Overexpression of a nuclear protein tyrosine phosphatase increases cell proliferation

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Abstract PTP-S2 is a widely expressed nuclear protein tyrosine phosphatase which shows increased expression upon mitogenic stimulation of a variety of cells. In order to elucidate the role of this enzyme in cell division, stable clones of HeLa cells expressing rat PTP-S2 were isolated and their growth properties analysed. Overexpressed PTP-S2 was located in the cell nucleus and there was no significant change in the total tyrosine phosphatase activity of PTP-S2 overexpressing cells. PTP-S2 overexpressing clones, D3 and B5, showed increased rate of cell division and lower serum requirement as compared with control cells. D3 and B5 cells formed larger colonies in soft agar, were not contact inhibited upon confluency, grew in multilayers, and showed altered morphology. These results are consistent with the suggestion that PTP-S2 may be a positive regulator of cell proliferation.

Key words: Tyrosine phosphatase; Cell proliferation; Nuclear phosphatase; Growth regulation

1. Introduction

The major product encoded by the rat *PTP-S* gene is the PTP-S2 isoform which is a PTPase that binds DNA in vitro and is located in the nucleus in association with chromatin [1–5]. Another isoform arising from the same gene by alternative splicing is PTP-S4 that differs from PTP-S2 in enzymatic activity, subcellular localization and inability to bind DNA [4,5]. Both PTP-S2 and PTP-S4 mRNAs are widely expressed in various tissues and cell lines [5]. In all cases, PTP-S2 mRNA level was found to be 2–10-fold higher than the PTP-S4 mRNA level [5]. Earlier work in our laboratory showed that PTP-S mRNA is induced in the absence of de novo protein synthesis, upon mitogenic stimulation of resting lymphocytes [6]. The time course of induction in stimulated lymphocytes, and serum-stimulated cells in culture showed that peak levels of mRNA are attained at late G1 phase of the cell cycle [6–8]. In these experiments PTP-S mRNA levels were compared by Northern blotting and individual isoforms were not analysed. Subsequently we have shown that both PTP-S2 and PTP-S4 isoforms are induced during liver regeneration after 6 h (G1 phase) of partial hepatectomy [5].

Since the level of PTP-S2 mRNA is transiently increased upon mitogenic stimulation, it was possible that this PTPase may play a role in cell proliferation. In order to explore this possibility, in this study we have analysed the effect of overexpression of PTP-S2 on the growth properties of established cell lines. HeLa cells overexpressing rat PTP-S2 grew faster, showed altered morphology and formed larger colonies in soft agar. Our results suggest that overexpression of PTP-S2 affects cell growth.

2. Materials and methods

2.1. Plasmids

Construction of expression plasmid pCB6-PTP-S2 has been described previously [5]. This construct expresses PTP-S2 protein under the control of cytomegalovirus promoter.

2.2. Cell culture, transfection and stable cell lines

Unless otherwise mentioned, all cell lines were normally maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS (fetal calf serum) in a humidified CO_2 incubator at 37°C. Transfections were performed using Lipofectin or Lipofectamine reagents (Gibco-BRL) as per the manufacturer's instructions. Briefly, cells were seeded 24 h prior to transfection to reach 70% confluency. Plasmid DNA (2 μg) and Lipofectin or Lipofectamine (12 μl) were mixed in 2 ml of serum-free medium, left for 30 min at room temperature and then overlayed on the cell monolayer. Six hours later, an equal volume of medium containing 20% FCS was added. To obtain stable cell lines, HeLa cells were transfected with pCB6-PTP-S2 (as described earlier) and after 48 h, medium was changed and cells maintained in DMEM containing 10% FCS and 500 μg/ml Geneticin (Sigma). The Geneticin-resistant colonies formed were transferred to 24-well plates and propagated. Expression of recombinant rat PTP-S2 was monitored by indirect immunofluorescence and Western blotting as described earlier using a monoclonal antibody, G11 which recognises rat PTP-S proteins but not the human PTP-S proteins [3,5]. Two clones that showed stable expression after repeated passages were selected for further study. HeLa cells and a HeLa cell line that is Geneticin resistant but shows no expression of recombinant PTP-S2 were used as controls (n^r HeLa 1).

2.3. Colony formation assay

Colony formation assay was performed as described by Hansen and Braithwaite [9]. About 3×10^5 COS-1 cells or 4×10^5 HeLa cells were plated in 60 mm petri dishes and 24 h later transfected with 2.5 μg of either pCB-6 or pCB6-PTP-S2 plasmid DNA using Lipofectin reagent. After 48 h of transfection, cells were maintained in DMEM containing 10% FCS and 400 μg/ml Geneticin for 14–16 days. Cells were then fixed and stained with 0.4% sulphorhodamine B (Sigma) in 1% acetic acid and the number and size of colonies monitored.

2.4. Determination of growth kinetics

PTP-S2 overexpressing clones and control cells (1×10^5) were seeded in duplicate 35 mm petri dishes in DMEM with 10% FCS. On consecutive days, cells were harvested with trypsin, dispersed and counted using a hemocytometer. For determination of saturation density, cells were fed regularly with fresh medium and allowed to grow post-confluence. Four days after attaining confluency, the number of viable cells was counted.

Serum requirement of various cell lines was monitored by assessing growth of cells in medium containing 0%, 1%, 2%, 4% or 10% serum. Cells (1×10^5) were plated in duplicate 35 mm dishes in DMEM with 10% FCS. After 24 h, plates were washed 5 times with PBS and replaced with medium containing different serum concentrations. Via-

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Abbreviations: PTPase, protein tyrosine phosphatase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum

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ble cell number was determined after 4 days of growth in the given serum concentration.

