em duas proteínas que ligam a PP1 e que foram recentemente identificadas no laboratório, denominadas I2L e Nek2C.

A I2L (Inhibitor 2-like) é >90% idêntica ao I2, tanto ao nível nucleotídico como ao nível da sequência de amino ácidos, e constitui uma isoforma nova do I2. Previamente tinha sido identificada como um pseudogene, no entanto apresentamos evidências que apoiam a sua expressão no testículo. A ausência da Thr-73 da I2L é muito significativa e pode ter consequências fisiológicas importantes. Esta ausência resulta numa inibição permanente da actividade da PP1γ2 pela I2L, promovendo potencialmente o desenvolvimento unidireccional da motilidade à medida que os espermtozóides viajam através do epidídimo. A Nek2C é uma nova variante de 'splicing' da Nek2A, diferenciando-se apenas pela ausência de 8 aminoácidos N-terminais à sequência consenso de ligação à PP1. Contudo a falta destes 8 aminoácidos tem consequências evidentes, resultando na expressão funcional de um NLS (sinal de localização nuclear). Deste modo, o mecanismo de 'splice' alternativo controla a translocação nuclear desta proteína cinase reguladora do ciclo celular, fornecendo um mecanismo pouco usual de modulação da localização da Nek2 e permitindo que a cinase desempenhe funções tanto nucleares como citoplamáticas. A PP1 encontra-se também em ambos os locais, regulando uma enorme variedade de processos celulares. No futuro espera-se que o presente trabalho possa contribuir para uma melhor compreensão dos eventos moleculares envolvidos no controlo da motilidade dos espermatozóides e na fertilização. Estas proteínas que interagem com a PP1 podem constituir alvos para interferir com diferentes funções específicas da PP1. Tanto a I2L como a Nek2C exibem características únicas, podendo constituir candidatos interessantes para futuros desenvolvimento no âmbito do diagnóstico e terapêutica baseadas na transdução de sinais para a contracepção e para o tratamento da infertilidade masculina.

keywords I2L, Nek2C, alternative splicing, sperm motility.

abstract Male infertility is a problem of growing concern in our society. Various factors contribute to this condition including motility defects in sperm. The molecular basis of sperm motility has not been fully elucidated, but non-motile immature sperm acquire motility when incubated with protein phosphatase inhibitors. Thus, protein phosphorylation is a key regulatory mechanism in sperm motility. In eukaryotic cells protein phosphorylation is a major general mechanism regulating signal transduction cascades. Consequently, protein kinases and protein phosphatases are central players in these reversible and dynamic processes. Among them, the serine/threonine-specific protein phosphatase 1 (PP1) appears to play a particularly important role in the control of sperm motility and in the testsis. In somatic cells, PP1 is known to be involved in many diverse processes, including cell cycle control, muscle contraction, gene expression, neuronal activity and glycogen metabolism. Functional specificity is provided by PP1 catalytic subunit interacting proteins, which bind the catalytic subunit and target it to a specific substrate or subcellular location, and/or modify its activity towards those substrates. To date more than 50 unrelated and biochemically diverse regulatory subunits have been described.
PP1 diversity can also arise from the expression of various isoforms: three PP1 genes occur in the human genome, termed PP1α, PP1β and PP1γ. Further complexity derives from alternative splicing events, known to occur at least for PP1α and PP1γ. The PP1γ gene produces a ubiquitously expressed PP1γ1 variant and the alternatively spliced PP1γ2 variant that is highly enriched in testis and is the main PP1 protein found in mammalian sperm. Therefore, it is the latter which has been implicated in the control of sperm motility. Hence, in this work we set out to identify and characterize PP1γ2 interactors expressed in human sperm, which could be targeted for male infertility therapeutics and contraception. Yeast two-hybrid screens of a human testis cDNA library were carried out using either PP1γ1 or PP1γ2 as bait. Several novel PP1 binding proteins were identified and interactions were validated using a variety of methods, both *in vivo* and *in vitro* (using purified recombinant PP1γ1 and PP1γ2 expressed in a bacterial expression system). Both recombinant PP1γ variants exhibited similar

Enzymatic profiles under the conditions tested, thus confirming that functional specificity is likely acquired by the binding of regulatory proteins. Here, we will address two recently identified novel PP1 binding proteins, namely I2L and Nek2C.

I2L (Inhibitor 2-Like) is >90% identical to I2, both at the nucleotide and amino acid levels, and constitutes a novel I2 isoform. Previously identified as a pseudogene, we present evidence supporting its testis-specific expression. The lack of Thr-73 from I2L is highly significant and may have important physiological consequences. The missing Thr-73 results in I2L being able to permanently inhibit PP1γ2 activity and potentially promote the unidirectional development of motility as sperm travel through the epidydimis. Nek2C is a novel splice variant of Nek2A, differing solely from the latter in missing 8 amino acids immediately N-terminal to the consensus PP1 binding motif. However, the lack of those 8 amino acids also appears to have marked consequences, resulting in the expression of a functional NLS (nuclear localization signal). Thus, alternative splicing controls nuclear translocation of this cell cycle regulated protein kinase, providing an unusual mechanism for modulating Nek2 localization and enabling it to undertake both nuclear and cytoplasmic functions. PP1 is known to occur in both locations, where it regulates many different cellular processes. In the future, it is hoped that the present work may contribute to a better understanding of the molecular events underlying sperm motility and fertilization. Clearly, such PP1 interacting proteins may provide interesting targets to interfere with specific PP1 functions. Thus, since both I2L and Nek2C exhibit unique characteristics, they may constitute interesting candidates for future developments in signal transduction diagnostics and therapeutics for contraception and the treatment of male infertility.

INDEX

PUBLICATIONS

This thesis contains experimental results included in the publications indicated below already published or under preparation. The author of this thesis declares that she participated in the planning and execution of the experimental work, as well as in data interpretation and in the preparation of the work for publication

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Wenjuan Wu, Ana Paula Vintém, Margarida Fardilha, Odete A. B. da Cruz e Silva and Edgar F. da Cruz e Silva (2007). I2L, a New Testis-Specific Inhibitor of Protein Phosphatase 1 (in preparation).

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Margarida Fardilha, **Wenjuan Wu**, Rosália Sá, Sara Fidalgo, Cristina Sousa, Catarina Mota, Odete A. B. da Cruz e Silva and Edgar F. da Cruz e Silva (2004). Alternatively spliced protein variants as potential therapeutic targets for male infertility and contraception. Ann N Y Acad Sci 1030, 468-478.

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ABBREVIATIONS

AATYK apoptosis-associated tyrosine kinase

AKAP A-kinaseanchoring anchoring protein

APC/C anaphase promoting complex/cyclosome

Bcl-2 B-cell lymphoma 2

BSA bovine serum albumin

BREK brain-enriched protein kinase

CA calyculin A

Cdk cyclin dependent kinase

CFTR cystic transmembrane conductance regulator

CIP calf intestinal phosphatase

CKII casein kinase 1

CKII casein kinase 2

C-Nap1 centrosome Nek associated protein 1

CPI-17 C-kinase-dependent phosphatase inhibitor of 17 kDa

Cprk cyclin-dependent kinase 5/p35-regulated kinase

DAPI 4,6-diamidino-2-phenylindole

DARPP-32 dopamine and cAMP-regulated protein of 32 kDa

D-box destruction box

DMSO dimethylsulfoxide

EDTA ethylenodiaminotetraacetic acid

Erk2 extracellular signal-regulated kinase 2

EST expressed sequence tag

FAK focal adhesion kinase.

G subunits glycogen targeting subunits, cGMP-dependent protein kinase substrate

Gac1 glycogen accumulation 1

GADDs growth arrest and DNA damage-inducible proteins

GIP1 Glc7-interacting protein 1

GIP2 Glc7-interacting protein 2

GL liver-type G subunite

Glc7 glycogen-deficient 7

GluR glutamate receptor

G_M the N-terminal domain of the muscle glycogen-targeting subunit

Grp78 glucose-regulated protein of 78 kDa，member of the HSP-70 family

GSK-3 glycogen synthase kinase-3

HCF host cell factor or human factor C1

HCG-V hemochromatosis candidate gene V

Hec highly expressed in cancer

His histidine

HMGA2 high mobility group protein A2

Hox11 homeodomain transcription factor

HSI2 human sperm protein phosphatase inhibitor-2

I-1 protein phosphatase inhibitor-1

I-1 ^{PP2A} (PHAP-I) inhibitor-1 of PP2A

I-2 ^{PP2A} (PHAP-I) inhibitor-2 of PP2A

I-2 protein phosphatase inhibitor-2

I-3 protein phosphatase inhibitor-3

I-4 protein phosphatase inhibitor-4

IP immunoprecipitation

IVT *in vitro* transcription

KEN-box protein degradation box, the amino acids sequence is KENIMRSEN

KEPI kinase-enhanced protein phosphatase type-1 inhibitor

KESTREL kinase substrate tracking and elucidation

KPI-2 kinase/phosphatase/inhibitor-2

LiAc lithium acetate

LTD long-term depression

LTP long-term potentiation

MAD mitotic arrest deficiency

MAPK mitogen-activivated protein kinase

MBS myosin binding subunit

MYPT myosin phosphatase targeting subunit

MT microtubule

MTOC microtubule organizing center

NCLK neuronal cdc2-like protein kinase

Nek2 NIMA-related protein kinase

NGF neuron growth factor

NIMA never in mitosis A

NKCC1 Na-K-Cl cotransporter 1

Nlp ninein-like protein

NIPP1 nuclear inhibitor of PP1

NMDA N-methyl-D-aspartate receptors

OA okadaic acid

ORF open reading frame

PBS phosphate-buffer saline

PCM pericentriolar matrix

PCR polymerase chains reaction

PEG polyethylene glycol

PFK Phosphofrutokinase

PHI phosphatase holoenzyme inhibitor

PKA protein kinase A

Plk1 pole-like protein kinase 1

PMSF phenyl methylsulfoxide

PRIP-1 phospholipase C-related inactive protein 1

PNUTS phosphatase 1 nuclear targeting subunit

PP1 protein phosphatase 1

PP1c catalytic subunit of PP1

PP2A protein phosphatase 2A

PP2B protein phosphatase 2B

PSF polypyrimidine tract-binding protein associated splicing factor

PTG protein targeting to glycogen

PTP protein tyrosine phosphatase

R subunit regulator subunit

Rb retinoblatoma protein

RIPP1 ribosomal inhibitor of PP1

RT-PCR reverse transcriptase - polymerase chain reaction

SARP Several ankyrin repeat protein

Scd5 suppressor of clathrin heavy-chain deficiency 5

Sds22 suppressor of the dis2 mutant; Sla1, synthetically lethal with ABP1

SDS sodium dodecyl sulfate

SDS-PAGE sodium dodecyl sulphate – polyacrylamide gel electrophoresis

Ser/Thr-PP serine threonine spicial protein phosphatase

SHP1 Scr homology domain 2 (SH2)-containing protein tyrosine phosphatase 1 (SHP-1)

TBS-T tris-buffered saline tween solution

Tris tryptophan

Ypi1 yeast phosphatase inhibitor 1

CHAPTER I:

INTRODUCTION

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.

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-PPs 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 apparently has not. In the past decade it has become apparent that functional diversity of the Ser/Thr-PPs 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.1.1 SER/THR-SPECIFIC PROTEIN 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 heat-stable inhibitors and that preferentially dephosphorylated the α-subunit of phosphorylase kinase (Cohen, 1989; Ingebritsen and Cohen, 1983). Type-2 phosphatases were further subdivided into cation independent (PP2A), Ca^{2+}-dependent (PP2B) and Mg^{2+}-dependent (PP2C) types. The use of okadaic acid, a specific phosphatase inhibitor, further facilitated the discrimination between the different classes (Cohen et al., 1989). Although widely in use, this classification does not reflect the currently known phylogenetic relationship between the different Ser/Thr-PPs. Molecular cloning revealed that PP2A was in fact much more related to PP1 than to PP2C (Berndt et al., 1987; Honkanen and Golden, 2002). 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., 1988b)] and PP2C in the unrelated family II or PPM (Fig. I.1). Here, only the PPP family will be further addressed.

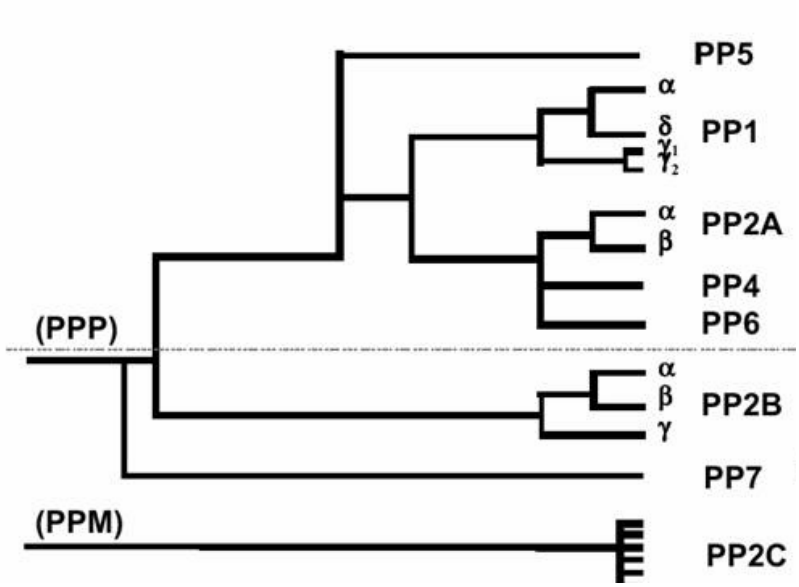

Figure I.1: Phylogenic tree depicting the evolutionary relationship between known phosphatases based on their primary amino acid sequence. PP1-PP7 belong to a single gene

family (PPP) that is structurally distinct and unrelated from the PP2C family (PPM). The phosphatases above the dashed line are highly sensitive to inhibition by naturally occurring toxins, such as okadaic acid, mycrocystin and calyculin A (Honkanen and Golden, 2002).

I.1.2 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; Cohen et al., 1988a; da Cruz e Silva and Cohen, 1987; da Cruz e Silva et al., 1987; da Cruz e Silva et al., 1988). Three genes are known to encode mammalian 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 $PP1\gamma_1$ isoform and a testis/sperm-enriched $PP1\gamma_2$ isoform (da Cruz e Silva et al., 1995b; da Cruz e Silva and Greengard, 1995). Two genes are known to encode mammalian PP2A catalytic subunits, termed PP2Aα and PP2Aβ, and the three known mammalian 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 previously unknown phosphatase catalytic subunit isoenzymes, that were termed novel phosphatases (da Cruz e Silva et al., 1988). For esample, 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* (Andreeva et al., 1998), and two *Drosophila* phosphatases appear to be exhibit tissue specific expression (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. An overall comparison of their structures is shown in Fig. I.2.

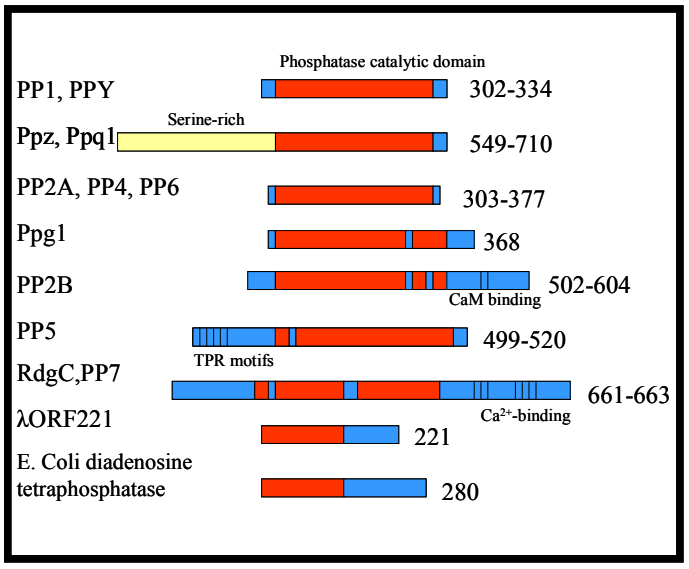

Figure I.2: Comparison of domain organization of PPP family members. The numbers of amino acids in each are indicated on the right.

I.1.3 PP1 - PROTEIN PHOSPHATASE 1

Eukaryotic genomes contain between one (*Saccharomyces cerevisiae*) and eight (*Arabidopsis thaliana*) genes that encode PP1 catalytic subunits. These isoenzymes typically show an overall sequence identity of approximately 90% and cannot be distinguished by either their *in vitro* substrate specificity or by their ability to interact with R subunits in vitro (Schillace and Scott, 1999; Zhang et al., 1993a). The sequence of the catalytic core of PP1 (corresponding to residues 41-269 of mammalian PP1α) 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).

I.1.3.1 PP1 Expression and distribution

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 in a distinct and characteristic manner within these cells. All the isoforms were present both in the cytoplasm and nucleus during interphase. Within the nucleus PP1α associates with the nuclear matrix, whereas PP1γ_1 concentrates in nucleoli in

association with RNA, and PP1β localizes to non-nucleolar chromatin. During mitosis PP1α is localized to 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 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).

Table I.1: Tissue distribution of the main PP1 isoforms (Fardilha 2004a)

	PP1α	**PP1γ1**	**PP1γ2**	**PP1β**
Brain	++	+++	-	+++
Heart	+	+		+
Liver	+	+		-
Intestine	+	+	-	+++
Kidney	+	+	-	+
Spleen	+	++	-	+
Adrenal gland	+	++	-	+
Lung	++	++		+++
Skeletal muscle	+	+		-
Testis	++	+	+++	++

-, below detection limit.

I.1.3.2 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 G, 1943). The discovery that this activity (PP1) was a phosphatase came at the same time as the discovery of phosphorylase kinase (Keller and Cori, 1955; Sutherland and Wosilait, 1955). These hallmark findings marked the beginning of an era: 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 (Bollen and Stalmans, 1992; Brautigan, 1994; Shenolikar, 1994; Shenolikar and Nairn, 1991), and progress in the isolation and characterization of PP1 activity was very slow. The study of the enzymology of this enzyme is still incomplete today and PP1 continues to provide many surprises, as well as stimulating new questions about its cellular functions.

I.1.3.2.1 PP1c crystal structure and catalytic mechanism

During the past decade and a half 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 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 of PP1 and PP2B are conserved [a review can be found in (Barford, 1996)], and that both contain a bimetal center at the active site which is structurally similar to that present in the purple acid phosphatase (Strater et al., 1995). PP1, like PP2B, is a metalloprotein possessing 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 cellular PP1 is unknown, although it can be speculated that it may be a Fe/Zn pair as in PP2B (King and Huang, 1984; Kissinger et al., 1995). The current views of the catalytic

mechanism 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. 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 H66, D64, D92 and H248 residues led to severe loss of catalytic function (Zhang et al., 1996a). Mutation of H125 and H173 did not result in readily expressed proteins. Although small amounts of the H125S and H125A mutant proteins could be isolated, these mutants were inactive. Mutation of D95, which is proposed to stabilize the protonation of H125 by a salt bridge, also resulted in significant reduction in catalytic activity. Mutations of R96 and N124 have also supported their proposed roles in phosphate binding.

I.1.3.2.2 Binding region for naturally occurring 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 okadaic acid (OA) (Shima et al., 1994). By comparing the amino acid sequence of PP1 and PP2A in this region (Fig. I.3), 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.

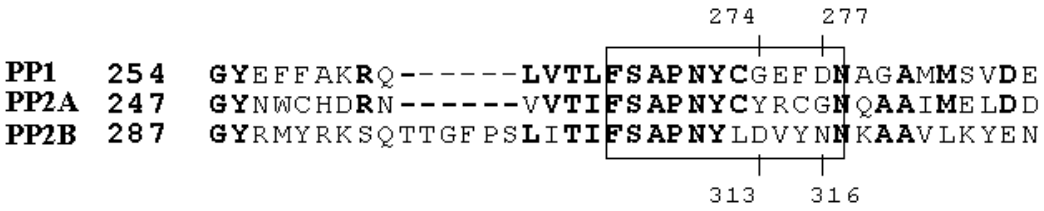

Figure I.3: Alignment of C-terminal regions of PP1, PP2A and PP2B. The boxed region shows the loop regions that connect beta sheets 12 and 13 in the structures of PP1 and PP2B.

The chimeric PP1 mutant in which GEFD was changed to YRCG resulted in increased sensitivity to OA (Zhang et al., 1994), consistent with the fact that PP2A is more sensitive to okadaic acid than PP1. The same occurred when F276 was mutated to Cys (Zhang et al., 1996a). 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 group within a few angstroms of 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.4).

If the toxins bind to the same site on PP1, this may reflect the possibility that these chemically 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. 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.

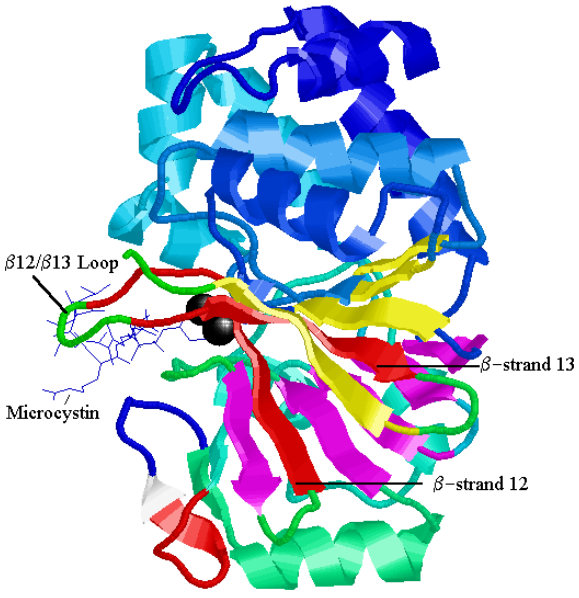

Figure I.4: The β12/β13 loop of PP1. The diagram shows a ribbon model of the PP1 structure. The two beta sheets that are the scaffold for the active site are shown in yellow (beta sheet 1) and magenta (beta sheet 2). Beta strands 12 and 13 are shown in red. Microcystin is shown in wireframe and the two metal ions as black spheres. (Goldberg et al., 1995).

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 (Fig. I.4). 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.

I.1.3.2.3 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 to 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 indicated that W206 and Y134 are well positioned to interact with the Ser or Thr carrying the phosphate residue (Egloff et al., 1995).

I.1.3.3 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 (Kuriyan and Cowburn, 1997; Lemmon and Schlessinger, 1994; Pawson, 1994), 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/heterotrimers 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 many 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 explains why the PP1 catalytic subunit exhibits a relatively nonspecific phosphatase activity *in vitro*. 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 PP1 with a R73C point mutation (Peng et al., 1990). This does 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 strain 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 identified and shown to be responsible for the involvement of PP1 in a number of diverse cellular functions [(Fardilha et al., 2004a).; 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 includes 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 binds 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 regulatory R subunits of PP1.

Regulatory subunit		General Function	Reference
I-1	PPP1R1A	PP1 inhibitors	(Huang and Glinsmann, 1976a)
DARPP-32	PPP1R1B		(Hemmings et al., 1984d)
I-2	PPP1R2		(Huang and Glinsmann, 1976a)
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)		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 signalling	(Schillace et al., 2001)

Yotiao		Synaptic transmission (NMDA receptor ion channel activity)	(Feliciello et al., 1999; Westphal 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 signalling?	(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)
SARP		?	(Browne et al., 2007)

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.

I.1.3.4 The consensus PP1 binding "RVxF motif"

Members of all 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]-(Agarwal-Mawal and Paudel, 2001){P}-[F/W] (Wakula et al., 2003). 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 highly homologous and structurally related phosphatases. Conversely, some proteins that bind PP1 but are also able to interact with the catalytic subunit of PP2A (HOX11, I-1^{PP2A} and I-2^{PP2A}) do not contain an RVxF sequence (Katayose et al., 2000; Kawabe et al., 1997).

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 (Beullens et al., 1999; Endo et al., 1996; Kwon et al., 1997). 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., 2000a). 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., 2000a). 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., 1997) 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 for 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 interaction, often mediated by 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; Liu et al., 2000; McAvoy et al., 1999). 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 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], where X denotes any residue and P any residue except proline (Wakula et al., 2003). This sequence is very similar to the consensus sequence previously proposed ([R/K]-[K/R]-X_{0-2}-V-[R/H]-[F/W]-X-[DE]) by panning of a random peptide display library (Zhao and Lee, 1997a). 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 thought to be 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 further 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 proposed, 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 various 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.1.4 PHOSPHATASE INHIBITORS

One of the most significant advances in the study of Ser/Thr-PPs, and for 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; Carmichael, 1994; Fujiki and Suganuma, 1993) and tautomycin (Hori et al., 1991; MacKintosh and Klumpp, 1990), to name a few. Another class of phosphatase inhibitors are the physiological protein inhibitors readily available inside the cell. The diverse specificity of the naturally occurring toxins for the different types of phosphatase has led to their use in the laboratory as key tools in the study of phosphorylation-dependent processes. However, below only the protein inhibitors are further addressed.

I.1.4.1 Inhibitor 1

Inhibitor 1 (I-1) and Inhibitor 2 (I-2) were originally identified by Huang and Glinsmann (Huang and Glinsmann, 1976b). 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). Differences between the rabbit, rat and human I-1 sequences were only detected in the C-terminus (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;

Elbrecht et al., 1990; 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., 1984c). 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 and has been used as a tool to study whether a process involves PP1. Amino acids ^{9}KIQF^{12} are conserved in rat, rabbit and human and seem to be crucial for binding and inhibition of PP1 (Egloff et al., 1997).

I.1.4.2 Inhibitor 2

I-2 from rabbit skeletal muscle comprises 204 amino acids and has a calculated mass of 22.9kDa (Holmes et al., 1986). Similarly to I-1, its apparent molecular mass on SDS-PAGE is much larger (31kDa). It binds to and inhibits PP1 regardless of its phosphorylation state. 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 (Zhang et al., 1996b). Interestingly, I-2 inhibition of PP1 can be reversed by GSK3 phosphorylation of I-2.

I.1.4.3 DARPP-32

DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein, Mr 32,000Da) resembles I-1 in function but is derived from a different gene. Further, DARPP-32 is expressed almost exclusively in the brain (Hemmings et al., 1984a), whereas I-1 exhibits a much more ubiquitous tissue distribution. It is a cytosolic protein and has a predicted molecular mass of 22.6kDa, but again a higher apparent molecular mass of 32kDa by SDS-PAGE (Williams et al., 1986). The same Thr residue on DARPP-32 is phosphorylated by cAMP-dependent protein kinase and 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, I-1 and DARPP-32 are dephosphorylated and inactivated by PP2A and even more efficiently by PP2B (Desdouits et al., 1995c; Hemmings et al., 1984b; Hemmings et al., 1990). The dephosphorylation by PP2B is dependent on the presence of calcium,

suggesting that this may represent a mechanism for Ca^{2+} levels to control I-1 and DARPP-32 phosphorylation and consequently PP1 activity (Hubbard and Cohen, 1989). Thiophosphorylated I-1 and DARPP-32 are relatively resistant to dephosphorylation and the corresponding synthetic peptides have been successfully used to study the physiological role of PP1-mediated processes.

I.1.4.4 Inhibitor 3

Inhibitor 3 (I-3) is another heat stable protein and potent inhibitor of PP1 that was more recently identified in a yeast two-hybrid screen (Zhang et al., 1998) It inhibits PP1 activity toward glycogen phosphorylase *a* with an IC_{50} in the nanomolar range, similar to the behavior of the other well-studied PP1 inhibitors: I-1, DARPP-32, and I-2. I-3 shares sequence homology with the yeast protein Yfr003c, also called Ypi1 (yeast phosphatase inhibitor 1). I-3 co-localizes with PP1γ1 in nucleoli and with PP1α in centrosomes (Huang et al., 2005a), possibly indicating that I-3 may act as modulator of PP1 functions in cytokinesis and nucleolar events.

I.1.4.5 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 some of the functionally important structures of I-2 and exhibits similar biochemical properties. I-4 inhibits the activity of the catalytic subunit of PP1 with an IC_{50} of 0.2nM, relatively more potent than I-2 (with an IC_{50} of approximately 2nM). Gel overlay experiments showed that I-4 binds PP1c directly through a multiple-point interaction.

I.1.5 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 low specificity inhibitors (e.g. calyculin A, microcystin or 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., 2000) have been employed to specifically suppress the expression of human PP5 and PP1γ1, respectively. More interesting, however, is data indicating 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 such regulatory proteins.

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 signalling pathways (Greengard et al., 1999). To date more than twenty primary signalling 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 and tissues. PP1 also appears to play a central role in the molecular mechanisms underlying the actions of several 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 Alzheimer's disease patients and in other neurodegenerative diseases.

I.2 PROTEIN PHOSPHATASE INHIBITOR-2

As mentioned above, two heat-stable phosphorylase phosphatase inhibitors were isolated from rabbit muscle (Huang and Glinsmann, 1976a). Inhibitor 1 (I1) was an active inhibitor only if in a phosphorylated form, while Inhibitor 2 (I2) did not need to be phosphorylated to inhibit PP1. The specificity of I2 led to its use for functionally defining type1 versus type 2 Ser/Thr protein phosphatases, since it is a potent inhibitor of the former but not the latter (Ingebritsen and Cohen, 1983). I2 is a highly charged protein of 204 amino acids with a predicted molecular mass of 22.9kDa, but an apparent molecular mass of 30-33kDa as determined by SDS-PAGE.

I.2.1 I2 AS A PHOSPHOPROTEIN AND MODULATOR OF PP1

The PP1-I2 complex was first identified as a Mg-ATP-dependent protein phosphatase activity (Hemmings et al., 1982). This inactive protein phosphatase needed to be preincubated with Mg-ATP and glycogen syntase kinase 3 (GSK-3) in order to exhibit biological activity (Goris et al., 1979; Hemmings, 1981; Hemmings et al., 1982). Activation results from the phosphorylation of I2 on a threonine residue and is accompanied by the time-dependent dissociation of the complex (Hemmings et al., 1982). Further studies indicated that GSK-3 phosphorylated I2 on Thr-72 and that casein kinase II (CKII) phosphorylated Ser-86, Ser-120, and Ser-121 (Aitken et al., 1984; Puntoni and Villa-Moruzzi, 1995). Interestingly, the *in vitro* phosphorylation of I2 Ser-86 by CKII enhances the phosphorylation of Thr-72 by GSK-3 (Park et al., 1994), which correlates with the synergistic activation of the ATP-Mg dependent protein phosphatase by GSK-3

and CKII (DePaoli-Roach, 1984). Comparing the two isoforms of GSK-3, it was found that GSK-3β was a significantly better I2 kinase than GSK-3α (Wang et al., 1994). I2 can also be phosphorylated by other kinases in biochemical assays, like cdc2-Cyclin B, ERK and CKI (Marin et al., 1994; Puntoni and Villa-Moruzzi, 1995; Wang et al., 1995). However, physiological regulation of Thr-72 phosphorylation in I2 is not yet clearly understood. Overexpression of GSK-3 and I2 in COS7 cells showed that the kinase could phosphorylate Thr-72 *in vivo*. In HeLa cells, phosphorylation of I2 on Thr-72 during mitosis was not carried out by GSK-3 but by a Cdk (cyclin dependent kinase), later shown to be cdk1/cyclinB1 (Leach et al., 2003). In neurons, a Cdk2-like protein kinase (Cdk5/p25) was shown to phosphorylate I2 on Thr-72 (Agarwal-Mawal and Paudel, 2001). On the other hand, PP1 activity in sperm maturation was correlated with changes in GSK-3 activity, supporting the PP1/I2/GSK-3 biochemical partnership (Vijayaraghavan et al., 1996). Moreover, the PP1/I2 complex was found to directly regulate GSK-3β in cultured mammalian cells (King et al., 2006). All these results suggest that I2 can be phosphorylated at Thr-72 by different kinases depending on the cellular environment. Interestingly, the highly species conserved I2 phosphorylatable motif containing Thr-72, can be phosphorylated by multiple Pro-directed kinases but with large differences in reactivity (Li et al., 2007). I2 was also reported to be phosphorylated on tyrosine residues by purified insulin receptor kinase *in vitro*, losing its inhibitory activity toward PP1 (Williams et al., 1995).

I2 may not only function as a PP1 inhibitor, but there is also experimental evidence indicating it may also function as a chaperone. That is, bacterial expressed recombinant PP1 protein exhibits some significant biochemical differences from native PP1 catalytic subunit, most notably recombinant PP1 is dependent on Mn^{2+} for activity. However, these differences were abolished after interaction of the expressed PP1 with I2 (Alessi et al., 1993; MacKintosh et al., 1996).

I.2.2 I2 INTERACTION WITH PP1

Different functional domains of I2 interact with distinct regions of PP1 (Park and DePaoli-Roach, 1994). Mutagenesis studies have shown that the N-terminal residues ^{10}IKGI^{13} are critical for I2 inhibition and that the C-terminal residues ^{144}KLHY^{147}

constitute a sequence analogous to the consensus RVxF PP1 binding binding motif (Huang et al., 1999; Yang et al., 2000b). Although the I2 structure is mostly random coil, it was possible to resolve the 3-D structure of the PP1-I2 complex showing three regions of order which corresponded to three sites of interaction (Hurley et al., 2007). The first and shortest region of interaction involves residues ^{12}KGILKN^{17} with hydrophobic associations and hydrogen bonds. The second interaction includes the I2 residues ^{44}KSQKWDEMNILAT^{56} of which ^{44}KSQKW^{48} bind to the RVxF groove of PP1. The previously identified ^{144}KLHY^{147} motif is included in the third stretch of interaction comprising residues 130-169. Residues 130-146 lie along the "acidic groove", to position amino acids 147-151 in the active site of PP1, while residues 152-169 exit the active site to the adjacent proposed "hydrophobic substrate-binding groove". Interestingly, the residues that interact with the catalytic center of PP1 displace essential metal ions, accounting for rapid inhibition and slower inactivation of PP1.

