
PTP-S2, a nuclear tyrosine phosphatase, is phosphorylated and excluded from condensed chromosomes during mitosis

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PTP-S2 is a tyrosine specific protein phosphatase that binds to DNA and is localized to the nucleus in association with chromatin. It plays a role in the regulation of cell proliferation. Here we show that the subcellular distribution of this protein changes during cell division. While PTP-S2 was localized exclusively to the nucleus in interphase cells, during metaphase and anaphase it was distributed throughout the cytoplasm and excluded from condensed chromosomes. At telophase PTP-S2 began to associate with chromosomes and at cytokinesis it was associated with chromatin in the newly formed nucleus. It was hyperphosphorylated and showed retarded mobility in cells arrested in metaphase. *In vitro* experiments showed that it was phosphorylated by CK2 resulting in mobility shift. Using a deletion mutant we found that CK2 phosphorylated PTP-S2 in the C-terminal non-catalytic domain. A heparin sensitive kinase from mitotic cell extracts phosphorylated PTP-S2 resulting in mobility shift. These results are consistent with the suggestion that during metaphase PTP-S2 is phosphorylated (possibly by CK2 or a CK2-like enzyme), resulting in its dissociation from chromatin.

1. Introduction

Phosphorylation and dephosphorylation regulate key cell cycle events in eukaryotic cells and this is achieved by the activities of protein kinases and phosphatases. Therefore, the timely regulation of these enzymes is crucial, with deregulated expression and activation leading to either inappropriate cell division, cell cycle arrest or apoptosis. Tyrosine-specific protein phosphorylation plays a significant role in regulating the activities of components of signal transduction pathways, the cyclin dependent kinases that determine phase transition during cell division and also the activities of transcription factors (Radha and Swarup 1997; Tonks and Neel 1996). In eukaryotes a large number of intracellular protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) have been identified but knowledge of the *in vivo* function of individual PTPs in most cases is lacking. The non-catalytic sequences of the PTPs have been shown to play a role in their regulation by modulating enzyme activity, determining substrate specificity and by effecting their subcellular localization

(Mauro and Dixon 1994; Fauman and Saper 1996; Radha and Swarup 1997).

PTP-S2 and PTP-S4 are two isoforms of the ubiquitously expressed intracellular PTP, PTP-S (Swarup *et al* 1991; Reddy and Swarup 1995). PTP-S2 is the predominant form which localizes to the nucleus and PTP-S4 is expressed at much lower levels and localizes to nuclear and cytoplasmic membranes (Radha *et al* 1994; Lorenzen *et al* 1995; Kamatkar *et al* 1996). PTP-S2 binds non-specific DNA whereas PTP-S4 does not and they differ in their substrate preferences *in vitro* (Kamatkar *et al* 1996). PTP-S2 expression is increased upon mitogenic stimulation of a variety of cells, with peak transcript levels present at a period corresponding to mid or late G1 phase (Rajendrakumar *et al* 1993; Tillman *et al* 1994; Nambirajan *et al* 1995; Kamatkar *et al* 1996). This increase was shown to be due to post transcriptional stabilization of mRNA rather than transcriptional activation (Rajendrakumar *et al* 1993). Stable clones of HeLa cells overexpressing rat PTP-S2 showed higher proliferation rates, lower serum dependency and ability to form larger colonies in soft agar assays,

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suggesting that PTP-S2 at moderate level of overexpression enhances cell proliferation (Radha *et al* 1997). Quantitative analysis of transcripts by reverse transcription-polymerase chain reaction (RT-PCR) assays from various rat tissues showed that spleen and thymus possess highest levels of PTP-S2 expression (Kamatkar *et al* 1996). PTP-S knockout mice exhibit specific defects in development of the hematopoietic system and survive for only 35 weeks after birth, suggesting a crucial role for PTP-S in the normal functioning of the hematopoietic system (You-Ten *et al* 1997). Transient overexpression of PTP-S2 in human tumour cell lines triggers apoptosis in a p53 dependent manner indicating that PTP-S2 plays a role in a pathway that is common to both cell proliferation and apoptosis (Radha *et al* 1999).

