Nuclear protein tyrosine phosphatases and control of cell proliferation

Vegesna Radha and Ghanshyam Swarup

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

Apart from playing a role in signal transduction pathways, tyrosine phosphorylation has more recently been shown to regulate nuclear events such as cell division cycle and transcription factor activity. Members of the family of protein tyrosine kinases and protein tyrosine phosphatases (PTPases) that may be responsible for this regulation have been shown to localize in the nucleus. The nuclear PTPases are of particular interest because of their role in regulating cell proliferation. One of them, PTP-S2 enhances cell proliferation and has the unique property of binding to DNA. Many of the nuclear PTPases are induced by mitogens and may act as either positive or negative regulators of cell proliferation, making them potential candidates for oncogenes and antioncogenes (tumour-suppressor genes), respectively.

PHOSPHORYLATION and dephosphorylation of proteins catalysed by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases) play a central role in cellular functions such as response to external stimuli, cell growth and differentiation[1]. Since tyrosine phosphorylation of proteins *in vivo* is a transient and reversible event, it is particularly important that the activities of these enzymes are stringently regulated. The PTPases have gained significance as regulatory enzymes because, apart from dephosphorylating substrates phosphorylated by the PTKs, the activities of many PTKs and protein serine (or threonine) kinases (PSKs) are dependent upon dephosphorylation of specific tyrosine residues[2]. The PTPases are conserved in evolution and are present in species as divergent as viruses, bacteria, yeast, invertebrates and mammals[3,4].

Structurally all members of protein tyrosine phosphatase superfamily share an active site motif consisting of a cysteine and an arginine separated by 5 other amino acids[5,6]. This motif Cx_5R (where x is any amino acid) forms a distinct cradle, the phosphate-binding loop, at the active site. Mutation of cysteine to any other amino acid inactivates the enzyme. Biochemically the PTPases are characterized by their sensitivity to vanadate and pervanadate, insensitivity to inhibitors of serine, threonine phosphatases (PSPases) such as okadaic acid, ability to hydrolyse *p*-nitrophenyl phosphate and lack of dependence on metal ions[3,7,8]. With the availability of sequence of over 150 members, a classification of PTPase superfamily based on amino acid sequence comparisons, three-dimensional structure and function has been proposed. PTPase superfamily can be divided into four families.

Tyrosine-specific phosphatase (PTP) family. These enzymes are divided into two groups – the transmembrane enzymes (such as CD45) located at the plasma membrane that serve to transmit signals from the external environment to the cell interior, and the intracellular enzymes that are targeted to specific subcellular locations and serve to regulate intracellular activities[9]. The intracellular PTPases are characterized by the presence of a catalytic domain of about 250 amino acids and non-catalytic sequences at the N or C-terminal that serve a regulatory function and attribute unique properties to various members of this class of enzymes[6]. Figure 1 depicts the localization of PTPases to various subcellular domains achieved by their interaction with different cellular organelles/components.

VHI-like dual specificity phosphatase family. Members of this family show very little sequence identity (5%) with PTP family members. This group of enzymes can dephosphorylate Ser, Thr and Tyr but prefer Tyr. VH1 phosphatase is coded by vaccinia virus late H1 gene and it was the first tyrosine phosphatase identified which could also hydrolyse phosphothreonine and phosphoserine[10]. This family of enzymes includes PAC-1, CL100 and other related proteins (MKP-2, MKP-3) which dephosphorylate MAP kinases[11]. Some of these enzymes have been found in the cell nucleus[12,13].

Cdc25 phosphatase family. The cell division cycle gene Cdc25 of fission yeast codes for a dual specificity phosphatase which dephosphorylates Tyr 15 of Cdc 2 protein kinase and activates it[14]. In mammalian cells, three Cdc25 enzymes Cdc25A, Cdc25B and Cdc25C are known which dephosphorylate both Tyr 15 and Thr 14 of cyclin-dependent kinases (CDKs) in the cell nucleus[15]. The active site sequence of Cdc25 enzymes is very different from those of VH1-like family, although both family members have Cx_5R motif.

The low-molecular weight phosphatase family. These enzymes were originally described as acid phosphatases which were subsequently found to dephosphorylate phos-