I.2.3 PHYSIOLOGICAL FUNCTION OF THE PP1-I2 COMPLEX: FORMATION OF TRIMERIC COMPLEXES WITH OTHER PP1 REGULATORY SUBUNITS

I.2.3.1 The cell cycle

Evidence suggests that I2 is involved in cell cycle regulation. I2 contains both a putative nuclear localization signal (^{137}KKRQFEMKRK^{147}) and a sequence resembling a leucine-rich nuclear export signal (^{155}LNIKLARQLI^{165}), and it can differentially localize to the cytoplasm or nucleus in different phases of the cell cycle of human HS68 fibroblast cells (Kakinoki et al., 1997). Starting from a low level, I2 expression increases during G1, peaking during S phase, dropping during G2 and then reaching its highest peak during mitosis (Brautigan et al., 1990; Kakinoki et al., 1997). During mitosis, I2 localizes to centrosomes and is highly phosphorylated at Thr-72 (Leach et al., 2003), suggesting that reversible phosphorylation of I2 may control centrosome-associated PP1 activity. Moreover, I2 is involved in the regulation of centrosome separation by forming a heterotrimeric complex with PP1 and Nek2 (Eto et al., 2002).

Aurora A kinase, that is necessary for centrosome maturation and is an oncogene, associates with and is negatively regulated by PP1. Thus, PP1 inhibition is potentially

involved in the activation of the kinase (Carmena and Earnshaw, 2003). However, I2 directly and specifically stimulated recombinant human Aurora-A activity *in vitro* (Satinover et al., 2004). The I2 stimulated increase in kinase activity was not simply due to inhibition of PP1, since it was not mimicked by other phosphatase inhibitors. Furthermore, activation of Aurora-A was unaffected by deletion of the I2 N-terminal PP1 binding motif but was eliminated by deletion of the I2 C-terminal domain. Aurora-A and I2 were recovered together from mitotic HeLa cells (Satinover et al., 2004) and it was further demonstrated that *Xenopus* extracts endogenous I2 and Aurora A regulate mitotic entry (Satinover et al., 2006).

Meiotic maturation of amphibian oocytes is induced by progesterone and regulated by protein phosphorylation. I2 caused over a 3-fold increase in the half-time for oocyte maturation, also implicating I2 in the control of meiosis by regulating PP1 activity (Foulkes and Maller, 1982). More recently it was suggested that I2 is one of the important endogenous regulators of PP1 during mouse oocyte development and meiosis (Wang et al., 2004).

I.2.3.2 Neuronal I2

The neuronal actin-binding proteins, Neurabin I and Neurabin II/Spinophilin are highly concentrated in dendritic spines and post-synaptic densities, being responsible for recruiting PP1 to the post-synaptic sites (Terry-Lorenzo et al., 2002). It was found that I2 could form a complex with the neurabins through PP1, which enhanced the targeting of PP1 and I2 to polymerized F-actin (Terry-Lorenzo et al., 2002). Immunocytochemistry in epithelial cells and cultured hippocampal neurons showed that endogenous neurabin II and I2 colocalized at actin-rich structures, consistent with the ability of neurabins to target the PP1-I2 complex to the actin cytoskeleton and regulate cell morphology.

NMDA (N-methyl-D-aspartate) receptors are critical for neuronal plasticity and survival, while their excessive activation produces excitotoxicity and may accelerate neurodegeneration. A recent study reported that stimulation of NMDA receptors in cultured rat hippocampal or cortical neurons, and in the adult mouse brain *in vivo,* activated GSK-3β which further stimulated PP1, through mediation of transient phosphorylation of I2 (Szatmari et al., 2005). In cortical neurons, inhibition of PP1 with

okadaic acid produced accumulation of phospho-I2, which could be blocked by LiCl, suggesting that GSK-3 phosphorylates I2 in intact neurons.

PP1 has also been involved in the hyperphosphorylation of tau in Alzheimer's disease. More particularly, it was found that oxidative stress promotes tau dephosphorylation via the complex PP1/cdk5/I2 (Zambrano et al., 2004).

As with Nek2 and Aurora, PP1 and I2 can form a trimeric complex with a transmembrane kinase termed KPI-2 (Kinase/Phosphatase/Inhibitor-2), also known as Lmr2, LMTK2, AATYK2, KIAA1079, Cprk, and BREK (Wang and Brautigan, 2002; Wang and Brautigan, 2006). The KPI-2 C-terminal domain associates with I2, with or without PP1, revealing that it can bind both proteins through different sites. The KPI-2 kinase domain could perform autophosphorylation and also phosphorylate PP1 at Thr-320, that leads to inhibition of PP1 activity. The substrate specificity for Ser/Thr residues was further demonstrated by using a microarray peptide analysis, which revealed two main protein substrates: cystic fibrosis transmembrane conductance regulator (CFTR) and glycogen phosphorylase (phos-*b*) (Wang and Brautigan, 2006). KPI-2 (as BREK) has been implicated in NGF signaling in PC12 cells, based on indirect evidence of cell shape changes in response to NGF after cells were stably transfected with active or inactive kinase (Kawa et al., 2004). The activity of KPI-2 was observed to decrease after adding NGF to living cells, consistent with the direct involvement of KPI-2 in the NGF signaling (Wang and Brautigan, 2006).

I.2.3.3 Cardiac contractility

In addition to the long known and relatively well characterized role for PP1 in muscle contraction, recent work also indicates it may also play an important role in cardiac contractibility (Carr et al., 2002). Transgenic mice overexpressing I2 in heart showed decreased PP1 activity and consequent enhanced contractility (Kirchhefer et al., 2005). Moreover, it was found that overactivated PP1 present in failing hearts could be due to inhibition of the activity of I1 and I2 (Gupta et al., 2005).

I.2.3.4 Spermatozoa maturation and motility

Mammalian sperm acquire the capacity for motility and fertilization during epididymal transit, which was associated with decreased GSK-3 and PP1γ2 activity (Vijayaraghavan et al., 1996). Heat stable I2-like activity was also detected in mammalian sperm, suggesting a biochemical mechanism for regulating sperm motility involving PP1/GSK-3/I2 (Smith et al., 2006; Vijayaraghavan et al., 1996).

Besides its ubiquitously expressed I2 family member, *Drosophila melanogaster* also expresses a testis-specific PP1 heat-stable inhibitor called inhibitor-t (Helps and Cohen, 1999; Helps et al., 1998). It was speculated that, as inhibitor-t is not the *D. melanogaster* homologue of mammalian I2, it may play an alternative role in the regulation of PP1c function in the testis. Further evidence for the existence of different testis-specific I2 isoforms was also found in rats (Osawa et al., 1996). $I2\alpha_1$ and $I2\alpha_2$ are alternatively spliced variants, while I2β is derived form a different gene. $I2\alpha_2$ and I2β are expressed exclusively in the testis and the expression of all three isoforms coincides with sperm cell maturation.

I.3 NIMA-RELATED KINASE 2 (NEK2)

Nek2 is a mammalian protein kinase structurally homologous to the yeast protein NIMA (never in mitosis A) and termed from NIMA-related kinase 2. It is a centrosomal serine/threonine kinase and is thought to modulate centrosome cohesion together with its substrate protein C-Nap 1 (centrosome Nek associated protein 1) and PP1 (Fry et al., 1999; Fry et al., 1998a; Uto et al., 1999). It is also involved in chromosome condensation and segregation, along with several related proteins found in mammalian cells. Until now, three alternative splice variants of Nek2 have been identified, which are Nek2A, Nek2B and Nek2C, originally called Nek2A-T (Fardilha et al., 2004b; Uto et al., 1999). These splice variants differ in their C-terminal exon 8 amino acid sequence (Fardilha et al., 2004b). Nek2A and Nek2B are expressed in different centrosome cycle phases (Fry et al., 2000a).

I.3.1 NEK2 IS THE HUMAN HOMOLOG OF NIMA

Nek2 kinase is the most closely related human gene to NIMA, being 44% identical over the amino acid sequence of the catalytic domain (Fry, 2002). The *in vitro* substrate specificity and many biochemical properties of Nek2 are similar to those of NIMA. Both Nek2 protein levels and activity are comparable to NIMA through the cell cycle (Fry et al., 1995). However, significant differences between Nek2 and NIMA have also been revealed. NIMA activity is low in G1 but increases though S and G2, to reach maximal levels in mitosis. The protein accumulates when cells are arrested in G2, but disappears as they progress through mitosis (Fry et al., 1995; Osmani et al., 1991b). However, Nek2 activity is high during S and G2 phases but low during M phase arrest (Fry et al., 1995). Unlike NIMA, Nek2 appears to have no function in the onset of centrosome entry into mitosis (Schultz et al., 1994) and contains no Cdc2 phosphorylation site motifs in the C-terminal region (Schultz et al., 1994).

I.3.2 NEK2 STRUCTURE

Glycerol gradient assays indicate that endogenous Nek2 is present in HeLa cells as a salt-resistant 6S complex, the predicted size of a Nek2 homodimer (Fig. I.6). Additionally, recombinant Nek2 overexpressed in insect cells also formed a 6 S complex. Nek2 can form dimers both *in vivo* and *in vitro*, as shown by yeast two-hybrid interaction analysis and co-immunoprecipitation assays. Dimerization specifically requires the leucine zipper motif and allows Nek2 trans-autophosphorylation. Deletion of the leucine zipper prevents the trans-autophosphorylation reaction on the C-terminal domain of Nek2 and strongly reduces Nek2 kinase activity on exogenous substrates (Fry et al., 1999)

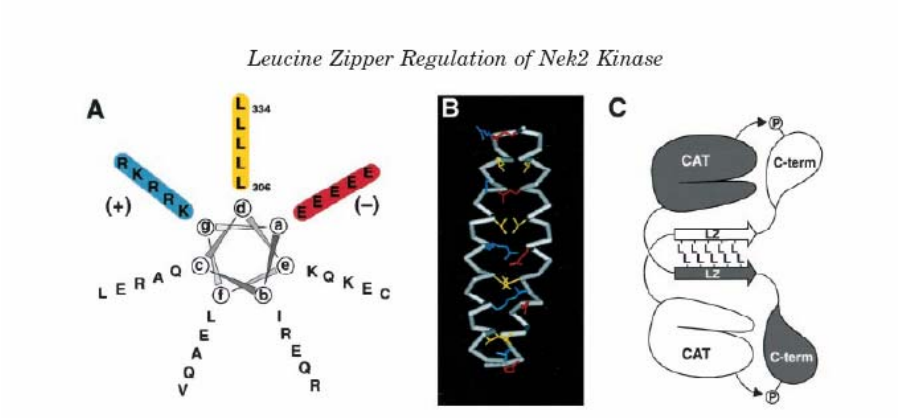

Figure I.6: Nek2 dimerization occurs through an unusual leucine zipper motif. A) helical wheel representation of amino acids 306–340 of the Nek2 protein showing the regular heptad repeat of leucine residues at position d flanked by a basic arm of residues at position g and by an acidic glutamate arm of residues at position a. B) a hypothetical wire diagram model of a Nek2 leucine zipper dimer The individual α-helices are right-handed and run parallel, and the coil of the helices is left-handed. Leucine residues in position d of each monomer are indicated in yellow, whereas the acidic glutamate residues in position α of one monomer are in red, and the basic lysine or arginine residues in position g of the other monomer are in blue. The symmetrical pair of glutamate and basic residues on the back side of the molecule is omitted for clarity. C) schematic model illustrating the interaction of two full-length Nek2 molecules is shown. Dimerization is shown to occur between parallel leucine zippers (LZ) such that it allows the C-terminal domain (C-term) of one molecule to be phosphorylated by the catalytic domain (CAT) of the second molecule. P, phosphate. (taken from (Fry et al., 1999)

I.3.3 NEK2 IS A CENTROSOME KINASE

The centrosome is the major microtubule (MT) organizing center (MTOC) in animal cells. It is composed of two cylindrical centrioles surrounded by a non-membranous amorphous mass termed the pericentriolar matrix (PCM). It influences all MT-dependent processes, including organelle transport, cell shape, polarity and motility, and plays an important role in the assembly and function of the bipolar mitotic spindle in mitosis, influencing the reliability of chromosome segregation. Furthermore, the centrosome anchors MT which acts as tracks for molecular motor based transport and positioning of vesicles and organelles in interphase. The centrosome cycle has been subdivided into a series of discrete events (Fig. I.7). Among them, centrosome cohesion plays a critical role in centrosome separation and is regulated by the balance of kinase and phosphatase activities and by proteolysis (Hames et al., 2001).

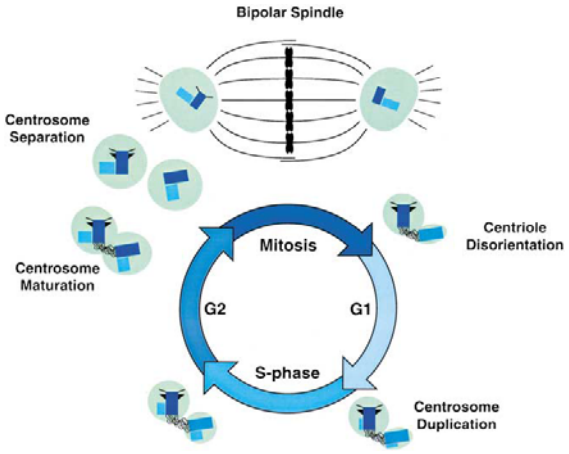

Figure I.7: Schematic view of the centrosome cycle in relation to the cell cycle. Mature centrioles are shown in dark blue, immature centrioles in blue, pro-centrioles in light blue and PCM in green. (taken from (Meraldi and Nigg, 2002).

Nek2 plays an important role in the regulation of centrosome separation. It was observed by both immunofluorescence microscopy and subcellular fractionation that Nek2 is a centrosome kinase (Fry et al., 1998b). Endogenous human Nek2 kinase is highly enriched at the MTOC and is associated with the centrosome throughout the cell cycle (Fry et al., 1998a; Fry et al., 1998b), including all stages of mitosis. After transfecting myc-Nek2 into cells, the exogenous Nek2 can be seen in centrosomes at low expression levels and also in the cytoplasm and nucleus at high expression levels. Ectopic expression of Nek2 affected centrosome structure by causing centrosome splitting or dispersion, or by leading to spindle pole defects, consistent with a loss of centrosome cohesion (Fry et al., 1998b). Similar effects could also be observed using antibodies against other centrosomal components. Transfection of a catalytically inactive Nek2 mutant (Nek2A-K37R) also caused dispersion of centrosome material, but did not trigger any centrosome splitting. This demonstrates that centrosome splitting depends on kinase activity. Nek2 activity is an earlier step in the process of centrosome separation that precedes the action of MTs and protein motors (Mayor et al., 1999). From either perspective, the experiments emphasize the importance of Nek2A as a critical scaffold element of the centrosome (Faragher and Fry, 2003).

The centrosome localization of Nek2 results in its interaction with *bona fide* components of the centrosome. PCM-1 is a major component of centriolar satellites and there is a partial overlap of PCM-1 and Nek2 localization in the cytoplasm. PCM-1 and proteasomal degradation also play important roles in the assembly and turnover of Nek2 at the centrosome (Hames et al., 2005). Down-regulation of PCM-1 using siRNA proved that

the recruitment of Nek2 and its substrate C-Nap1 to the centrosome was dependent on PCM-1. PCM-1 is a major component of centriolar satellites, 70-100nm cytoplasmic granules that are found in the vicinity of the centrosome although not at the centrosome itself (Dammermann and Merdes, 2002; Kubo et al., 1999). Trafficking of certain proteins to the centrosome is known to be dependent on the PCM-1 protein, and recruitment of Nek2 to the centrosome also depends on its localized degradation (Hames et al., 2005). APC/C is a key regulator of mitotic destruction, catalyzing the covalent attachment of ubiquitin molecules to a substrate and thereby targeting it for degradation by the multiple peptidase activities of ATPase-dependent 26S proteasome (Hershko and Ciechanover, 1998). The substrates for APC/C are not all targeted for destruction at the same time, but in a sequential mode. What regulates this sequential destruction of proteins by the APC/C is still not clear (Hames et al., 2001). Two kinases, Cdc20 and Cdh1, have been related to the activation of APC/C by phosphorylation of APC subunits. Nek2A itself is destroyed upon entry into mitosis, in a process mediated by the proteasome and is dependent on the APC/C-Cdc20 ubiquitin ligase. Nek2 activity is not required for APC/C catabolism instead of Nek2 degradation. But Nek2 destruction depends upon a cyclin A-like destruction box (D-box) motif or KEN-box in its C-terminus. Human Nek2A contains putative versions of both motifs: a KEN-box (KENIMRSEN) at amino acids 391-399 and a poorly defined extended D-box consisting of the C-terminal 25 amino acids (residues 420-445). Further work has indicated that the Nek2A MR-tail (444-445) binds directly to the APC/C and interferes with APC/C activity (Hayes et al., 2006). Truncation of the Nek2A C-terminus delays its degradation until late mitosis. D-box and KEN-box motifs in Nek2A are targeted by APC/C-Cdc20 in early mitosis, whereas the KEN-box is targeted by APC/C-Cdh1 in mitosis and G1 (Hames et al., 2001; Pfleger and Kirschner, 2000). This degradation may somehow be necessary to allow re-establishment of the intercentriolar linkage in late mitosis (Hames et al., 2001).

The MT cytoskeleton undergoes intracellular framework during cells dynamic actions. Besides localizing to the centrosome, Nek2A is present in small particles distributed throughout the cytoplasm, which partially localize with MT. These Nek2A particles were disseminated along the MT, upon which Nek2 trafficks towards or backwards the centrosomes. *In vitro* MT binding assays indicated that Nek2 binds MT through a motif in its C-terminal domain (amino acids 335-370) and this MT binding motif

is also important for Nek2 centrosome localization. It is hypothesized that Nek2 may be anchored to the centrioles via its microtubule binding domain (Hames et al., 2005). Nek2 was also proposed to have a function in organization of the microtubule (Rapley J, 2005). It is a primer kinase for the Plk1 substrate Nlp (ninein-like protein), which is a microtubule minus end binding protein. After being first phosphorylated by Nek2, Nlp can be further phosphorylated by Plk1 and removed from the centrosome at the G2/M transition.

Protein phosphorylation constitutes a major mechanism regulating centrosome cohesion during the cell cycle. PP1α was reported to have a marked localization at centrosomes during mitosis (Andreassen et al., 1998). A balance between Nek2 and the interacting PP1α is particularly critical. The immunoprecipitation of endogenous Nek2A with a Nek2A specific antibody and subsequent detection of PP1 activity in the cell lysate immunoprecipitate indicated that both Nek2A and PP1 form a complex *in vivo* (Helps et al., 2000). The amino acids sequence KVHF, corresponding to the consensus RVxF PP1 binding motif, is responsible for the binding of Nek2A to PP1. *In vivo* co-expression of PP1α with Nek2A strongly suppressed centrosome splitting and this effect was specific for Nek2A. These results indicated that PP1α and Nek2 regulate centrosome cohesion by counteracting each other (Meraldi and Nigg, 2001). Co-expression of PP1 also antagonizes Nek2A function via dephosphorylation of Nek2A, itself and the Nek2A substrate C-Nap1, indicating that PP1 acts as a negative regulator for centrosome separation (Helps et al., 2000; Meraldi and Nigg, 2001). On the other hand, phosphorylation of PP1c by Nek2A reduces the phosphatase activity of the complex (Helps et al., 2000). The kinase-phosphatase complex is expected to function as a bistable switch, potentially creating a double-negative feedback circuit for regulation of centrosome dynamics (Ferrell J.E., 2002).

The protein C-Nap1, isolated through a two-hybrid screen using Nek2A as bait, has been identified as a Nek2A substrate (Fry et al., 1998a). The C-terminal domain of C-Nap1 could be readily phosphorylated by Nek2A both *in vitro* and *in vivo* (Fry et al., 1998a). It has a predicted molecular mass of 281kDa and comprises extended domains of coiled-coil structure. It was found concentrated at the centrosome in all interphase cells, with its immunoreactivity being found at mitotic spindle poles. In agreement with a role in the maintenance of centrosome cohesion after its duplication in S phase, C-Nap1 associates with the proximal ends of both mother and daughter centrioles, but dissociates from these

structures at the onset of mitosis. It has been suggested that C-Nap1 participates in providing a link that hold the old centrioles together. After centrosome duplication, this linkage is dissolved upon phosphorylation of C-Nap1 by Nek2A, which in turn, triggers the observed loss of C-Nap1 from centrosomes to allow the separation of the duplicated centrosomes before mitosis. Further work points to C-Nap1 as a key component of centrosome dynamics, including centriole-centriole cohesion (Mayor et al., 2000).

Nek2A was further observed to form a complex with the catalytic subunit of PP1 and the large coiled-coil C-Nap1 protein (Helps et al., 2000). The kinase-phosphatase complex (Nek2A-PP1) can influence the phosphorylation state of C-Nap1, which has implications in the regulation of centrosome separation and cell cycle progression. C-Nap1 phosphorylated by Nek2A could be dephosphorylated by the endogenous Nek2A-PP1 complex (Fry et al., 1998a). Nek2A is also able to phosphorylate itself, PP1 and C-Nap1, whereas PP1 can dephosphorylate both Nek2A and C-Nap1 (Faragher and Fry, 2003; Fry et al., 1999; Fry et al., 1998a), and can autodephosphorylate after being phosphorylated by Nek2.

One model for Nek2A regulation of cohesion between mother and daughter centrosome involves phosphorylation of C-Nap1 at G2/M transition. This phosphorylated C-Nap1 may trigger its displacement from centrioles, promoting their separation and subsequent bipolar spindle formation (Fig. I.8) (Faragher and Fry, 2003).

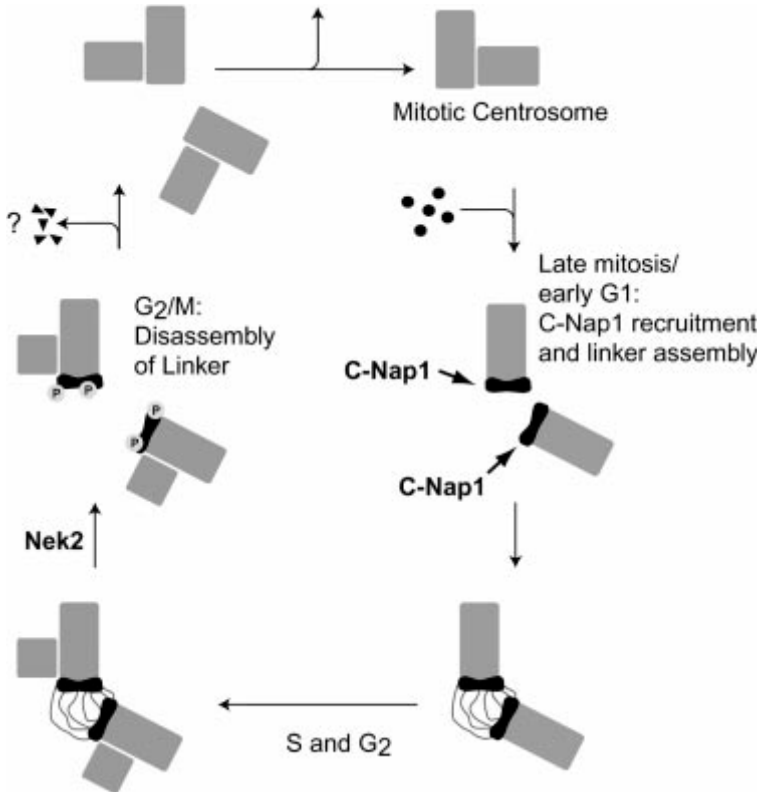

Figure I.8: Cell cycle-regulated centrosome association of C-Nap1. This schematic model summarizes current information about the function and precise subcellular localization of C-Nap1. (taken from (Mayor et al., 2000).

PP1 interacts with Nek2 and can dephosphorylate both Nek2 and C-Nap1 (Fry et al., 1998b). Phosphorylation may regulate C-Nap1 function, and the phosphorylation state of C-Nap1 may depend on the balance between Nek2 and PP1 activity. In support of such a model, centrosome splitting can be induced by overexpression of active Nek2 (Fry et al., 1998b), as well as by drug-induced inhibition of PP1 (Meraldi and Nigg, 2001).

C-Nap1 has a tendency to aggregate when expressed in cultured cells (Mayor et al., 2002). These aggregates are reduced in the presence of increased levels of active Nek2A protein kinase, but only around the centrosomal region suggesting that C-Nap1 aggregation is locally regulated by Nek2A. The mitotic C-Nap1 signal is reduced as a consequence of M-phase-specific phosphorylation, and the dissociation of C-Nap1 from mitotic centrosomes is regulated by localized phosphorylation rather than generalized proteolysis. As C-Nap1 is an *in vivo* substrate of Nek2A, Nek2A activity may thus be responsible for the dissociation of C-Nap1 from mitotic spindle poles (Mayor et al., 2002). Using inducible stable cell lines expressing moderate levels of active Nek2, these authors have

shown that only half of the C-Nap1 protein remains at the centrosome after its separation. This suggests that total removal of C-Nap1 from centrosome might be regulated by other mechanisms and/or that Nek2A might phosphorylate other centrosome proteins to trigger centrosome separation (Faragher and Fry, 2003).

Several lines of evidence suggest an involvement of I2 in cell cycle regulation. The expression levels of I2 fluctuate during the cell cycle and are enhanced at mitosis (Brautigan et al., 1990). I2 is a regulator of the Nek2A-PP1 complex and of centrosome separation in cells (Eto et al., 2002). In a yeast two-hybrid system, I2 was seen to interact with PP1c and Nek2A. Indeed, I2, PP1 and Nek2A form a trimeric complex *in vitro* (Eto et al., 2002). Endogenous I2 was seen to occur concentrated into a cloud in the perinuclear region, and centrosomes were embedded within this cloud of I2 (Eto et al., 2002). Co-expression of PP1 with Nek2A suppresses centriole splitting, whereas ectopic expression of I2 stimulates splitting (Fry et al., 2000b; Meraldi and Nigg, 2001). I2 can enhance the kinase activity of the Nek2-PP1c complex via inhibition of the phosphatase activity, in order to initiate centrosome separation (Eto et al., 2002). Hence, I2 is a regulator of the Nek2-PP1c complex and centrosome separation in cells, creating new potential links for coordination of cell cycle signals. Using yeast conjugation and other assays, Eto et al. (2002) have demonstrated that the I2 interaction with the Nek2 C-terminus requires the bindings of both PP1c and the C-terminal domain of I2. I2 is capable to interact with PP1c on Nek2. Interaction between I2 and Nek2 requires PP1c, further supporting the notion that PP1c forms a bridge binding to both proteins at the same time (Agostino et al., 2002).

I.3.4 NEK2 AND CHROMOSOMES

The mitogen-activated protein kinase (MAPK) pathway is known to be required for chromosome condensation and it triggers Nek2A activation at the same time (Agostino et al., 2002). Extracellular signal-regulated kinase 2 (Erk2) is a serine/threonine kinase which belongs to MAPK kinase family (Boulton et al., 1991; Tanoue et al., 2000). Nek2A and Erk2 co-localize in the centrosomes and can form a complex in the centrosomes of mitotic cells via the Nek2A C-terminal ^{414}KKR^{416} sequence (a typical docking motif for Erk2) (Tanoue et al., 2000). The centrosome localization of Nek2A is independent of Erk2 kinase activity, whereas the centrosomal localization of Erk2 depends on Nek2A, suggesting that Nek2A links extracellular signaling cascades to the centrosome via Erk2 (Lou et al.,

2004a). Nevertheless, further studies are required to evaluate precisely the significance of the interaction between Nek2A and Erk2 in the centrosome.

High mobility group protein A2 (HMGA2) belongs to the HMG superfamily which is associated with DNA (Giancotti et al., 1991; Postnikov and Bustin, 1999a). It is an abundant component of chromatin that modulates DNA conformation and gene expression (Bustin, 1999; Di Agostino et al., 2004b; Herrera et al., 1999; Postnikov and Bustin, 1999b). HMGA2 is highly expressed in male meiotic cells (Di Agostino et al., 2004b) and directly and specifically interacts with Nek2A, after which it is activated by the MAPK pathway in mouse pachytene spermatocytes. This interaction requires the C-terminal regulatory region of the kinase, but it does not account for the activity of Nek2A and seems consititutive. Nek2A directly phosphorylates HMGA2 *in vitro*, and decreases the affinity of HMGA2 for DNA (Di Agostino et al., 2004b; Fardilha et al., 2004b).

The protein encoded by the human gene Hec (Highly expressed in cancer) contains 642 amino acids and a long series of leucine heptad repeats at its C-terminus . It is most abundantly expressed in the S and M phases of rapidly dividing cells, but not in terminally differentiated cells. Hec localizes to the nuclei of interphase cells and a portion distributes to centromeres during M phase. It apparently plays an important role in chromosome segregation during M phase (Chen et al., 2002). Nek2 was first found to interact with Hec1 in a yeast two-hybrid screen using Hec1 as bait (Chen et al., 2002; Zheng et al., 1999). Human Hec1 is a serine phosphoprotein that binds specifically to the mitotic regulatory kinase Nek2A during G2/M. Nek2A phosphorylates Hec1 on the serine residue 165, both *in vitro* and *in vivo*, with a peak of activity during G2/M phase (Chen et al., 2002). Cell cycle-regulated serine phosphorylation of Hec1 by Nek2A is critical and essential for faithful chromosome segregation and cell survival (Chen et al., 2002). The *in vitro* binding of Hec1 to Nek2A was established using GST pull-down assays. Using Hec1 deletion constructs and yeast two-hybrid assays, it was found that Hec1 interacts with Nek2A via the first (aa 251-431) or second (aa 361-547) coiled-coil domain (Chen et al., 2002). Co-immunoprecipitation of Nek2A and Hec1 occurred specifically during G2 and M phases. The initiation of Hec1 phosphorylation corresponded to the same time period during which Nek2 was most abundant, suggesting that Nek2 may phosphorylate Hec1 *in vivo* during G2/M phase (Lou et al., 2004b). All these results indicated an involvement of Nek2 in the chromosome condensation and segregation process.

MAD (mitotic arrest deficiency) proteins are spindle checkpoint components transiently associated with the kinetochore (Lou et al., 2004b). The immunoprecipitation of FLAG-MAD1 and GFP-Nek2A in co-transfected 293T cells first indicated an interaction between MAD1 and Nek2A. Interaction of Nek2A with MAD1 further demonstrated that Nek2A plays a critical role in kinetochore dynamics, in addition to its function in centrosome regulation. This is in accordance with the observation that knock-down of Nek2A with siRNA leads to a typical chromosome segregation defect. Full-length Nek2A does not bind MAD1, but binds with MAD2. Mutated Nek2A induced inability to assemble MAD2 onto kinetochores or leads to insufficient assembly of MAD2 to kinetochores. Hence, Nek2A is a kinetochore-associated protein kinase essential for faithful chromosome segregation, and it was hypothesized that Nek2A links MAD2 molecular dynamics to spindle checkpoint signaling. However, it remains unclear whether MAD2 is a substrate or not for Nek2 (Lou et al., 2004b).

All these results implicate Nek2 kinase in the control of chromosome condensation, conformation and separation.

I.3.5 ALTERNATIVELY SPLICED VARIANTS OF NEK2

Nek2A and Nek2B were the first alternatively spliced variants to be identified and are known to be expressed in vertebrates (Fry et al., 2000a; Hames and Fry, 2002; Twomey et al., 2004; Uto et al., 1999; Uto and Sagata, 2000). Both Nek2A and Nek2B localize to centrosomes (Hames and Fry, 2002). These two variants differ in that the C-terminal 384-445 amino acids are absent from Nek2B, resulting from an alternative exon 8 splice event. In human adult somatic cells, Nek2A is the predominant isoform although Nek2B is also present (Hames and Fry, 2002).

Nek2A is required for separation of duplicated centrosomes and formation of a bipolar mitotic spindle (Faragher and Fry, 2003). A number of important regulatory motifs have been identified within the Nek2A molecule. It has a N-terminal catalytic domain and a C-terminal regulatory domain within which are found a leucine zipper dimerization motif, a PP1 binding motif and two degradation motifs recognized by the anaphase promoting complex/cyclosome (APC/C) (Fig. I.9) (Fry, 2002).

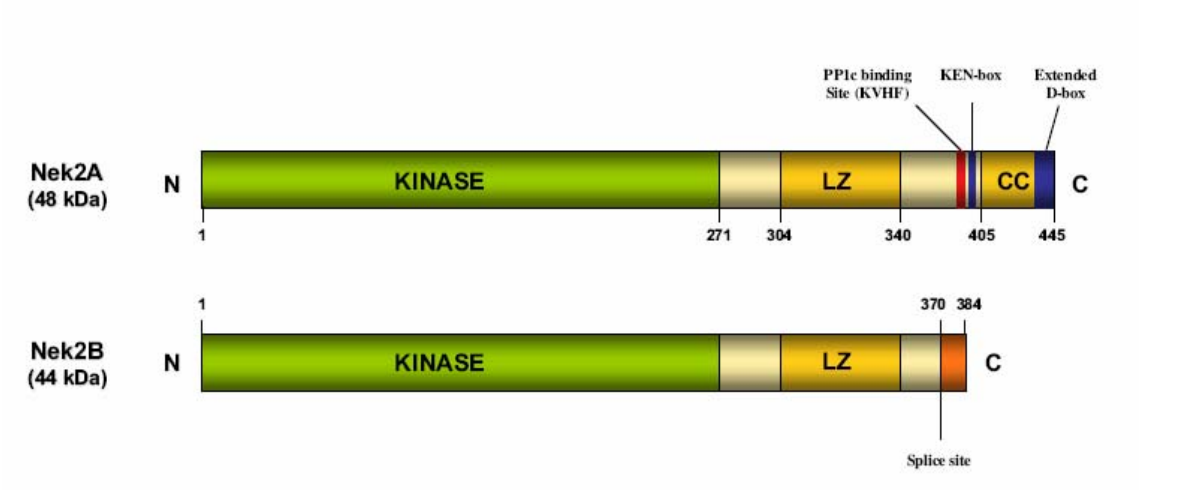

Figure I.9: Human Nek2 protein structure. A schematic representation of human Nek2A and Nek2B is shown. The relative positions of the catalytic domain (kinase), leucine zipper (LZ), coiled coil (CC), splice site, PP1 binding motif, KEN-box and extended cyclin A-type destruction box are indicated. Numbers above and below the structures indicate amino acid positions. (taken from (Fry, 2002).