PTP-S2 is present mostly in the cell nucleus and an analysis of subnuclear distribution of PTP-S2 had shown that a part of it (about 30%) is associated with chromatin. A small fraction is tightly associated with the nuclear matrix (Radha *et al* 1994; Kamatkar *et al* 1996). Many regulatory nuclear proteins show changes in subnuclear distribution in response to physiological stimuli and/or during cell division cycle. In the present study we have analysed changes in the distribution of PTP-S2 during cell division particularly its interaction with chromatin. We have also attempted to explore the underlying molecular mechanism that may be responsible for the observed alterations in subcellular location of PTP-S2 during mitosis. During metaphase PTP-S2 showed specific phosphorylation, a stage at which it was excluded from the condensed chromosomes. Using recombinant protein, we have shown that CK2 phosphorylates PTP-S2 *in vitro* in its C-terminal non-catalytic domain, suggesting that CK2 or a CK2-like enzyme may be regulating PTP-S2 during mitosis.

2. Materials and methods

2.1 Cell lines

Rat fibroblast cell line F-111 and D3, a stable clone of HeLa cells expressing rat PTP-S2, were maintained in DMEM containing 10% fetal bovine serum. D3 cell line has been described previously (Radha *et al* 1997). Cells in exponential cultures were used as interphase cells. Metaphase-arrested cells were obtained by treating exponentially growing cultures with 1 µg/ml nocodazole for 16 h and rounded cells collected by mitotic shake off. Cells remaining attached to the flask after nocodazole treatment were also used as additional control for nonmitotic interphase cells that are exposed to the drug.

2.2 Immunoblotting

Whole cell lysates were prepared by washing cells in cold PBS and lysing them by boiling in Laemli's sample buffer. Proteins were electrophoresed on 10% SDS-gels and

Western blotting was performed as described earlier using the monoclonal G11 antibody that recognizes the murine PTP-S isoforms (Radha *et al* 1994).

2.3 *In vitro* phosphorylation of PTP-S2

PTP-S2 and its deletion mutant expressed in *Escherichia coli* were purified as described previously (Kamatkar *et al* 1996). *In vitro* phosphorylation was performed by incubating 500 ng of purified PTP-S2 or the deletion mutant in a 50 µl reaction mix containing 25 mM Tris pH 7.5, 10 mM MgCl₂, 20 mM NaCl, 20 µM [³²P]ATP and 0.2 units of purified kinases CK1 or CK2 (obtained from Promega, USA) at 30°C for 30 min. The reaction was stopped by adding 25 µl of 3 × SDS sample buffer and fractionated on SDS-PAGE. The dried gels were exposed for autoradiography. Similar conditions were used when purified PTP-S2 was phosphorylated *in vitro* by mitotic cell extracts except that incubation was for 10 min at 25°C. This was followed by immunoprecipitation of PTP-S2 as described in the following section.

2.4 *In vivo* labelling and immunoprecipitation

Cell labelling was performed by growing cells for 2 h in serum and phosphate free medium. [³²P]orthophosphate (200 µCi/ml) was added along with nocodazole for 16 h in serum containing medium. Floating and attached cells were lysed and subjected to immunoprecipitation. Immunoprecipitation of PTP-S2 from mitotic and non-mitotic cells was performed by extracting cells in 2 × IP buffer (1 × IP buffer contains 20 mM Tris pH 7.2, 1% Triton X-100; 150 mM NaCl, 0.5% sodium deoxycholate, 2 mM EDTA, 2 mM PMSF, 2 µg/ml STI, leupeptin and aprotinin, 1 mM sodium orthovanadate and 50 mM sodium fluoride) on ice for 20 min and centrifuged at 16,000 g for 20 min. Preswollen Protein A agarose beads were incubated with G11 antibody or control antibody for 30 min at 4°C by gentle shaking. The extract was diluted to make it in 1 × IP buffer and added to the pelleted beads and further incubated for 1 h at 4°C. The beads were washed with IP buffer and boiled in SDS sample buffer before electrophoresing on SDS-PAGE.

2.5 Immunofluorescence and confocal microscopy

Cells grown on coverslips for 24 h were fixed and stained for PTP-S expression as described (Radha *et al* 1994). To identify cells at various phases of mitosis, cellular DNA was stained with 0.5 µg/ml of DAPI added to the mountant. Confocal microscopy was performed by taking optical sections of 0.5 µm depth using Optima confocal system from Meridian Instruments Inc, USA.