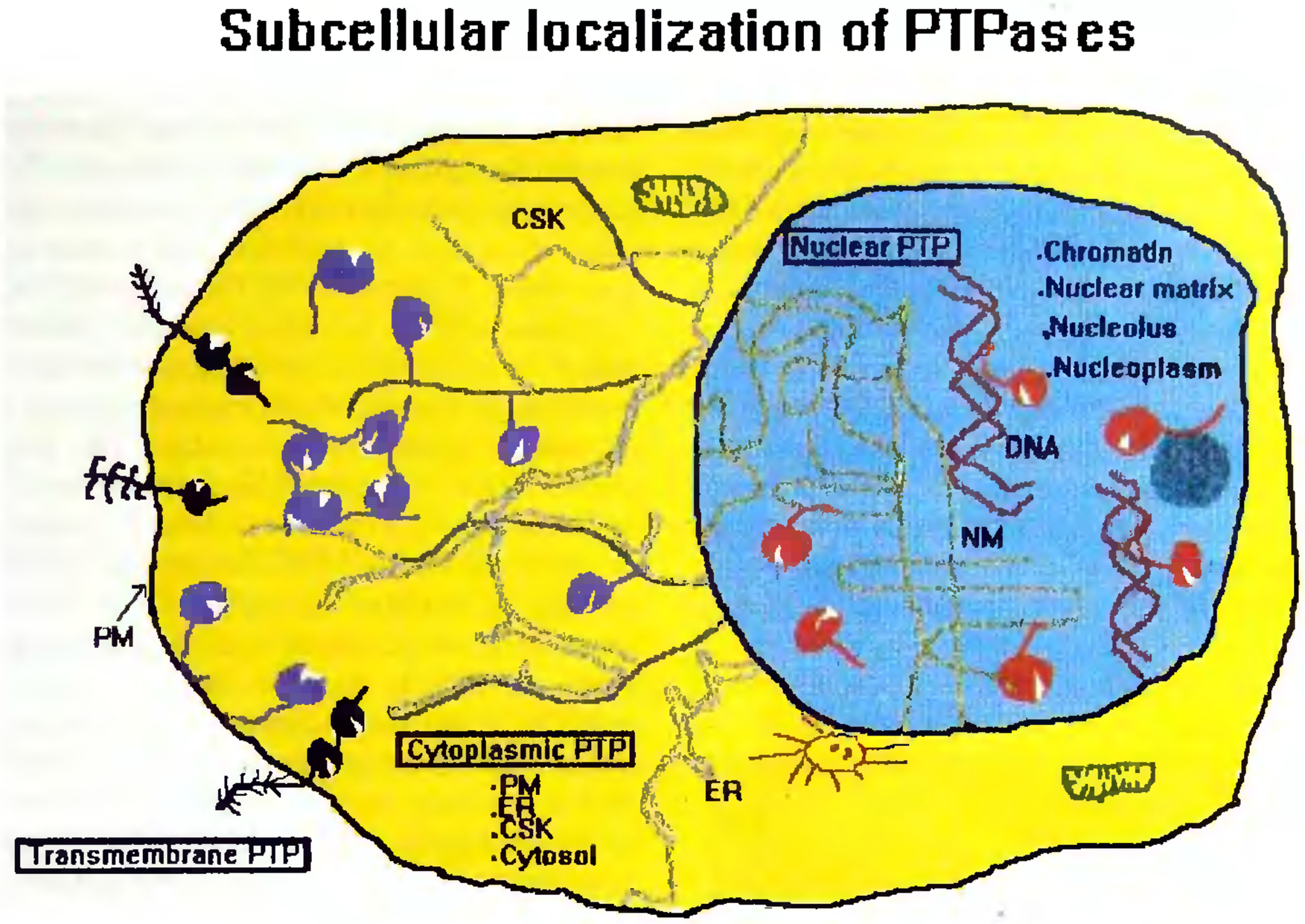

Figure 1. Localization of PTPases in the cell: Cartoon of an eukaryotic cell depicting the localization of transmembrane and intracellular PTPases. The transmembrane enzymes (depicted black) have extracellular ligand binding and intracellular catalytic domains. The intracellular enzymes localize to the cytoplasm (depicted purple) or nucleus (depicted red) and associate with various intracellular structures as ER (endoplasmic reticulum), Csk (cytoskeleton), PM (plasma membrane), NM (nuclear matrix), DNA or chromatin and nucleolus.

photyrosine preferentially[16]. These enzymes show no sequence similarity to other PTPases except for the presence of Cx_5R motif. The active site structure of these enzymes shows similar arrangement of secondary structure elements as found in PTPases, suggesting that they possibly represent convergent evolution[17].

The earliest members of PTKs and PTPases that were identified were the membrane bound or cytoplasmic enzymes leading us to believe that tyrosine phosphorylation plays a role in receiving external stimuli and transmission across the cytoplasm. But in the present decade, considerable progress has been made towards our understanding of the role played by tyrosine phosphorylation in nuclear events such as cell division cycle and transcription. Examples of PTKs and PTPases that localize to the nucleus and thereby regulate nuclear functions have been identified and the properties of nuclear PTKs have been recently reviewed[18]. In the present review, we wish to highlight the unique features of nuclear tyrosine phosphatases and their role in control of cell proliferation.

Nuclear proteins phosphorylated at tyrosine

Many nuclear proteins that play a role in cell cycle control or as transcription factors have been shown to be regulated by tyrosine phosphorylation (Table 1). Phosphorylation at tyrosine controls their interaction with other molecules, translocation to the nucleus from the cytoplasm, enzymatic activity, DNA binding and transcriptional activity. Table 1 shows that most of the nuclear proteins phosphorylated at tyrosine function as either protein kinases or transcription factors. Wherever identified, the kinase and phosphatase responsible is indicated. The dual dephosphorylation of CDKs at Tyr and Thr serves to activate them and overcome checkpoints for transition from G_1 to S and G_2 to M phases of the cell cycle[14]. In eukaryotic cells, many members of the CDK family have been identified, but the specific phosphatases that act on them are, in most cases, not known.

Signal transduction across the cytoplasm in response to growth factors, cytokines or stress, could follow two alternate pathways[19,20]. In one pathway a chain of protein kinases is serially activated that results in the translocation of MAP kinases (mitogen-activated protein kinases) to the nucleus. Within the nucleus these MAP kinases phosphorylate transcription factors leading to transactivation of several genes. In the alternate pathway, the transcription factor or its associated molecules are directly phosphorylated by tyrosine kinases at the cell

membrane or cytosol, which are then translocated to the nucleus as in the case of signal transducer and activator of transcription (STAT) proteins which serve the dual functions of signal transducers and activators of transcription[21]. The DNA binding and transcriptional activation of STATs are modulated by tyrosine phosphorylation. Within the nucleus, the MAP kinases which are phosphorylated at Tyr and Ser/Thr, are inactivated by MAP kinase phosphatases (MKP-1,2,3) which are dual specificity enzymes that act specifically on the various homologues of MAP kinases[11].

Because tyrosine phosphorylation is required for nuclear translocation and transactivation potential of STATs, a nuclear tyrosine phosphatase is a likely candidate to mediate shutting off of the transcriptional response. Using inhibitors, the involvement of PTPases has been shown, but no specific enzyme that dephosphorylates STATs has been identified[22]. Inhibition of tyrosine dephosphorylation of STAT 1 results in enhancement of the antiproliferative activity of γ interferon, indicating that inactivation of the signal requires the activity of tyrosine phosphatases[23].

Table 1. Nuclear proteins phosphorylated at tyrosine

Substrate class	Kinase	Phosphatase
Cyclin-dependent kinases Cdk1 (cdc2) Cdk2, Cdk3, Cdk4, Cdk5, Cdk6	Wee1, Mik1, Myt1, c-Src, Lyn, Lck	Cdc25A, B, C
Mitogen-activated protein (MAP) kinases ERK1, ERK2, ERK3, JNK/SAPK RK/p38	MEK	PAC1, MKP1, MKP2
Signal transducer and activator of transcription (STAT) proteins Stat 1, Stat 2, Stat 3, Tyk2 Stat 4, Stat 5a, Stat 5b, Stat 6	Jak1, Jak2, Jak3,	Unknown
Steroid hormone receptors Estradiol receptor Glucocorticoid receptor Retinoic acid receptor Thyroid hormone receptor	Src, estradiol receptor kinase	Unknown
RNA polymerase II	C-Abl	Unknown
HNF-4 (hepatocyte nuclear factor)		Unknown
Protein tyrosine kinases p140/c-Abl, $p56^{lyn}$, $p53^{lyn}$	Unknown	

Tyrosine phosphorylation modulates ligand binding and dimerization of the estradiol receptor and it has been shown that Src and a kinase purified from the cytosol phosphorylate the receptor[24–27]. Though a nuclear tyrosine phosphatase activity has been shown to be required for estradiol receptor inactivation[28], the specific enzyme involved has not been identified. Other hormone receptors (Table 1) have also been shown to be phosphorylated at tyrosine, but the enzymes responsible are not known[29–31]. Tyrosine phosphorylation of RNA polymerase II carboxy terminal domain has been implicated in the control of cell cycle dependent transcription of specific genes[32]. The nuclear PTK, c-abl has been shown to phosphorylate RNA polymerase II[33]. Abl itself is a nuclear tyrosine kinase that is phosphorylated on tyrosine and this modification is essential for its DNA binding[34]. Tyrosine phosphorylation determines the localization of HNF4 to specific intranuclear compartments and affects its DNA binding and transactivation potential[35]. In most instances of transcription factor activity being regulated by tyrosine phosphorylation, phosphorylation enhances transcription and dephosphorylation decreases it. But tyrosine dephosphorylation has been shown to enhance the binding of TGFβ responsive factor to its elements and increase α2(I) collagen gene expression, indicating that in this case, phosphorylation at tyrosine is inhibitory to transcriptional activation[36].