Nek2B is the only isoform present and required for the assembly and maintenance of centrosomes in *Xenopus* early embryos (Fry et al., 2000a; Uto and Sagata, 2000). It is rapidly recruited to sperm basal bodies in a microtubule (MT)-independent manner during formation of the zygotic centrosome (Twomey et al., 2004). Fragmentation of centrosomes was observed following either injection of anti-Nek2 antibodies or expression of a dominant-negative variant of the XINek2 kinase in *Xenopus* embryos (Uto and Sagata, 2000). Nek2B contains the same kinase domain and leucine zipper motif as Nek2A, but lacks the PP1 binding site and degradation signals (Hames and Fry, 2002). Immunodepletion of *Xenopus* Nek2B from cell-free extracts led to a failure of centrosome components to be recruited to sperm centrioles (Fry et al., 2000a).

I.4 AIMS

The aim of this work was to express, purify and characterize two recently discovered new regulators of PP1, obtained by yeast two-hybrid screening of a human testis cDNA library, and to study how these new interactors regulate the activity of PP1γ1 and PP1γ2 (the main PP1 isoforms expressed in human testis and sperm) both *in vitro* and *in vivo*.

Some of the specific aims included the following:

1. To express recombinant PP1γ1 and PP1γ2 isoforms in bacteria both with and without a His-tag, and to optimize their purification.
2. To express and purify the new PP1 regulators Nek2C (or Nek2A-T) and I2-L.
3. To investigate the properties of I2-L and Nek2C.
4. To compare I2-L and Nek2C with I2 and Nek2A in terms of their putative physiological roles as regulators of endogenous PP1γ1 and PP1γ2 in mammalian sperm and testis.
5. To address the importance of these new regulators of PP1 in the control of sperm motility.

CHAPTER II

EXPRESSION AND PURIFICATION OF PROTEIN PHOSPHATASE 1 GAMMA ISOFORMS

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II EXPRESSION AND PURIFICATION OF PROTEIN PHOSPHATASE 1 GAMMA ISOFORMS

II.1 INTRODUCTION

The serine/threonine-specific protein phosphatase-1 (PP1) is a major eukaryotic protein phosphatase that regulates a diverse range of cellular processes, such as cell cycle progression, protein synthesis, muscle contraction, carbohydrate metabolism, transcription, neuronal signaling and the splicing of RNA (Aggen et al., 2000; Llorian et al., 2005; Shenolikar, 1994; Shenolikar and Nairn, 1991; Wera and Hemmings, 1995). PP1 regulates cellular processes by dephosphorylation of specific serine and threonine residues in key target proteins (Bollen and Stalmans, 1992; Cohen, 1989). The pleiotropic functions of PP1 depend on its association with a host of function-specific and substrate-specific targeting and regulatory proteins. The catalytic subunit of PP1 (PP1c) associates with many different regulatory subunits *in vivo* that may target PP1c to specific subcellular locations, alter its substrate specificity and allow its activity to be modified by extracellular signals (Helps et al., 2000).

Three genes are known to encode mammalian type 1 phosphatase catalytic subunits, termed PP1α, PP1β and PP1γ (da Cruz e Silva et al., 1995a; da Cruz e Silva et al., 1995b; Strack et al., 1999). PP1γ_1 and PP1γ_2 are alternatively spliced isoforms generated from the PP1γ gene (Sasaki et al., 1990; Wera and Hemmings, 1995), that are identical except for their extreme C-terminus. It is interesting that the unique 21 amino acid carboxy terminus of PP1γ2 is conserved in all mammalian species (Mishra et al., 2003). In contrast to PP1γ1 that is ubiquitously expressed, although PP1γ2 was found to be present in the brain (Strack et al., 1999), it is most highly expressed in germ cells, being the predominant PP1 isoform in testis and sperm (Smith et al., 1996; Strack et al., 1999).

Recombinant PP1 has been reported to be expressed and purified from a variety of systems, including *E. coli* and insect cells (Berndt and Cohen, 1990; Watanabe et al., 2003; Zhang et al., 1992; Zhang et al., 1993). PP1α was first expressed in insect cells (Berndt and Cohen, 1990), where 5% was soluble and represented a 15-fold increase over the endogenous activity. The remaining 95% recombinant insoluble PP1α was fully reactivated by denaturation with 6M guanidinium chloride followed by extensive and rapid dilution

with buffers containing Mn^{2+} (Berndt and Cohen, 1990). Recombinant PP1α, PP1γ1 and PP1γ2 were also expressed in *E. coli* (Zhang et al., 1992; Zhang et al., 1993). The enzymatic properties of such recombinant proteins were examined in terms of substrate specificity and sensitivity to OA (okadaic acid) and I2 (inhibitor-2). Furthermore, expression of recombinant His-tagged PP1α and PP1γ1 was also reported in Sf9 insect cells and *E. coli*-DH5α cells. *E. coli* expressed PP1α exhibits high specific activity, comparable to native PP1c (usually prepared from rabbit skeletal muscle as a mixture of isoforms) (Watanabe et al., 2003; Zhang et al., 1992; Zhang et al., 1993). The *E. coli* expressed enzyme is also normal in terms of inhibition by a variety of well characterized inhibitors, including microcystin, calyculin A (CA) and OA. However, recombinant *E. coli* PP1α exhibits several anomalous properties in comparison to the non-recombinant enzyme. These include the fact that it is dependent on Mn^{2+} for activity and that it is able to dephosphorylate phospho-tyrosine-containing substrates and paranitrophenyl phosphate. Recombinant *E. coli* PP1α is also relatively insensitive to inhibition by phospho-inhibitor-1 and phospho-DARPP-32 (Watanabe et al., 2003). It is notable that recombinant *E. coli* PP1α also exhibits a marked ability to auto-dephosphorylate phospho-Thr320 when incubated with cdc2 kinase, in contrast to native or recombinant Sf9 expressed PP1c (Watanabe et al., 2003). In contrast, recombinant *E. coli* PP1α appears to be inhibited normally by full length inhibitor-2 (Watanabe et al., 2003). Recombinant Sf9 expressed PP1α and PP1γ1 also exhibited high specific activities under standard assay conditions (Watanabe et al., 2003). The two enzyme preparations produced in Sf9 insect cells were also normal in terms of inhibition by microcystin, CA and OA. They did not require Mn^{2+} for activity and, similarly to native PP1c, addition of 1 mM Mn^{2+} inhibited their activities. Notably, Sf9 expressed PP1α and PP1γ1 did not dephosphorylate phospho-DARPP-32 or phospho-inhibitor-1 (Watanabe et al., 2003).

In this work methods were developed to express in *E. coli* and to purify the two alternatively spliced variants known to be produced from the human PP1γ gene (PP1γ1 and PP1γ2). To facilitate the purification process, a poly-histidine tag was added to the N-terminus of the recombinant proteins. Furthermore, the purified recombinant proteins were compared by a variety of methods.

II.2 MATERIAL AND METHODS

II.2.1 PLASMIDS CONSTRUCTION

To generate the expression plasmid pTAC-His-PP1γ2, the primers "His-PP1 gamma I" and "PP1 gamma2 T" were used for PCR amplification using pAS-PP1γ2 (Fardilha, 2004) as the template. The "His-PP1 gamma I" oligonucleotide (5'-GGAATTCATATGCATCATCATCATCATCACATGGCGGATTTAGAT-3') was designed to contain a *Nde* I endonuclease restriction site at the initiating methionine codon and 6 histidine codons. The "PP1 gamma2 T" primer (5'-GCTCTAGACTGCAGGTCGACGGATC-3') introduced a *Xba* I endonuclease restriction site following The stop codon. The His-tagged PP1γ2 DNA fragment amplified by PCR was detected by agarose gel electrophoresis, and inserted into the pBlueScript vector (pKS-His-PP1γ2), where its sequence was fully confirmed. It was then recovered from pKS-His-PP1γ2 and inserted into *Nde* I and *Xba* I cut pTACTAC vector to yield pTAC-His-PP1γ2, whose sequence was also fully verified by automatic DNA sequencing.

To construct the pKS-His-PP1γ1 plasmid, a C-terminal fragment was excised from pKS-His-PP1γ2 with *Pst* I and *Hind* III restriction enzymes and replaced with the corresponding *Pst* I-*Hind* III fragment from pKS-PP1γ1 (da Cruz e Silva and Greengard, 1995). pKS-His-PP1γ1 was digested with *Nde* I and *Hind* III and the insert recovered and then also subcloned into pTACTAC cleaved with the same restriction enzymes to yield pTAC-His-PP1γ1, whose sequence was fully verified by DNA sequencing.

II.2.2 IMMUNOBLOT SCREENING OF RECOMBINANT PROTEINS

After transformation into *E. coli* strain DH5α, single clones were grown in 3 ml of LB medium plus 100 μg/ml ampicillin and 1 mM Mn^{2+} (LB/Amp/Mn^{2+}) at 37ºC overnight with shaking. Overnight cultures (30 μl) were transferred into 3 ml of new LB/Amp/Mn^{2+} and grown at 37ºC with vigorous shaking to on A_{600} of 0.6. IPTG was then added to 0.5 mM final concentration to induce recombinant protein expression. After 3 hours of induction, the cells were collected by centrifuging at 14,000 rpm for 1 min. Cultured cells (1 ml) were resuspended in 100 μl of 1 % SDS. Samples (10 μl) were loaded onto SDS-

PAGE gels after adding sample buffer. Electrophoresis was performed at 100 V for 2 hours. Proteins were transfered to nitrocellulose membrane, and the expression of recombinant proteins was analysed by immunobloting with appropriate antibodies.

II.2.3 MEDIAL AND LARGE SCALE EXPRESSION OF RECOMBINANT PROTEINS

A single clone was taken from a fresh plate and grown in 10 ml LB/Amp/ Mn^{2+} medium at 37ºC overnight with shaking at 250 rpm. 500 µl or 5 ml of the overnight culture were transferred into 50 ml or 500 ml respectively of LB/Amp/Mn^{2+} medium and grown at 37ºC with shaking to the A_{600} of 0.6. IPTG was then added to 0.5 mM final concentration and incubation was continued at 26ºC for 15 hours to induce expression of the recombinant protein.

II.2.4 PURIFICATION OF THE TWO ISOFORMS OF HIS-TAGGED PP1 GAMMA UNDER DIFFERENT CONDITIONS

For recovery of the induced recombinant His-tagged PP1γ isoforms, the bacterial suspension was centrifuged at 4,000 rpm for 20 min and the cell pellet was resuspended in lysis buffer. Different concentrations of imidazole (5 mM, 10 mM and 30 mM) lysis buffer were tried in a pH 8.0 buffer system (10 mM Tris-HCl，300 mM NaCl，1 mM $MnCl_2$，10% Glycerol). After binding the His-tagged PP1γ isoforms with the resin separately, the resin was washed with 30 mM imidazole (pH 8.0) buffer and the recombinant protein was eluted with 500 mM imidazole (pH 8.0) elution buffer.

Different pH buffer systems (10 mM Tris-HCl, 300 mM NaCl, 30 mM imidazole, 1 mM $MnCl_2$, 10% Glycerol) were also used to purify the His-tagged PP1γ isoforms. In these cases, the cells were lysed with 30 mM imidazole (pH 5.0, pH 6.0 or pH 7.0) buffer, respectively. After binding the His-tagged PP1γ isoforms to the resin, it was washed with the same buffer used for cell lysis. Then the protein was eluted with 500 mM imidazole buffer of the same pH as was used for the lysis and wash buffers.

II.2.5 THE PROTEIN PHOPHATASE ACTIVITY ASSAY

II.2.5.1 Preparation of 32P-labelled phosphorylase a

20 mg Phosphorylase *b* (Sigma) was phosphorylated with 16 µg phosphorylase kinase (Sigma) in 1.6 ml reaction buffer containing 31 mM ATP (Sigma), and [γ-^{32}P] ATP (Amersham) (500 µCi). A final concentration of 100 nM microcystin was added to inhibit endogenous protein phosphatases. The reaction mixture (Table II.1) was incubated at 30ºC for 2 hours, then 1.6 ml of 90 % saturated ammonium sulfate was added (to 45 % saturation), kept on ice for 30 min and centrifuged at 19,000×g for 15 min at 4ºC. The pellet was resuspended in 0.4 ml of Buffer C [50 mM Tris-HCl (pH 7.0), 0.1% (v/v) 2-mercaptoethanol] and 0.4 ml of 90 % saturated ammonium sulfate was added. The mixture was incubated on ice for another 30 min and centrifuged at 19,000×g for 15 min at 4ºC. The pellet was resuspended in 0.4 ml Buffer C and dialyzed against 4×1 L of Buffer D [10 mM Tris-HCl (pH 7.0), 0.1% (v/v) 2-mercaptoethanol] for 24 hours at 4ºC. Precipitated phosphorylase *a* was taken from the dialysis tube and left at 4ºC overnight. After centrifuging at 19,000×g for 15 min at 4ºC, the precipitated phosphorylase *a* was resuspended in 0.5 ml of Buffer E [50 mM Tris-HCl (pH 7.0)].

Table II.1: Reaction mixture for Preparation of ^{32}P-labelled phosphorylase a

Component	Volume (µl)
Phosphorylase kinase (20 mg/ml)	16
Magnesium acetate (100mM)	32
$CaCl_2$ (100 mM)	2
Microcystin (50 µM)	3.2
Phosphorylase *b* (100mg/ml)	200
Buffer B*	846.8
[γ-^{32}P] ATP (250 µCi – 3000 Ci/mmol)	500
Total	1600

*125 mM Na-2-glycerophosphate (Sigma) pH 8.5

II.2.5.2 Assay of protein phosphatase1 activity

The prepared ^{32}P-labelled phosphorylase *a* was diluted with 0.1 ml of caffeine and 0.4 ml of Buffer C to a final concentration of 30 μM. Recombinant PP1 isoforms were diluted in Buffer A [50 mM Na-2-glycerophosphate (pH 7.5), 10% (v/v) glycerol, 0.1 mM EGTA, 0.2% (v/v) 2-mercaptoethanol] and 10 μl of diluted PP1 was mixed with 10 μl of Buffer B [125 mM Na-2-glycerophosphate (pH 8.5)]. The blank control consisted of 10 μl of Buffer A mixed with 10 μl of Buffer B. After incubating the mixtures at 30ºC for 2 min, 10 μl of ^{32}P-labelled phosphorylase *a* was added. After 10 min, the reaction was stopped by the addition of 100 μl of 20 % TCA. The mixture was centrifuged at 15,000×g for 2 min at room temperature and 100 μl of the supernatant was transferred to another microtube. The ^{32}P released from the substrate was quantified using a Scintillation Counter, and all samples were assayed in duplicate three times.

Unit Definition - 1 unit of PP1 activity releases 1 μmol phosphate from phosphorylase *a* per minute in a standard assay.

Therefore the 10 min assay used

$$\text{c.p.m. released} = \text{SAMPLE c.p.m.} - \text{Blank c.p.m.} \qquad (1)$$

$$\text{Activity (mU/mL)} = \frac{c.p.m.released}{c.p.m.TOTAL} \times \frac{0.3}{10} \times 100 \times \frac{130}{100} \qquad (2)$$

The number 0.3 refers to the number of nanomoles of phosphorylase in the assay, 10 is the incubation time in minutes, 100 is the factor necessary to convert the results for 1 ml rather than 10 μl of enzyme, and 130/100 corrects for the fraction of the TCA supernatant that is counted. All calculations made use of Microsoft Excel.

II.2.6 ASSAY OF PROTEIN PHOSPHATASE 1 ACTIVITY INHIBITED BY I2

I2 was diluted in Buffer B to yield a range of different concentrations. 10 µl of each I2 dilution solution were mixed with 10 µl of His-tagged PP1γ isoform, diluted in buffer A, separately. A control was prepared by mixing 10 µl of Buffer B with 10 µl of the corresponding His-tagged PP1γ isoform diluted in buffer A. The blank was prepared by mixing 10 µl of buffer A with 10 µl of buffer B. These mixtures were then incubated at 30ºC for 15 min and 10 µl of ^{32}P-labelled phosphorylase *a* substrate was added. The reaction was stopped after 10 min by adding 100 µl of 20 % TCA. The mixture was centrifuged at 15,000×g for 2 min at room temperature and 100 µl of the supernatant was transferred to another microtube. The ^{32}P released from the substrate was quantified and the phosphatase activity calculated as described above. All samples were assayed in duplicate three times.

II.3 RESULTS

II.3.1 CONSTRUCTION OF RECOMBINANT PLASMIDS FOR EXPRESSION OF HIS-TAGGED PP1 GAMMA 1 AND PP1 GAMMA 2

PCR amplification of the pAS-PP1γ2 template using "His-PP1 gamma I" and "PP1 gamma2" primers yielded a fragment of the expected size (Fig. II.1):

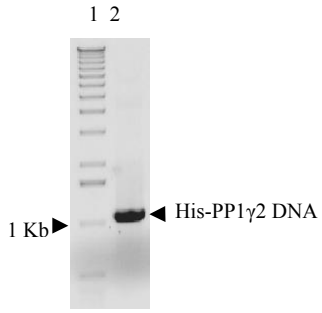

Figure II.1: Agarose gel analysis of the PCR amplification of His-tag PP1γ2. Lane1. 1 Kb DNA molecular weight. Lane2. PCR amplified His-tag PP1γ2 cDNA fragment with the expected molecular weight of 1.1 kb.

This DNA fragment was then inserted into pBlueScript to yield pKS-His-PP1γ2, and the construct was completely verified by automatic DNA sequencing. The *Nde* I/*Xba* I fragment containing the His-PP1γ2 sequence was then transferred into pTACTAC to yield pTAC-His-PP1γ2. The recombinant expression plasmid was screened by restriction analysis with *Nde* I and *Xba* I. After transforming *E. coli* strain DH5α, several clones were analyzed by inducing small scale expression. Both SDS-PAGE (Fig. II.2A) and immunoblot (Fig. II.2B) confirmed His-PP1γ2 expression:

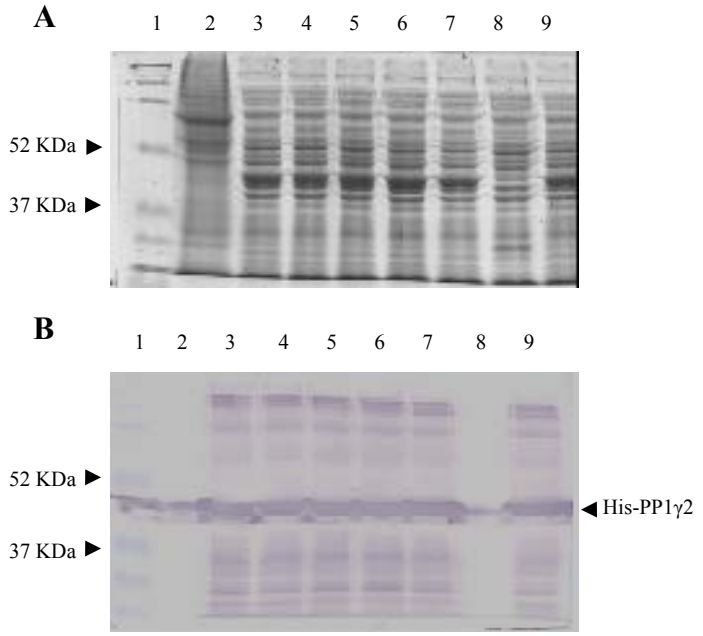

Figure II.2: Small scale expression for detection of His-tagged PP1γ2 **A**. Coomassie blue stained. SDS-PAGE gel; **B**. Immunoblot with Anti-PP1γ2 antibody. Lane 1: Protein Molecular Weight Marker; Lane 2: Positive control of rat testis; Lane 3-9: Bacterial clones 1-7.

The Coomassie blue stained SDS-PAGE gel showed a strong band of approximately 40 KDa, except for lane 8 (Fig. II.2A), that was absent from the non-induced and non-transformed controls (not shown). The corresponding immunoblot with a specific anti-PP1γ2 antibody (Fig. II.2B) confirmed that the expression of the 40 KDa protein likely corresponded to the expression of His-tagged PP1γ2. These results confirmed that the pTAC-His-PP1γ2 recombinant expression plasmid was constructed and that this plasmid can express the recombinant His-PP1γ2.

The correct construction of the recombinant plasmid pKS-His-PP1γ1 was also confirmed by sequencing, as previously described. After transferring the insert into the pTACTAC vector, the resulting construct was screened with the restriction enzymes *Nde* I and *Hind* III (Fig. II.3):

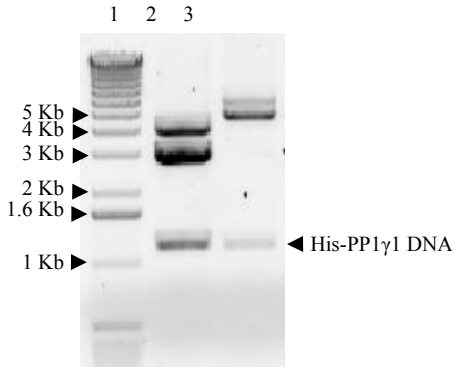

Figure II.3: Restriction enzyme analysis of pKS-His-PP1γ1 and pTAC-His-PP1γ1 by agarose gel electrophoresis. Lane 1: 1 Kb DNA molecular weight; Lane 2: pKS-His-PP1γ1/Nde I+Hind III; Lane 3: pTAC - PP1γ1-His /Nde I+Hind III.

Small scale expression of His-tagged PP1γ1 in DH5α and immunoblot analysis also indicated that His-tagged PP1γ1 can be expressed by the resulting pTAC-His-PP1γ1 expression plasmid (Fig. II.4).

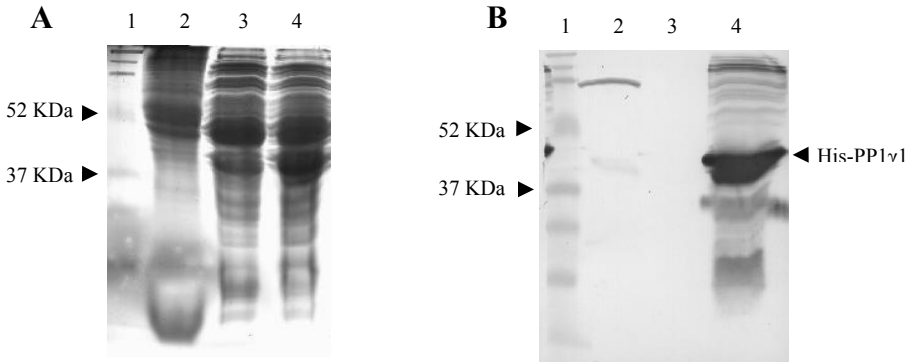

Figure II.4: SDS-PAGE (A) and immunoblot with Anti-PP1γ1 antibody (B) detected the expression of His-tagged PP1γ1. Lane 1: Protein molecular Marker; lane 2: Positive Control of rat cotex; Lane 3: Negative contro.l Lane 4. Expresszion of pTAC-His-PP1γ1 in DH5α.

II.3.2 PURIFICATION OF HIS-TAGGED PP1 GAMMA 1 AND PP1 GAMMA 2 USING NI-NTA RESIN

After induction of recombinant PP1 expression from pTAC-His-PP1γ1 and pTAC-His-PP1γ2 in DH5α with IPTG at 26ºC in 50 ml LB, His-tagged PP1γ1 and PP1γ2 were purified under different conditions to determine the best method to obtain pure and active His-tagged PP1γ1 and PP1γ2 phosphatase enzymes. His-tagged PP1γ1 and PP1γ2 were

purified in 5 mM, 10 mM and 30 mM imidazole (Fig. II.5). After scanning the SDS-PAGE gel using a Bio-Rad GS-710 Calibrated Imaging Densitometer, the density of the expressed protein bands was analyzed using Quantity One software. The percentage purification of His-tagged PP1γ1 and PP1γ2 obtained for each method are listed below (Table II.2). The results obtained indicated that the highest degree of purification was obtained in 30 mM imidazole, both for His-PP1γ1 and His-PP1γ2.

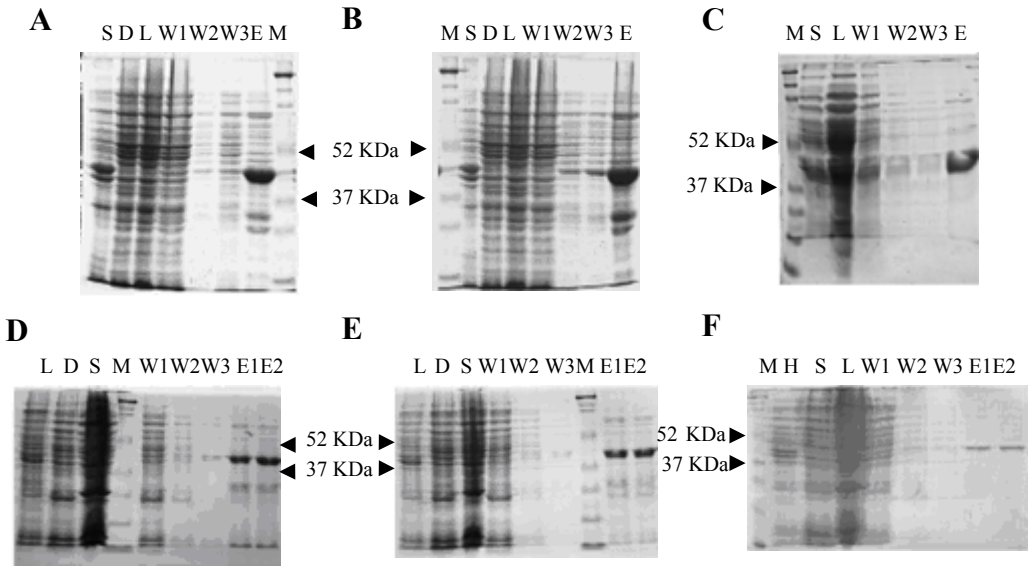

Figure II.5: SDS-PAGE analsis of expression proteins for His-PP1γ1 (A-C) and His-PP1γ2 (D-E), under different concentrations of imidazole (A and D 5 mM; B and E 10 mM; C and F 30 mM). D, cell debris; E, eluted protein; E1, eluted fraction with 250 mM imidazole (pH 8.0); E2, eluted fraction with 500 mM imidazole (pH 8.0); H, cell homogenate; L, loading flow through; M, molecular weight markers; S, supernatant from cell lysis; W1, first wash flow though; W2, second wash flow though; W3, third wash flow though.

Table II.2: Yield of purified His-PP1γ 1 and His-PP1γ2 with different concentration of imidazole

Method of purification	Concentration of PP1γ1(OD)	% PP1γ1	Concentration of PP1γ2(OD)	% PP1γ2
5mM imidazole, pH 8.0 lysis	3295	58.39	5181	57.61
10mM imidazole, pH 8.0 lysis	4866	49.38	3889	54.67
30mM imidazole, pH 8.0 lysis	2745	88.61	1743	60.43

Furthermore, His-tagged PP1γ1 and PP1γ2 were also purified under different pH conditions in 30 mM imidazole (Fig. II.6). Following SDS-PAGE analysis, gels were scanned with a Bio-Rad GS-710 Calibrated Imaging Densitometer and the density of the protein bands was analyzed using Quantity One software. The percentage of His-PP1γ1 and His-PP1γ2 recovered for each method are listed (Table II.3). The results indicate that using 30 mM imidazole (both pH 7.0 and pH 8.0 buffer systems), the quantity and quality of His-PP1γ1 recombinant protein recovered was better than using other pH buffer systems. Thus, for large scale purification the two different methods were compared to purify His-PP1γ1.

In the case of His-PP1γ2, the best method appeared to be 30mM imidazole (pH 5.0). Thus, for the large scale purification of His-PP1γ2, the 5 mM imidazole (pH 8.0) and 30 mM imidazole (pH 5.0) conditions were compared.

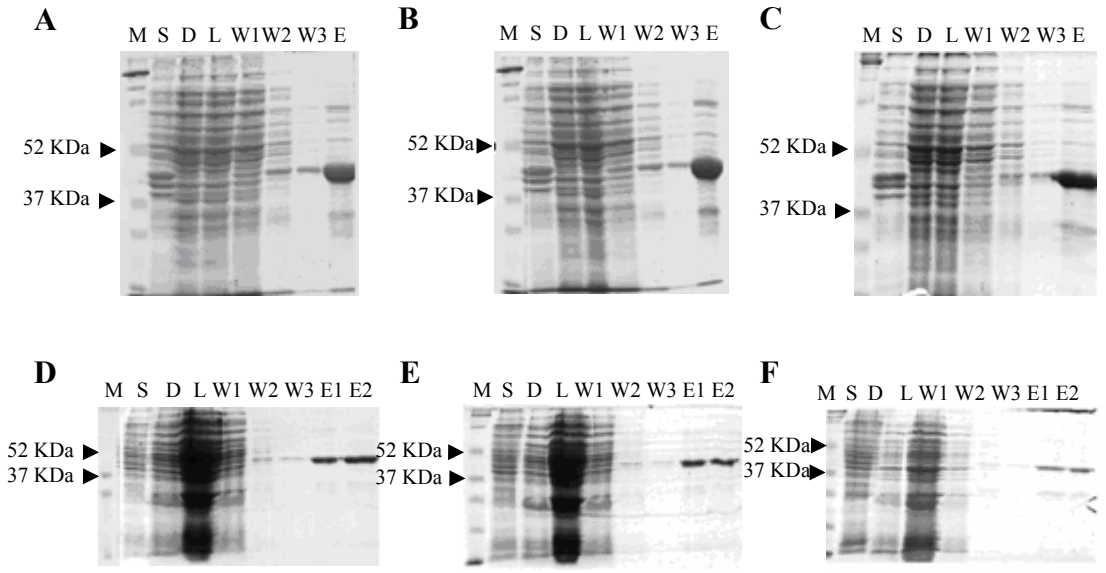

Figure II.6: SDS-PAGE analysis of purification reactions for His-PP1γ1 (A-C) and His-PP1γ2 (D-F) under different conditions of pH (A and D, pH 5.0; B and E, pH 6.0; C and F, pH 7.0). D, cell debris; E, eluted protein; E1, eluted fraction with 250 mM imidazole (pH 8.0); E2, eluted fraction with 500 mM imidazole (pH 8.0); L, loading flow through; M, molecular weight markers; S, supernatant from cell lysis; W1, first wash flow though; W2, second wash flow though; W3, third wash flow though.

Table II.3: Yield of purified His-PP1γ1 and His-PP1γ2 with 30 mM imidazole and different pH condition.

Method of purification	Concentration of PP1γ1(OD)	% PP1γ1	Concentration of PP1γ2(OD)	% PP1γ2
pH 5.0 buffer	2474	69.64	3854	93.14
pH 6.0 buffer	3113	69.05	2702	85.49
pH 7.0 buffer	4478	72.20	1273	73.25

II.3.3 LARGE SCALE PURIFICATION OF HIS-PP1 GAMMA 1 AND HIS-PP1 GAMMA 2

Given the previous results, it seemed sensible to test two different methods for the large-scale expression and purification of His-PP1γ1 (comparing imidazole buffer systems of pH 7.0 and pH 8.0). Using 30 mM imidazole (pH 8.0), the eluted fractions were recovered from the purification column and analyzed by SDS-PAGE followed by Coomassie blue staining (Fig. II.7):

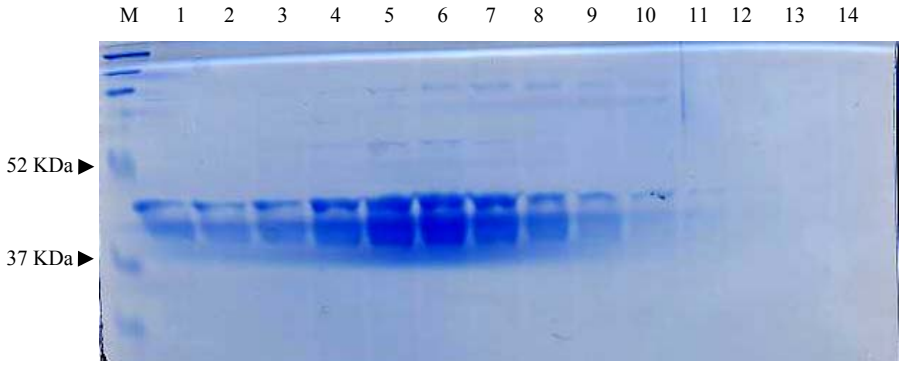

Figure II.7: SDS-PAGE gel analsis of eluted His-PP1γ1 for large scale purification in pH 8.0 buffer system Coomassie blue (Sigma) stained gel of eluted fractions. M, Protein Molecular Weight Marker; Lanes 1-14: Elution fractions 1 to 14.

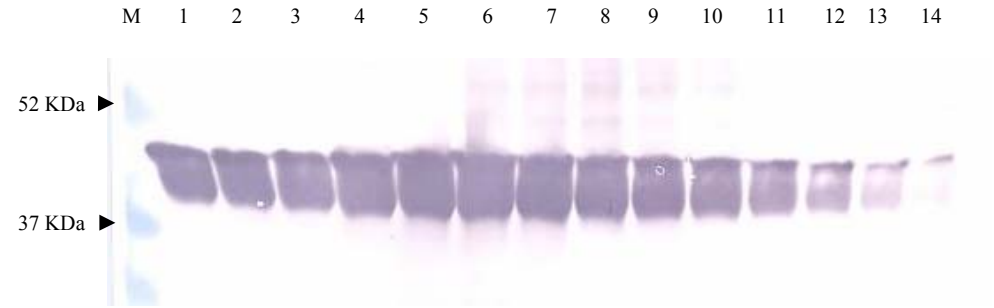

Figure II.8: Immunoblot analysis of eluted fractions of His-PP1γ1 from pH 8.0 buffer system. Immunoblot analysis was performed using a anti-PP1γ1 antibody (CBC-3C). M, Protein Molecular Weight Marker. Lanes 1-14: Elution fractions 1 to 14.