3. Results

3.1 PTP-S2 dissociates from chromatin during mitosis

Earlier studies have shown that PTP-S2 localizes to the nucleus in interphase cells, with prominent nucleolar staining. Within the nucleus a fraction of PTP-S2 is associated with chromatin and the interaction is perhaps stabilized by its ability to bind DNA (Radha *et al* 1993, 1994; Kamatkar *et al* 1996). Using indirect immunofluorescence staining of exponentially growing D3 cells (a stable clone of HeLa cells expressing rat PTP-S2), we followed the localization of PTP-S2 in cells at various stages of mitosis, which were identified by DNA staining. In D3 cells only the transfected rat PTP-S2 isoform is detectable by the monoclonal antibody G11 which recognizes rat PTP-S proteins but not human PTP-S proteins present in D3 or HeLa cells. PTP-S2 showed nuclear staining with more prominent staining of nucleoli in interphase cells. As cells entered mitosis with the beginning of chromatin condensation, at prophase, PTP-S2 was distributed throughout the cytoplasm (figure 1). This redistribution took place prior to complete breakdown of the nuclear envelope. During metaphase and anaphase PTP-S2 was completely dissociated from chromatin and was found excluded from the chromosomes. In telophase cells, PTP-S2 appeared to colocalize with DNA once again, though staining was still seen throughout the cell. At cytokinesis, PTP-S2 localized to the newly formed nucleus, with very little extranuclear staining. Similar redistribution of the nuclear PTP-S2 was observed during mitosis in F111 cells and other murine fibroblast cell lines.

To confirm that no PTP-S2 was present along with condensed chromosomes, we analysed the distribution of PTP-S2 at various stages of mitosis by using a confocal microscope. For this purpose we used HeLa cells transiently transfected with PTP-S2 since the cells gave stronger staining. Optical sections showed that PTP-S2 in interphase cells was localized to the nucleus along with DNA but it did not colocalize with DNA in metaphase or anaphase (figure 2) confirming that it is excluded from the chromosomes. At telophase, most of this protein is still present throughout the cell, though a small amount seems to reappear along with chromatin. But during cytokinesis, majority of the PTP-S2 colocalized with DNA (yellow colour in dual staining in figure 2) showing that at this stage PTP-S2 reenters the newly forming nucleus.

3.2 Modification of PTP-S2 during mitosis

We have attempted to understand the molecular mechanism underlying the observed changes in subcellular location of PTP-S2 during mitosis. We found that in metaphase cells (D3) enriched by nocodazole block PTP-S2 protein migrated with retarded mobility on SDS-PAGE as compared with that

in interphase cells (figure 3B). This was not an artefact due to nocodazole treatment since PTP-S2 in non-mitotic cells, remaining attached to the flask after nocodazole treatment, showed normal mobility (figure 3B). Similar results were obtained with nocodazole arrested F111 fibroblast cells suggesting that retarded mobility of PTP-S2 in metaphase arrested cells is not an unusual feature of D3 cells (figure 3A).

When the metaphase arrested cells were released into nocodazole free medium, they reentered the cell cycle