PTP-S: A nuclear tyrosine phosphatase that binds DNA

The first intracellular tyrosine phosphatase to be cloned, TC-PTP and its rat and mouse homologues – PTP-S and MPTP are ubiquitously expressed enzymes[37–39]. These proteins have a conserved catalytic domain at the N-terminus and non-catalytic domain at the C-terminus that exhibits several unique properties. Analysis of the PTP-S gene structure and other experiments showed that alternate splicing gives rise to four different transcripts in murine cells while only two forms are generated in human cells due to a mutant splice site[40]. The four rat isoforms have been denominated PTP-S1, S2, S3 and S4 and will be referred to with this nomenclature. MPTP, the mouse form and TC45, the human form are homologous to PTP-S2 and human TC48 or TC-PTP is homologous to PTP-S4. PTP-S2 is the major form in most tissues and cell lines examined and PTP-S4 is present at 2–10 fold lower levels than PTP-S2 (ref. 41). The last six amino acids of PTP-S2 are replaced by 34 amino acids (mostly hydrophobic) in PTP-S4 (Figure 2). PTP-S1 and PTP-S3 are minor transcripts similar to PTP-S2 and PTP-S4 forms respectively, but lack 19 amino acids (exon E1) in the non-catalytic domain arising from alternate splicing[40].

Subcellular localization of PTP-S gene products has

been analysed in detail. In indirect immunofluorescence experiments, cellular PTP-S polypeptides are predominantly localized to the nucleus[42,43]. Subcellular fractionation showed that they are present in the nucleus as well as cytosol; within the nucleus PTP-S polypeptides are largely associated with chromatin and a small fraction with the nuclear matrix[42]. These studies were performed using a monoclonal antibody that recognizes both the major forms, PTP-S2 and PTP-S4. But when the localization of these forms was examined individually in transient transfection experiments, PTP-S2 was localized exclusively to the nucleus with prominent nucleolar staining[41]. PTP-S4 localized to cytoplasmic membranes (such as endoplasmic reticulum) with prominent staining of the nuclear periphery (Figure 3). Fractionation of cells expressing exogenous PTP-S2 or PTP-S4 forms showed that PTP-S2 is chromatin bound in the nucleus, while a majority of PTP-S4 is present in the post-nuclear supernatant and the small amount of PTP-S4 in the nucleus is associated with the nuclear matrix. PTP-S4, but not PTP-S2 interacts strongly with the isolated nuclear matrix[41].

An interesting and unique property of PTP-S2 is that it binds to double-stranded and single-stranded DNA[44]. The DNA-binding domain has been localized to C-terminal 57 amino acids which are essential and sufficient for DNA-binding. PTP-S4 does not bind to DNA even though it possesses the DNA binding domain, except for the last six amino acids present in PTP-S2 (ref. 41). Experiments performed using a deletion construct of PTP-S4 lacking the C-terminal 34 amino acids (ΔPTP-S4) (Figure 2) showed that the absence of this domain makes the PTP-S4 form capable of binding to DNA and localizing to the nucleus just like the PTP-S2 form[41]. It appears as if the hydrophobic tail of PTP-S4 overrides the nuclear location signals (NLS) and DNA-binding domain by masking it. This possibility appears likely as it has been shown that the C-terminal 34 amino acids is not sufficient for targeting to endoplasmic reticulum in the case of the human protein[45]. It has also been found that while PTP-S2 exists as a monomer, PTP-S4 forms a multimer, the hydrophobic C-terminal sequences being responsible for its multimerization[41]. It is possible that in the process of intermolecular and intramolecular interaction, the DNA-binding domain and NLS get masked.

The hydrophobic sequences of PTP-S4 have also been shown to regulate the substrate preferences of this isoform; PTP-S2 prefers acidic substrate whereas PTP-S4 shows preference for basic substrate[41]. Though all these properties have not been studied using the human homologues it has been shown that TC-PTP p48 and p45

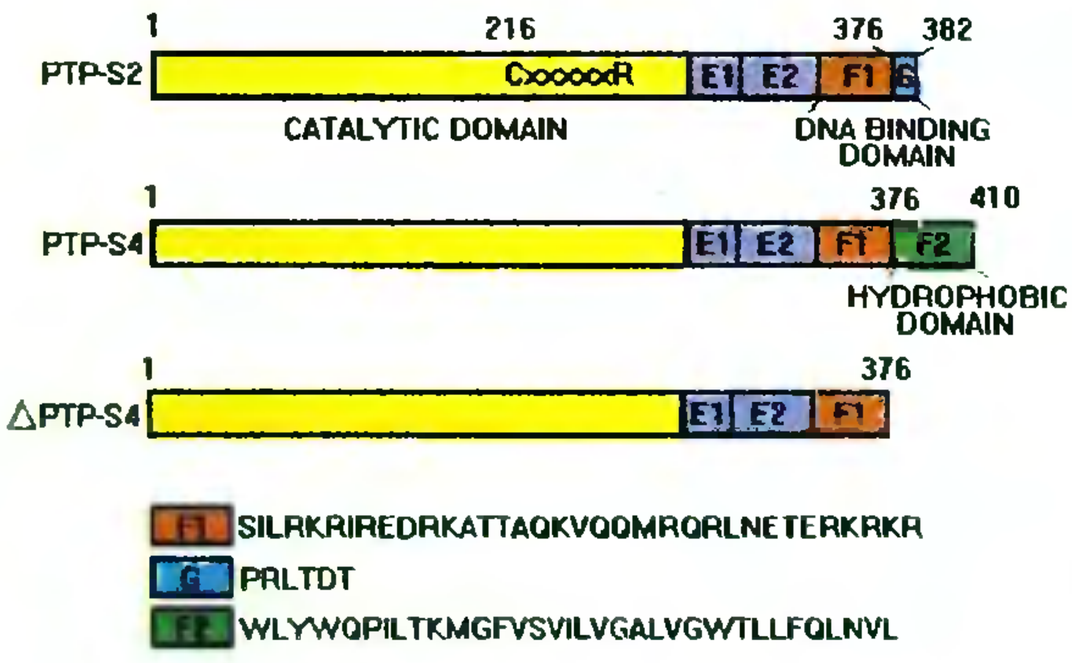

Figure 2. Schematic presentation of PTP-S2, PTP-S4 and a deletion mutant. Numbers at the top (1, 216, 376, 382, 410) indicate amino acid position. Exons in the C-terminal domain E1, E2, F1, F2 and G are indicated. Amino acid sequence of C-terminal exons is shown in single-letter code. Last six amino acids of PTP-S2 (exon G) are replaced by 34 amino acids (exon F2) in PTP-S4. C216 indicates cysteine at position 216 which is essential for catalysis.