The identity of the eluted protein was confirmed by immunoblotting using a specific antibody (Fig. II.8).

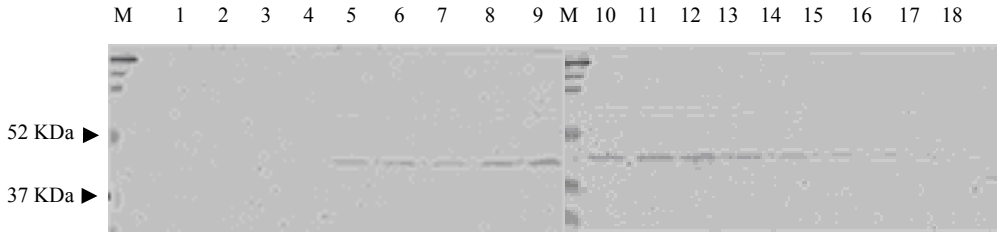

Figure II.9: SDS-PAGE gel analysis of large scale purification of His-PP1γ1 in pH 7.0 buffer system Coomassie blue (Sigma) stained gel of eluted fractions. M, Protein Molecular Weight Marker; Lanes 1-18: Eluted fractions 1 to 18.

The results obtained seem to indicate that the pH 8.0 buffer system is more efficient than the pH 7.0 buffer system for large scale purification of His-PP1γ1. Specifically, the yield of purified protein obtained with the pH 8.0 system appears to be better, as judged by Coomassie staining (see Figs. II.7 and 9), although the same expression condition were used. This suggests that, for His-PP1γ1, the optimal method for large scale purification involves suspension of cells in 30 mM imidazole (pH 8.0) lysis buffer, followed by the addition of 1 μg/ml lysozyme on ice for 30 min. After sonication, the supernatant is collected by centrifugation. The resin is packed into the column and equilibrated with the lysis buffer used. The collected supernatant is loaded into the column and the column is

washed with the same buffer. His-PP1γ1 is eluted with a linear concentration gradient of imidazole from 30 mM to 500 mM with 30 ml total volume.

For large scale purification of His-PP1γ2, the two methods compared consisted of 30 mM imidazole (pH 5.0) and 5 mM imidazole (pH 8.0) for the lysis of the cells. After loading the cell lysis supernatant, each column was washed with the corresponding buffer. His-PP1γ2 was eluted with a linear concentration gradient of imidazole from 30 mM to 500 mM. The eluted fractions were analyzed by SDS-PAGE (Figs. II.10 and 11):

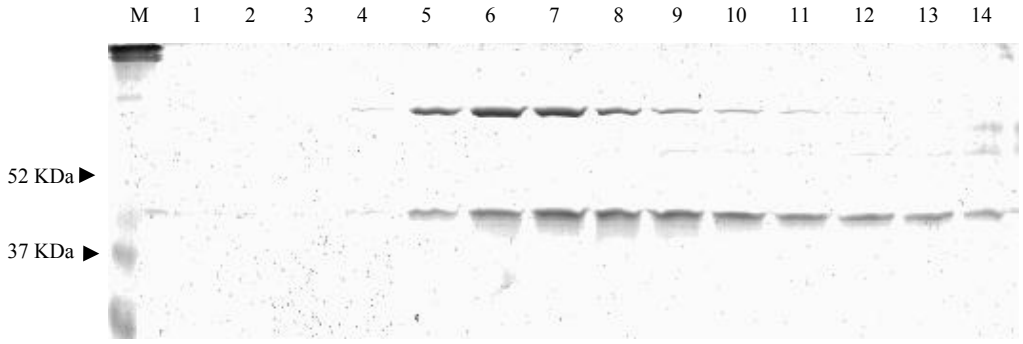

Figure II.10: SDS-PAGE analysis eluted fractions of His-PP1γ2 with 5 mM imidazole (pH 8.0) buffer. M, Protein Molecular Weight Marker; Lanes 1-14: Eluted fractions 1 to 14.

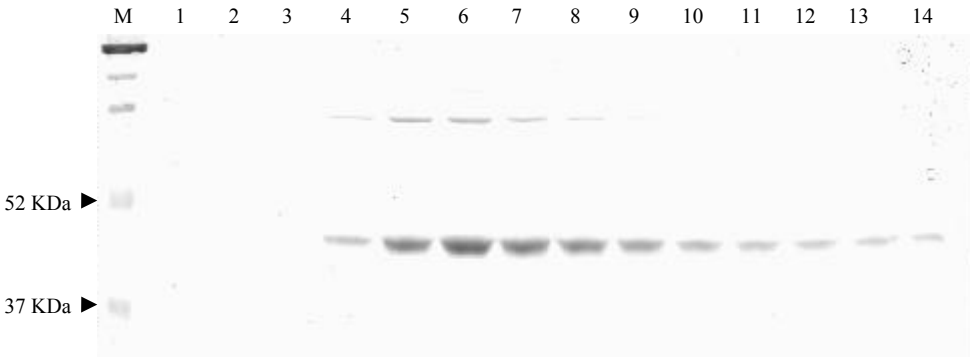

Figure II.11: SDS-PAGE gel analysis of eluted fractions of His-PP1γ2 using 30 mM imidazole (pH 5.0). M, Protein Molecular Weight Marker; Lanes 1-14: Eluted fractions 1 to 14.

Immunoblot analysis of the eluted fractions using a specific anti-PP1γ2 antibody (CBC-G502) confirmed the expression and purification of His-PP1γ2 (Fig. II.12).

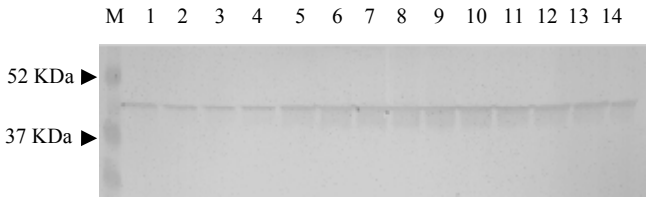

Figure II.12: Immunoblot analysis of eluted fractions obtained from large scale purification of His-PP1γ2. M, Protein Molecular Weight Marker; Lanes 1-14. Eluted fractions 1 to 14.

The result obtained (compare Figs. II.10 and 11) indicated that the second method, 30 mM imidazole (pH 5.0), appears to be more efficient for the purification of His-PP1γ2, as judged by recovery of a relatively abundant higher molecular weight contaminating protein using the other method (Fig. II.10). This contaminating protein was largely eliminated with the method of choice (Fig. II.11). Thus, this second method was chosen for routine large scale purification of His-PP1γ2.

II.3.4 PHOSPHATASE ACTIVITY OF RECOMBINANT HIS-TAGGED PP1 GAMMA 1 AND PP1 GAMMA 2

Activity of confirm the biological recombinant His-tagged PP1γ1 and PP1γ2, the phosphatase activity presenting each eluted fraction was assayed using ^{32}P-phosphorylase *a* as a substrate (Fig. II.13).

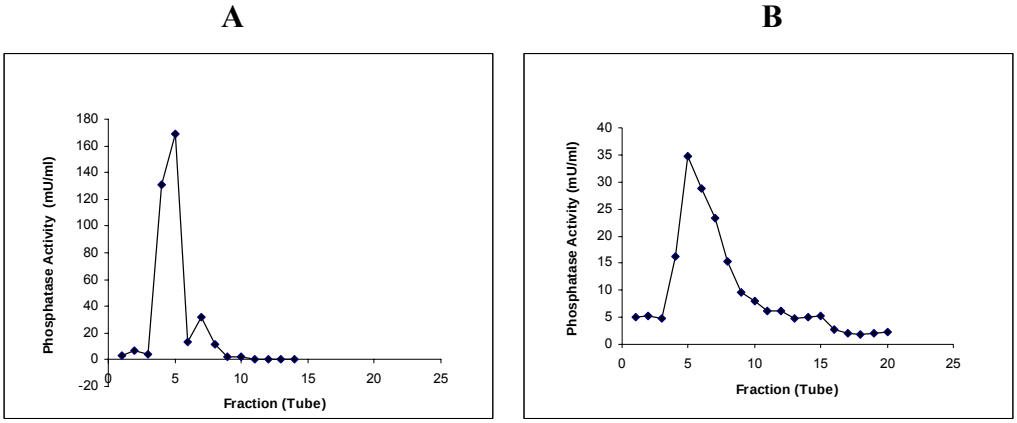

Figure II.13: Phosphatase activity of His-PP1γ1 (A) and His-PP1γ2 (B) eluted fractions, using ^{32}P-phosphorylase a as substrate. U, Unit of protein phosphatase.

The above results indicated that both recombinant His-tagged PP1γ1 and PP1γ2 were expressed in the bacterial system and could be purified essentially in their active forms in a high degree of homogeneity. Approximately 1.4 g (wet weight) of bacterial cells was collected from a 500 ml starting bacterial culture. Following the purification protocols described above, about 0.7 mg of PP1γ1 and 0.8 mg of PP1γ2 were obtained on average, exhibiting specific activities of around 10.7 U/mg and 9.2 U/mg for PP1γ2, respectively. After scanning Coomassie blue stained SDS-PAGE gels containing samples of the purified proteins, using a Bio-Rad GS-710 Calibrated Imaging Densitometer, the density of visible protein bands was analyzed using the Quantity One software and the purity of the recombinant PP1 preparations was estimated to be, on average, approximately 81% for His-PP1γ1 and 92% for PP1γ2.

In order to compare the properties of recombinant proteins to their physiological counterparts, their sensitivity to inhibitor by I2 was also assayed (Fig. 14 and Table II.4).

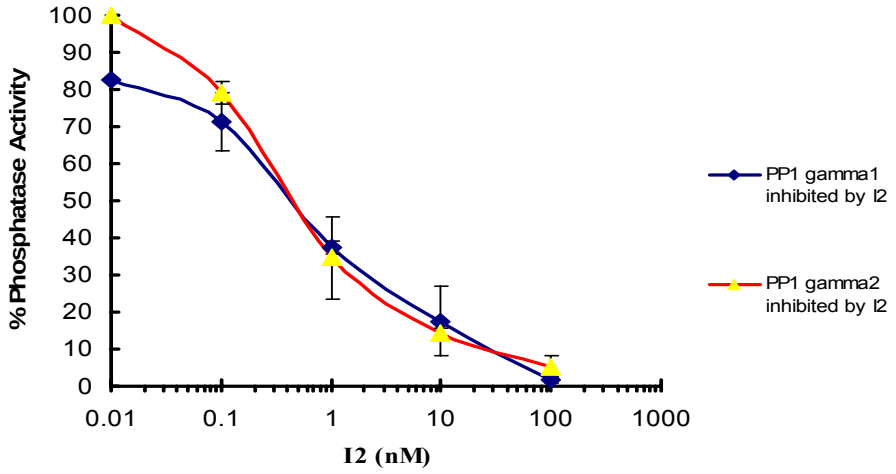

Figure II.14: I2 inhibition curves for recombinant His-pp1γ1 and His-PP1γ2, using phosphorylase a as substrate.

Table II.4: Inhibitory potency of I2 against His-PP1γ1 and His-PP1γ2.

Phosphatase	IC_{50}
His-PP1γ1	0.141 nM
His-PP1γ2	0.145 nM

.

The results obtained indicated that recombinant His-PP1γ1 and His-PP1γ2 exhibited near normal sensitivity to inhibition by I2, comparable to that previously determined for rabbit skeletal muscle PP1 preparations. The respective IC_{50} values were calculated to be approximately 0.41 nM for His-PP1γ1 and 0.415 nM for His-PP1γ2.

II.4 DISCUSSION

PP1 is generally thought to be one of the most important protein phosphatases, being ubiquitously expressed and involved in the regulation of wide variety of cellular signal transduction mechanisms. However, mammals possess three genes encoding highly homologous PP1 isoforms (PP1α, PP1β and PP1γ) exhibiting different abundance and tissue distributions. Furthermore, PP1 complexity is further enhanced by the process of alternative splicing. Splice variants have been identified and partially characterized, at least for the PP1α and PP1γ genes. The latter is known to exhibit tissue-specific alternative splicing to yield two main variants: the ubiquitous PP1γ1 and PP1γ2 that is predominantly expressed in testis and sperm. Indeed, PP1γ2 is thought to be the main PP1 isoform expressed in mammalian sperm, including humans. Therefore, while PP1γ1 has a broad range of roles, including relatively well characterized roles in neuronal function, PP1γ2 function appears to be relatively more restricted. Given its relative abundance and preponderance in testis and sperm, PP1γ2 is probably involved in the control of many of the underlying reproductive cellular processes. Thus, in our laboratory, we have been interested in the identification and isolation of PP1γ1 and PP1γ2 interacting proteins, the two main PP1 phosphatases expressed in human testis. Several such proteins were identified using the yeast two-hybrid system (Fardilha et al., 2004), including various previously unknown and novel PP1 regulators. The biochemical characterization and physiological importance of the latter is currently being addressed using a variety of approaches. However, in order to facilitate such studies, it is of the utmost importance to have at our disposal a ready source of pure PP1γ1 and PP1γ2 proteins. Thus, to this end, we set up to develop and establish large scale expression and purification methods for obtaining high quality recombinant PP1γ1 and PP1γ2, an essential step for the *in vitro* study of the newly identified PP1 regulators.

Initially, we attempted to obtain recombinant PP1γ1 and PP1γ2 from different expression systems, including the *Pichia pastoris* expression system. Although the *Pichia* system has been used to express a wide variety of proteins in very large amounts, in our hands PP1 was only expressed in relatively small quantities. However, most significant was the fact that the expressed recombinant PP1 isoforms migrated with larger apparent molecular masses than expected, as determined by SDS-PAGE and immunoblotting (data

not shown). After rechecking the expression plasmids by DNA sequencing, it was concluded that the observed anomalous *Pichia* expressed PP1 migration might be due to an unexpected post-translation modification (possibly glycosylation), and the Pichia method was not further pursued.

Next, we turned to the relatively well characterized bacterial expression systems. Different expression methods were attempted for these proteins, including expression of the native proteins without any tags, in order to avoid possible interference of such tags with the properties of the enzymes. Expression of native untagged PP1γ2 in bacteria, although yielding a reasonable amount of expressed recombinant soluble protein, was eventually abandoned as the routine method of choice. Without a tag to facilitate the process, the purification method was very complex and morose, requiring several complex and lengthy steps. The final yield and the degree of purification of recombinant PP1γ2 obtained were not the desirable for downstream biochemical applications (data not shown).

In previous studies, the baculovirus expression system was used to express both untagged PP1α and PP1γ1 (E. F. da Cruz e Silva, unpublished results) and His-tagged PP1α (Watanabe *et al.*, 2003), revealing that the recombinant PP1 isoforms obtained from insect cells exhibited virtually identical biochemical properties to the native enzyme. However, since bacterial expression of His-tagged PP1γ1 (Watanabe *et al.*, 2003) also led to the isolation of recombinant protein whose properties were similar (except for the dependence on Mn^{2+}), given the much easier and straightforward bacterial expression methodology, we also elected to develop large scale expression methods for obtaining high quality His-tagged PP1γ1 and PP1γ2 using the bacterial system.

Various previous studies found that expression of PP1γ1 in pET28a, or other T7 promotor vectors, led the expression of large quantities His-PP1γ1 but that it was mostly expressed as an insoluble protein (only a small fraction was recoverable as a soluble protein). Most likely, such results derive from the strong nature of the T7 promotor (Watanabe et al., 2003). Thus, in order to try to minimize this problem, we decided to express His-tagged (to aid purification) PP1γ1 and PP1γ2 using the pTACTAC vector. The relatively weaker nature of its promoter might be expected to lead to the expression of larger quantities of soluble PP1. Different concentrations of IPTG were also tried to induce the expression of PP1γ1 and PP1γ2 (data not shown here), in an attempt to optimize the

conditions for obtaining maximal amounts of both isoforms in the soluble fraction (0.5 mM IPTG was found to be the best concentration to use for both).

Using the methods described, both His-PP1γ1 and His-PP1γ2 were successfully expressed in the DH5α bacterial host and purified using Ni-NTA resin. As expected, the activity of the purified phosphatase isoforms was also dependent on the presence of Mn^{2+}, as was previously observed for other PP1 isoforms expressed in bacteria. From 500 ml bacterial culture approximately 05-1 mg of relatively homogeneous recombinant protein could be obtained (similar yields and specific activities were obtained for His-PP1γ1 and His-PP1γ2). Comparing His-PP1γ1 and His-PP1γ2 in the presence of their physiological counterpart I2, indicated that both exhibited near normal sensitivity to inhibition by I2, giving IC_{50} values in the nanomolar range (similar to native PP1). These recombinant His-tagged phosphatase isoforms produced in this study will be very important for the study the new regulators of PP1 identified in the laboratory.

CHAPTER III:

I2-L, A NEW TESTIS-SPECIFIC ISOFORM OF PP1 INHIBITOR-2

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III I2L, A NEW TESTIS-SPECIFIC ISOFORM OF PP1 INHIBITOR-2

III.1 INTRODUCTION

Mature spermatozoa are particularly specialized cells as they are highly compartmentalized, transcriptionally inactive and unable to synthesize new proteins. Therefore, it is likely that post-transcriptional events, like protein phosphorylation, play a major role in controlling sperm function, including motility, capacitation and the acrosome reaction. Sperm motility defects appear to be one of the main underlying causes of male infertility, a growing concern in industrialized nations. The biochemical mechanisms underlying sperm maturation and the development of motility are still far from being completely understood. However, Ser/Thr protein phosphorylation by protein phosphatase 1 (PP1) and glycogen synthase kinase-3 (GSK3), seem to be one of the controlling processes. PP1γ2, a testis-specific PP1 isoform, was found to be the only PP1 isoform present in bovine, rhesus monkey and human sperm (Smith et al., 1996; Vijayaraghavan et al., 1996). Its direct involvement in sperm motility is consistent with the fact that PP1 has two-fold higher activity in immotile bovine caput epididymal sperm compared to mature motile caudal sperm (Vijayaraghavan et al., 1996). Moreover, inhibition of PP1γ2 activity by okadaic acid and calyculin A causes both initiation and stimulation of motility (Vijayaraghavan et al., 1996).

The evolutionary conservation and the importance of Ser/Thr phosphatases in regulating flagellar motility is highlighted by the involvement of a PP1 homolog in the regulation of rooster sperm motility (Ashizawa et al., 1995; Ashizawa et al., 1994) and by the involvement of a Ser/Thr phosphatase in the regulation of microtubule sliding velocity in *Paramecium* and *Chlamydomonas* (Habermacher and Sale, 1995; Habermacher and Sale, 1996; Habermacher and Sale, 1997; Klumpp et al., 1990; Klumpp and Schultz, 1991). Bovine sperm immunocytochemistry showed that PP1γ2 is present along the entire flagellum including the mid-piece, consistent with a role in sperm motility, but also in the posterior and equatorial regions of the head, suggesting a role in the acrosome reaction (Huang et al., 2002). PP1γ2 is expressed during germ cell differentiation in testis (da Cruz e Silva et al., 1995b; Kitagawa et al., 1990; Sasaki et al., 1990). It has also been involved

in the onset of hyperactivated motility and the acrosome reaction (Si and Okuno, 1999; Smith et al., 1996; Smith et al., 1999; Vijayaraghavan et al., 1996).

PP1 activity towards different substrates appears to be mediated via binding to specific regulatory proteins. The increasing diversity of such PP1 regulatory subunits and their tissue specificity make them attractive pharmacological targets. An example of such a regulatory protein involved in sperm motility is a homologue of the yeast protein phosphatase binding protein sds22 (Huang et al., 2002). In caudal sperm PP1γ2 was found to be complexed with sds22 and to be catalytic inactive, whereas in caput sperm PP1γ2 and sds22 do not interact (Mishra et al., 2003). Another mechanism of PP1 regulation likely to be directly involved in sperm motility involves the long-known PP1 inhibitor 2 (I2) and GSK3. Interestingly, an I2-like activity detected in sperm could be reversed by purified GSK3 (Vijayaraghavan et al., 1996). PP1 and I2 form a stable but catalytically inactive complex. Phosphorylation of rabbit I2 at Thr72 by GSK3, releases the inhibition and the complex becomes active (Hemmings et al., 1982). Additionally, the phosphorylation of I2 by GSK3 is enhanced when Ser86, 120 and 121 are also phosphorylated by casein kinase II (CKII), particularly Ser86 (DePaoli-Roach, 1984; Park et al., 1994). Endogenous sperm GSK3 activity was also demonstrated by activation of purified PP1-I2 complex, and immotile caput sperm was shown to contain six-fold higher GSK3 activity than motile caudal sperm (Vijayaraghavan et al., 1996). The presence and activity of GSK3 in sperm has been further characterised sustaining its role in sperm motility regulation (Payaningal et al., 2004; Vijayaraghavan et al., 2000). However, the role of I2, which would make the regulating bridge between PP1 and GSK3, has remained more elusive.

In order to find out which PP1 partners might be involved in the control of sperm motility, we embarked on a study aimed at defining the testis specific interactomes of PP1γ1 and PP1γ2 (the two known alternatively spliced variants of PP1γ). To this end, exhaustive screens were performed on a human testis cDNA library using the Yeast Two Hybrid method (Fardilha et al., 2004a). Here we report the discovery of a new PP1 binding protein, termed Inhibitor 2-like (I2L) that is 95% identical to I2, including the human I2 substitutions of the main PP1 regulatory residues Thr73 and Ser87. The presence of I2 in sperm was confirmed by both immunoblot and immunocytochemistry and the importance of both I2 and I2L in regulating sperm PP1 is discussed.

III.2 MATERIALS AND METHODS

III.2.1 LIBRARY SCREENING

Microbial strains and methods for performing yeast two-hybrid screens of human testis cDNA libraries using human PP1 cDNAs as bait have been described (Fardilha et al., 2004a). Positive colonies were obtained on selective media from which pACT plasmids were recovered into *E. coli*. DNA sequence analysis was performed in-house using an Automated DNA Sequencer (Applied Biosystems). The DNA sequences obtained were compared to the GenBank data base, using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/), to identify the corresponding encoded proteins. Protein sequence analysis used PROSITE at the ExPASy site (http://www.expasy.org) and ELM (http://elm.eu.org). Multiple sequence alignments were performed using the ClustalW program (http://www.ebi.ac.uk/clustalw/).

III.2.2 I2L CLONING, EXPRESSION AND PURIFICATION

In order to correct the nucleotide sequence of clone 48, a single adenine insertion was made by PCR using appropriate oligonucleotide primers and the complete corrected I2L sequence was verified by DNA sequencing. For expression and purification purposes, the I2L cDNA was inserted into pET28c expression vector (Novagen), between *EcoR* I and *Xho* I restriction sites, adding a histidine tag to the N-terminal of the protein. The pET-I2L construct sequence was fully verified by DNA sequencing and transformed into *E. coli* Rosetta (DE3) strain (Novagen). The expression of recombinant His-tagged I2L (His-I2L) was induced with 1mM IPTG for 3h at 37ºC and the protein was purified using Ni-NTA resin (Qiagen), according to the supplier's instructions. Briefly, the cells were lysed in 10 mM imidazole, sodium phosphate buffer (pH 8.0), centrifuged at 15,000×g for 30 min at 4ºC, and the supernatant was applied to the resin. The resin was washed with 20mM imidazole and His-I2L was eluted with 500 mM imidazole. The eluate was further purified from a 12% SDS-PAGE gel. A portion of the lane containing His-I2L was stained with Commassie blue and the corresponding band containing the remaining protein cut out. The gel band was washed three times with water and cut into smaller pieces. After adding 1 ml

of 100 mM Tris-HCl (pH 8.5), 0.1% SDS it was frozen at -20ºC overnight. The slurry was frozen and thawed another three times and then passed through a 0.22 μm filter membrane. The gel-free filtrate was then dialyzed against 4x 500 ml of 10 mM Tris-HCl (pH 7.5) buffer for 24 hours at 4ºC. The protein concentration of His-I2L was determined by the BCA method (Pierce).

The I2L cDNA was also inserted in the pTACTAC expression vector (Zhang et al., 1993b), using *Nde* I and *Xba* I restriction sites. The sequence was verified by DNA sequencing and then transformed into the *E. coli* Rosetta strain mentioned above. The expression of recombinant holo-I2L, without any tags, was induced with 0.4 mM IPTG for 3 h at 37ºC. The protein was partially purified from a bacterial heat extract as previously described for I2 (Helps et al., 1994).

III.2.3 CONSTRUCTION OF AN I2L-GFP FUSION AND EXPRESSION IN MAMMALIAN CELLS

Using appropriate oligonucleotide primers, PCR amplification was used to sub-cloned I2L into the vector pEGFP-N1 (Novagen) between the *EcoR* I and *BamH* I sites, and with the elimination of the original STOP codon. After confirming the correctness of the construct by DNA sequencing, the fusion vector for expression of I2L-GFP was then transfected into cultured HeLa and COS-7 cells, as previously described (Rebelo et al., 2007). The fluorescent recombinant fusion protein was visualized using an Olympus IX-81 inverted epifluorescence microscope.

III.2.4 YEAST CO-TRANSFORMATION WITH PLASMID DNA

Competent AH109 yeast cells were prepared by the lithium acetate method. In a microtube 0.1 μg of the PP1 bait plasmid and 0.1 μg of the I2L prey plasmid were added to 100 μg of herring testes carrier DNA. Then, 100 μl of freshly prepared competent cells were added, followed by 600 μl of sterile PEG/LiAc (40% PEG 4000/1X TE/1X LiAc). The solution was incubated at 30ºC for 30 min with shaking (200 rpm). After adding 70 μl of DMSO, the solution was mixed gently and heat shocked for 15 min at 42ºC. The cells were pelleted after being chilled on ice, centrifuged for 5 sec at 14,000 rpm and resuspended in 0.5 ml of 1X TE buffer. These cells were plated in the appropriate selection

media, incubated at 30ºC for 2 days, and replated in selective media containing X-α-Gal (Clontech, USA).

III.2.5 BLOT OVERLAY ANALYSIS

For blot overlay analysis, 0.3 μg of purified I2 (NEB) and His-I2L were run on a SDS-PAGE gel and then transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with purified recombinant PP1γ1 or PP1γ2 diluted in TBS-T/BSA. After washing to remove excess protein, the bound PP1 isoforms were detected by incubating the membrane with specific anti-PP1γ and anti-PP1γ2 antibodies. Immunoreactive bands were revealed by incubating with horseradish peroxidase conjugated secondary antibody and developed by ECL (Amersham).

III.2.6 PHOSPHATASE ACTIVITY ASSAYS

The IC_{50} values of I2 and I2L for purified PP1γ1 and PP1γ2 isoforms were determined using ^{32}P-phosphorylase *a* as substrate. The substrate was prepared from phosphorylase *b* (Sigma) using [γ-^{32}P]ATP (Amersham) and phosphorylase kinase (Sigma), as previously described (Watanabe et al., 2003). An appropriate range of concentrations of purified I2 and I2L were incubated with the purified PP1 catalytic subunits and the phosphatase activity determined. The IC_{50} was calculated using BioDataFit 1.02 software.

III.2.7 PREPARATION OF HUMAN SPERM HEAT EXTRACT OF I2/I2L

Human sperm samples [kindly provided by Dr. Mário de Sousa (Clínica de Reprodução Dr. Alberto Barros, Porto, Portugal)] were used to partially purify endogenous I2/I2L based on published procedures (Vijayaraghavan et al., 1996). Briefly, human sperm samples were centrifuged at 1,200 rpm for 10 min at 4ºC and washed two times with PBS. The pellet was resuspended in homogenization buffer [50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 1 mM EGTA, supplemented with 6 μg/mL Pepstatin A, 2 μM Leupeptin, 10 mM Benzamidine, 10 μg/mL Aprotinin and 1 mM PMSF] and boiled for 10 min. The mix was sonicated twice for 15 sec, boiled again for 30 min and centrifuged at 16,000×g for 15 min. The supernatant was adjusted to 10% TCA and, after centrifugation, the precipitate was

dispersed in 0.5 M Tris base and then dialysed overnight against 5 mM Tris-HCl (pH 7), 1 mM EDTA and 1mM EGTA. The dialysate was boiled for 10 min and, after centrifugation, the supernatant (termed human sperm heat extract or HSI2) was stored in aliquots at -20ºC.

III.2.8 IMMUNOBLOT ANALYSIS

I2/I2L containing samples were separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in TBS-T for 1h, followed by incubation with sheep polyclonal anti-I2 antibody [diluted 1:5000; kindly provided by PT Cohen (Medical Research Council Protein Phosphorylation Unit, Dundee, Scotland) or DL Brautigan (Center for Cell Signalling, Virginia, USA)] for 1h at room temperature with shaking. After washing, the blots were incubated with anti-sheep secondary antibody conjugated with horseradish peroxidase (diluted 1:1000; Dako) for 1h at room temperature. Blots were washed and developed with an ECL chemiluminescence kit (Amersham).

III.2.9 PHOSPHORYLATION OF I2 AND I2L

Recombinant I2, or bacterial heat extract of recombinant I2L, or human sperm heat extract of native I2/I2L [about 200 ng of inhibitor protein in 50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA and 0.03% Brij-35] were phosphorylated by GSK-3β (Calbiochem) or by CK-II (Calbiochem), or both, as previously described (Park et al., 1994). The phosphorylation reaction was performed at 30ºC for 90 min and then terminated with the addition of 4X SDS loading buffer. Aliquots (1/10 and 9/10) of the reaction volume were separated in two separate 12% SDS-PAGE gels. The 1/10 reaction was analysed by immunoblot analysis. The other gel was dried and autoradiographed. When the phosphorylation was carried out in the presence of PP1, the inhibitor proteins were pre-incubated at 30ºC for 15 min with 200 ng of purified His-tagged PP1γ1 or PP1γ2 in the presence of 1 mM $MnCl_2$.

III.2.10 DEPHOSPHORYLATION OF HSI2

HSI2 was incubated overnight with either Protein Tyrosine Phosphatase 1B (Upstate Biosystems) at 37ºC, or Calf Intestinal Phosphatase (NEB) at 37ºC, or with PP1γ1,

at 30ºC, in the respective assay buffers. The reactions were stopped with the addition of 4x SDS loading buffer and analysed by immunoblotting as described above.

III.2.11 TWO DIMENSIONAL (2D) GEL ELECTROPHORESIS ANALYSIS

Both HSI2 without and with the addition of 10 ng of purified I2 were acetone precipitated and the pellets resuspended in 250 µl of 2D rehydratation solution (8 M Urea/2 M Thiourea/2% CHAPS/0.002% bromophenol blue) and supplemented with 2.5 µl of IPG buffer (in the pH 4-7 range) and 14 mg of DTT. The first dimension separation conditions were the following: 1 h at 30 V, 2 h at 150 V, 1 h at 500 V, 1 h at 1000 V and 2 h at 8000 V. For the second dimension the samples were separated on 12% SDS-PAGE gels and, after electrophoretic transfer to nitrocellulose, the results were analysed by immunoblotting.

III.2.12 IMMUNOCYTOCHEMISTRY OF SPERMATOZOA

Human sperm samples were centrifuged at 1,200 rpm for 10 min at room temperature and washed twice with PBS. Spermatozoa were then resuspended in PBS and were attached to poly-ornithine coated coverslips for 15min. After fixation with 4% paraformaldehyde and washing with PBS, spermatozoa were permeabilized with methanol for 2 min and immediately washed with PBS. Sperm cells were incubated overnight 4ºC with anti-I2 antibody (diluted 1:500 in 3% BSA in PBS). After washing with PBS, the secondary antibody (FITC-conjugated rabbit anti-sheep at 1:50 dilution) was placed over the sperm cells for 1 h at room temperature. Following further washing with PBS, the procedure was repeated with the anti-PP1γ2 antibody (diluted 1:300) and Texas Red-conjugated goat anti-rabbit secondary antibody (diluted 1:300; Molecular Probes). Coverslips were mounted in one drop of DAPI (Vector Laboratories) in a glass slide. Sperm cells were visualized using an Olympus 1X-81 epifluorescence microscope.

III.2.13 IMMUNOPRECIPITATION OF HUMAN SPERM SAMPLES

Human sperm samples (~1 mg of protein) were centrifuged at 1,200 rpm for 7 min at room temperature and washed twice with PBS. The pellet was then resuspended in 1 ml

lysis buffer [50 mM Tris-HCl (pH 8.0)/120 mM NaCl/4% CHAPS/0.1 mg/ml Pepstatin A/0.03 mM Leupeptin/145 mM Benzamidine/0.37 mg/ml Aprotinin/4.4 mM PMSF] for 15 min on ice and sonicated for 30 sec. The lysates were precleared with 25 µl of Protein A Sepharose slurry (Pharmacia) for 1 h at 4ºC with orbital agitation. After centrifuging the sample for 1 min at 4ºC and 10,000×g, the supernatant was transferred to a new tube. Protein A Sepharose slurry (50 µl) and 1 µl of anti-PP1γ2 antibody were added and the mixture incubated overnight at 4ºC with orbital agitation. The mixture was centrifuged for 1min at 4ºC and 10,000×g and the pellet washed twice for 15 min with 500 µl of 50 mM Tris-HCl (pH 8.0)/120 mM NaCl. The pellet was resuspended in SDS-PAGE loading buffer and analysed by immunoblotting with anti-I2 antibody.