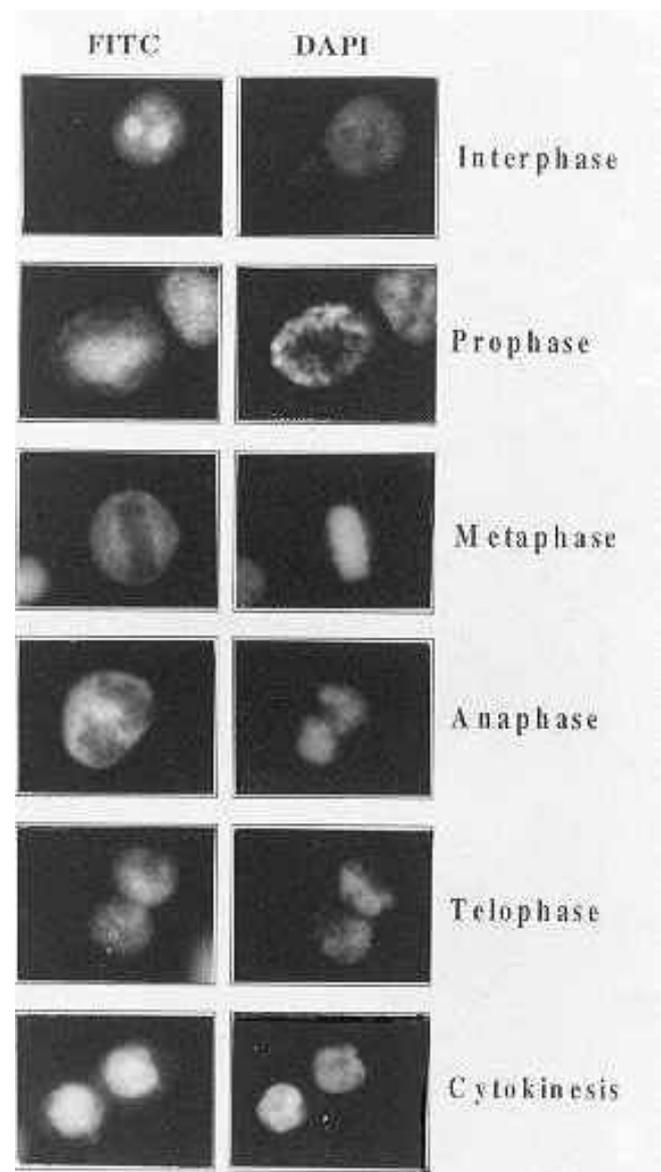


Figure 1. Distribution of PTP-S2 during mitosis. Cells at various phases of mitosis among exponentially growing D3 cells were identified after staining for PTP-S (left panel) and DNA (right panel) and photographed using 100 × objective on a fluorescence microscope.

after cytokinesis into G1 phase. We followed the PTP-S2 protein at 3 and 6 h after release from nocodazole arrest and found that the normal mobility of PTP-S2 was restored in cells that have entered the G1 phase (figure 3). These observations suggested that PTP-S2 undergoes a transient and reversible modification during mitosis. Since

phosphorylation of a protein can lead to a change in its mobility on SDS-PAGE, it was possible that change in mobility of PTP-S2 in metaphase cells might be due to its phosphorylation. Treatment of immunoprecipitate (using G11 antibody) from mitotic cells with alkaline phosphatase abolished the mobility shift as determined by Western