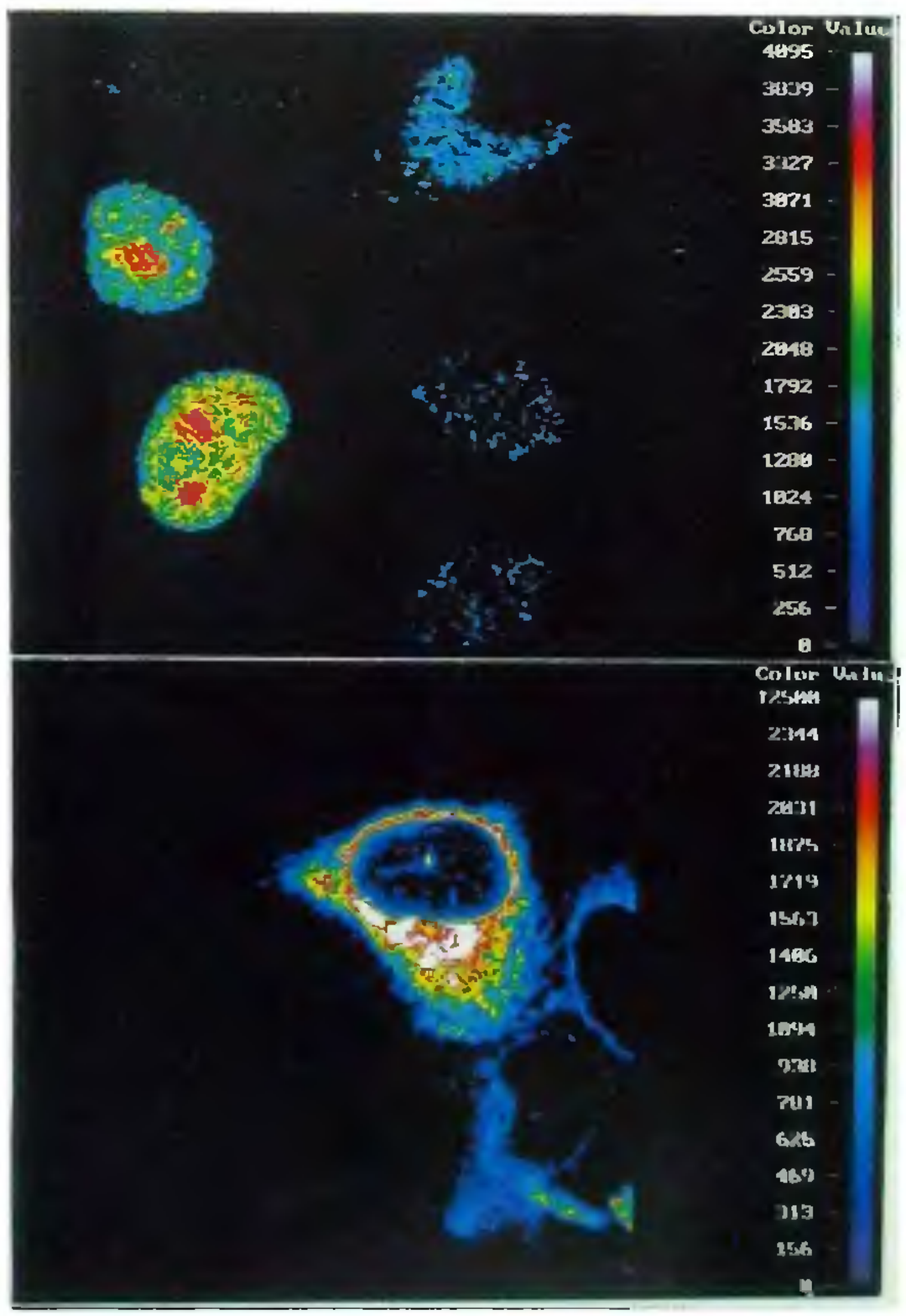

Figure 3. An optical section showing subcellular localization of PTP-S2 and PTP-S4, as revealed by using a laser confocal scanning microscope. Cells were transfected with appropriate plasmids and then stained with a monoclonal antibody against PTP-S. FITC conjugated second antibody was used for visualization. Upper panel shows HeLa cells transfected with PTP-S2. Within the nucleus prominent staining of nucleoli is seen. Lower panel shows PTP-S4 in COS-1 cells.

show localization patterns similar to the rat forms with the exception that the human homologue is excluded from the nucleoli[45]. The possible significance of nucleolar localization of murine PTP-S2 is not evident. The primary structure does not indicate the presence of any known nucleolar localization signals. However, it is possible that a signal for nucleolar localization may be present in rat and mouse PTP-S2, but it may be nonfunctional in the human homologue. Nuclear localization signals have been identified in mouse and human PTP-S2 which are located in the basic DNA-binding domain[43,45]. Table 2 lists some of the properties of the two forms PTP-S2 and PTP-S4.

The domain structure of non-catalytic region of PTP-S4 (and human TC PTP) has striking resemblance with those of sequence specific DNA-binding proteins. The basic domain with sequences that can bind DNA have been shown to have homology with transcription factors Fos and Jun[38]. This domain is followed by a hydrophobic tail which functions as an oligomerization domain. Although PTP-S4 does not bind to non-specific double-stranded or single-stranded DNA, the possibility that it could interact with specific sequences of DNA has not been explored.