III.3 RESULTS

III.3.1 IDENTIFICATION OF A NEW I2 ISOFORM FROM A YEAST TWO HYBRID SCREEN

To identify putative regulatory subunits of PP1γ1 and PP1γ2, yeast two-hybrid screens were performed using the respective phosphatases isoforms as baits to screen a human testis cDNA library (Fardilha et al., 2004a). One of the 120 positive clones (clone 48) isolated from the PP1γ1 screen, encoded the complete coding sequence of a new protein exhibiting a high degree of identity with the known PP1 regulator PPP1R2 or inhibitor 2 (I2). We named it Inhibitor 2-like or I2L. By alignment with GenBank database (Fig. III.1A) clone 48 had a missing adenine at position 206 which would change the reading frame and thus result in an early STOP codon. Given the occurrence of the missing nucleotide in the corresponding genomic sequence, we concluded that it must represent a cloning artefact and re-introduced the missing A by PCR amplification using appropriate oligonucleotide primers. The clone 48 sequence aligned with sequence NR_002168, localized in chromosome 5, classified as an I2 pseudogene 3, and with a human testis mRNA sequence from the Mammalian Gene Collection program (nucleotide: BC066922; protein: Q6NXS1) (Fig. III.1A). Moreover, there is a human testis EST (BU854595) that aligns 99% to chromosome 5 at the I2L site, from nucleotide 3 to 680 of a total of 840 (high quality sequence stops at nucleotide 539). Figure III.1B shows the alignment of I2, I2L and the testis EST. Where other species are concerned, the *Pan troglodytes* genome at chromosome 5 encodes a predicted one-exon protein 97% similar to human I2L (nucleotide: XM_001134994; protein: XP_001134994) (Fig. III.1C), having the same Thr and Ser substitutions characteristic of human I2L (see text below and Table III.1). Interestingly, the genome localization of *Pan troglodytes* I2 (chromosome 3) and I2L (chromosome 5) are similar to the corresponding human genes. There is also a testis EST from Pigtailed macaque (DY743031) that encodes a protein 94% similar to I2L (Fig. III.1C), having the characteristic Thr substitution but not the Ser one.

I2L is 95% identical to I2 at both the nucleotide and the amino acid levels (Fig. III.1B and III.1C)， but with some important differences (Table III.1). I2 is encoded by 6 exons in chromosome 3, while I2L is a one-exon protein encoded in chromosome 5. Thr73

and Ser87, phosphorylation sites for GSK3 and CKII, respectively, are absent in I2L. These residues are the main regulatory targets for PP1 regulation by I2. Using Prosite and ELM software, several putative CKII phosphorylation sites were identified in I2L (35-38 SvdE, 121-124 SsgE, 122-125 SgeE, 130-133 SpeE, 193-196 TpsD). The 35-38 SvdE and 193-196 TpsD sites represent new putative CKII phosphorylation sites in I2L that do not exist in I2. I2L also contains three additional putative protein kinase C (PKC) phosphorylation sites (7-9 ShR, 41-43 SkK, 44-46 SqK) and two protein kinase A (PKA) phosphorylation sites (33-35 RrS, 87-89 RdT), in comparison to I2.

```
        SeqA Name              Len(nt)   SeqB Name              Len(nt)   Score
          1    I2Lgene_chr5     2043      2    I2L_Clone48       863       98
          1    I2Lgene_chr5     2043      3    I2L_cloneIMAGE    2007      99
          2    I2L_Clone48      863       3    I2L_cloneIMAGE    2007      92

I2Lgene_chr5        CTCTGCTGTGCCGACCCTTCTCTTCGCGGACCCCACGCCAAGCAGCGACCCTGAGGCGAC 60
I2L_cloneIMAGE      ------------------------------------------------------GAGGGGAC 8
I2L_Clone48         CTCTGCTGTGCCGACCCTTCTCTTCGCGGACCCCACGCCAAGCAGCGACCCTGAGGCGAC 60
                                                                      **** ***
I2Lgene_chr5        AGCCGGAGCGCCCGGCAATGGCGGCCTCGACGGCCTCCCACCGGCCCATCAAGGGGATCT 120
I2L_cloneIMAGE      AGCCGGAGCGCCCGGCAATGGCGGCCTCGACGGCCTCCCACCGGCCCATCAAGGGGATCT 68
I2L_Clone48         AGCCGGAGCGCCCGGCAATGGCGGCCTCGACGGCCTCCCACCGGCCCATCAAGGGGATCT 120
                    ************************************************************
I2Lgene_chr5        TGAAGAACAAGACCTCTACGACTTCCTCTATGGTGGCGTCGGCCGAACAGCCCCGCAGGA 180
I2L_cloneIMAGE      TGAAGAACAAGACCTCTACGACTTCCTCTATGGTGGCGTCGGCCGAACAGCCCCGCAGGA 128
I2L_Clone48         TGAAGAACAAGACCTCTACGACTTCCTCTATGGTGGCGTCGGCCGAACAGCCCCGCAGGA 180
                    ************************************************************
I2Lgene_chr5        GTGTCGACGAGGAGCTGAGCAAAAAATCCCAGAAGTGGGATGAAATTAACATCTTGGCGA 240
I2L_cloneIMAGE      GTGTCGACGAGGAGCTGAGCAAAAAATCCCAGAAGTGGGATGAAATTAACATCTTGGCGA 188
I2L_Clone48         GTGTCGACGAGGAGCTGAGCAAAAA-TCCCAGAAGTGGGATGAAATTAACATCTTGGCGA 239
                    ************************ ***********************************
I2Lgene_chr5        CCTATCATCCAGCAGACAAAGGCTATGGTTTAATGAAAATAGATGAACCAAGCCCTCCTT 300
I2L_cloneIMAGE      CCTATCATCCAGCAGACAAAGGCTATGGTTTAATGAAAATAGATGAACCAAGCCCTCCTT 248
I2L_Clone48         CCTATCATCCAGCAGACAAAGGCTATGGTTTAATGAAAATAGATGAACCAAGCCCTCCTT 299
                    ************************************************************
I2Lgene_chr5        ACCATAGTATGATGGGTGATGATGAAGATGCGTGTAGGGACACCGAGACCACTGAAGCCA 360
I2L_cloneIMAGE      ACCATAGTATGATGGGTGATGATGAAGATGCGTGTAGGGACACCGAGACCACTGAAGCCA 308
I2L_Clone48         ACCATAGTATGATGGGTGATGATGAAGATGCGTGTAGGGACACCGAGACCACTGAAGCCA 359
                    ************************************************************
I2Lgene_chr5        TGGCGCCAGACATCCTAGCCAAGAAATTAGCTGCTGCTGAAGGCTTGGAGCCAAAGTACC 420
I2L_cloneIMAGE      TGGCGCCAGGCATCCTAGCCAAGAAATTAGCTGCTGCTGAAGGCTTGGAGCCAAAGTACC 368
I2L_Clone48         TGGCGCCAGACATCCTAGCCAAGAAATTAGCTGCTGCTGAAGGCTTGGAGCCAAAGTACC 419
                    ********* ****************************************************
I2Lgene_chr5        GGATTCAGGAACAAGAAAGCAGTGGAGAGGAGGATAGTGACCTCTCACCTGAAGAACGAG 480
I2L_cloneIMAGE      GGATTCAGGAACAAGAAAGCAGTGGAGAGGAGGATAGTGACCTCTCACCTGAAGAACGAG 428
I2L_Clone48         GGATTCAGGAACAAGAAAGCAGTGGAGAGGAGGATAGTGACCTCTCACCTGAAGAACGAG 479
                    ************************************************************
I2Lgene_chr5        AAAAAAAGCGACAATTTGAAATGAGAAGGAAGCTTCACTACAATGAAGGACTCAATATCA 540
I2L_cloneIMAGE      AAAAAAAGCGACAATTTGAAATGAGAAGGAAGCTTCACTACAATGAAGGACTCAATATCA 488
I2L_Clone48         AAAAAAAGCGACAATTTGAAATGAGAAGGAAGCTTCACTACAATGAAGGACTCAATATCA 539
                    ************************************************************
I2Lgene_chr5        AACTAGCCAGACAATTAATTTCAAAAGACCTACATGATGATGATGAAGATGAAGAAATGT 600
I2L_cloneIMAGE      AACTAGCCAGACAATTAATTTCAAAAGACCTACATGATGATGATGAAGATGAAGAAATGT 548
I2L_Clone48         AACTAGCCAGACAATTAATTTCAAAAGACCTACATGATGATGATGAAGATGAAGAAATGT 599
                    ************************************************************
I2Lgene_chr5        TAGAGACTGCAGATGGAGAAAGCATGAATACGGAAGAATCAAATCAAGGATCTACTCCAA 660
I2L_cloneIMAGE      TAGAGACTGCAGATGGAGAAAGCATGAATACGGAAGAATCAAATCAAGGATCTACTCCAA 608
I2L_Clone48         TAGAGACTGCAGATGGAGAAAGCATGAATACGGAAGAATCAAATCAAGGATCTACTCCAA 659
                    ************************************************************
I2Lgene_chr5        GTGACCAACAGCAAAACAAATTACGAAGTTCATAGAAGAGATTTGTTCAACACTGCAATT 720
I2L_cloneIMAGE      GTGACCAACAGCAAAACAAATTACGAAGTTCATAGAAGAGATTTGTTCAACACTGCAATT 668
I2L_Clone48         GTGACCAACAGCAAAACAAATTACGAAGTTCATAGAAGAGATTTGTTCAACACTGCAATT 719
                    ************************************************************
I2Lgene_chr5        GTTTGTTAGATATAAACCCTGTGACTATAATACATTGCTTCTTGTTCTCCACAATTCATG 780
I2L_cloneIMAGE      GTTTGTTAGATATAAACCCTGTGACTATAATACATTGCTTCTTGTTCTCCACAATTCATG 728
I2L_Clone48         GTTTGTTAGATATAAACCCTGTGACTATAATACATTGCTTCTTGTTCTCCACAATTCATG 779
                    ************************************************************
I2Lgene_chr5        ACTTAAGTACCAAAATGCATACCAGTTATTATATATTGCCAAGAATTAAATGATAAACTT 840
I2L_cloneIMAGE      ACTTAAGTACCAAAATGCATACCAGTTATTATATATTGCCAAGAATTAAATGATAAACTT 788
I2L_Clone48         ACTTAAGTACCAAAATGCATACCAGTTATTATATATTGCCAAGAATTAAATGATAAACTT 839
                    ************************************************************
I2Lgene_chr5        AGAGACTAATTAGACTGAAAATGCCTAATTGATATATATATTCTTATGCCTAGTACTTTA 900
I2L_cloneIMAGE      AGAGACTAATTAGACTGAAAATGCCTAATTGATATATATATTCTTATGCCTAGTACTTTA 848
I2L_Clone48         AGAGACTAAAAAAAAAAAAAAAA----------------------------------- 863
                    *********  * *   ****
I2Lgene_chr5        TATTCCTCTAACGCAGTAAGAATTATGTGGAATATTTTCCTTAAACGAAGTGCAGGAAAG 2040
I2L_cloneIMAGE      TATTCCTCTAACGCAGTAAGAATTATGTGGAATATTTTCCTTAAACGAAGTGCAGGAAAG 1988
I2L_Clone48         ------------------------------------------------------------

I2Lgene_chr5        CCC---------------- 2043
I2L_cloneIMAGE      CCCAAAAAAAAAAAAAAAA 2007
I2L_Clone48         --------------------
```

Figure III.1A. A new Inhibitor 2-like (I2L) isoform. ClustalW alignment of the I2L gene sequence in human chromosome 5 (NC000005) with clone 48 (isolated from the yeast two-hybrid screen) and a cDNA sequence subsequently entered in the database (BC066922). (//) indicates a gap in the sequence alignment at the 3'-end. The initiation and stop codons are in bold and the putative poly(A) addition signals are underlined. The alignment scores are indicated on top.

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	I2	3355	2	I2Lgene_chr5	2043	95
1	I2	3355	3	EST_testis	840	88
2	I2Lgene_chr5	2043	3	EST_testis	840	90

```
I2                CTTTAGCCCTGAGCGGGATCTGCGGCTGCCTGCGAGTCTCTGCTGTGCCGACCCTTCTCT 180
I2Lgene_chr5      -------------------------------------CTCTGCTGTGCCGACCCTTCTCT 23
EST_testis        ------------------------------------------------------------

I2                TCGCGGACCCCACGCCAAGCAGCGACCCTGAGCCGACAGCCGGAGCGCCCGGCAATGGCG 240
I2Lgene_chr5      TCGCGGACCCCACGCCAAGCAGCGACCCTGAGGCGACAGCCGGAGCGCCCGGCAATGGCG 83
EST_testis        ------------------------------------------------------------
                                                 *
I2                GCCTCGACGGCCTCGCACCGGCCCATCAAGGGGATCTTGAAGAACAAGACCTCTACGACT 300
I2Lgene_chr5      GCCTCGACGGCCTCCCACCGGCCCATCAAGGGGATCTTGAAGAACAAGACCTCTACGACT 143
EST_testis        ------------------------------------------------------------

I2                TCCTCTATGGTGGCGTCGGCCGAACAGCCCCGCGGGAATGTCGACGAGGAGCTGAGCAAA 360
I2Lgene_chr5      TCCTCTATGGTGGCGTCGGCCGAACAGCCCCGCAGGAGTGTCGACGAGGAGCTGAGCAAA 203
EST_testis        ------------------------------------------------------------
                                               *   *
I2                AAATCCCAGAAGTGGGATGAAATGAACATCTTGGCGACGTATCATCCAGCAGACAAAGAC 420
I2Lgene_chr5      AAATCCCAGAAGTGGGATGAAATTAACATCTTGGCGACCTATCATCCAGCAGACAAAGGC 263
EST_testis        ------------------------------------------------------------
                                     *                                   *
I2                TATGGTTTAATGAAAATAGATGAACCAAGCACTCCTTACCATAGTATGATGGGGGATGAT 480
I2Lgene_chr5      TATGGTTTAATGAAAATAGATGAACCAAGCCCTCCTTACCATAGTATGATGGGTGATGAT 323
EST_testis        ------------------------------------------------------------
                                           *                       *
I2                GAAGATGCCTGTAGTGACACCGAGGCCACTGAAGCCATGGCGCCAGACATCTTAGCCAGG 540
I2Lgene_chr5      GAAGATGCGTGTAGGGACACCGAGACCACTGAAGCCATGGCGCCAGACATCCTAGCCAAG 383
EST_testis        ------------------------------------------------------------
                         *               *                       *      *
I2                AAATTAGCTGCAGCTGAAGGCTTGGAGCCAAAGTATCGGATTCAGGAACAAGAAAGCAGT 600
I2Lgene_chr5      AAATTAGCTGCTGCTGAAGGCTTGGAGCCAAAGTACCGGATTCAGGAACAAGAAAGCAGT 443
EST_testis        ----------GGGCTGAAGGCTTGGAGCCAAAGTACCGGATTCAGGAACAAGAAAGCAGT 50
                            ********************** ************************
                                               *
I2                GGAGAGGAGGATAGTGACCTCTCACCTGAAGAACGAGAAAAAAAAGCGACAATTTGAAATG 660
I2Lgene_chr5      GGAGAGGAGGATAGTGACCTCTCACCTGAAGAACGAGAAAAAAAAGCGACAATTTGAAATG 503
EST_testis        GGAGAGGAGGATAGTGACCTCTCACCTGAAGAACGAGAAAAAAAAGCGACAATTTGAAATG 110
                  ************************************************************

I2                AAAAGGAAGCTTCACTACAATGAAGGACTCAATATCAAACTAGCCAGACAATTAATTTCA 720
I2Lgene_chr5      AGAAGGAAGCTTCACTACAATGAAGGACTCAATATCAAACTAGCCAGACAATTAATTTCA 563
EST_testis        AGAAGGAAGCTTCACTACAATGAAGGACTCAATATCAAACTAGCCAGACAATTAATTTCA 170
                  * ************************************************************
                   *
I2                AAAGACCTACATGATGATGATGAAGATGAAGAAATGTTAGAGACTGCAGATGGAGAAAGC 780
I2Lgene_chr5      AAAGACCTACATGATGATGATGAAGATGAAGAAATGTTAGAGACTGCAGATGGAGAAAGC 623
EST_testis        AAAGACCTACATGATGATGGTGAAGATGAAGAAATGTTAGAGACTGCAGATGGAGAAAGC 230
                  ****************** ***************************************

I2                ATGAATACGGAAGAATCAAATCAAGGATCTACTCCAAGTGACCAACAGCAAAACAAATTA 840
I2Lgene_chr5      ATGAATACGGAAGAATCAAATCAAGGATCTACTCCAAGTGACCAACAGCAAAACAAATTA 683
EST_testis        ATGAATACGGAAGAATCAAATCAAGGATCTACTCCAAGTGACCAACAGCAAAACAAATTA 290
                  ************************************************************

I2                CGAAGTTCATAGACGAGATTTGTTCAACACTGCAATTGTTTGTTAGATGTAAACCCTGTG 900
I2Lgene_chr5      CGAAGTTCATAGAAGAGATTTGTTCAACACTGCAATTGTTTGTTAGATATAAACCCTGTG 743
EST_testis        CGAAGTTCATAGAAGAGATTTGTTCAACACTGCAATTGTTTGTTAGATATAAACCCTGTG 350
                  ************ ********************************** ***********
                               *                                   *
I2                ACTATAGTACGTTGCTTCTTGTTCTTCACAATTCATGACTTAAGTACCAAAATGCATACC 960
I2Lgene_chr5      ACTATAATACATTGCTTCTTGTTCTCCACAATTCATGACTTAAGTACCAAAATGCATACC 803
EST_testis        ACTATAATACATTGCTTCTTGTTCTCCACAATTCATGACTTAAGTACCAAAATGCATACC 410
```

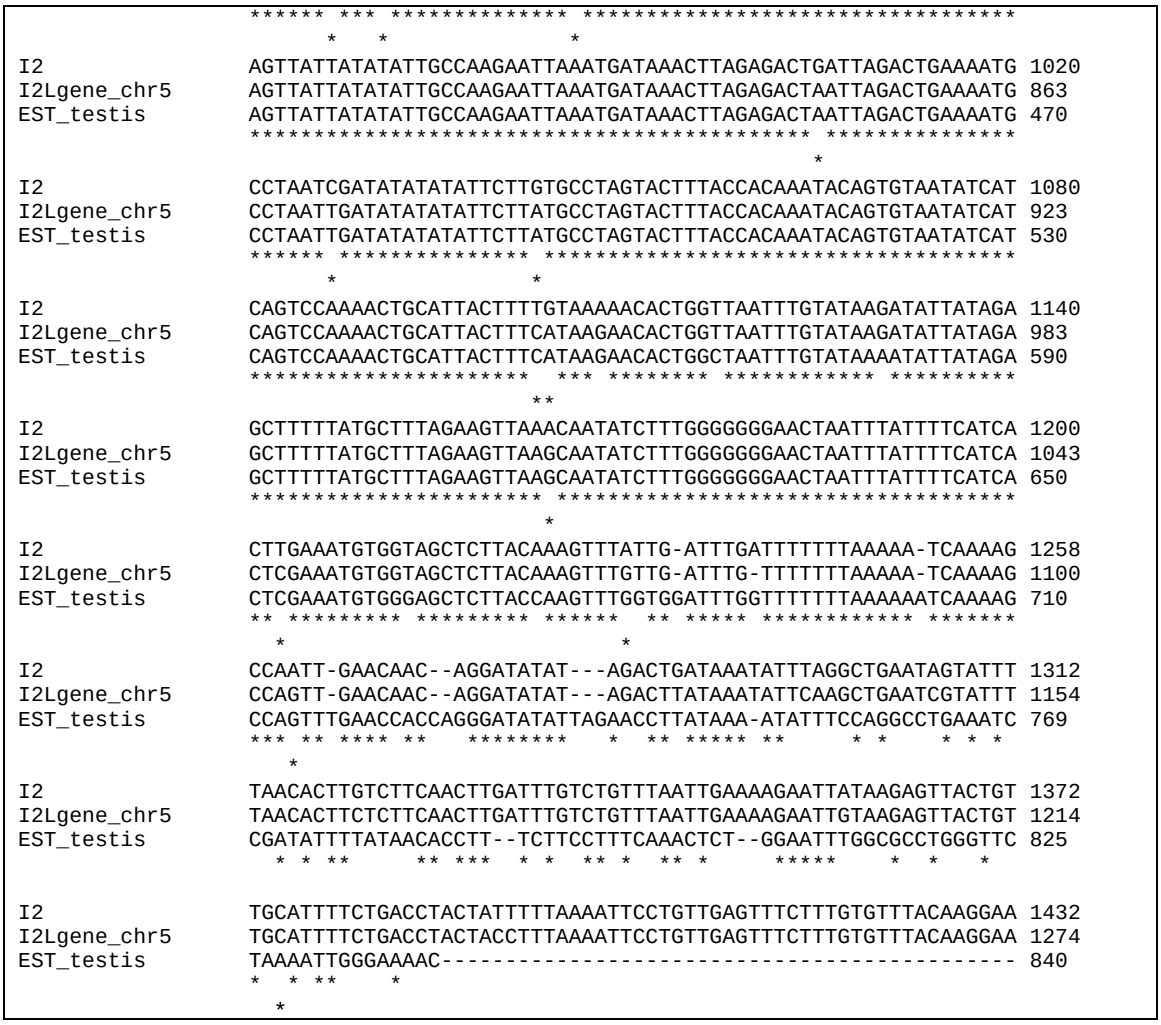

Figure III.1B. A human testis EST of I2L. ClustalW alignment of I2 (NM_006241) with the I2L gene sequence (NC000005) and a human testis EST sequence (BU854595). The second line * correspond to mismatches between I2 and I2L. The two-sequence alignment at the 3'-end is omitted. The initiation and stop codons are in bold. The alignment scores are indicated on top.

```
SeqA Name                       Len(aa)  SeqB Name                       Len(aa)  Score
1    I2_Human                   205      2    I2L_Human                  205      95
1    I2_Human                   205      3    I2L_Pan_Troglodytes_Pred   205      94
1    I2_Human                   205      4    I2L_Pigtailed_macaque_EST  150      95
2    I2L_Human                  205      3    I2L_Pan_Troglodytes_Pred   205      97
2    I2L_Human                  205      4    I2L_Pigtailed_macaque_EST  150      94
3    I2L_Pan_Troglodytes_Pred   205      4    I2L_Pigtailed_macaque_EST  150      92

I2_Human                  MAASTASHRPIKGILKNKTSTTSSMVASAEQPRGNVDEELSKKSQKWDEM 50
I2L_Human_chr5            MAASTASHRPIKGILKNKTSTTSSMVASAEQPRRSVDEELSKKSQKWDEI 50
I2L_Pan_Troglodytes       MAASTASHRPIKGILKNKTSTTSSMVAWAEQPRGSVDEELSKKSQKWDEI 50
I2L_Pigtailed_macaque     MAASTASHRPIKGILKNKTSTTSSMVASAEQPRGSVDEELSKKSQKWDEM 50
                          ************************** *****  ************** 
                                                          *              *
I2_Human                  NILATYHPADKDYGLMKIDEPSTPYHSMMGDDEDACSDTEATEAMAPDIL 100
I2L_Human_chr5            NILATYHPADKGYGLMKIDEPSPPYHSMMGDDEDACRDTETTEAMAPDIL 100
I2L_Pan_Troglodytes       NILATYHPADKGYGLMKIDEPSAPYHSMMGDDEDACRDTETTEAMAPDIL 100
I2L_Pigtailed_macaque     NILATYHPADKDYGLMKIDEPSPPYHSMMGDDEDACSDTETTEAMAPDIL 100
                          ********** ********** ************* *** *********
                                     *                        *
I2_Human                  ARKLAAAEGLEPKYRIQEQESSGEEDSDLSPEEREKKRQFEMKRKLHYNE 150
I2L_Human_chr5            AKKLAAAEGLEPKYRIQEQESSGEEDSDLSPEEREKKRQFEMRRKLHYNE 150
I2L_Pan_Troglodytes       AKKLAAAEGLEPKYRIQEQESRGEEDSDLSPEEREKKRQFEMRRKLHYNE 150
I2L_Pigtailed_macaque     ANKLAAAEGLEPKYRIQEQESSGEEDSDLSPEEREKKLLFEMKRKLHYYE 150
                          * ****************** ***************  *** ***** * 
                           *
I2_Human                  GLNIKLARQLISKDLHDDDEDEEMLETADGESMNTEESNQGSTPSDQQQN 200
I2L_Human_chr5            GLNIKLARQLISKDLHDDDEDEEMLETADGESMNTEESNQGSTPSDQQQN 200
I2L_Pan_Troglodytes       GLNIKLARQLISKDLHDDDEDEEMLETADGESMNTEESNQGSTPSDQQQN 200
I2L_Pigtailed_macaque     --------------------------------------------------

I2_Human                  KLRSS 205
I2L_Human_chr5            KLRSS 205
I2L_Pan_Troglodytes       KLRSP 205
I2L_Pigtailed_macaque     -----
```

Figure III.1C. Amino acid sequence comparison between human I2 and human and primate I2L. ClustalW alignment of human I2 (NP_006232), human I2L (Q6NXS1), *Pan troglodytes* I2L (XP_001134994) and translated *Pigtailed macaque* testis EST (nucleotide: DY743031). The second line * corresponds to mismatches between I2 and I2L. The known I2 phosphorylatable Thr and Ser and their I2L substitutions are in bold. The alignment scores are indicated on top.

Table III.1. Characteristic gene and primary protein sequence differences between I2 and I2L

I2	I2L
Chr3	Chr5
5 exons	1 exon
Thr73 (GSK3 site)	Pro73
Ser87 (CKII site)	Arg87
4 putative CKII sites	5 putative CKII sites
No PKC sites	3 putative PKC sites
No PKA sites	2 putative PKA sites

III.3.2 I2L AND I2 INTERACT SIMILARLY WITH THE VARIOUS PP1 ISOFORMs

I2L interaction *in vitro* with the different PP1 isoforms was confirmed using several techniques. The sequential co-transformation of yeast, confirmed the interaction of I2L with PP1α, PP1γ1, PP1γ2 and the unique C-terminal sequence of PP1γ2 (Fig. III.2A). In order to compare the binding of I2 and I2L to the two known PP1γ isoforms, a blot overlay analysis was performed. An immunoblot containing the same amount of I2 and His-I2L was incubated either with PP1γ1 or PP1γ2, and the interaction detected using PP1 isoform -pecific antibodies (Fig. III.2B). The binding of I2L by both PP1 isoforms was confirmed and did not appear to differ significantly in relation I2.

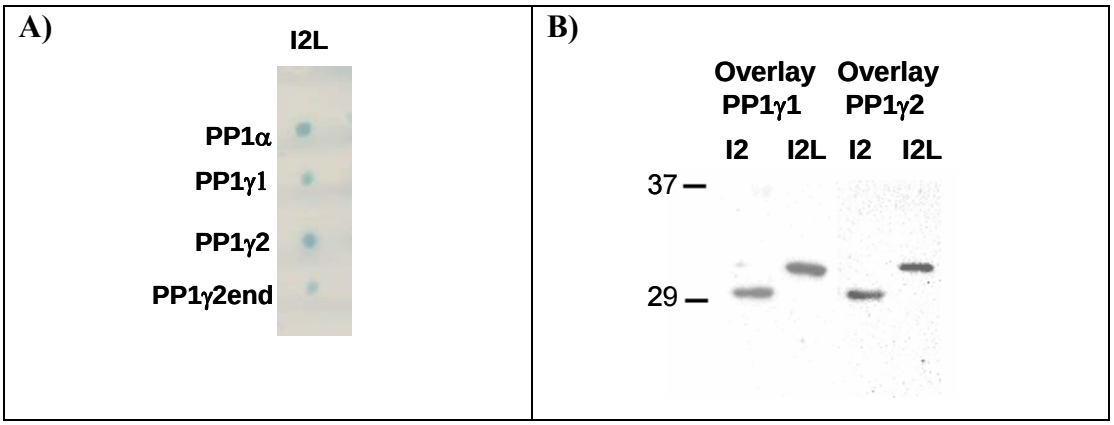

Figure III.2. Interaction of I2L with different PP1 isoforms. A) Sequential transformation of yeast AH109 with bait plasmid (either pAS2-PP1α, pAS2-PP1γ1, pAS2-PP1γ2 or pAS2-PP1γ2end) and the prey plasmid (pACT2-I2L). PP1γ2end is the unique C-terminal tail of PP1γ2 produced by alternative splicing of the PP1γ gene. **B)** Overlay immunoblot detection of I2 and His-I2L with PP1γ1 and PP1γ2. I2 (inhibitor 2) and I2L (His-tagged recombinant I2L) were separated by SDS-PAGE, transferred to nitrocellulose and overlaid with recombinant PP1γ1 or PP1γ2, as indicated. Immunoblotting was performed with the respective specific antibodies.

Since I2 is a potent inhibitor of PP1, we decided to determine if I2L also exhibited PP1 inhibitory activity, and if so, how its potency compared to that of I2. The IC_{50} values of I2L and I2 for both recombinants His-tagged PP1γ isoforms were determined using the phosphorylase phosphatase assay. The results presented in Table III.2 indicate that recombinant His-I2L is also a potent PP1 inhibitor, similar to I2, exhibiting IC_{50} values in the subnanomolar range.

Table III.2. Comparison of PP1γ isoform inhibition by I2 and I2L

	IC_{50} (nM)[a]	
Isoform	**I2**	**His-I2L**
His-PP1γ1	0.141 nM	0.73 ± 0.10
His-PP1γ2	0.145 nM	0.09 ± 0.08

[a] The values are expressed as the mean ± S.E.M. of at least three independent experiments.

III.3.3 I2L SUBCELLULAR DISTRIBUTION

In order to investigate its cellular distribution, I2L was fused on the N-terminal side of GFP and resulting expression vector was transfected into different mammalian cell lines (HeLa and COS-7 cells). After allowing sufficient time for the recombinant fusion I2L-GFP protein to be expressed, the cells were fixed and the nucleus was stained with DAPI (Fig. III.3). I2L-GFP appears to be relatively enriched in the nucleus of human HeLa cells, but excluded from the nucleolus. Similar results were obtained with monkey COS-7 cells (data not shown).

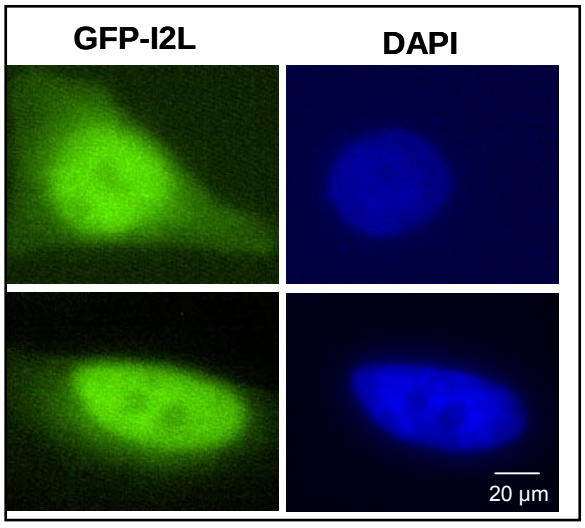

Figure III.3. I2L localization in HeLa cells. HeLa cells were transfected with I2L-GFP and the green fluorescence resulting from GFP was visualized in an epifluorescence microscope. DAPI was used to stain the nucleus.

III.3.4 COMPARISON OF I2 AND I2L PHOSPHORYLATION BY GSK3

The absence from I2L of the important I2 regulatory GSK3 phosphorylation site (Thr73) and CKII phosphorylation site (Ser87), led us to investigate the phosphorylation of both proteins by those kinases. Both I2 and I2L were incubated with GSK3 or CKII (or both), in the presence of radioactive-labeled ATP. I2 is known to be a poor *in vitro* substrate for GSK3, but if phosphorylated previously by CKII, synergistic phosphorylation by GSK3 can be elicited (Park et al., 1994). In our hands this was also observed (Fig. III.4). However, under the exact same conditions, GSK3 did not phosphorylate I2L and the synergistic effect produced by the previous phosphorylation with CKII was also not observed (Fig. III.4). The same experiments were also performed following pre-incubation of I2 and I2L with either PP1γ1 or PP1γ2. This resulted in a significant decrease in the level of phosphorylation of I2 by GSK3 and by both kinases compared to CKII alone. In contrast, the phosphorylation of I2L was unaffected by the pre-incubation with either PP1 isoform.

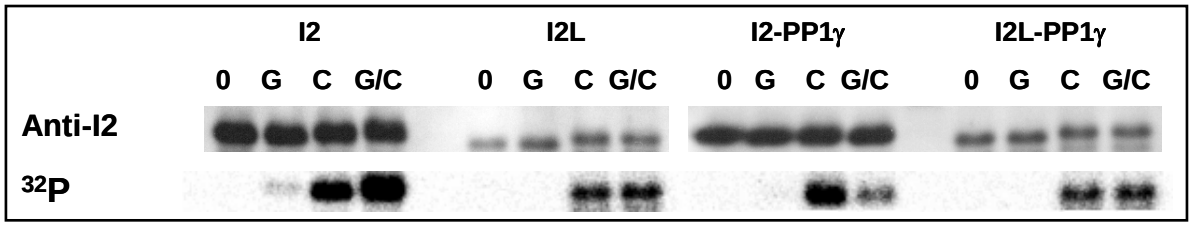

Figure III.4. Comparison of I2 and I2L phosphorylation by GSK3 and CKII. Recombinant I2 and I2L were incubated in kinase buffer with radioactive ATP (0); or in the presence of GSK3β kinase (G); or CKII kinase (C); or both kinases (G/C), for 90min at 30ºC. Protein phosphorylation was detected by autoradiography (^{32}P) following SDS-PAGE. Immunoreactivity detected with a specific anti-I2 antibody is shown for comparative purposes. The same experiment was repeated with a 10min pre-incubation of I2 and I2L with either PP1γ1 or PP1γ2.

III.3.5 PHOSPHORYLATION OF ENDOGENOUS I2/I2L PRESENT IN HUMAN SPERM

Immunoblot analysis of human sperm heat extract (HSI2) revealed two bands migrating above both I2 and I2L (Fig. III.5A). In order to investigate the nature of the observed relatively slow mobility of the endogenous proteins in comparison to their

recombinant counterparts, human sperm heat extract was separated by two-dimensional electrophoresis and transferred to nitrocellulose. Immunoblot analysis revealed three isolated spots of different apparent molecular mass and pI (Fig. III.5B), suggesting the possible occurrence of different phosphorylated forms of I2 and/or I2L in human sperm.