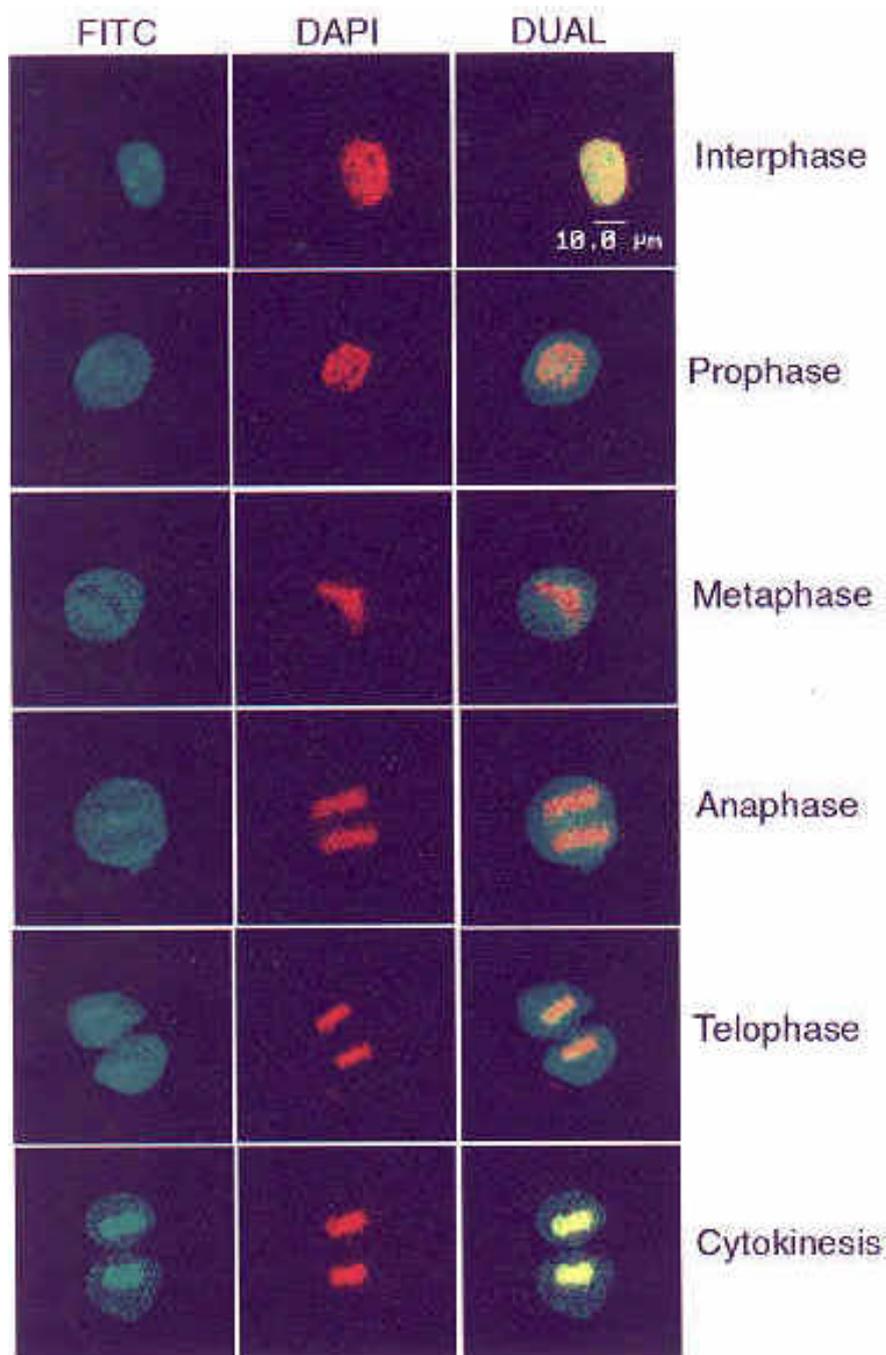


Figure 2. Confocal analysis of PTP-S2 distribution during mitosis. PTP-S2 expressing cells in interphase and various phases of cell division were examined by confocal microscopy and analysed for the staining pattern of PTP-S2 (FITC) and DNA (DAPI) and colocalization of the 2 stains (dual). Single optical section of cells at various phases of cell division are shown.

blotting (not shown). Examination of the amino acid sequence of PTP-S2 showed several putative phosphorylation sites for Ser/Thr kinases. Therefore F-111 cells were treated with nocodazole and simultaneously labelled with [³²P]orthophosphate. Floating and attached cells were collected and PTP-S2 was immunoprecipitated from cell extracts. It was observed that in mitotic cells PTP-S2 was heavily phosphorylated (figure 4). The nonmitotic cells showed much less phosphorylation on PTP-S2. These results indicate that PTP-S2 from interphase cells is also phosphorylated but this modification does not result in a mobility shift as happens in the case of the phosphorylation specific to mitotic cells. PTP-S2 in mitotic cells showed two bands perhaps due to partial dephosphorylation during immunoprecipitation.

3.3 Phosphorylation of PTP-S2 by CK2 in C-terminal domain

Sequence analysis of PTP-S2 showed several putative sites for phosphorylation by Ser/Thr kinases CK1 and CK2 but none for cdc2 kinase. Using purified recombinant PTP-S2, we performed *in vitro* phosphorylation reactions using either purified CK1 or CK2. As shown in figure 5 purified PTP-S2 could be phosphorylated by CK2, but very weakly by CK1. The phosphorylation by CK2 also resulted in retarded mobility of PTP-S2 similar to that observed in mitotic cells. *In vitro*, PTP-S2 could not be phosphorylated by cAMP dependent kinase, though one putative phosphorylation site is present. Under conditions of histone H1 phosphorylation, mitotic cdc2 did not

phosphorylate PTP-S2 *in vitro* (data not shown). The plausible sites of phosphorylation on PTP-S2 by CK2 were examined by using a C-terminal deletion mutant. Full length PTP-S2 and a truncated variant with 68 amino acids deleted from the C-terminal end were purified and used for *in vitro* phosphorylation by CK2 as the kinase. f68PTP-S2 was enzymatically active indicating that the deletion does not grossly alter its conformation. As shown in figure 6A, full length PTP-S2 was phosphorylated, but not f68PTP-S2 suggesting that CK2 phosphorylation sites are present in the C-terminal 68 amino acids of PTP-S2. Therefore we could tentatively identify the CK2 phosphorylation sites to be either or both Ser 320 and Thr 335 (figure 6B).