Drosophila nuclear tyrosine phosphatase

dPTP61F, a *Drosophila* gene encodes two forms of intracellular tyrosine phosphatases, generated by alternate splicing that localize to the nucleus or the cytoplasmic membranes[46]. The 3′ end of this gene (that localizes to 61F position of polytene chromosomes) contains an alternate splice site in exon VII that generates a form with either a hydrophobic tail of 24 amino acids (p61/62 m) or a hydrophilic tail of 11 amino acids (p61/62 n). Using pNPP as a substrate, it was shown that the catalytic activity of the two forms do not differ. Transfection of Cos-1 cells with expression vectors containing the two cDNAs showed that p61/62m is localized to the reticular network and mitochondria-like organelles whereas p61/62n showed nuclear staining, the nucleoli being excluded. Using chimeric molecules it was shown that the 24 C-terminal amino acids unique to p61/62m could target to cytoplasmic membranes, but the 11 amino acids unique to p61/62n could not target a fusion protein to the nucleus, though the C-terminal 53 amino acids are capable of conferring nuclear localization. There are three small stretches of basic amino acids common to both forms that could function as NLS, but the presence of the hydrophobic tail in p61/62m may be acting dominantly to retain the protein on cytoplasmic membranes.

The C-terminal non-catalytic sequence of these enzymes has five proline-rich motifs that align with the consensus for SH3 domain binding. Using the nuclear form of dPTP in a yeast 2 hybrid system, *Dock*, a novel adaptor protein homologous to mammalian oncoprotein Nck was identified[47]. dPTPn interacts through its proline containing sequence with the SH3 domains of *Dock*. In the Drosophila embryo, the expression of *Dock* and dPTP overlap, localizing predominantly to the central nervous system. Though *Dock* is not a substrate of dPTP, two other tyrosine phosphorylated proteins of 190 and 145 kDa that may be likely substrates have been identified to complex with *Dock*. Drosophila *Dock* is a protein essential in photoreceptor axon guidance and tyrosine phosphorylation plays a crucial role in nervous system development suggesting a role for dPTP in nervous system guidance mechanism.

Table 2. Properties of PTP-S2 and PTP-S4

PTP-S2	PTP-S4
Monomer	Probably tetramer, no monomer seen
Binds to non-specific DNA, RNA, through basic domain	Does not bind to DNA, RNA although it has the basic domain
Prefers acidic substrate, basic substrate is a poor substrate	Prefers basic substrate
Present in the nucleus	Perinuclear and cytoplasmic staining
Induced during liver regeneration after 6 h	Induced after 6 h but to a greater extent than PTP-S2
Deletion of non-catalytic domain increases activity towards poly Glu, Tyr	Deletion of C-terminal 34 amino acid decreases activity towards basic substrate but increases activity towards acidic substrate
Overexpression increases cell proliferation	Not yet known. Human homologue has no effect on cell proliferation
Binds with very poor affinity to isolated nuclear matrix	Binds well to isolated nuclear matrix

PRL-1: Phosphatase of regenerating liver

One of the genes identified as an immediate early gene in regenerating liver and mitogen treated fibroblasts was PRL-1, an intracellular tyrosine phosphatase[48]. This enzyme differs from other members of the family in possessing essentially only the catalytic phosphatase domain of 173 amino acids. Expression of PRL-1 is high in several tumour cell lines, but low in proliferating nontumourigenic cells. The endogenous protein as well as exogenously expressed PRL-1 localize to both the nucleus and cytoplasm. Subcellular fractionation showed that it is present in a Triton insoluble nuclear fraction and could be solubilized by high salt. These features suggest that it is not a soluble nucleoplasmic protein, but is associated with nuclear structures, possibly chromatin. PRL-1 is a basic protein with highly basic stretches (that could serve as NLS) raising the possibility of its association with DNA.

Expression of PRL-1 in NIH 3T3 cells resulted in

multinucleate cells of abnormal morphology. Stable clones expressing moderate levels of the protein showed enhanced growth rate, increased saturation density and anchorage independent growth. The potential substrates of PRL-1 are yet to be identified. More recent studies have shown that PRL-1 is significantly expressed in intestinal epithelia and in contrast to its expression pattern in liver, expression in the intestine is associated with cellular differentiation[49]. It is expressed in the villus (but not in crypt enterocytes) and in confluent differentiated, but not in proliferating undifferentiated Caco-2 colon carcinoma cells. These findings suggest that this enzyme has differing functional roles in different tissue systems.

FLP1 (Fetal liver phosphatase)

This is a novel member of the intracellular tyrosine phosphatases that has an N-terminal catalytic domain followed by a C-terminal 160 amino acid non-catalytic domain[50]. Its expression is restricted to the thymus during the fetal stage, but is expressed in the kidney and hematopoietic tissues in adult mice. This enzyme is localized to the nucleus in Ba/F3 lymphoid cells. Ectopic expression of a catalytically inactive mutant inhibited TPA induced differentiation of the progenitor cell line K562, suggesting a role for this enzyme in differentiation.

PEST containing phosphatases

This is a subfamily of intracellular phosphatases that is characterized by the presence of PEST (proline, glutamic acid, serine, threonine) enriched sequences in the non-catalytic region. One of them, PEP, in transfected HeLa cells, localized to the nucleus with no nucleolar staining and the C-terminal 18 amino acids were identified by deletion and domain swap experiments to confer nuclear localization[51]. But in Cos-1 cells, full length PEP showed cytoplasmic localization upon transfection indicating that subcellular localization may vary with cell type, transfection protocol and physiological status of the cells[52]. PEP expression does not change during the cell cycle, but in B lymphocytes, PEP mRNA is induced upon B cell receptor ligation and PEP protein in the nucleus increased considerably, though the levels in cytoplasm did not change. Transfection of antisense RNA particularly reduced the nuclear protein and counteracted the anti-IgM induced growth arrest and apoptosis. Three other closely related phosphatases PTP-PEST, PTP-K1 and BDP-1 possess NLS similar to PEP but localize predominantly to the cytoplasm, though PTP-K1 also shows nuclear localization[53–55]. Another member of this family cloned from hematopoietic progenitor cells is homologous to PTP-PEP in the region involved in nuclear localization and is localized to the nucleus in these cells[56]. This phosphatase is expressed at minimal levels in many adult tissues, but is expressed at high levels in several murine hematopoietic progenitor cell lines. Upon differentiation, its mRNA is down regulated indicating a role for this enzyme in maintenance of the undifferentiated state of the hematopoietic stem cells. CSK, a cytoplasmic PTK has been shown to associate with PEP, but as yet no physiological role has been attributed to this association[52].