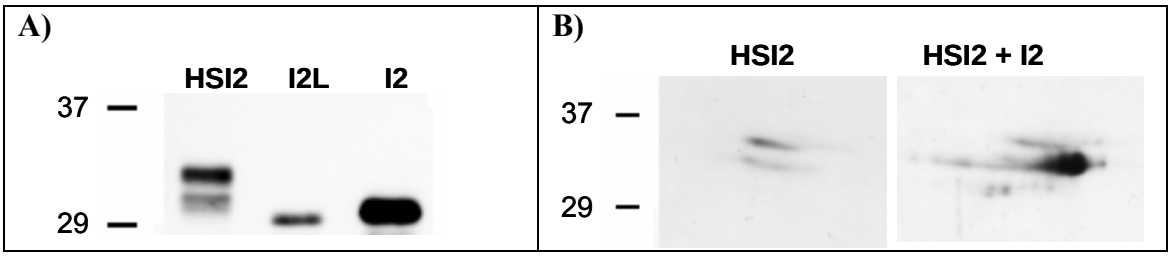

Figure III.5. Immunoblot analysis of endogenous I2/I2L from human sperm. A) Comparison of endogenous human sperm I2/I2L with recombinant I2 and I2L using an anti-I2 antibody. HSI2, Heat stable human sperm extract; I2L, Heat stable *E. coli* extract of recombinant I2L; I2, Purified recombinant I2. **B)** Anti-I2 immunoblot of 2D separation of heat stable human sperm extract. Left panel, Heat stable human sperm extract; Right panel, Heat stable human sperm extract supplemented with purified recombinant I2.

Besides the well known threonine and serine phosphorylation sites, I2 can also be phosphorylated tyrosine (Williams et al., 1995). Thus, in order to investigate whether the abnormal relative mobility of endogenous I2/I2L might be attributable to phosphorylation, we attempted to dephosphorylate the human sperm heat extract protein with a variety of phosphatases : protein tyrosine phosphatase 1B (PTP), calf intestinal phosphatase (CIP) and PP1γ1 (Fig. III.6). Under the conditions used, only CIP, an unspecific phosphatase, yielded significant dephosphorylation of HSI2, as judged by the observed increase in electrophoretic mobility. However, the observed mobility was still significantly slower than that of recombinant dephosphorylated I2 (Fig. III.6A). When HSI2 was incubated with GSK3 or CKII (or both), only GSK3 was able to induce a mobility shift, which was abolished by pre-incubation of HSI2 with purified recombinant PP1γ (Fig. III.6B).

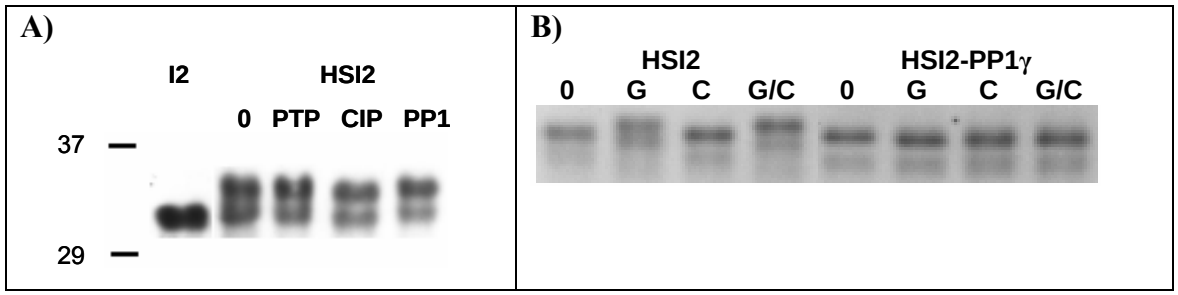

Figure III.6. Comparison of dephosphorylation and phosphorylation of endogenous human sperm I2/I2L. **A)** HSI2 was incubated in the presence of different phosphatases, in the respective buffers at 30ºC for 3h. 0, no phosphatase control; PTP, incubation with Protein Tyrosine Phosphatase 1B; CIP, incubation with Calf Intestinal Phosphatase; PP1, incubation with Protein Phosphatase 1γ1. **B)** HSI2 was incubated in the presence of GSK3 (G) or CKII (C), or both (G/C), as previously described. Resulting changes in protein migration, probably reflecting alterations in phosphorylation, were detected by immunoblot analysis using an anti-I2 antibody.

III.3.6 INTERACTION OF HUMAN SPERM ENDOGENOUS I2/I2L WITH PP1

An inhibitor-2 like activity can be measured and was reported to exist in mammalian sperm (Smith et al., 1996; Vijayaraghavan et al., 1996). Interestingly, human sperm PP1γ2 co-immunoprecipitates endogenous I2/I2L proteins that co-migrate with the two abnormally migrating species described above (Fig. III.7A). In addition, a third faster migrating species was also detected, although further work will be required to assess whether it may be the result of proteolytic degradation. Our results indicate that irrespective of their nature, all endogenous I2/I2L proteins bind and therefore probably regulate human sperm PP1γ2 (Fig. III.7A). Here we also demonstrate by immunocytochemistry the co-localization of I2/I2L with PP1γ2 in human sperm (Fig. III.7B). Like PP1γ2, I2/I2L was also detected in the sperm neck and tail, suggesting a potential regulatory role in the control of sperm motility.

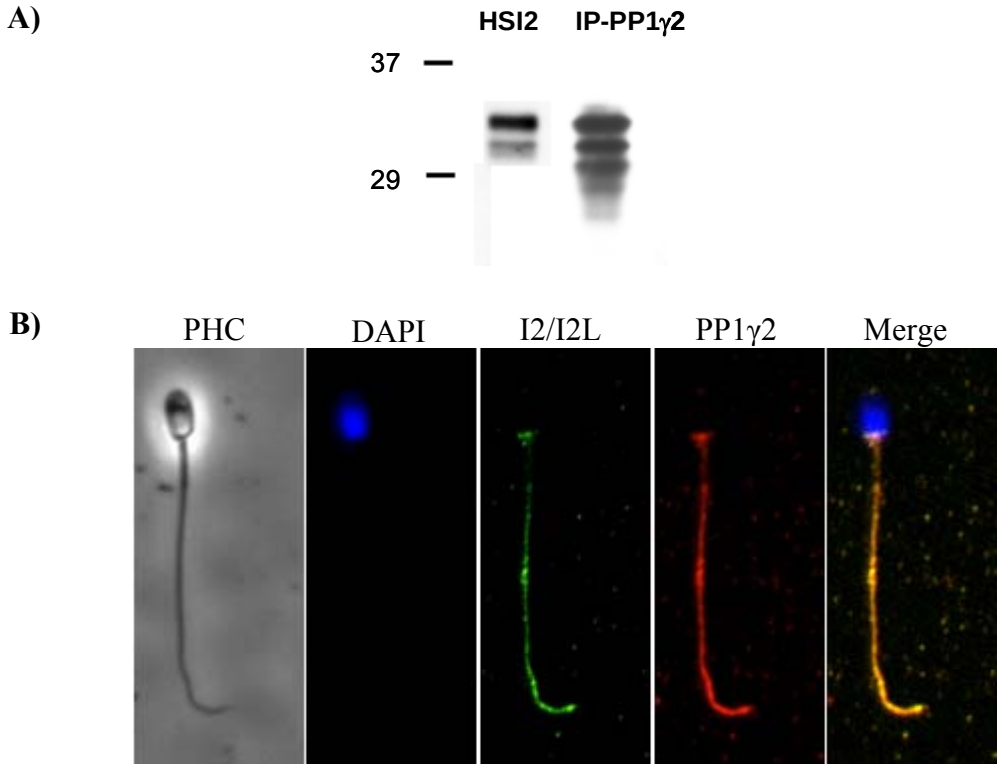

Figure III.7. Interaction of I2/I2L with PP1 in human sperm. A) HSI2, Heat stable human sperm extract; IP-PP1γ2, Anti-I2 immunoblot of the anti-PP1γ2 immunoprecipitate from human sperm. **B.** Co-localization of I2/I2L and PP1γ2 in human spermatozoa using anti-I2 and anti-PP1γ2 antibodies, and specific secondary antibodies conjugated with FITC and Texas Red, respectively. DAPI was used to stain the nucleus.

III.4 DISCUSSION

I2 and I1 were the first PP1 inhibitory regulators to be discovered (Huang and Glinsmann, 1976a). Here, we report the finding of a novel isoform of I2, which we termed I2L, corresponding to one of the nine Genbank entries identified as processed pseudogenes of I2 (Ceulemans et al., 2002). Processed pseudogenes (retropseudogenes) are sequences present in the genome similar to functional genes that resulted from the process of reverse transcription of mRNA transcript followed by integration into genomic DNA, presumably in the germ line (Maestre et al., 1995). Retropseudogenes are characterized by a complete lack of introns, functional promoters and other regulatory elements, thus commonly being thought to be nonfunctional gene copies (Mighell et al., 2000). Due to the release of selection pressure, over time, retropseudogenes accumulate deleterious mutations (stop codons or frameshifts) that will disrupt their ORF. Analysis of human genome retropseudogenes sequences showed that there was a burst of retroposition during the primate lineage and that approximately 18% were "intact", i.e. had no deleterious mutations in their ORF (Marques et al., 2005). Searching genomes database, we found I2L type sequences to occur solely in primate and human genomes, placing its origin at the above mentioned burst of retroposition in primates. Moreover, the I2L gene contains the complete ORF and is 95% similar to its parental gene, both at the nucleotide and protein level, suggesting a high functional constraint by natural selection. In addition to a few examples that started to emerge, recent studies have shown that a significant amount of retropseudogenes are expressed and have functional roles (retrogenes) (Marques et al., 2005; Vinckenbosch et al., 2006). Interestingly, when the expression patterns of retrogenes were experimentally determined, they were found to be expressed predominantly or exclusively in testis, whereas the parents genes are all expressed ubiquitously (Marques et al., 2005). A broader study found that the proportion of testis ESTs that mapped to retrogenes is over twice that of multiexon genes, supporting the notion that retrogenes emerge "out of the testis" (Vinckenbosch et al., 2006). Our data suggest that I2L is another such example, as we found an additional I2L transcript in the database (clone IMAGE in Fig. III.1A) following the identification of our cDNA isolate (clone 48), both originating from testis libraries, as well as an I2L testis EST (Fig. III.1B). I2L appears to be under the same low level of transcription tendency with high incidence in the testis of young

transcribed retrogenes that are absent from the mouse genome, in contrast to the high number of ESTs and lower testis transcription of older transcribed retrogenes that have an ortholog in the mouse (Vinckenbosch et al., 2006). These facts can be explained by the initial facilitated transcription of retrogenes in the testis enabling them to obtain a functional role in this tissue, which is known to evolve rapidly (Swanson and Vacquier, 2002). Although we could not find a TATA box or a promoter enhancer in the immediate 5' vicinity of the I2L start codon, further analysis and more distant scanning will be required to assess this question. However, retrogene transcription is often driven by nearby genes, one of the mechanisms being the insertion into chromosomal domains that favour transcription through long-range regulatory sequences (Spitz et al., 2005; Vinckenbosch et al., 2006). Both I2L transcripts identified (ours and the database) possess poly(A) tails but inserted at different sites each apparently having used different polyadenylylation signals (Fig. III.1A). ATTAAA for clone 48 and possibly AATATT for the Genbank clone. Both are slightly atypical variants of the canonical AATAAA poly(A) signal, which usually are associated with tissue-specific polyadenylation (Zhao et al., 1999). Interestingly, AATTAAA is the most frequent variant of the typical poly(A) signal and is highly used in germ cell transcripts (Wallace et al., 1999; Zhao et al., 1999).

As I2L is highly similar to I2 and with mostly single aminoacid substitutions, the available techniques for protein differentiation are very restricted. However, due to the Ser87 substitution for an arginine, in future work should be possible to identify I2L specific trypsin digested peptides from human sperm samples through mass spectrometry.

Not surprisingly, I2L retains the characteristic I2 interaction with PP1, as evaluated through binding and phosphatise inhibition assays (Fig. III.2 and Table III.2). This is due to the high degree of sequence conservation observed over the three well characterized I2 interaction regions with PP1 (Fig. III.1C): ^{11}IKGILKN^{14}, the main motif responsible for the PP1 inhibitory activity of I2; ^{43}KSQKW^{47}, that binds to the RVxF PP1 groove; and residues 147-169, which bind to the PP1 active site and occupy the hydrophobic substrate binding groove (Hurley et al., 2007).

I2 contains both a putative nuclear localization signal (^{137}KKRQFEMKRK^{147}) and a sequence resembling a leucine-rich nuclear export signal (^{155}LNIKLARQLI^{165}), and it can differentially localize to the cytoplasm or nucleus in different phases of the cell cycle of human HS68 fibroblast cells (Kakinoki et al., 1997). I2L exhibits a conserved substitution

of Lys145 for Arg, that therefore does not affect significantly the putative nuclear localization signal of I2L. Indeed, the functionality of this signal appears to be confirmed since I2L-GFP was observed to be relatively enriched in the nucleus of transfected cell lines (Fig. III.3). During mitosis, I2 has been reported to be localized in the centrosome and to be highly phosphorylated at Thr73 (Leach et al., 2003), suggesting that reversible phosphorylation of I2 may control centrosome-associated PP1 activity. As I2L is missing the important regulatory Thr73, it can not be phosphorylated at this site like I2 and it remains to be seen how this affects its localization in centrosomes during mitosis. Indeed, the most significant difference between I2L and I2 is the absence of the phosphorylatable residues Thr73 and Ser87, as these are the main players in the regulation of the PP1-I2 complex. We found that, as expected and contrary to I2, I2L is not phosphorylated by GSK3. Further, although I2L is phosphorylated by CKII, no synergistic effect was observed in the presence of the two kinases (Fig. III.4), as is characteristic for I2. Although I2L contains two different putative CKII sites from I2 (Table III.1), our results indicate therefore that those can not functionally substitute the missing Ser87. Pre-incubation of I2 with PP1 either prevents the phosphorylation by GSK3 or, more likely, dephosphorylates Thr73 (Fig. III.4). Thus, under the same conditions, I2L phosphorylation is not affected by the presence of PP1. This *in vitro* result does not represent clearly the *in vivo* dynamic process of phosphorylation/dephosphorylation in the complex GSK3/I2/PP1, but suggests that PP1 can affect it own regulation by I2, through the Thr73, but not the regulation by I2L.

The 1D and 2D immunoblot analysis of human sperm samples suggested that endogenous I2/I2L may occur in different phosphorylated forms (Fig. III.5). However, *in vitro* assays of dephosphorylation of human sperm I2/I2L by several phosphatases and of phosphorylation by GSK3 and CKII suggested that PP1 does not dephosphorylate the human sperm I2/I2L and that GSK3, but not CKII, is capable of phosphorylating it (Fig. III.6). Thus, the results obtained are consistent with the occurrence of I2 protein in human sperm, but did not allow us to reach any conclusions regarding the occurrence or not of the I2L protein.

Through immunoprecipitation and immunocytochemistry we showed that human sperm PP1γ2 interacts endogenously with I2/I2L and that they co-localize in the sperm tail (Fig. III.7), consistent with a regulatory role for PP1γ2 and I2/I2L in the control of sperm

motility. Indeed, since sperm are terminally differentiated cells essentially devoid of transcriptional and translational activity, 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. The absence from I2L of the most important regulatory residues of I2 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 GSK3 phosphorylation. However, further work will be required to determine whether I2L may be subject to an alternative regulatory mechanism. Indeed, since I2L has putative PKA phosphorylation sites not present in I2 may represent such an alternative regulatory mechanism. PKA is known to have an important role in mammalian sperm capacitation and hyperactivation of sperm motility in the female reproductive tract by regulating downstream tyrosine phosphorylation events (Burton and McKnight, 2007). Nevertheless, by regulating I2L it would also be regulating the effect of PP1 in human sperm hyperactivation motility (Si and Okuno, 1999). It is interesting to speculate that PP1γ2 inhibition by I2L may represent an irreversible, unidirectional event, essentially paralleling the acquisition of motility as human sperm travel through the epidydimis.

The notion that newly emerged retrogenes may evolve new functional roles through adaptive evolution of encoded proteins and/or by developing new spatial or temporal expression patterns, leads us to suggest that I2L expression in testis favours human sperm motility and enhances the chances for successful reproduction.

CHAPTER IV

ALTERNATIVE SPLICING CONTROLS NUCLEAR TRANSLOCATION OF THE CELL CYCLE REGULATED NEK2 KINASE

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IV ALTERNATIVE SPLICING CONTROLS NUCLEAR TRANSLOCATION OF THE CELL CYCLE REGULATED NEK2 KINASE

IV.1 INTRODUCTION

Cell division involves the replication and segregation of genetic material between daughter cells. During mitotic and the second meiotic division, duplicated sister chromatids are segregated, whereas during the first meiotic division homologous chromosomes are segregated. Mitosis and meiosis are both highly complex processes involving condensation of chromatin, breakdown of the nuclear envelope and reorganization of the microtubule cytoskeleton into a spindle capable of bipolar segregation. These events are known to be regulated, in large part, by cell cycle-dependent protein kinases, most notably members of the Cdk (cyclin-dependent kinase), Plk (polo-like kinase), Aurora and Nek (NIMA-related kinase) families (Nigg, 2001).

The Neks represent a comparatively large family (11 members in humans) of serine/threonine kinases that are related to the NIMA kinase of *Aspergillus nidulans* (O'Connell et al., 2003). NIMA was first identified in a screen for conditional loss-of-function mutants that were unable to enter mitosis (Oakley and Morris, 1983). *nimA* mutants remain blocked in G2 despite activation of Cdk1 (Osmani et al., 1991a), while overexpression of wild-type NIMA drives cells into mitosis from any point in the cell cycle with premature condensation of chromosomes and spindle formation (Osmani et al., 1988). Interestingly, overexpression of *Aspergillus* NIMA in fission yeast, *Xenopus* oocytes and cultured human cells induces aspects of a premature mitosis, including condensation of chromosomes, suggesting that similar pathways may be conserved in higher eukaryotes (Lu and Hunter, 1995; O'Connell et al., 1994). More recently, it has been reported that NIMA activity is essential forphosphorylation of histone H3 thereby promoting chromatin condensation in *Aspergillus* (De Souza et al., 2000).

So far, it remains unclear which, if any, of the human Neks represents the closest functional homologue of NIMA. Mitotic functions have been proposed for Nek2, Nek6, Nek7 and Nek9 (a.k.a. Nercc1) (Belham et al., 2003; Faragher and Fry, 2003; Roig et al., 2002; Yin et al., 2003). Nek2 is the most closely related of the human proteins to NIMA

based on amino acid conservation within the N-terminal catalytic kinase domain (Fry, 2002). However, depletion studies in human cells do not support an essential role for Nek2 in mitotic entry (Fletcher et al., 2005). Rather there is strong evidence that, in mitotic cells, Nek2 contributes to centrosome separation, and perhaps reorganization of the microtubule network, at the G2/M transition (Faragher and Fry, 2003; Fry et al., 1998b; Rapley et al., 2005). Nek2 may have additional roles in chromatin condensation, at least in meiotic cell division, as it has been shown to phosphorylate the chromatin associated protein, high mobility protein A2 (HMGA2), and be activated during the Erk1/p90Rsk2-stimulated condensation of chromatin in mouse pachytene spermatocytes (Di Agostino et al., 2004b; Di Agostino et al., 2002).

In human cultured cells, Nek2 is predominantly expressed as two alternative splice variants, Nek2A and Nek2B (Hames and Fry, 2002). These differ at their non-catalytic carboxyl (C)-termini as a result of an alternative polyadenylation signal within intron 7 which prevents splicing to the terminal exon 8 in Nek2B. Nek2A therefore encodes a protein of 445 amino acids (48 kDa), while Nek2B encodes a protein of 384 amino acids (44 kDa) with the sequence diverging after amino acid 370. Downstream of the kinase domain, both Nek2A and Nek2B share a leucine zipper motif (amino acids 304-340) that promotes dimerization, autophosphorylation and activation of the kinase (Fry et al., 1999; Rellos et al., 2007). This is followed by a short motif (amino acids 333-370) shown to be required for centrosome targeting and microtubule binding (Hames et al., 2005). However, the position of splicing means that a binding site for PP1 (protein phosphatase 1; amino acids 383-386), and two APC/C-dependent degradation motifs, a KEN box (amino acids 391-399) and an MR-tail (amino acids 444-445), are present in Nek2A, but missing from Nek2B (Hames et al., 2001; Hayes et al., 2006; Helps et al., 2000). This falls in line with observations that Nek2A is degraded upon mitotic entry, whereas Nek2B remains stable. It also suggests that Nek2B may not be subject to regulation by PP1, although this hypothesis is complicated by the fact that Nek2A and Nek2B can heterodimerize via their leucine zippers, at least *in vitro* (Hames and Fry, 2002).

Recently, a third splice variant of Nek2 was identified during a yeast two hybrid screen using PP1γ1 and PP1γ2 as baits (Fardilha et al., 2004b). This isoform is identical to Nek2A except that it lacks amino acids 371-378. This 8 amino acid deletion starts at the splice donor position of the Nek2ANek2B splice site but uses a downstream splice

acceptor sequence within exon 8. This variant was originally named Nek2A-T as it was isolated from testis mRNA. However, we prefer now to call it Nek2C because Nek2A is also expressed in the testis (Arama et al., 1998; Fardilha et al., 2004b; Rhee and Wolgemuth, 1997; Tanaka et al., 1997) while Nek2C is clearly not exclusively expressed in testis (see results). In this study, we investigated the properties of Nek2C in comparison to those of Nek2A and Nek2B. As one would predict from the position of the regulatory motifs described above, Nek2C is an active kinase that can undergo autophosphorylation, bind microtubules, localize to the centrosome and be degraded in early mitosis. However, upon overexpression, there is a significant increase in accumulation of Nek2C in the nucleus, as compared to Nek2A or Nek2B, and, through mutagenesis, we show that this is the result of the 8 amino acid deletion creating a strong nuclear localization sequence (NLS) in Nek2C, that is weaker in Nek2A and absent in Nek2B. We speculate that the nuclear accumulation of Nek2C may be essential to catalyze the phosphorylation of nuclear substrates that promote mitotic or meiotic entry.

IV.2 MATERIAL AND METHODS

IV.2.1 PLASMID CONSTRUCTION

pCMV-mycNek2A, pCMV-mycNek2A-K37R and pCMV-mycNek2B have been previously described (Fry et al., 1998b; Hames and Fry, 2002). To generate pCMVmycNek2C, a C-terminal fragment was excised from pCMV-mycNek2A with *BspE*I and *Xba*I restriction enzymes and replaced with the corresponding fragment from pACT-Nek2C (Fardilha et al., 2004b). Since *BspE*I is blocked by overlapping *dam* methylation, *dam- E. coli* JM110 were used for transformation. Mutations were introduced using the Gene Tailor^{TM} Site-Directed Mutagenesis System (Invitrogen, Paisley, U.K.). The GST-PP1α and His-Nek2A-CTD bacterial expression constructs were generated by subcloning human PP1α from pBS-mycPP1α into the pGEX-4T1 vector, and amino acids 255-445 of Nek2A into the pET22b vector, respectively. All constructs were confirmed by DNA sequencing by Lark Technologies, Inc. (Saffron Walden, U.K.).

IV.2.2 CELL CULTURE, TRANSFECTION AND EXTRACTION

All media and additives were obtained from Invitrogen. Human cervical epithelia HeLa cells were cultured in Minimal Essential Media with 1% Non-Essential Amino Acids, 10% heatinactivated Fetal Bovine Serum (FBS) and 1% antibiotic/antimycotic (AA) mix. Human osteosarcoma U2OS cells and human embryonic kidney cells, HEK 293, were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and 1% AA. Cells were cultured at 37ºC in a 5% CO2 atmosphere. Transient transfections were carried out using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cell extracts were prepared as previously described (Fry and Nigg, 1997).

IV.2.3 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was isolated from cultured cells using TRI Reagent (Sigma, Poole, U.K.) according to manufacturer's instructions. Extracted RNA (5 μg) was used for first strand

synthesis using SuperScript II reverse transcriptase (Invitrogen) and oligo-(dT)20 at 50ºC for 60 mins. PCR amplification of Nek2A and Nek2C specific fragments of 153 bp and 129 bp, respectively, was performed in a 50 µl reaction using Taq polymerase (Invitrogen), 2 µl of first strand cDNA synthesis mixture as template and the following primers: 1077F: 5'- GGAACGGAAGTTCCTGTC-3' and 1229R: 5'-CACTTGGACTTA GATGTGA-3'. Amplified products were purified using a PCR purification kit (Qiagen Ltd., Crawley, U.K.), subcloned into a pBlueScript vector, linearized with *Sma*I, and sequenced.

IV.2.4 IN VITRO TRANSLATION (IVT)

Nek2, Rab4 and Lamin A proteins were generated by IVT from appropriate vectors using the TnT-coupled transcription/translation kit (Promega, Southampton, U.K.) according to manufacturer's instructions. For radioactive detection of proteins, reactions were carried out in the presence of [^{35}S]-methionine, separated by SDS-PAGE and exposed to X-ray film.

IV.2.5 IMMUNOPRECIPITATION (IP)-KINASE ASSAYS

For IP of proteins generated by IVT, 10 µl prewashed protein G-sepharose beads (Sigma) were added to 490 µl Neb buffer (Fry and Nigg, 1997) containing 9 µl non-radioactive IVT reaction and mixed on a rotating wheel for 30 mins at 4ºC, before centrifugation at room temperature (RT) for 10 secs. The pre-cleared supernatant was transferred to a fresh tube, 1 µl mouse anti-myc monoclonal antibody (Cell Signaling Technology, Beverly, Mass.) was added and incubated on ice for 60 mins. Meanwhile, 40 µl protein G-sepharose beads were blocked with 5 µl rabbit reticulocyte lysate in 500 µl Neb buffer on a rotating wheel for 30 mins. The blocked beads were centrifuged at RT for 10 secs, the supernatant removed and the beads washed three times with 100 µl Neb buffer. The blocked beads were resuspended in 50 µl Neb buffer and added to the antibody-IVT mixtures. This bead slurry was placed on a rotating wheel for 60 mins at 4ºC, before centrifugation for 10 secs at RT. The supernatant was removed and the beads washed twice with 100 µl Neb buffer and finally resuspended in 100 µl Neb buffer. A 25 µl aliquot was transferred to a fresh tube for Western blot analysis of the efficiency of the IP. The

remaining 75 µl aliquot was spun down and the bead pellet used for kinase assays. For IP of cell lysates, essentially the same protocol was followed except that to pre-clear the extract, 50 µl pre-washed protein G-sepharose beads were added to 40 µl cell lysate diluted in 500 µl icecold Neb buffer, and then 30 µl protein Gsepharose beads were added to the antibodyextract mixtures. Immune complexes generated by IP were washed three times with 100 µl kinase assay buffer (Fry and Nigg, 1997). The beads were resuspended in a final volume of 50 µl kinase assay buffer (containing ^{32}P-γ-ATP and β-casein), and incubated at 30ºC for 30 mins. The reaction was stopped by addition of 50 µl 2x Laemmli sample buffer. After separation by SDS-PAGE, the gel was stained with Coomassie Brilliant Blue, dried and exposed to X-ray film.

IV.2.6 PP1 BINDING, DIMERIZATION AND MICROTUBULE BINDING ASSAYS

GST, GST-PP1α and His-Nek2A-CTD were expressed in BL21 *E. coli* and purified from bacterial lysates on glutathione-sepharose (GE Healthcare, Chalfont St. Giles, U.K.) or Ni^{2+}-NTA agarose beads (Invitrogen) according to standard protocols. Purified proteins were then dialysed against PBS for storage at -800C. For pull-down assays, purified proteins were rebound to glutathione-sepharose or Ni^{2+}-NTA agarose beads for 60 mins followed by three washes with NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris.HCl, pH 8, 0.5% Nonidet-P40). The His-Nek2A-CTD bead complexes were blocked by first washing three times with 0.5% Marvel in PBS and then incubating with rabbit reticulocyte lysate for 30 mins, before three washes in NETN buffer. Protein-bead complexes were then incubated with IVT reaction mixes for 60 mins, before washing three times in NETN buffer containing 450 mM NaCl. Bound proteins were analysed by SDS-PAGE and autoradiography. Microtubule pull-down assays with proteins generated by IVT were performed as previously described (Hames et al., 2005).

IV.2.7 DEGRADATION ASSAYS

Preparation of *Xenopus laevis* CSF egg extracts, *in vitro* degradation assays and addition of MG132 were performed as previously described (Hames et al., 2001).

Generation of S- and M-phase arrested U2OS cells was also as described (Hames et al., 2001).

IV.2.8 IMMUNOFLUORESCENCE MICROSCOPY

Cells grown on glass coverslips were fixed at -20ºC for 30 mins using 100% methanol (precooled at -20ºC). Antibody staining and epifluorescence microscopy was performed as previously described (Faragher and Fry, 2003; Hames et al., 2005). Primary antibodies used were rabbit anti-γ-tubulin (0.15 g/ml; Sigma), mouse anti-myc (1:2,000; Cell Signaling Technology), anti-PP1α antibodies (Amador et al., 2004), rabbit anti-Nek2 (2 g/ml; Invitrogen) and mouse antiphospho- H3 (1:400; Cell Signaling Technology) and mouse anti-nucleophosmin (1:500; (Shu et al., 2005)). Secondary antibodies were Alexa 488-conjugated goat anti-rabbit and Alexa 594-conjugated goat anti-mouse secondary antibodies (1 g/ml; Invitrogen). DNA was stained with 1 μg/ml DAPI (4,6-diamidino-2-phenylindole) or 0.2 μg/ml Hoechst 33258 dye (Calbiochem, San Diego, Calif.), incubated together with the secondary antibody. Coverslips were mounted on slides in a drop of FluoroGuard anti-fade reagent (Bio-Rad, Hemel Hempstead, U.K.). Images were captured on Nikon TE300 or Olympus IX81 fluorescence microscopes. To score whether transfected cells had predominantly nuclear accumulation of recombinant protein, cytoplasmic accumulation or relatively equal distribution, pixel levels were measured in representative areas of the nucleus and cytoplasm using analySIS software. If these values were >10% different, then the cell was judged to have the protein predominantly in one compartment or the other.

IV.2.9 NUCLEAR-CYTOPLASMIC FRACTIONATION

Nuclear and cytoplasmic fractions of HeLa cells transfected with myc-tagged Nek2 constructs was performed using the Nuclear/Cytosol Fractionation Kit (Cambridge BioScience Ltd., Cambridge, U.K.) according to manufacturer's instructions. Western blots were performed as described (Faragher and Fry, 2003) using anti-myc (1:1,000; Cell Signaling Technology), anti-Lamin A/C (1:30; Chemicon, Chandlers Ford, U.K.) and anti-γ-tubulin (1:3,000; Sigma) antibodies.

IV.2.10 KINASE SUBSTRATE TRACKING AND ELUCIDATION (KESTREL) ANALYSIS

HEK 293 cells were lysed in extraction buffer (EB; 50 mM Tris.HCl, pH 7.5, 5% glycerol, 1% Tritin X-100, 14 mM β-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 10 g/ml leupeptin, 1 mM Pefabloc) and the nuclear fraction sedimented by centrifugation for 20 min at 15,000×g. The nuclear pellet was incubated for 20 min with EB + 0.5 M NaCl and protein extracted by sedimenting insoluble material by centrifugation for 20 min at 28,000×g. The supernatant was filtered, desalted on Sephadex G25 fine and chromatographed on heparin sepharose. Aliquots of the desalted extracts and eluted fractions were diluted 10-fold in 50 mM Tris.HCl, pH 7.5, 7.5 mM β-mercaptoethanol, 1 mM EGTA, 10 g/ml leupeptin, 1 mM Pefabloc). These aliquots were incubated for 5 mins with 3 mM $MnCl_2$, 2 KBq/ml [γ-^{32}P]-ATP in the absence or presence of Nek2, Nek6, Nek7 or Nek9 recombinant kinases (Invitrogen) and then separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane and autoradiographed.

IV.3 RESULTS

IV.3.1 EXPRESSION OF NEK2C IN HUMAN CELL LINES

Three splice variants of the cell cycle-regulated Nek2 protein kinase have been described, Nek2A, Nek2B and Nek2C (originally called Nek2A-T; Fig. IV.1A). To confirm the expression of Nek2C, primers were designed for use in RT-PCR analysis that spanned the splice site and, when used on plasmids containing Nek2A and Nek2C cDNAs, amplified distinct bands of 153 bp and 129 bp, respectively (Fig. IV.1B). Using these primers, a major band of 153 bp, representing Nek2A, was amplified using mRNA isolated from HeLa, U2OS and HEK 293 human cell lines. However, a 129 bp band, at the expected size of Nek2C, was also reproducibly detected, although this was ~10-fold less abundant (Fig. IV.1B & C). To confirm the identity of this band, it was excised from the gel, re-amplified using the same primers, purified and subcloned into a pBlueScript vector. DNA sequencing confirmed that the 129 bp product contains the predicted sequence of Nek2C and lacks the 24 nucleotides present in Nek2A (Fig IV.1D). Hence, Nek2C is a *bona fide* splice variant whose expression can be detected, albeit consistently more weakly than Nek2A, in different human cell lines.