The presence of PTP-S2 phosphorylating kinase activity was also examined in mitotic cells. Cellular extracts were made from nocodazole arrested F-111 cells and used in

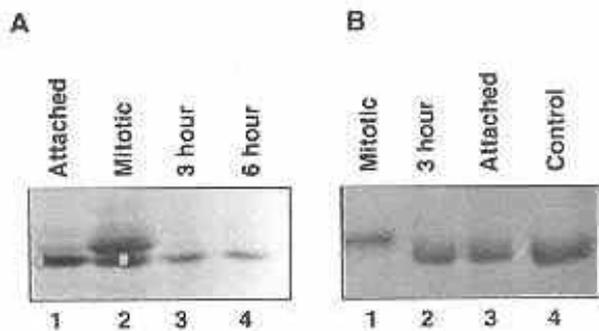


Figure 3. PTP-S2 from metaphase arrested cells shows retarded mobility. Exponentially growing F-111 (A) or D3 cells (B) were treated overnight with nocodazole and rounded cells collected by shake off (mitotic, lanes A2 and B1) and remaining attached cells (attached, lanes A1 and B3) scraped from the flasks. Western blot was performed with whole cell lysates using anti PTP-S antibody. Control lane (B4) shows PTP-S2 from exponentially growing cells. Cells collected by mitotic shake off were washed to remove nocodazole and replated to reenter the cell cycle and attached cells collected after 3 h and 6 h (lanes A3 and 4 and

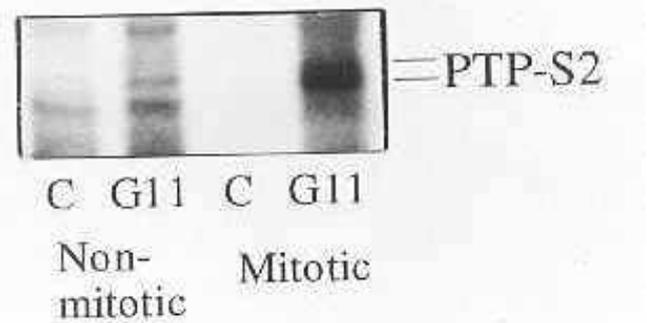


Figure 4. PTP-S2 is phosphorylated during mitosis. F-111 cells were arrested in metaphase and simultaneously labelled with [³²P]orthophosphate. Extracts made from mitotic and attached cells (non-mitotic) were immunoprecipitated using control or anti PTP-S antibody, fractionated on SDS-PAGE and autoradiographed. PTP-S2 bands with native or retarded mobility are indicated.

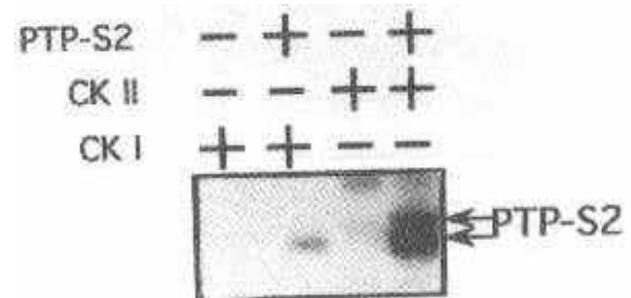


Figure 5. Phosphorylation of PTP-S2 by CK2 results in retarded mobility. Recombinant purified PTP-S2 was phosphorylated *in vitro* using either CK1 or CK2 as described in §2 and the phosphoproteins observed after autoradiography of SDS-PAGE gels.

in vitro kinase reactions to phosphorylate purified PTP-S2. As seen in figure 7, mitotic extracts phosphorylated PTP-S2 causing a retardation of the polypeptide on SDS-PAGE. It was also seen that part of the PTP-S2 polypeptides that did not show a mobility shift were also phosphorylated suggesting that PTP-S2 may be phosphorylated at multiple sites, with only some of them resulting in retarded mobility. Similar result was obtained with extracts from non-mitotic cells (results not shown) suggesting that the kinase that phosphorylates PTP-S2 giving rise to mobility shift is present in mitotic as well as non-mitotic cells. It is possible that the activity of this kinase is regulated *in vivo* and therefore specific phosphorylation of PTP-S2 is seen only during mitosis.

The kinase phosphorylating PTP-S2 was also characterized

by its sensitivity to heparin as it is known that CK2 is strongly inhibited by heparin (Pinna 1990). As seen in figure 7, the presence of 1 $\mu\text{g/ml}$ heparin in the kinase reaction totally inhibited that phosphorylation on PTP-S2 which resulted in retarded mobility. But it was also observed that there was a considerable increase in the amount of phosphorylation seen on PTP-S2 in the presence of 1 $\mu\text{g/ml}$ heparin without effecting its mobility. In the presence of 10 $\mu\text{g/ml}$ heparin, this increase in phosphorylation is not seen, suggesting that the kinase involved may be activated or inhibited by heparin in a concentration dependent manner. These results further strengthen our hypothesis that PTP-S2 is phosphorylated at multiple sites, by more than one protein kinase, one of which is inhibited by heparin.