Dual specificity phosphatases

Cdc25 was first identified in yeast as a gene essential for entry into mitosis and was later found to be a dual specificity phosphatase[15]. Cdc25 is a predominantly nuclear protein in mammalian cells and is excluded from condensed chromosomes during mitosis[57]. Mammalian cells were later found to have three homologues A, B and C showing differences in subcellular localization and functioning at different phases of the cell cycle[15]. Mouse fibroblasts stably transfected with Ha-Ras and Cdc25A show that 5–10% of the Cdc25 protein is associated with the plasma membrane and the majority of the protein which is nuclear is not extracted by 1% NP-40 (ref. 58). This suggests that Cdc25A is not a nucleoplasmic protein, but is associated with nuclear structures like chromatin or nuclear matrix. In interphase cells, Cdc25B shows granular intranuclear staining. It accumulates in the cytoplasm in late G2 and functions to activate the cytoplasmic pool of Cdc2/Cyclin B responsible for microtubule nucleation at the centrosomes[59]. But, by itself, Cdc25B does not associate with either the microtubule network or centrosomes. Exogenously expressed Cdc25B in HeLa cells, Cos cells or fibroblasts does accumulate in the nucleus, the nucleolus remaining excluded. Cdc25C is present in the nucleus, but mostly in the nucleoplasm, not tightly associated with nuclear structures and therefore easily extracted under mild lysis conditions. It is presumed that Cdc25C acts on the nuclear pool of Cdc 2/Cyc B whereas Cdc25B acts on the centrosomal Cdc2.

Cdc25B shows changes in expression during the cell cycle, rapidly accumulating at prophase and turning over at the end of mitosis, unlike Cdc25A and C isoforms which are present at constant levels throughout the cycle in growing HeLa cells, though they act at specific points in the cell cycle[57,59,60]. But when quiescent fibroblasts are stimulated, expression of Cdc25A is growth factor responsive, peaking by 3 h after stimulation. Cdc 25A has been shown to act on the G1 phase kinase Cdk4 and reverse its tyrosine phosphorylation[61].

MAP kinase phosphatases

Members of the MAP kinase phosphatases (MKPs) family of enzymes possess the canonical signature sequence of tyrosine phosphatases, but exhibit catalytic activity towards both phosphotyrosine and phosphothreonine in MAP kinase substrates. They share a conserved catalytic domain at the C-terminal, but exhibit considerable sequence diversity at the N-terminal. Differential expression in a variety of cell types in response to various extracellular stimuli suggests that each member plays a distinct role in regulation of the various MAP kinase family members involved in different signal transduction pathways[10]. Sequence analysis showed the presence of putative NLS in these enzymes. One of the first members to be identified, PAC-1 was shown to be a nuclear protein with uniform pattern of strong punctate staining with the nucleolus excluded[12]. Other members of this class have been isolated from diverse group of organisms including viruses, yeast and mammals and have been shown to be nuclear enzymes, but studies on their subnuclear localization are lacking[11,13]. Another common property shared by these enzymes is rapid induction upon mitogenic stimulation.

More recently, other members of this family of dual specificity phosphatases have been identified that have substrate specificity towards MAP kinases and its homologues, but are not inducible by either mitogens or stress (*pyst* 1 and MKP-3)[62,63]. They are localized to the cytoplasm and unlike the nuclear enzymes, are unable to block either the stress-mediated activation of JNK *in vivo* or to inhibit nuclear signalling events. These enzymes are likely to act on the cytoplasmic pool of the MAP kinase family members.

The requirement for phosphorylation at both Tyr and Thr for activation of MAP kinases has been shown *in vitro*. Dephosphorylation at either Tyr or Thr is sufficient to inactivate MAP kinases. In some instances it has been shown that protein serine, threonine phosphatases (PSPs) and PTPs also act on MAP kinases and dephosphorylate either the Thr or the Tyr residues, thereby inactivating them[64,65]. *In vivo*, this could result in integration of signalling pathways at the level of MAP kinase.

Induction of nuclear tyrosine phosphatases upon mitogenic stimulation

Mitogenic stimulation of resting cells or exposure of responsive cells to cytokines or stress results in rapid induction of several genes prior to the initiation of fresh protein synthesis[66]. Such genes have been classified as immediate early genes and majority of them encode nuclear proteins like transcription factors and proteins required for progression through the cell cycle. Screens set up to identify such genes resulted in the identification of a novel class of tyrosine phosphatases that localize to the nucleus and dephosphorylate proteins at both Ser/Thr or Tyr residues (MKP family)[12,13]. Further studies showed that these enzymes function to dephosphorylate MAP kinase and its homologues and are induced upon mitogenic stimulation and in stress response to arrest the transcriptional activation initiated by the MAP kinases. Their properties have recently been reviewed[11].

Apart from the dual specificity phosphatases, some of the intracellular tyrosine phosphatases are also induced upon mitogenic stimulation. Con A stimulation of rat splenocytes results in the stabilization of PTP-S mRNA leading to increased levels of PTP-S in stimulated cells[67]. Studies on regenerating liver and cells in culture have shown that PTP-S isoforms are present at very low level in resting cells and increase several fold upon entry into G1 phase, reaching maximum levels prior to the start of S phase[68]. More recently using RT-PCR (reverse transcription-polymerase chain reaction), it has been shown that both the major forms of PTP-S, PTP-S2 and PTP-S4 are induced during liver regeneration by 6 h after partial hepatectomy[41]. Though the time course of induction suggests that PTP-S is a delayed early gene, its mRNA is superinduced upon treatment of lymphocytes with cycloheximide[67]. While many genes show a transient spate of induction, mRNA and protein levels of other mitogen-induced genes remain elevated for extended periods during the cell cycle. PTP-S levels have been shown to change from G0 to S transition, but remain essentially constant in all phases of cycling cells[68].

PRL-1 is the other nuclear tyrosine phosphatase that is induced in mitogen-treated cells and also in the presence of protein synthesis inhibitors[48]. It is an immediate early gene and in regenerating liver its mRNA is induced within 30 min reaching peak levels after 3 h of partial hepatectomy. Its expression is not limited to the G1 phase of the cell cycle and remains elevated throughout the growth response. PRL-1 nuclear protein levels in regenerating liver parallel those of its mRNA, though its peak occurs just before S phase. PTP-PEST shows induction of mRNA within 6 h of partial hepatectomy, decreases to normal levels by 24 h and increases again at 48–72 h (ref. 69). Analysis of the genes for some of the mitogen-induced phosphatases showed the presence of sequences (in the promoter region) responsible for the regulated induction of these genes[70].

The fact that many tyrosine phosphatases are immediate early genes suggests that these genes are likely to be primary targets of mitogenic signal transduction pathways. While some of these nuclear enzymes are triggered to arrest the signalling pathways (MKP family) and act as negative regulators of cell proliferation, others like PTP-S2, PRL-1 and Cdc25 act as positive regulators. On the other hand, growth inhibitory signals could also

act by suppressing the phosphatases acting as positive regulators. Treatment of Daudi cells with Ifnα results in sharp reduction in Cdc25A mRNA and this may be the mechanism accounting for the rapid reduction in activity of Cdk2 complexes[71].