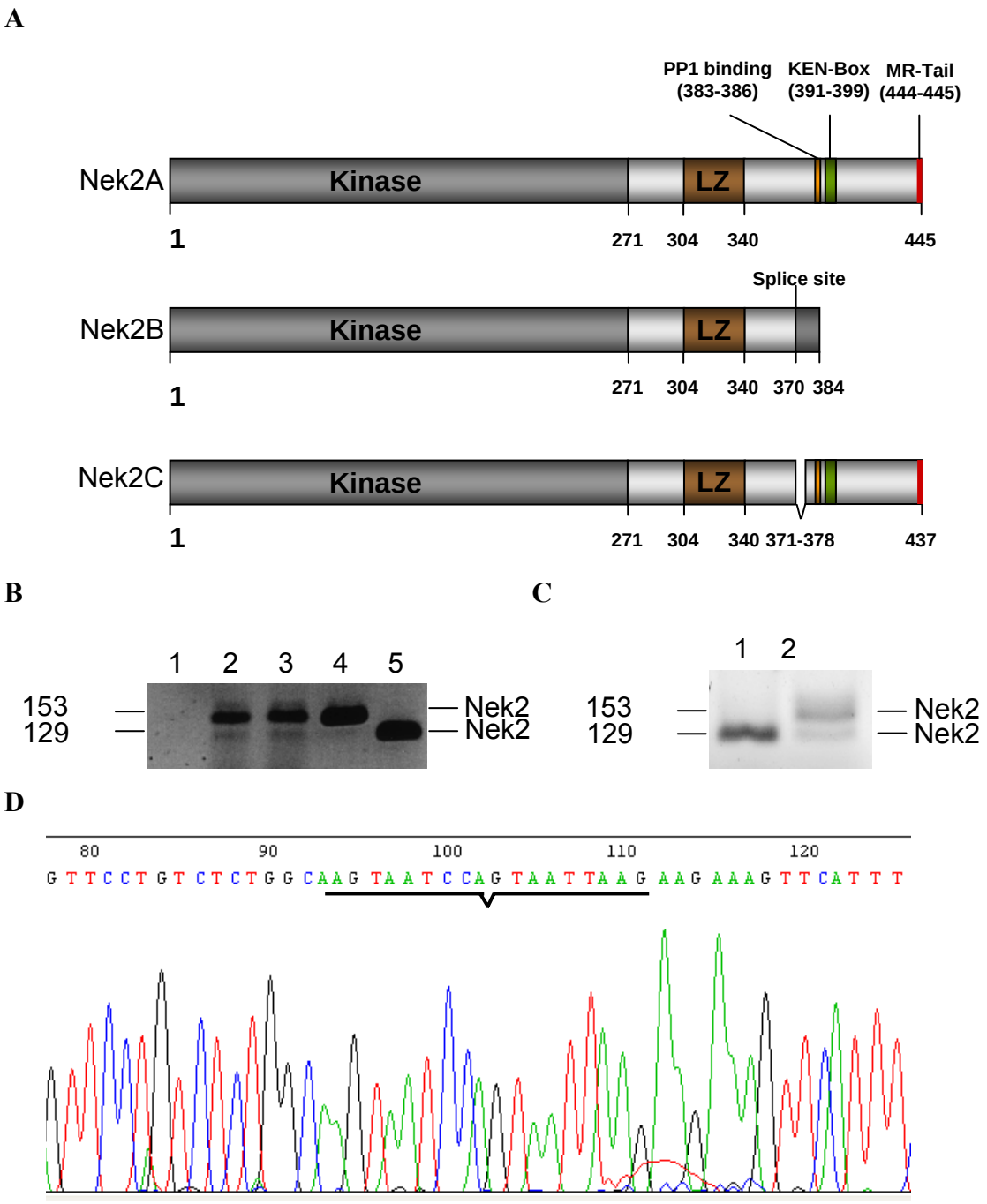


Figure IV.1. Expression of Nek2C in human cell lines **A.** Schematic representation of the Nek2A, Nek2B and Nek2C proteins highlighting the positions of the catalytic domain (Kinase), leucine zipper motif (LZ), PP1 binding site, KEN-box and MR-tail. Amino acids numbers are indicated underneath. **B**. Agarose gel analysis of RT-PCR reactions performed on RNA extracted from cell lines or Nek2-containing plasmids. No RNA (*lane 1*), HeLa mRNA (*lane 2*), U2OS mRNA (*lane 3*), pCMV-mycNek2A plasmid (*lane 4*) and pCMV-myc-Nek2C plasmid (*lane 5*). Position of the Nek2A (153 bp) and Nek2C (129 bp) products are indicated. **C**. The 129 bp band from the HeLa RT-PCR reaction was excised and re-amplified using the same primers (*lane 1*). RT-PCR reaction products from mRNA extracted from HEK 293 cells were separated alongside for comparison (*lane 2*). **D**. Following subcloning into pBlueScript, the 129 bp product from HeLa cells was sequenced. The resulting chromatogram confirms the expected sequence for Nek2C. Nek2A has an additional 24 nucleotides inserted at the position indicated (arrow).

IV.3.2 NEK2C IS AN ACTIVE KINASE THAT BINDS PP1A AND UNDERGOES DIMERIZATION

Nek2C contains the complete catalytic kinase domain (Fig. 1A). It also contains the leucine zipper motif that is required for dimerization, and autophosphorylation, and the PP1 binding site (Fry et al., 1999; Helps et al., 2000). However, the 8 amino acid deletion removes two serine residues (S377 and S378) present in Nek2A that could act as sites for regulation (Fardilha et al., 2004b). First, to compare Nek2A and Nek2C kinase activity, full-length myc-tagged Nek2C, Nek2A and Nek2A-K37R (catalytically inactive mutant) proteins were either translated *in vitro* (data not shown) or expressed in HEK 293 cells by transient transfection (Fig. IV.2A). Nek2 proteins were then immunoprecipitated with anti-myc antibodies and equal protein recovery confirmed by Western blotting with an anti-Nek2 antibody. Kinase assays performed on the immune complexes revealed that Nek2AK37R was inactive as expected, whereas Nek2A and Nek2C were both capable of phosphorylating the control substrate β-casein to a similar extent (Fig. IV.2A, and data not shown). Equivalent levels of autophosphorylation of Nek2A and Nek2C were also detected.

Second, to test PP1 binding, a GST-PP1α fusion protein was expressed in *E. coli* and used to pull-down *in vitro* translated proteins (Fig. IV.2B). Both Nek2A and Nek2C proteins bound GST-PP1α but not GST alone. In contrast, Nek2B, which lacks the PP1 binding site, Nek2A-F386A, which has a mutation in the PP1 binding site, and Lamin A did not bind GST-PP1α. Third, to test dimerization, *in vitro* translated Nek2A, Nek2B, Nek2C and the control protein Rab4, were mixed with a His-tagged Nek2A C-terminal domain fusion protein that contains the entire non-catalytic domain including the leucine zipper motif. As predicted, all Nek isoforms, but not Rab4, were pulled down by nickel-affinity chromatography (Fig. IV.2C). Hence, the 8 amino acid deletion in the non-catalytic domain does not interfere with the kinase activity, PP1 binding or dimerization of the Nek2C protein.

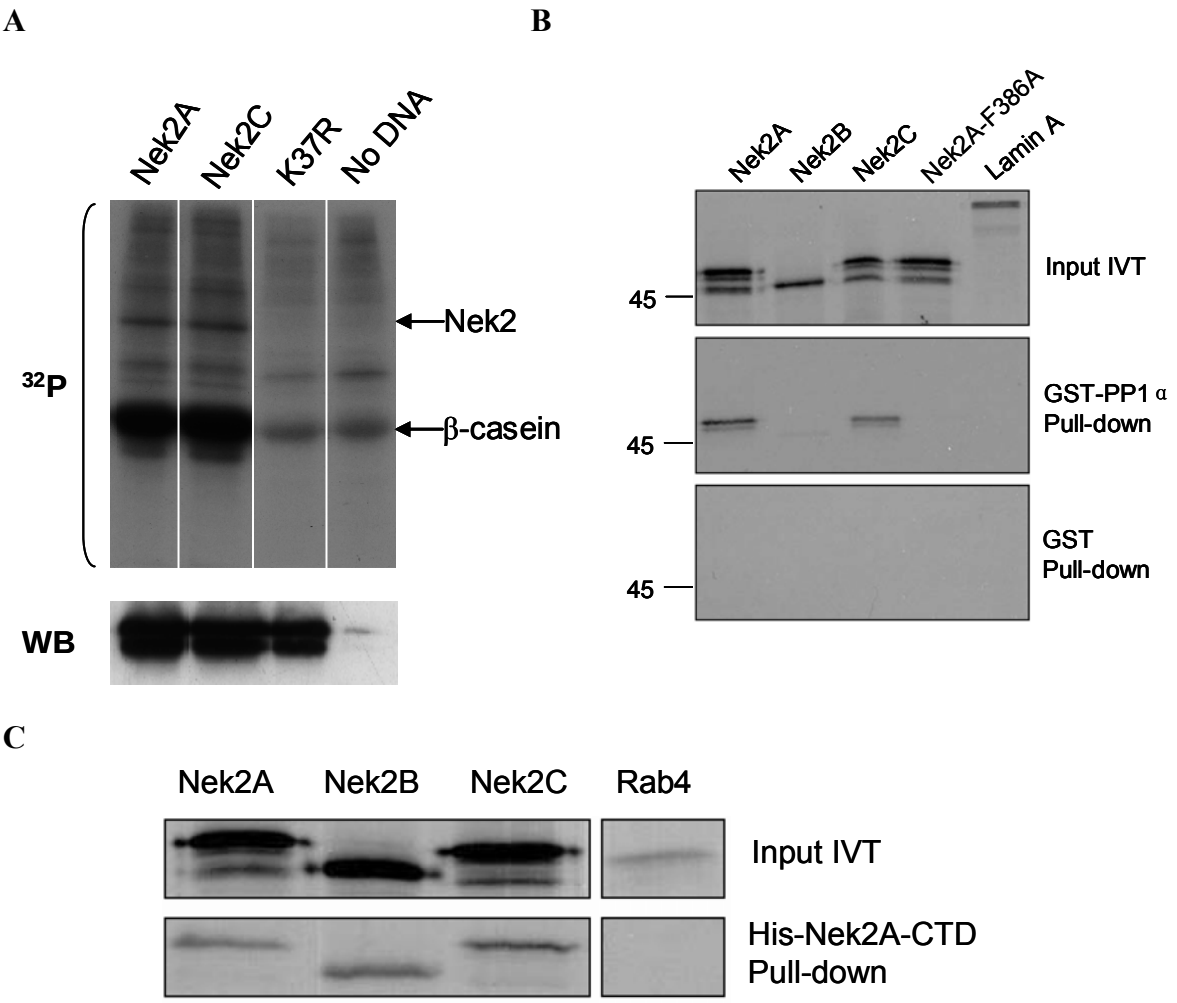


Figure IV.2. Nek2C is an active protein kinase that binds PP1α and undergoes dimerization **A**. Myc-tagged Nek2A, Nek2C and Nek2A-K37R proteins were expressed in HEK 293 cells. Following immunoprecipitation with an anti-myc antibody, an in vitro kinase assay was performed using ^{32}P-γ-ATP and β-casein as substrates. Phosphorylated products were detected by SDS-PAGE and autoradiography (upper panel, ^{32}P); the positions of β-casein and autophosphorylated Nek2 proteins are indicated. Equal levels of immunoprecipitated proteins were confirmed by Western blotting with an anti-Nek2 antibody (lower panel, WB). **B**. ^{35}S-labeled Nek2A, Nek2B, Nek2C, Nek2A-F386A and lamin A proteins were generated by *in vitro* translation (top panel) before binding with either GST-PP1α (middle panel) or GST alone (bottom panel). Proteins were analysed by SDS-PAGE and autoradiography. The position of the 45 kDa M.wt marker is indicated. **C**. ^{35}S-labeled Nek2A, Nek2B, Nek2C and Rab4 proteins were generated by *in vitro* translation (top panel) before binding to His-Nek2A-CTD (bottom panel). Proteins were analysed by SDS-PAGE and autoradiography.

IV.3.3 NEK2C IS DEGRADED IN MITOTIC EGG EXTRACTS AND PROMETAPHASE-ARRESTED CELLS

Total Nek2 exhibits cell cycle-dependent mRNA and protein expression with low levels in G1 and increased expression in S and G2 (Fry et al., 1995; Hayward et al., 2004).

After mitotic entry, Nek2A is specifically targeted for proteasomal degradation as a result of APC/C-mediated ubiquitylation, whereas Nek2B remains stable as it lacks the degradation motifs present in the C terminus of Nek2A (Hames et al., 2001; Hayes et al., 2006). These degradation motifs, a KEN-box and the C-terminal MR dipeptide, are present in Nek2C and we predicted that this isoform would also be degraded in mitosis. To test this we first generated Nek2A and Nek2C proteins by IVT and added them to mitotic extracts prepared from cytostatic factor-arrested *Xenopus* eggs. These extracts contain all the necessary machinery for APC/C-mediated ubiquitylation and proteasomal degradation (Murray, 1991). In untreated extracts, which represent metaphase II of meiosis, both proteins were slowly degraded, whereas upon addition of calcium, which releases the extracts into anaphase, both proteins were rapidly degraded (Fig. IV.3A & B). This degradation was proteasome-dependent as it was inhibited by MG132 (Fig. IV.3C). Moreover, both Nek2A and Nek2C were degraded in U2OS cells arrested with nocodazole in prometaphase of mitosis but not in S-phase arrested cells (Fig. IV.3D). Hence, Nek2C shows identical degradation kinetics to Nek2A both *in vitro* and *in vivo* and is most likely also an APC/C target.

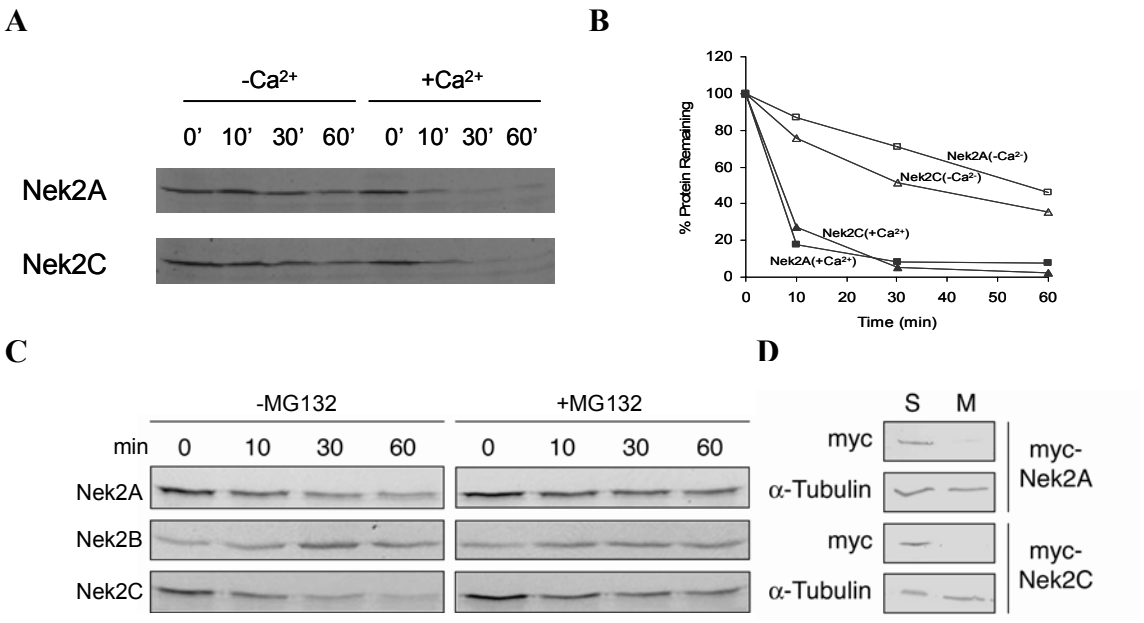


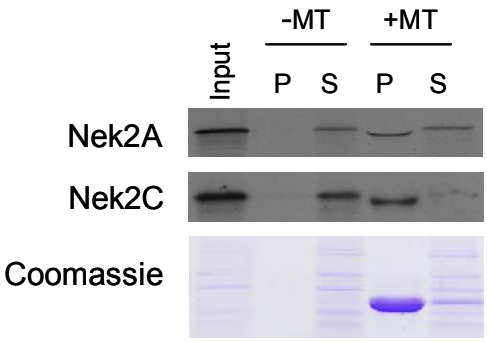
Figure IV.3. Nek2C is degraded in mitotic egg extracts and mitotic cells **A.** ^{35}S-labeled Nek2A and Nek2C proteins were generated by *in vitro* translation and degradation assays performed in *Xenopus* CSF-arrested egg extracts without (-Ca^{2+}) or with (+Ca^{2+}) calcium. Samples were taken at the times indicated (min), separated by SDS-PAGE and exposed to autoradiography. **B**. The Xray films were analysed by densitometry and the amount of protein remaining at each timepoint was plotted. Open triangles, Nek2A –Ca2+; open squares, Nek2C –Ca2+; solid triangles, Nek2A +Ca^{2+}; solid squares, Nek2C +Ca^{2+}. **C**. Degradation assays were carried out in cytostatic factor extracts +Ca^{2+} as described above in the absence (-MG132) or presence (+MG132) of the proteasome inhibitor, MG132. **D**. Myc-tagged Nek2A and Nek2C proteins were transfected into U2OS cells before arresting the cells either in S-phase with hydroxyurea (S) or in M-phase with nocodazole (M) for 16 h. Cell extracts were analysed by Western blotting with myc and α-tubulin antibodies, as indicated.

IV.3.4 NEK2C CAN BIND MICROTUBULES AND LOCALIZE TO CENTROSOMES

The C-terminal domain of Nek2 and specifically the region encompassing amino acids 333-370 is essential for the protein to interact with microtubules and localize to the centrosome (Hames et al., 2005). This region is present in Nek2C as well as Nek2A and Nek2B. Microtubule sedimentation assays confirmed that Nek2C could indeed bind to microtubules *in vitro* (Fig. IV.4A), whereas transfection revealed colocalization of myc-tagged Nek2C with γ-tubulin at the centrosome (Fig. IV.4B). As has been previously described, Nek2 associates with proteins at the proximal ends of centrioles, whereas γ-tubulin is distributed throughout the pericentriolar material, thus explaining why the two signals only partially overlap at high resolution. Previous localization of PP1 isoforms had

shown that PP1α and PP1γ1 are present at the centrosome in mitotic cells (Andreassen et al., 1998). Because Nek2C had been isolated in a yeast two hybrid screen with PP1γ as bait and Nek2C retains the PP1 binding site ($KVHF^{386}$), we stained cells transfected with Nek2C with antibodies against PP1α. Again, we observed colocalization of the signals at interphase centrosomes (Fig. IV.4C). These centrosome localizations patterns were identical to those of Nek2A (Figs. IV.4B & C). Thus, as predicted from the known motifs, Nek2C can associate with microtubules and localize to the centrosome probably in a complex with PP1

A



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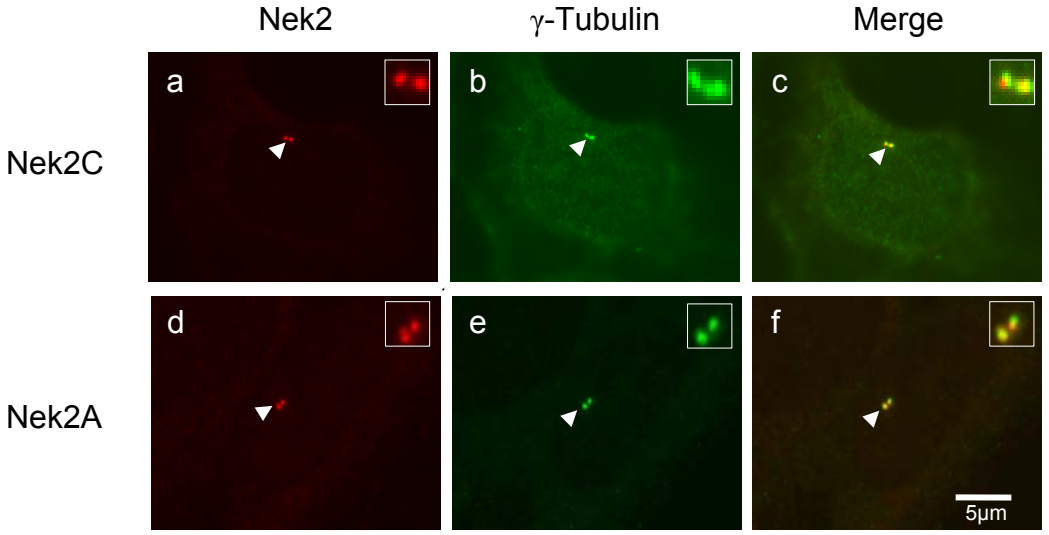

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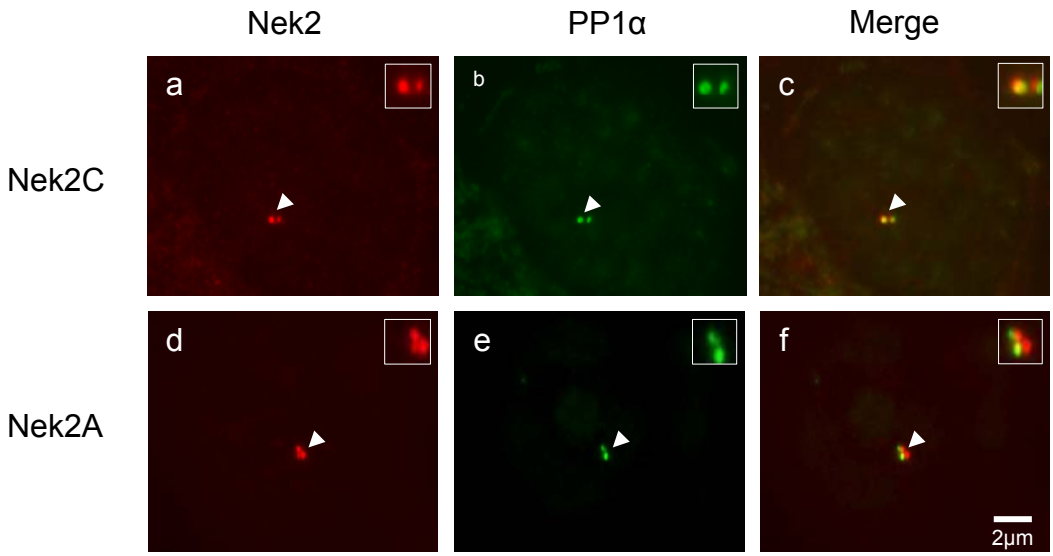


Figure IV.4. Nek2C binds microtubules in vitro and associates with centrosomes in cells **A.** Nek2A and Nek2C proteins were produced by *in vitro* translation in the presence of 35S-methionine and analysed by SDS-PAGE and autoradiography either before (Input) or after sedimenting at

35,000 rpm in the absence (-MT) or presence (+MT) of taxol-stabilized microtubules. Pellet (P) and supernatant (S) fractions were analysed. The Coomassie Blue-stained gel (CB) is also shown indicating the presence of tubulin in the pellet fraction in the presence of microtubules. **B & C.** Immunofluorescence microscopy of U2OS cells transfected with myc-tagged Nek2A or Nek2C and stained with anti-myc (red) and either anti-γ-tubulin (B) or anti-PP1α (C) antibodies (green). Merged images are shown and centrosomes indicated with arrowheads; enlargements of the centrosomes are also included. Scale bar, 5 μm.

IV.3.5 NON-CENTROSOMAL NEK2C ACCUMULATES IN THE NUCLEUS

While examining U2OS cells transfected with Nek2C, we noticed that in most cells (82%) the non-centrosomal protein was predominantly present in the nucleus (Fig. IV.5A & B). This was in contrast to U2OS cells transfected with Nek2A in which the protein showed a variable localization, sometimes mainly nuclear (35%), sometimes mainly cytoplasmic (29%) and sometimes distributed evenly throughout the cell (36%). Almost identical results were obtained in HeLa cells (Fig. IV.5A & B). For comparison, we decided to determine the cytoplasmic-nuclear distribution of the third splice variant, Nek2B. Strikingly, Nek2B was rarely detected predominantly in the nucleus (7%) with the majority of cells having the transfected protein predominantly in the cytoplasm (59%). To corroborate these microscopy experiments, transfected HeLa cells were also subject to subcellular fractionation and analysis by Western blotting (Fig. IV.5C). Again, Nek2C was predominantly found in the nuclear fraction (70%), whereas Nek2A was present in equal amounts in the nuclear (49%) and cytoplasmic (51%) fractions. Using this approach, Nek2B also appeared to be equally distributed between nuclear (48%) and cytoplasmic (52%) fractions. This discrepancy with the microscopy data for Nek2B is most likely due to the microscopy being a measure of individual cells, while the Western blot is a measure of total transfected protein. It was clear from the microscopy that the most strongly expressing cells were the ones that tended to show equal staining throughout the cell (i.e. N=C). However, counting individual cells, very few showed accumulation of NekB in the nucleus, but there were clearly cells with protein accumulated in the cytoplasm (see Fig. IV.5A). Hence, we conclude that there is a preferential uptake of Nek2C into the nucleus, whereas Nek2A tends to be more evenly distributed and Nek2B remains more in the cytoplasm, at least in the less strongly expressing cells. Thus, nuclear translocation of Nek2 is regulated by alternative splicing.

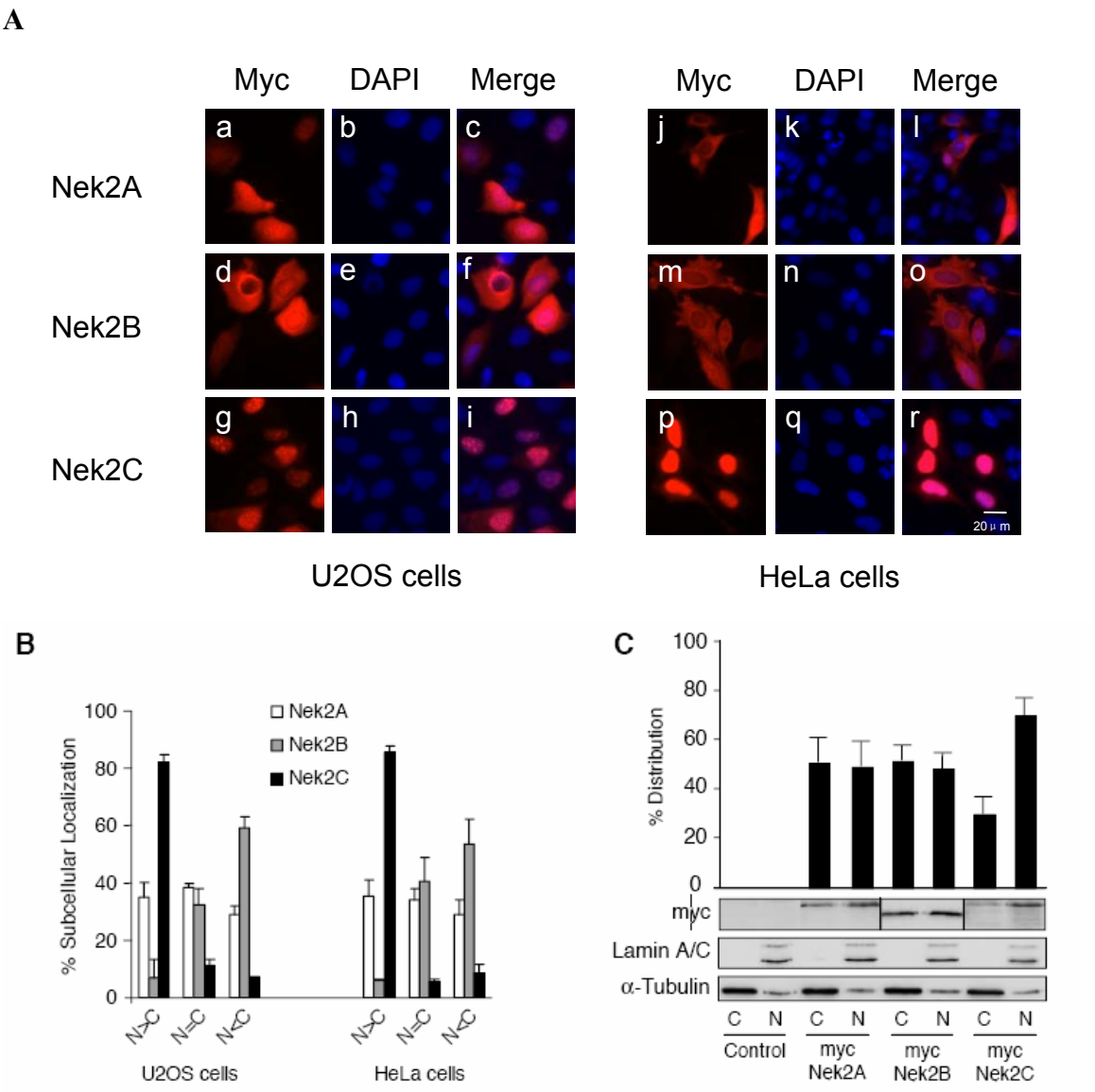


Figure IV.5. Recombinant Nek2C is predominantly nuclear **A**. Immunofluorescence microscope images of U2OS (**a-i**) and HeLa (**j-r**) cells transfected with myctagged Nek2A, Nek2B or Nek2C as indicated and stained with anti-myc antibodies (red) and with DAPI to highlight the nucleus (blue). Merge images are shown. Scale bar, 20 µm. **B.** The subcellular distribution of transfected protein was scored as either predominantly nuclear (N>C), evenly distributed throughout the cell (N=C) or predominantly cytoplasmic (N<C) in 100 cells in three independent experiments. Errors bars, s.d. **C**. Cytoplasmic (C) and nuclear (N) fractions were prepared from HeLa cells transfected with myc-tagged Nek2A, Nek2B or Nek2C before analysis by SDS-PAGE and Western blotting with antibodies against myc to detect transfected Nek2 proteins, Lamin A/C (alternatively spliced nuclear proteins) and α-tubulin (a cytoplasmic protein). The histogram shows the relative fraction of each myctagged protein in the cytoplasmic and nuclear fractions; mean and standard error from three independent experiments are shown.

IV.3.6 NUCLEAR TRANSLOCATION OF NEK2C DEPENDS UPON AN NLS THAT FLANKS THE SPLICE SITE

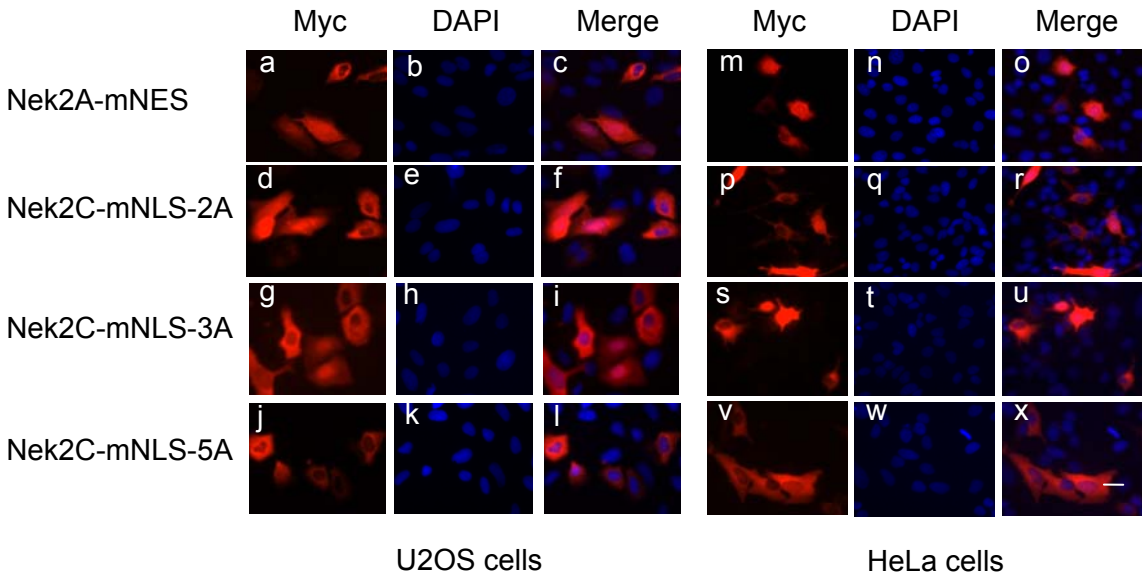
To explain the unexpected nuclear accumulation of Nek2C, but not Nek2A or Nek2B, we scanned the Nek2 protein sequence for potential nuclear localization signals (NLSs) and nuclear export signals (NESs) (Gorlich and Kutay, 1999; Mattaj and Englmeier, 1998). We identified in Nek2A a putative bipartite NLS at residues 361-383 and a weak NES at residues 372-375 (Fig. IV.6A). The NLS comprises two patches of basic residues, RK (361-362) and KKK (381-383). Intriguingly, these two patches span the eight amino acids missing from Nek2C, resulting in a linker of 18 amino acids in Nek2A but a linker of only 10 amino acids in Nek2C. Because most bipartite NLSs have a linker of 10-12 amino acids (Robbins et al., 1991), we hypothesized that, as a result of the eight amino acid deletion, the NLS may be better recognized by the nuclear transport machinery in Nek2C than in Nek2A. Nek2B lacks the second basic patch so does not contain a bipartite NLS. Meanwhile, the leucine rich NES sequence LLNL (372-375) falls within the eight amino acid stretch missing from Nek2C and so could act as a functional NES only in Nek2A.

To test whether either of these signals was functional, we generated a series of mutants in the predicted NES and NLS (Fig. IV.6A). All mutants localized correctly at the centrosome, suggesting that protein folding in the C-terminal domain was largely unaffected (data not shown). Mutations to abolish the putative NES (L372A/L373A/L375A) in the Nek2A protein had no effect with the protein again showing a fairly even mix of distributions. In contrast, mutation in the Nek2C protein of either the first basic patch (R361A/K362A) or the second basic patch (K373A/K374A/K375A) led to proteins that were rarely predominantly nuclear (<18%) (Fig. 6B & C). A combined mutant with all five basic residues mutated was even more effective at keeping the protein from accumulating in the nucleus (<2%). Thus, mutation of the NES did not lead to an accumulation of Nek2A in the nucleus, but mutation of the NLS did substantially reduce uptake of Nek2C into the nucleus. We conclude that a functional bipartite NLS is present in Nek2C, is less effective in Nek2A, and is completely absent in Nek2B. Hence, as a result of the NLS spanning the splice site, the cell can regulate the overall abundance and the particular isoform distribution of Nek2 in the cytoplasm and nucleus.