4. Discussion

In this study we have analysed the changes in subcellular location of PTP-S2, a nuclear protein tyrosine phosphatase, during cell division cycle. PTP-S2, which is present in the nucleus in interphase cells, becomes cytoplasmic as the cells enter mitosis. When cells enter mitosis, the chromosomes become condensed and at metaphase and anaphase PTP-S2 is seen excluded from chromosomes. Towards the end of cell division or slightly before completion of cytokinesis, PTP-S2 becomes associated with chromatin in the newly formed nucleus.

Complex nuclear and cytoplasmic events that occur during mitosis are primarily regulated by phosphorylation. Over 50 proteins including the products of several cellular oncogenes, antioncogenes and transcription factors have been shown to be phosphorylated during mitosis (Davis *et al* 1983; Stukenberg *et al* 1997). Cdc2, a cell division kinase which is activated at metaphase, phosphorylates a wide range of substrates, initiating many of the structural and functional changes that take place at mitosis (Nurse

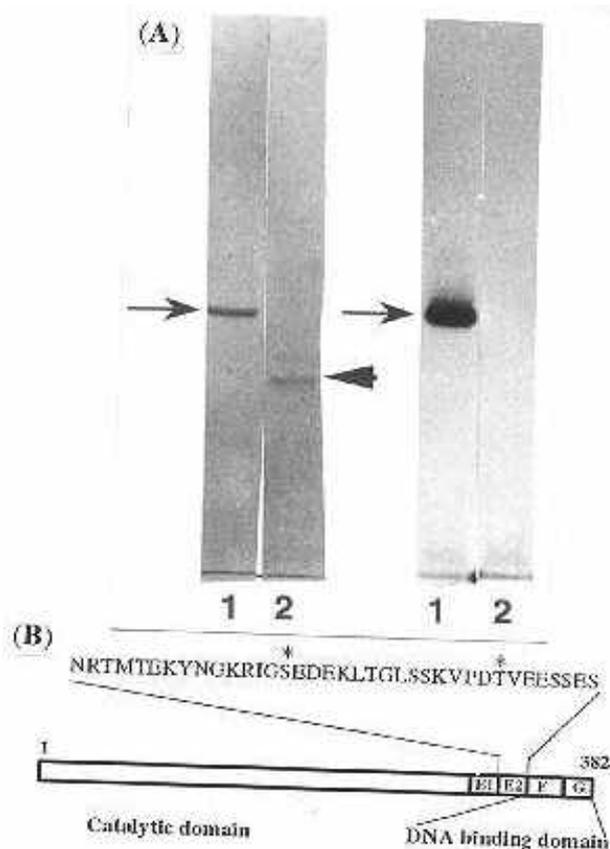


Figure 6. CK2 phosphorylates PTP-S2 in the C-terminal domain. (A) CK2 was used to phosphorylate either full length PTP-S2 or $\Delta 68$ PTP-S2, in *in vitro* kinase assay; fractionated by SDS-PAGE and subjected to autoradiography (right panel). Left panel shows Coomassie blue stained gel of the purified proteins. (B) Line diagram of PTP-S2 showing the two potential CK2 phosphorylation sites (Ser 320 and Thr 335) (indicated by asterisk) in the C-terminal domain of the protein.

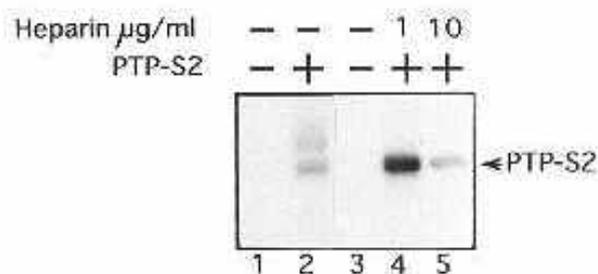


Figure 7. PTP-S2 is phosphorylated by mitotic cell extracts. Purified PTP-S2 was phosphorylated *in vitro* using mitotic cell extracts in the presence or absence of heparin. After immunoprecipitation with PTP-S2 antibody the samples were subjected to SDS-PAGE followed by autoradiography.