Regulation of cell proliferation by PTPases

The physiological role of many of the intracellular phosphatases has been investigated by overexpressing them in cell lines and monitoring the consequences on cell proliferation. Since the level of tyrosine phosphorylation is crucial to maintenance of cellular homeostasis, it has been relatively difficult to obtain stable cell lines that overexpress tyrosine phosphatases. In most instances, high levels of overexpression lead to toxicity and thereby surviving stable clones are usually moderate expressors of the enzymes.

Overexpression of a PTPase can lead to a variety of effects on cell growth and morphology depending on the cell type and the PTPase gene employed in the experiment. In general, overexpression of nuclear PTPases results in increase in cell proliferation, whereas dual specificity phosphatases can increase or decrease cell growth. PRL-1, when overexpressed in NIH 3T3 cells, resulted in cells with abnormal morphology and multiple nuclei[48]. The stable PRL-1 expressing clones showed enhanced growth rates and higher saturation density and were capable of forming colonies in soft agar and tumours in nude rice, though not with the same efficiency as V-src transfected cells.

Expression of TCPTP, the human homologue of PTP-S4 in BHK cells does not show any altered phenotype, but expression of a truncated enzyme that is active *in vivo* leads to cytokinetic failure with the cells exhibiting a multinucleate phenotype[72]. But when this truncated form is expressed in V-fms oncogene transformed Rat2 cells, there is a suppression of the transformed phenotype as evident from the loss of anchorage independent growth and reduced tumour formation in nude mice[73]. The full length protein does not show these effects. Therefore the effects of truncated TC-PTP (which is cytosolic) on cell physiology are unlikely to mimic the cellular function of full length TCPTP. Rat PTP-S2, the nuclear isoform of the same gene, when transfected into either COS-1 or HeLa cells, gave rise to a greater number of large G418 resistant colonies compared to the control plasmid indicating that it enhances cell proliferation. Stable cell lines of HeLa expressing the full length rat PTP-S2 show enhanced growth rates and lower serum requirement compared to control cells[74]. These clones show altered morphology in confluent cultures and develop larger colonies in soft agar assays.

The dual specificity MKP family members dephosphorylate MAP kinases and inactivate them. Constitutive expression of MKP-1 blocks G1 specific gene transcription and therefore inhibits DNA synthesis and cell proliferation[75]. In several systems it has been shown that down regulation of MKP-1 is necessary for proliferation of cells[76]. But the other class of dual specificity phosphatases involved in cell cycle regulation, Cdc25 homologues are positive regulators of cell proliferation and cooperate with oncogenes in transforming primary rodent fibroblasts[77]. Cdc25A gene contains Myc/Max-binding sites and its mRNA is induced following activation of Myc (ref. 78). Using deletion mutants of Myc, it has been shown that the oncogenic properties of Myc depend on its ability to induce Cdc25A mRNA, establishing a link between oncogenes and cell cycle machinery.

Though some enzymes can be clearly defined as either positive or negative regulators of cell proliferation, other enzymes show opposing effects when expressed in different cell lines. For e.g., PTP-1C, an SH2 domain containing phosphatase is predominantly expressed in hematopoietic cells where it negatively regulates cellular signalling from receptors[79]. But when expressed in non-hematopoietic cells, it plays a positive role in EGF or serum-stimulated mitogenesis[80].

In addition to nuclear PTPases, some other PTPases have been shown to affect cell proliferation. For example receptor type PTPase PTPα increases cell proliferation whereas LAR-PTP and DEP-1 cause reversion of transformed phenotype and reduction of cell growth[81–83]. CD45 has an important role in lymphocyte activation[84]. PTP-1, PTP-1B, PTP-MEG are non-receptor type PTPases which reduce cell proliferation[85–88]. Overexpression of low molecular weight phosphatase causes reduction in cell growth of NIH3T3 cells[89].

PTPases in human disorders

Accumulating evidence has shown that the PTPases play as significant a role as the PTKs in maintaining cellular homeostasis. Therefore it is not surprising to find that deregulation of tyrosine phosphatases can lead to neoplastic and non-neoplastic disorders. A few examples where the disease phenotype has been related to altered expression or mutations in tyrosine phosphatases are discussed below. In instances where altered expression is correlated with studies on overexpression in cell lines that lead to higher or lower proliferation rate, one could envisage that the malignant phenotype is a consequence of the deregulated enzyme. Table 3 lists the nuclear PTPases known to be associated with malignancy in humans and their chromosomal localization.

Human CDC25A or CDC25B but not CDC25C phosphatases are capable of transforming primary rodent fibroblast in cooperation with either Ha-RAS or loss of RB1 (ref. 77). CDC25B is overexpressed in 32% of human primary breast cancers tested. Tumour-specific

Table 3. Nuclear PTPases which may be involved in human disorders

PTPase	Chromosomal localization	Abnormality	Associated disorder	Ref.
PTEN	10q 23	Deletions/mutation	Multiple human cancers	93, 94
OV-1 (PRL-1)	17q	LOH	Sporadic breast and ovarian cancers	95
Cdc25A	3p21		Abnormal in renal, small cell lung carcinoma and salivary gland tumours	77
Cdc25B	20p13	Overexpression	Breast cancer	77
MKP-1 (CL-100)	5q35	Overexpression	Epithelial tumours	90
		Deletion/ translocation	Adult leukaemias and solid tumours	91
PAC-1	2q11	Translocation	Myeloproliferative disorders and adenocarcinomas	91
hVH-4	10q11		Region of MEN syndrome and adult leukaemia	91

expression of CDC25B in human breast carcinomas correlates with less favourable prognosis and survival. Studies carried out so far suggest that CDC25 phosphatases may contribute to the development of human cancer.

MKP-1 is induced by several oncogenes in the Ras-dependent pathways and can inactivate both proliferative (through ERKs) and apoptotic signals (through JNKs). MKP-1 RNA and protein expression were analysed in human epithelial tumours and was found to be over-expressed in the early phases of prostate, colon and bladder carcinogenesis, with loss of expression in metastasized tumours[90]. But in breast carcinomas MKP-1 expression was found to be high in all stages. No deletions or mutations were found in the MKP-1 gene indicating that MKP-1 does not behave as a tumour-suppressor in epithelial tumours. MKP-1 expression was examined in prostrate cancers, either treated or not treated by androgen ablation[91]. In untreated cases, MKP-1 was overexpressed in the preinvasive stage, but its expression decreased with advanced disease stage. In the treated cases, MKP-1 expression was down regulated and was correlated with apoptosis, indicating that MKP-1 levels could serve as a prognostic marker in monitoring prostrate cancers. Overexpression of MKP-1 in human tumours is surprising since this phosphatase causes growth inhibition upon overexpression in cell lines. It is likely that MKP-1 has positive or negative regulatory roles depending upon the type of cell. Three members of the MKP family (PAC-1, CL100 and hVH-4) have been found to map to regions frequently involved in deletions or translocations in myeloproliferative disorders and adeno carcinomas[92]. But it is yet to be established whether the transformed phenotype is due to mutations in the phosphatase genes.