A

Nek2A	359-KE**RK**FLSLASNPELLNLPSSVI**KKK**VHF
Nek2B	359-KE**RK**FLSLASNPGMRINLVNRSWCYK
Nek2C	359-KE**RK**FLSLASNPVI**KKK**VHF
Nek2A-mNES	359-KE**RK**FLSLASNPEaaNaPSSVI**KKK**VHF
Nek2C-mNLS-2A	359-KE**aa**FLSLASNPVI**KKK**VHF
Nek2C-mNLS-3A	359-KE**RK**FLSLASNPVI**aaa**VHF
Nek2C-mNLS-5A	359-KE**aa**FLSLASNPVI**aaa**VHF

B



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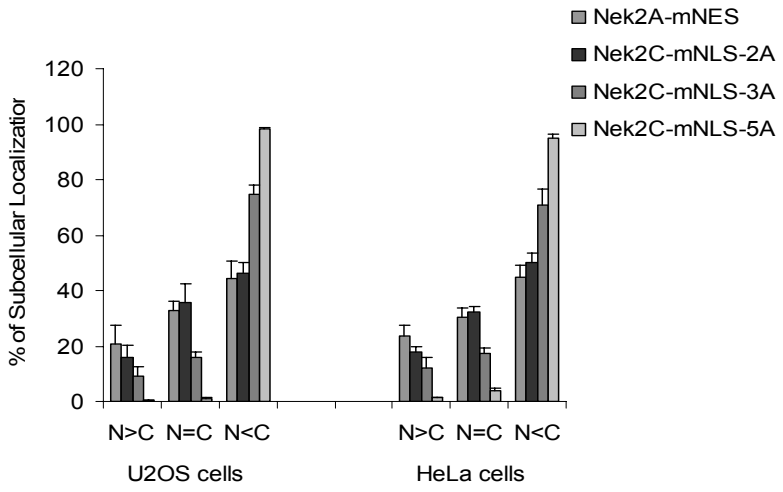

Figure IV.6. Mutation of the bipartite NLS leads to loss of Nek2C from the nucleus A. The amino acid sequences of Nek2A, Nek2B and Nek2C starting at K359 and encompassing the proposed NLS are indicated. The two basic patches of the bipartite NLS are in bold and the eight amino acid insertion is underlined. For comparison, the sequences within this region of the four mutants, Nek2AmNES and Nek2C-mNLS-2A, -3A and –5A, are shown with the mutated residues in lower case. **B**. Immunofluorescence microscope images of U2OS (**a-l**) and HeLa (**m-x**) cells transfected with the myctagged Nek2 mutants as indicated stained with anti-myc antibodies (red) and with DAPI to reveal the nucleus (blue). Merge images are shown. Scale bar, 20 μm. **C.** The subcellular distribution of mutant protein was scored as either predominantly nuclear (N>C), evenly distributed throughout the cell (N=C) or predominantly cytoplasmic (N<C) in 100 cells in three independent experiments. Errors bars, s.d.

IV.3.7 DETECTION OF A NOVEL NEK2 NUCLEAR SUBSTRATE

Aspergillus NIMA promotes chromatin condensation through phosphorylation of histone H3 (De Souza et al., 2000). We therefore tested whether nuclear accumulation of Nek2C might similarly promote this event. However, although untransfected prophase cells had clearly condensed chromatin that stained with phospho-H3 antibodies, interphase cells with strong nuclear accumulation of transfected Nek2C exhibited neither condensed chromatin nor phospho-H3 staining (Fig. IV.7A). Nek2A has also been reported to colocalize with Nek11 in nucleoli and to interact with the nucleolar protein, nucleophosmin (Noguchi et al., 2004; Yao et al., 2004). We therefore examined whether Nek2C was concentrated in nucleoli and colocalized with nucleophosmin. As expected, the bulk of transfected cells contained myc-Nek2C in the nucleus, but there was almost complete exclusion from the nucleoli and hence no colocalization with nucleophosmin (Fig. IV.7B). With a nuclear function for Nek2C still unclear, we therefore, decided to perform an unbiased search for nuclear substrates of Nek2 using the KESTREL approach for substrate detection (Cohen and Knebel, 2006). This method has been developed for identifying *bona fide* substrates of kinases by making use of short incubation times, high concentrations of added kinase, and high specificity of radioactivity combined with extracts that have been fractionated to separate endogenous kinases from their substrates and concentrate the substrates. Cytoplasmic and nuclear extracts were prepared from HEK 293 cells and fractionated by heparin sepharose chromatography. These fractions were then incubated for 5 min with Mn-ATP, the preferred substrate of Nek2 (Fry et al., 1995), and either no kinase or recombinant Nek2A, Nek6, Nek7 or Nek9 before separation by SDS-PAGE and autoradiography (Fig. IV.7B). A 28 kDa protein in Fraction 3 of the nuclear extract was specifically phosphorylated by the Nek2 kinase but not the closely related

Nek6, Nek7 and Nek9 kinases. This protein was not detected in Fraction 3 of the cytoplasmic extract. Although substantial further work will be required to validate this observation, the specificity of the phosphorylation and the stringent nature of the KESTREL approach leads us to propose that this 28 kDa protein is likely to represent a novel nuclear substrate of Nek2.

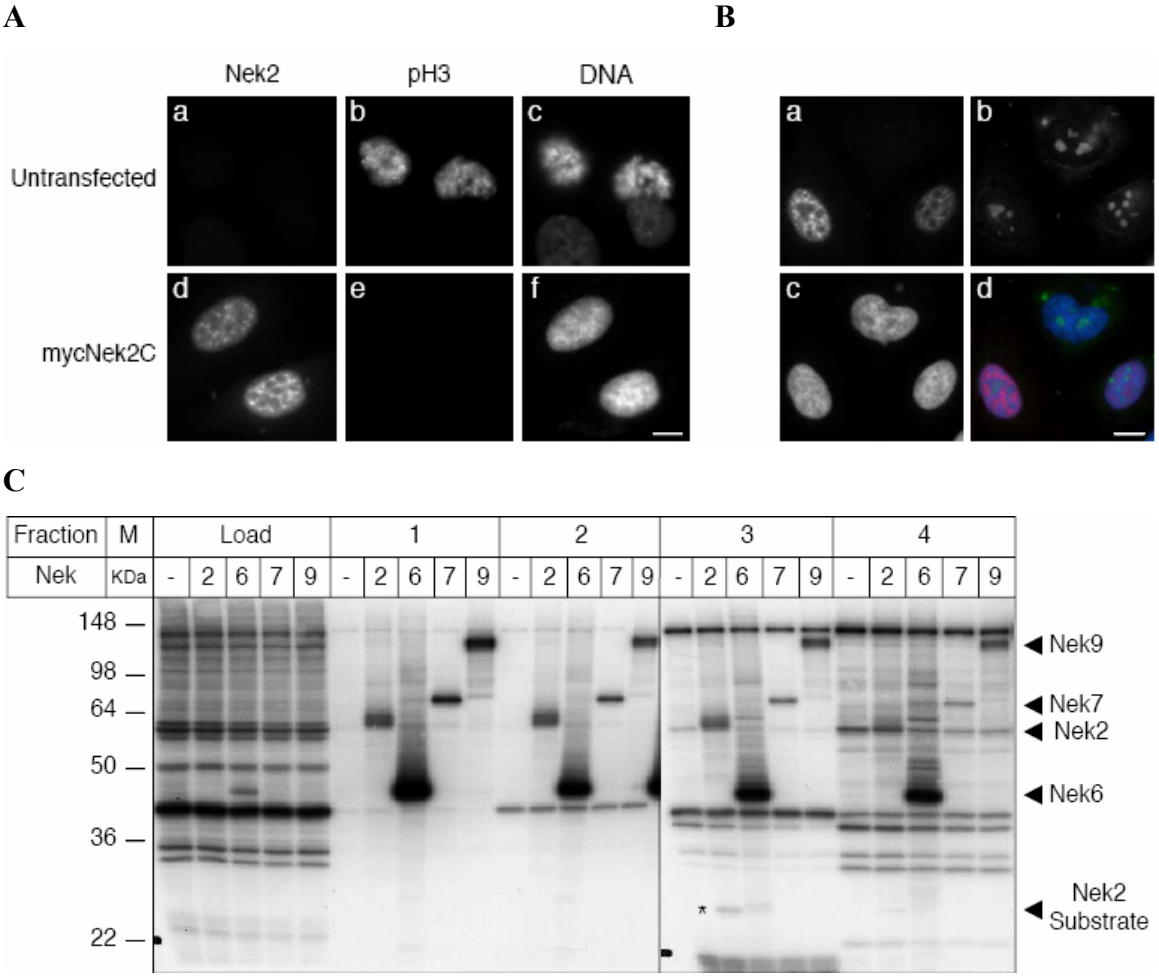

Figure IV.7. Detection of a 28 kDa Nek2 substrate in HEK 293 nuclear extracts **A**. U2OS cells that were either untransfected (**a**-**c**) or transfected with mycNek2C for 24 hours (**d**-**f**) were processed for immunofluroescence microscopy with Nek2 (**a**,**d**) and phospho-H3 (**b**,**e**) antibodies and DNA stained with Hoechst 33258 (**c**,**f**). Scale bar, 5 µm. **B**. U2OS cells transfected for 24 hours with mycNek2C were stained with anti-Nek2 (**a**) and anti-nucleophosmin (**b**) antibodies and DNA stained with Hoechst 33258 (**c**). A merged image is shown (**d**). **C**. Nuclear extracts from HEK 293 cells were desalted and chromatographed on heparin sepharose. Aliquots of the desalted extract (load) and the fractions (numbered) were incubated with Mn-ATP in the absence (-) or presence of Nek2, Nek6, Nek7 or Nek9 kinases as indicated. The reactions were separated by SDS-PAGE, transferred to PVDF and analysed by autoradiography. The positions of molecular weight markers (kDa) and autophosphorylated Nek kinases are indicated. The 28 kDa protein in fraction 3, phosphorylated only in the presence of Nek2, is indicated (*).

IV.4 DISCUSSION

Nek2A was originally isolated in a degenerate PCR-based screen for human kinases related to *Aspergillus* NIMA (Schultz et al., 1994). The first alternative splice variant, Nek2B, was identified in *Xenopus* oocytes (Uto et al., 1999), and subsequently in cultured human cells (Hames and Fry, 2002). Most recently, Nek2C (originally called Nek2A-T) was described following a yeast two hybrid screen performed with a testis library and PP1γ1 and PP1γ2 as baits (Fardilha et al., 2004b). These three splice variants differ only in their non-catalytic C-terminal domains, and hence, not surprisingly, all encode active protein kinases. Indeed, Nek2C differs from Nek2A only in so much as it lacks eight amino acids (371-378) in the middle of the non-catalytic domain. Nevertheless, our results suggest that this small change has significant consequences on subcellular localization, which in turn may allow the different splice variants to undertake distinct functions during cell division.

Although several independent clones representing Nek2C were identified in the two hybrid screen with a testis library, the majority of clones isolated in that study were Nek2A (Fardilha et al., 2004b). Likewise, Nek2A cDNAs were isolated from mouse testis libraries in three independent laboratories (Arama et al., 1998; Rhee and Wolgemuth, 1997; Tanaka et al., 1997). Hence, both Nek2A and Nek2C are present in testis, although they may have distinct functions to play during meiotic cell division. By using common primers that span the splice site and amplify products of distinct sizes, we were able to detect Nek2C mRNA in three different human cell lines (U2OS, HeLa and HEK 293), although at levels ~10-fold lower than Nek2A. Previous studies revealed elevated expression of Nek2 in cancer cell lines and primary tumors without distinguishing between Nek2A and Nek2C (Hayward and Fry, 2006). It will be interesting to determine whether there is elevation of both splice variants.

Clearly, at the sequence level, there is very little difference between Nek2A and Nek2C, and one would predict that they share many of the same properties. With respect to regulation of kinase activity, we confirmed that Nek2A and Nek2C phosphorylate the exogenous substrate β-casein equally, show similar levels of autophosphorylation, have the capacity to undergo dimerization, and can both bind PP1α. Moreover, the extreme C terminus, which contains two APC/C-dependent degradation motifs, is identical in the two

proteins, and we found no difference in their destruction in mitotic extracts or prometaphase-arrested cells. Finally, Nek2A and Nek2C both retain the region encompassing amino acids 333-370 required for microtubule binding and centrosome localization, and experimentally, we were able to confirm that Nek2C retains these properties. Thus, our data not only confirm that Nek2A and Nek2C are biological very similar but provide further evidence for the correct mapping of these different functional motifs.

Despite the high degree of sequence identity, we were able to identify an important difference in the behavior of the two variants. After expression of recombinant proteins, it was clear that Nek2C was preferentially taken up into the nucleus, whereas Nek2A was more evenly distributed throughout the cell. Mechanistically, this can be explained by the loss of the eight amino acids from Nek2C that result from splicing. Nek2C contains an $RKX_{10}KKK$ sequence, where X is an uncharged linker, that conforms perfectly to the well defined bipartite NLS motif (Robbins et al., 1991). Mutation of the NLS led to loss of nuclear Nek2C, whereas mutation of a putative NES located within the eight amino acids missing from Nek2C had no effect. Furthermore, Nek2B, which only has the first basic patch and so lacks a functional NLS altogether, was present within the nucleus less than either Nek2A or Nek2C in single cell analysis. Because Nek2 proteins are generally around 45 kDa, they are close to the cut-off for proteins that can diffuse passively into the nucleus. This would explain why proteins were sometimes found in both compartments, particularly in the most strongly expressing cells. Localization of the endogenous protein, which is present at much lower levels, is likely to be more discretely controlled.

Structural analysis of NLS-containing peptides bound to the cargo binding domain of importin-α show that there is space to accommodate two lysine or arginine residues in the upstream basic patch and five lysine or arginines in the second basic patch; these are ideally separated by a linker of 10 residues (Conti and Kuriyan, 2000; Conti et al., 1998). Studies on the NLS sequence of nucleoplasmin revealed that linkers shorter than 10 amino acids are not tolerated, whereas longer linkers are tolerated but can have reduced activity (Robbins et al., 1991). In Nek2A, the linker is extended to 18 amino acids and includes additional hydrophobic and proline residues that could alter the conformation of the linker and interfere with its ability to function as a bipartite NLS. Indeed, there is support for interaction of the linker backbone with importin-α residues (Conti and Kuriyan, 2000).

However, there are cells in which Nek2A is concentrated in the nucleus, indicating that the longer linker does not completely abrogate NLS activity (Fry et al., 1998b). Indeed, there may be an additional level of regulation at which the distribution of Nek2A is controlled. It is intriguing that the second basic patch overlaps with the PP1 binding site (383-386) raising the possibility that nuclear uptake via importin-α interaction and PP1 binding are mutually exclusive. On the other hand, simultaneous binding to importin-α and PP1 would be possible for opposing polypeptides of dimeric Nek2.

The function of Nek2 in the nucleus remains to be determined. Conflicting data exists on whether endogenous Nek2 is localized to condensed chromatin in early stage meiotic cells (Rhee and Wolgemuth, 1997; Tanaka et al., 1997). However, persuasive studies on mouse spermatocytes suggest that Nek2 plays an active role in chromatin condensation during the first meiotic division through phosphorylation of the chromatin-associated protein, high mobility A2 (Di Agostino et al., 2004b; Di Agostino et al., 2002). Nuclear entry of Nek2C may be necessary for providing access not only to downstream substrates, such as high mobility protein A2, but also to upstream regulators, including activated extracellular signal-regulated kinase 1/p90Rsk2, before nuclear envelope breakdown. This, coupled with the fact that NIMA regulates chromatin condensation in *Aspergillus* (De Souza et al., 2000), seems the most promising explanation for nuclear Nek2. However, in contrast to NIMA, we found that nuclear accumulation of Nek2C did not promote phosphorylation of histone H3, indicating that, alone, it is not sufficient to trigger premature chromatin condensation. Interactions have been described for Nek2 with nuclear proteins, including nucleophosmin (also known as numatrin or NPM/B23) and, interestingly, another NIMA-related kinase, Nek11 (Noguchi et al., 2004; Yao et al., 2004). However, we found that nuclear Nek2C was excluded from nucleoli and did not co-localize with nucleophosmin, raising questions over the significance of these reported interactions. Because the nuclear function of Nek2C remains obscure, we opted instead to initiate a search for novel nuclear substrates of Nek2 using the unbiased KESTREL chromatographic approach. This led to identification of a 28 kDa protein as a likely physiological substrate of Nek2. Future efforts will be required to scale up this process to identify this protein by mass spectrometry. In conclusion, besides providing support for a nuclear role of Nek2, this work neatly illustrates how alternative splicing can modulate the structure, and thereby functionality, of a bipartite NLS motif.

CHAPTER V

DISCUSSION

V DISCUSSION

Infertility is a growing problem in the Western world, presently affecting around 20% of couples. Growing evidence from clinical and epidemiological studies suggests that, at least in part, this is paralleled by the increasing incidence of male reproductive problems (Iammarrone et al., 2003). Although male infertility is complex and results from a variety of underlying causes, defects in sperm motility appear to be one of the main culprits. However, sperm biology is a complex process, as cells go from the male reproductive tract into the female reproductive tract and through processes of acquisition of progressive motility, capacitation, hyperactivation of motility, directed motility and induction of the acrosome reaction. In contrast, the worldwide market for male contraceptives is also worth billions of euros. The demand for a reliable non-hormonal oral male contraceptive is phenomenal, since male-directed options, in what concerns contraception, are almost solely limited to condoms and vasectomy. Due to various shortcomings of both methods, researchers have been looking for hormonal based contraceptives that also have their limitations (Amory et al., 2006). Both male infertility and contraception would benefit greatly from a more rational approach based on targeting sperm-specific processes. However, the underlying molecular mechanisms which regulate sperm motility are still not fully understood, though protein phosphorylation is an attractive model (Ren et al., 2001; Smith et al., 1996; Vijayaraghavan et al., 1996; Vijayaraghavan et al., 1997; Visconti and Kopf, 1998; Smith et al., 1999; Huang et al., 2002). The latter is the most common regulatory mechanism for triggering intracellular events, and abnormal protein phosphorylation profiles have been associated with various disease processes and dysfunctional states. Consequently, kinases and phosphatases represent attractive targets for novel approaches in drug design.

Protein phosphorylation is the main post-translational modification in eukaryotic cells, regulating numerous physiological processes. The phosphorylation of target regulatory proteins, controlled by a variety of extra- and intra-cellular signals, is a dynamic and reversible process where the phosphate groups are incorporated into target proteins by the kinases and removed by the phosphatases. Several studies have demonstrated the involvement of cyclic nucleotides, bicarbonate, cholesterol and protein phosphorylation in the acquisition of progressive motility, capacitation, hyperactivation, directed motility and

the acrosome reaction (Cross, 1998; Garbers and Kopf, 1980; Huang et al., 2002; Mishra et al., 2003; Smith et al., 1996; Smith et al., 1999; Vijayaraghavan et al., 1996; Vijayaraghavan et al., 1997; Visconti and Kopf, 1998). Therefore, the main focus of this work concerned sperm protein phosphatases and their involvement in the control of sperm signal transduction mechanisms.

Recent sequencing projects of entire genomes has revealed that protein kinases and protein phosphatases constitute approximately 3% of all eukaryotic genes (Plowman et al., 1999). However, there are at least 2-5 times fewer phosphatases than kinases, and this imbalance is even more pronounced when one considers only the Ser/Thr-specific phosphatases and kinases. For example, the human genome encodes approximately 20x fewer Ser/Thr phosphatases than kinases. Thus, whereas the diversity of the Ser/Thr-specific protein kinases has kept pace with the increasing complexity of evolving organisms, that of the Ser/Thr phosphatases has not. Over the past decade it has become apparent that phosphatase diversity has been achieved not only by the evolution of new catalytic subunits, but mainly by the ability of each single catalytic subunit to interact with multiple regulatory (R) subunits. Phosphatases may be fewer in number, but they are still capable of dephosphorylating thousands of proteins with a tight, specific and independent regulation. This is explained by the formation of complexes between the phosphatases (the catalytic subunit) and the regulatory protein subunits in a mutually exclusive manner (Ayllon et al., 2002). The occurrence of large multimeric complexes, sometimes containing both phosphatase and kinase activities has recently began to be appreciated.

Germ cell-specific PP1γ2 phosphatase is the predominant Ser/Thr phosphatase in spermatozoa and its activity is inversely correlated with sperm motility (Smith et al., 1996; Vijayaraghavan et al., 1996). PP1 inhibition by okadaic acid or calyculin A induces motility initiation and stimulation. Indeed, inhibition of immotile caput sperm PP1 activity with specific inhibitors, results in them acquiring normal motility parameters. Intriguingly, these effects are completely independent of calcium. Sperm are terminally differentiated cells, constituting 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 different aspects 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 the phosphatases and their regulation have only recently started to be elucidated.

It is now well established that in somatic cells PP1 activity relies on its binding to several phosphatase regulators to provide the necessary functional, subcellular and cell type-specific diversity (we now know that the same mechanism also operates in germ cells). With this in mind, we have previously undertaken an in-depth survey using the yeast two hybrid approach to identify proteins expressed in human testis capable of interacting specifically with the alternatively spliced isoforms of PP1γ. Around 120, 160 and 85 positive clones were identified, purified, partially sequenced and compared to the Genbank database for PP1γ1, PP1γ2 and the unique PP1γ2 C-terminus, respectively. The PP1 interactomes thus defined comprised 26, 29 and 42 different proteins from human testis capable of binding to PP1γ1 PP1γ2 and the PP1γ2 C-terminus (Fardilha, 2004). The validity of the results obtained was confirmed by a variety of criteria, including the isolation of bona fide PP1 regulators (Fardilha et al., 2004) and the presence of a consensus PP1 binding motif in the newly identified proteins. The most abundant interacting protein identified was Nek2A (Fry et al., 1998). Although it is already known that Nek2A interacts with PP1, which dephosphorylates Nek2A itself and other Nek2A substrates (Helps et al., 2000), a new alternatively spliced isoform of Nek2A was identified, that we named Nek2C (Fardilha et al., 2004; Wu et al., 2007). The existence of testis-specific protein isoforms seems to be a recurrent event which can be used for specific targeting in relation to therapeutic interventions.

The importance of PP1γ1 and PP1γ2 function has been addressed with the targeted disruption of the PP1γ gene. Knock out mice were viable and homozygous females were fertile. On the contrary, disruption of the PP1γ gene caused 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 PP1 isoforms can compensate for the lack of PP1γ1 in somatic cells and for the lack of PP1γ2 in germ cells but only until the final stages of spermatogenesis, where PP1γ2 becomes indispensable (Varmuza et al., 1999). Furthermore, it was shown that PP1γ2 has a role in sperm morphogenesis, being required for normal flagellar integrity and structures (Chakrabarti et al., 2007). It is interesting to note that even though PP1α, PP1β, PP1γ1 and PP1γ2 are virtually identical, they can compensate for each others absence in relation to some functions but not in all. That is, there are some processes that are absolutely PP1 isoform specific.

Besides the two splice variants, Nek2A and Nek2B, identified in primary blood lymphocytes and adult transformed cells (Hames and Fry, 2002) that differ in their non-catalytic carboxyl-termini, we have now described a third Nek2 variant, Nek2C (Fig. IV.1A). Nek2C was first identified in a human testis library (Fardilha et al., 2004; Wu et al., 2007), where Nek2 is also known to be highly expressed in the adult. Nek2 is expressed in a stage specific pattern during spermatogenesis and intense signals were also observed in oocytes (Tanaka et al., 1997). Alternative splicing is an unusual mechanism for modulating Nek2 localization and it supports the hypothesis that Nek2 has both nuclear and cytoplasmic functions, and that it might bind different PP1 isoforms depending on its function. Although Nek2A, Nek2B and Nek2C are likely to exhibit distinct functional properties, it may be difficult to specifically target Nek2A and Nek2C for therapeutics, since the only difference is that Nek2C lacks 8 amino acids present in Nek2A. However, the effectiveness of siRNA directed against each, needs to be fully explored to assess the feasibility of this approach. The kinase/phosphatase complex of Nek2-PP1 functions as a bistable switch (Sohaskey and Ferrell, 2002). The binding and regulation of the Nek2-PP1 complex by I2 opens new avenues for PP1 regulation, showing the existence of PP1 trimeric complexes. Indeed, further complexity is provided by the expression of both Nek2 variants (Nek2A and Nek2C) and both I2 isoforms (I2 and I2L) in sperm. It is tempting to speculate that in vivo they may express some degree of functional specificity. Future work in the laboratory will attempt to address this important issue.

One particularly interesting mechanism for controlling PP1 activity involves its inhibition by heat-stable inhibitors, particularly protein phosphatase inhibitors I1 and I2

(phosphoproteins whose state of phosphorylation dictates their PP1 inhibitory activity). I1 is phosphorylated by cAMP-dependent protein kinase (PKA) and dephosphorylated by the calcium/calmodulin-dependent protein phosphatase calcineurin (PP2B). Thus, PP1 is involved in the cross-talk between the intracellular messengers calcium and cAMP. I2 is also capable of inhibiting the catalytic subunit of PP1 leading to the production of a stable PP1-I2 complex. GSK-3 kinase phosphorylates I2 in the PP1-I2 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 (Smith et al., 1996; Vijayaraghavan et al., 1996). Immotile bovine caput epididymal sperm contain two fold higher levels of protein phosphatase activity, identified as PP1γ2, and six fold higher GSK-3 activity, compared to mature motile caudal sperm. Thus, high GSK-3 activity keeps PP1γ2 active in immotile caput sperm, but the complex PP1γ2/I2 forms and remains largely inactive in motile caudal sperm (Smith et al., 1996; Vijayaraghavan et al., 1996).

In this work, we have now identified a new I2 isoform, that we called I2L. The two are highly similar, except for some important substitutions in key regulatory amino acids (Fig. III.1C and Fig. V.1). The identified differences between I2 and I2L may be crucial for their inhibition of PP1γ2 and consequent regulation of human sperm motility (Fardilha et al., 2004). Indeed, I2L exhibits an amino acid substitution of the key threonine residue that is phosphorylated by GSK-3 and that relieves PP1 inhibition. Consequently, I2L is not subject to regulation by this kinase and therefore can be viewed as a constitutive inhibitor of PP1. The I2L gene is only present in the primate and human genomes, suggesting that it may represent an evolutionary important event.

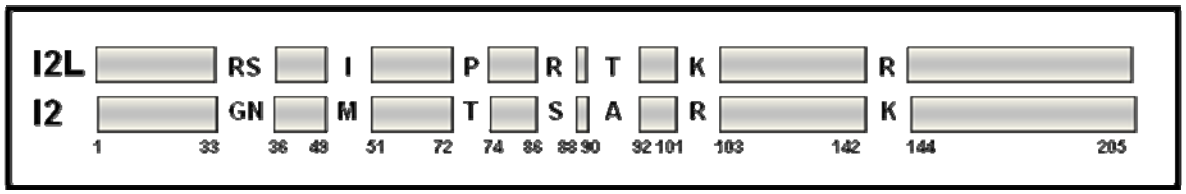

Figure V.1A. Diagramatic comparison of I2L and I2. Boxed areas represent identical residues. Different amino acids are shown. The main difference is the Thr for Pro substitution at position 73. Drawn to scale.

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

I2-L    61 KGYGLMKIDEPSPPYHSMMGDDEDACRDTETTEAMAPDILAKKLAAAEGLEPKYRIQEQE
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 V.1B: Alignment of amino acid sequences of I2L and I2. Highlighted residues represent important regulatory sites; the underlined region represents the most highly conserved region of the protein. Amino acid differences are shown in colour.

I2L and I2 are both heat-stable protein inhibitors of PP1 that were shown to inhibit its activity with nanomolar affinity (see Chapter III). The inactive complex PP1-I2 can be reactivated by phosphorylation of Thr73 on I2 by GSK-3 (DePaoli-Roach, 1984). Phosphorylation at this site can be enhanced by CK2 phosphorylation of Ser87 (Fig. III.4; Park and DePaoli-Roach, 1994). PP1γ2 and GSK3 are both present in sperm and their activities are 2- and 6-fold higher, respectively, in immotile caput compared to motile caudal epididymal sperm (Smith et al., 1996; Vijayaraghavan et al., 1996). Given that I2 is also expressed in sperm, PP1γ2 activity is also probably regulated by its interaction with I2, in a manner analogous to that described for somatic cells. However, although the expression of I2L in sperm remains to be demonstrated experimentally, it provides an interesting and hitherto unknown mechanism for the irreversible and unidirectional inhibition of PP1 that might correlate with the development of sperm motility during epidydimal transit. The absence of the most important regulatory residue of I2 from I2L (it has a Thr for Pro substitution at position 73) must have consequences on the regulation of PP1 and ultimately on the acquisition of sperm motility.

In general, since *in vivo* protein 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 their catalytic activity. Indeed, the diversity of such phosphatase regulatory subunits explains not only the need for few catalytic subunit types, but also make the former attractive targets for pharmacological

intervention. A major question remaining is how the regulators exert their discrimination between virtually identical mammalian PP1 isoforms. The regulatory subunits are usually unrelated, but most possess a consensus RVxF motif, a canonical PP1 binding domain. However, resolution of the crystal structure of the complex between PP1γ and a 34kDa N-terminal domain of the myosin phosphatase targeting subunit MYPT1 (Hartshorne et al., 1998) demonstrated the importance of other structural elements amino- and carboxy-terminal to the RVxF motif of MYPT1 to the reshaping of the catalytic cleft of PP1 (Terrak et al., 2004). These interactions also contribute to the increased myosin specificity of the complex. Another interaction was also observed between MYPT1 and the C terminus of PP1γ, involving residues that are not present in the other PP1 isoforms, thus giving an indication of at least one mechanism by which isoform specificity can be achieved.

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/I1 system in other brain regions. PP1 has also been suggested to play a central role 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. 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. Since I1 and DARPP-32 are not expressed in mammalian sperm, it is interesting to speculate that the PP1/I2 and PP1/I2L systems may function to regulate the diversity of sperm signalling cascades.

In conclusion, we have demonstrated the occurrence of two hitherto unknown PP1 holoenzymes in testis/sperm (PP1/I2L and PP1/Nek2C), that may constitute potential targets for pharmaceutical intervention (Fig. V.2). However, before interfering with these protein complexes, a better understanding is required of their involvement in sperm motility and other sperm functions. Nevertheless, the newly identified PP1 regulators exhibit novel and unusual properties. I2L is missing critical amino acid residues required for GSK-3 regulation, converting it into an irreversible PP1 inhibitor, whereas Nek2C is likely to have important nuclear functions given the functional NLS created by the specific splicing out of 8 amino acids. Therefore, both proteins may represent interesting specific targets to treat infertility and to develop new non-hormonal contraceptives. The characterization of testis- and sperm-specific PP1 regulatory proteins should aid in the development of novel signal transduction therapeutic approaches to male infertility and male contraception.

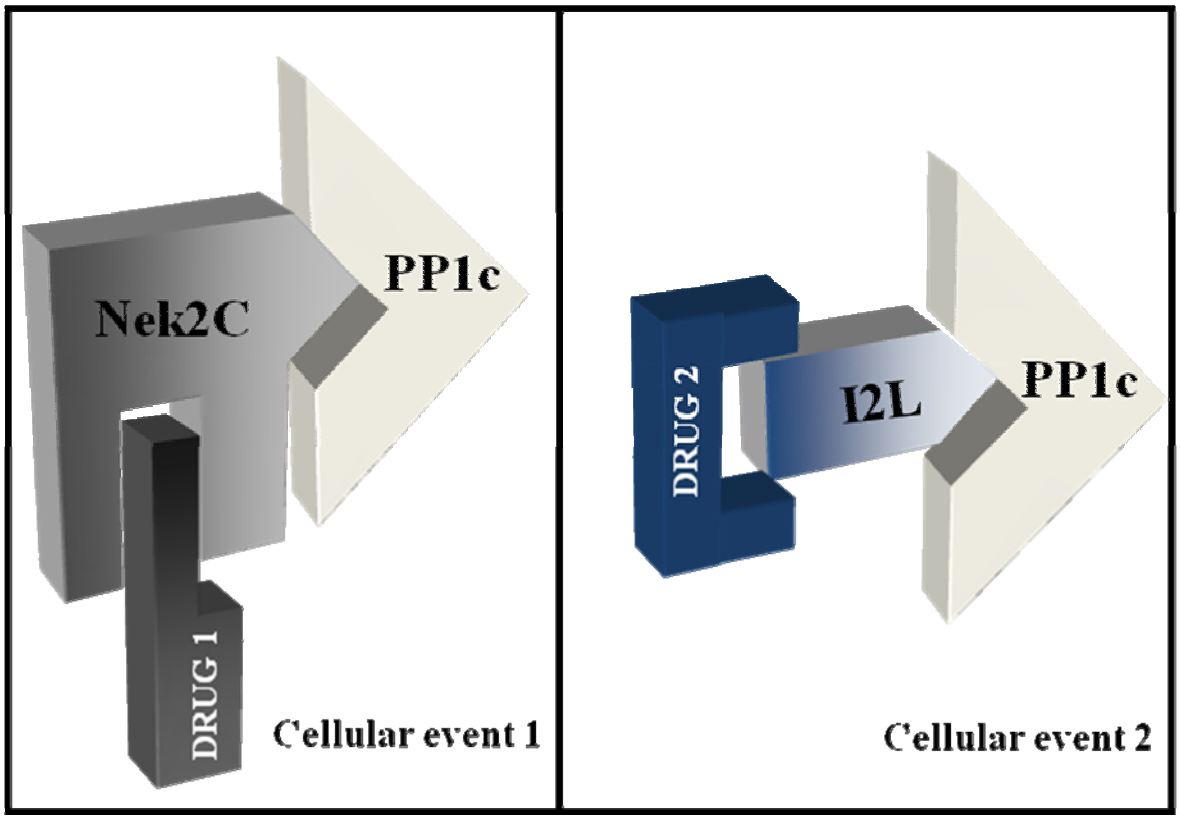

Figure V.2. Signal transduction therapeutics for specific cellular events based on targeting of different PP1 regulators.

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