1990). We have shown that PTP-S2, a nuclear protein that has a role in cell proliferation is phosphorylated specifically at mitosis resulting in retarded mobility. Even though putative sites for cAMP dependent kinase and CK1 are present, PTP-S2 does not get phosphorylated *in vitro* by these enzymes, but only by CK2 showing that there is considerable amount of substrate specificity for these kinases even *in vitro*. Not all phosphorylations alter the mobility of a polypeptide on SDS gels (Luscher *et al* 1990). Our experiments show that phosphorylation by CK2 results in altered mobility. Though we do not have evidence for *in vivo* phosphorylation of PTP-S2 by CK2, *in vitro* experiments suggest that CK2 or a CK2-like enzyme phosphorylates this protein *in vivo* during mitosis.

CK2 is present in the nucleus, nucleolus and cytoplasm of all eukaryotic cells and its activity increases upon association with cdc2/cyclin B complex (Allende and Allende 1995; Meggio *et al* 1995; Li *et al* 1996). CK2 is activated transiently and shows oscillatory changes when cells are stimulated to enter the cell cycle (Carroll and Marshak 1989). A role for CK2 like enzyme has also been suggested in the prophase/metaphase transition of meiotic cell division in xenopus oocytes (Mulner-Lorillon *et al* 1988). In a screen set up to identify mitotic phosphoproteins, it was found that a majority of them are direct substrates of cdc2 (Stukenberg *et al* 1997). Examination of PTP-S2 sequence showed no consensus phosphorylation site for cdc2 kinase (S/TPXK) and, *in vitro* PTP-S2 does not serve as a substrate for cdc2. Also cdc2 is not known to be inhibited by heparin suggesting that other kinases may play a significant role in mitotic regulation of PTP-S2.

We have used nocodazole arrest for enrichment of mitotic cells. To ensure that PTP-S2 hyperphosphorylation is not drug induced, mitotic cells were collected from exponentially growing cultures by mechanical shake off. Western blotting showed that in these cells also, PTP-S2 has retarded mobility, but to a lesser extent than that seen in nocodazole arrested cells (not shown). That phosphorylation and mobility retardation are not an artefact of nocodazole treatment is evident from the observation that cells remaining attached to the flask after the treatment do not show a band of retarded mobility. Though these cells have been exposed to the drug, they are not in the mitotic phase and therefore we have used this population as non-mitotic or interphase cells in most of our experiments. We have observed that mobility shift of PTP-S2 is reversed after removal of nocodazole, as cells enter the next G1 phase suggesting that PTP-S2 is dephosphorylated at the end of mitosis.

Many nuclear proteins exhibit difference in their localization as cells undergo division. For example, Wee 1, the tyrosine kinase that phosphorylates and inhibits Cdc 2, colocalizes with chromatin during prophase, is cytoplasmic during anaphase and present only at the cleavage plane during telophase (Baldin and Ducommun 1995). Phosphorylation is known to regulate the subcellular location of certain

transcription factors, thereby affecting their activity during mitosis (Martinez-Balbas *et al* 1995; Muchardt *et al* 1996). In some instances it has been shown that altering the subcellular location serves to regulate the activities of both the enzyme and its substrate. The activation of cdc 2 at the onset of mitosis appears to be regulated by the localization of cdc 25 and cyclin B (Girard *et al* 1992). In our experiments PTP-S2 appears to become cytoplasmic being excluded from condensed chromosomes concomitant with hyperphosphorylation during mitosis.

Phosphorylation is known to affect the activities of many enzymes (Swarup and Radha 1992; Chackalaparampi and Shalloway 1988; Laird *et al* 1995). We investigated whether the activity of mitotic form of PTP-S2 differs from that of the interphase form but did not find a significant difference in activity. It has been suggested that in the case of many transcription factors, mitotic phosphorylation serves to dissociate the proteins from chromatin (Luscher and Eisenman 1992; Stukenberg *et al* 1997). It is possible that phosphorylation of PTP-S2 during mitosis may result in the dissociation of PTP-S2 from chromatin. The data presented in this paper are consistent with this hypothesis.

Cytoplasmic localization of PTP-S2 during mitosis may be due to (i) a function for this protein in the cytoplasm, (ii) to sequester it away from its nuclear targets or (iii) due to the requirement for its dissociation from chromatin. These possibilities are not mutually exclusive and further work would be required to delineate the functional significance of the difference in localization of PTP-S2 during mitosis.

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