In the above instances, it is not clear whether over-expression of the phosphatases is the cause or the effect of malignant transformation and which of these enzymes actually play an oncogenic role. Phosphatases that play a growth-suppressive role in normal cells may lead to transformation when either transcriptionally down-regulated or deleted (or mutated). Recently, a gene mutated in multiple human cancers that is localized to the long arm of chromosome 10 (10q23), was identified to encode a protein tyrosine phosphatase, PTEN[93]. This enzyme shows closest homology to PRL-1 and Cdc14, which are enzymes involved in cell growth regulation. Many of the mutations identified mapped to the catalytic domain of the enzyme. Mutations and loss of heterozygosity observed in patients indicate that PTEN is a candidate tumour-suppressor gene. Further evidence for the role of PTEN as a tumour-suppressor gene comes from studies showing germline mutations in this gene in Cowden disease, an inherited breast and thyroid cancer syndrome[94]. Human PRL-1 (OV-1) gene is localized to long arm of chromosome 17, which is a region that shows loss of heterozygosity in sporadic breast and ovarian cancers[95].

In addition to the above mentioned nuclear PTPases, certain other intracellular PTPases such as PTP-1B, PTP-G1, PTP-N6 and SAP-1 (stomach cancer associated phosphatase) may have a role in the development of malignancy[96–100]. The receptor type PTPases, PTP-γ and HPTPα, are potential candidates for tumour-suppressor genes in lung and renal carcinomas[101,102].

Mapping of tyrosine phosphatases to certain aberrant loci does not directly imply their involvement in the malignant phenotype, but given the role played by these enzymes in growth regulation, they are likely to contribute to the development of human cancer. The PTPases

also have a role to play in non-malignant disorders as it has been found that the intracellular domains of certain transmembrane phosphatases are the major targets of the autoimmune response in insulin-dependent diabetes mellites[103,104]. The intracellular PTPases also appear to modulate insulin responsiveness by acting on the insulin receptor. Mutations in a putative PTPase gene (MTM1) have been found in X-linked myotubular myopathy, a congenital muscular disorder[105]. It is proposed that this gene product is required to mediate action of growth factors in myogenic differentiation.

Concluding remarks and future prospects

Till the early nineties it was believed that tyrosine phosphorylation generally does not occur in the cell nucleus. Since then several tyrosine kinases (such as c-Abl, Lyn, Wee 1, Mik), tyrosine phosphatases and tyrosine phosphorylated proteins have been identified in the nucleus. Work in various laboratories including ours has clearly shown that nuclear protein tyrosine phosphorylation is widespread[106]. In addition to identification of novel PTKs and PTPases in the nucleus, some of the previously known cytosolic PTKs have been found to be associated to some extent, with the nucleus. For example $p56^{lyn}$ and $p53^{lyn}$ (ref. 106).

It was believed that *in vitro* there is no large difference in substrate specificity of PTPases, and subcellular localization restricts or determines substrate specificity *in vivo*. However recent observations from our laboratory and others have shown that there are intrinsic differences in substrate specificity even *in vitro*. A particularly striking example is provided by the products of PTP-S gene, PTP-S2 and PTP-S4, which show very small difference in structure but differ greatly in substrate specificity and other properties[41] (Table 2).

Although several transcription factors have been identified which are phosphorylated on tyrosine (and Tyr phosphorylation in many cases has been shown to affect transcription) the role of nuclear PTPases in the regulation of transcription is poorly understood. Several PTPases and tyrosine-phosphorylated proteins are known to be present in the nucleus, but a link between these proteins and individual PTPases is yet to be established.

Nuclear (and other) PTPases may have more complex and multiple roles since a single PTPase is likely to have many substrates. An indication of complexity is provided by PTP-1C which acts as positive and negative regulator in different types of cells. In Drosophila, genetic evidence shows that protein phosphatase 2A positively and negatively regulates the same Ras1 mediated signalling pathway involved in photoreceptor development[107]. How a phosphatase can have positive and negative regulatory effects in the same pathway remains to be elucidated.

Identification of the role of nuclear and other PTPases in signalling pathways would require identification of immediate targets (substrates) and distant targets (genes/proteins affected by the substrates). Newly emerging strategies based on identification of interacting proteins by using yeast two hybrid system and substrate trapping catalytically inactive mutants should help in identifying substrates and distant targets of PTPases. These and other approaches are likely to help in defining the roles of PTPases in signalling pathways, transcriptional control, cell proliferation and transformation.

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Soft X-ray microscope: A new biological tool

Satish Vitta

Department of Metallurgical Engineering and Materials Science, Indian Institute of Technology, Mumbai 400 076, India

Soft X-ray microscopy is a technique which has tremendous application potential for biological imaging in the *native aqueous environment*. It offers a structural resolution of ~ 50 nm, which is between that of an electron microscope and the conventional optical microscope. The main advantage of this technique, however, is that it facilitates imaging of relatively thick (1–10 μm) samples at high resolution in the *wet* condition without the necessity of external contrast-enhancing agents. Although this technique has been proved to be highly useful for biological imaging, it is still not widely used because of the technological difficulties. This paper deals with the technological issues of soft X-ray microscopy *vis-à-vis* the recent developments in these areas which will widen the application potential of this technique.

Soft X-ray microscopy

The optical and electron microscopy techniques are being used successfully for the characterization and design of materials in various applications. These techniques, however, are not extensively used for characterization in biological sciences. The optical microscopy technique is simple in nature, offers flexible conditions for observation and the radiation used for imaging is benign to the cells. However, the maximum resolution that can be achieved and the depth of focus are poor, which limit the usage of this technique. The recently developed confocal optical microscope facilitates 3D imaging, which is not possible with the conventional optical microscope. However, the point-to-point resolution is only ~ 0.2 μm, an order of magnitude lower than that offered by soft X-ray microscopy. Electron microscopy, on the other hand, is a technique capable of resolving features down to subnanometer sizes. This technique, however, is not widely used for imaging biological materials because of the following reasons: (i) The specimen thickness should be < 100 nm to realize resolutions of the order of ≤ 5 nm. This means careful and complex specimen preparation is required which can cause irreversible damage to the sample; (ii) The high energy electron beam used for imaging can also lead to an irreversible damage to the sample; (iii) The weak dependence of scattering cross-section for electrons on atomic number makes artificial staining a necessity for contrast enhancement; (iv) The samples can be observed only in dried state which means imaging in the native aqueous environment is not possible.

The soft X-ray microscopy technique is ideally suited for imaging biological materials as it bridges the