2.5. Soft agar assay

Cells (1×10^5) were suspended in 1.5 ml of DMEM with 10% FCS and 0.33% agar (Sigma) and plated in 60 mm dishes containing a basal layer of 0.5% agar in DMEM with 10% FCS. Plates were incubated for 2 weeks at 37°C in 5% CO_2. Cultures were fed weekly with 1.5 ml of DMEM containing 10% FCS and 0.33% agar. At the end of 2 weeks, colonies were photographed on an inverted light microscope at 50× magnification.

2.6. Other methods

Immunoblotting, immunoprecipitation and immunofluorescence staining with anti-PTP-S monoclonal antibody was carried out as described [3]. Protein tyrosine phosphatase assays were carried out as described [10] using ^{32}P-labelled poly(Glu^4,Tyr^1) as substrate except that during the assay 1 mM dithiothreitol was added instead of 2-mercaptoethanol.

3. Results

To identify the effects of expression of exogenous PTP-S2 on cell survival and growth, COS-1 and HeLa cells were transfected with either the control plasmid or plasmid encoding full-length PTP-S2. Transfected cells were selected with Geneticin and 14–16 days later cell growth was assessed by counting the number of large colonies which arose from transfected cells. (Fig. 1). In case of COS-1 cells, PTP-S2 plasmid gave 13.0 ± 1.7 (mean ± SE, $n = 6$) large colonies and control plasmid gave 6.2 ± 1.1 ($n = 6$) large colonies. With HeLa cells PTP-S2 plasmid gave 13.0 ± 0.9 ($n = 3$) large colonies whereas control plasmid gave 5.7 ± 0.3 ($n = 3$) large colonies. Thus the number of large colonies was about 2-fold higher when the cells were transfected with PTP-S2 plasmid as compared with the control plasmid. This increase in the number of large colonies with PTP-S2 plasmid is not due to higher efficiency of transfection since the total number of Geneticin-resistant colonies (large and small) was essentially the same with control or PTP-S2 plasmid. This result, therefore, indicated that PTP-S2 overexpression promotes growth of cells.

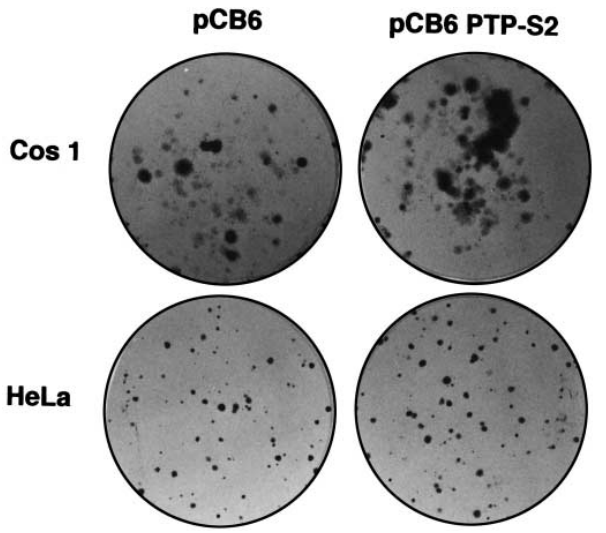

Fig. 1. Colony formation assay of COS-1 and HeLa cells transfected with either pCB6 control plasmid or pCB6-PTP-S2. After 14–16 days of selection in Geneticin, cells were stained with sulforhodamine B and cell growth was assessed by observing the number and size of colonies.

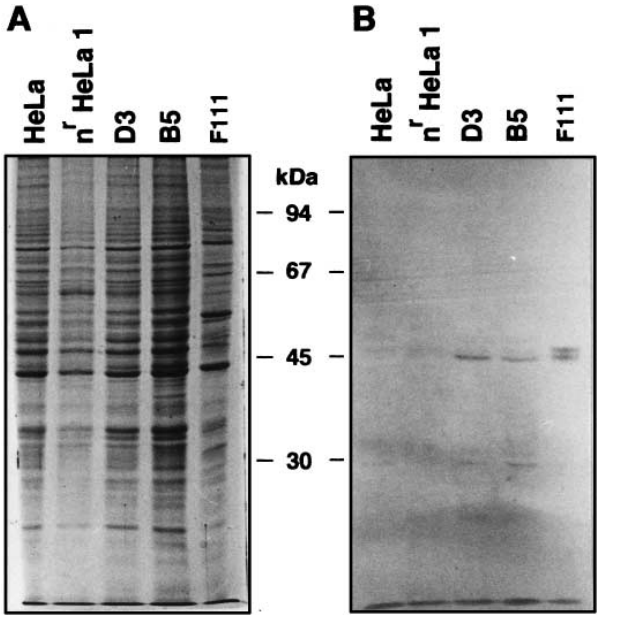

Fig. 2. Immunoblot showing expression of rat PTP-S2 in overexpressing and control cells. A: Coomassie blue-stained gel showing protein profile of lysates used for blot in (B). The doublet seen in lane marked F111 are the endogenous rat PTP-S polypeptides.

HeLa cells were transfected with the full-length cDNA encoding the rat PTP-S2 isoform in an expression vector pCB6, and Geneticin-resistant cell lines were isolated. PTP-S2 expression in the clones was tested by immunofluorescence and Western blotting using a previously characterised monoclonal antibody which recognises the rat as well as the mouse PTP-S proteins but not human proteins [3]. Majority of the clones expressed the recombinant protein but only in about 1–5% of the cells. The clones D3 and B5 that expressed PTP-S2 in more than 30% of the cells were selected for further study. Fig. 2 shows the expression of PTP-S2 in HeLa, n^r HeLa1, D3 and B5 clones as determined by immunoblotting. For the purpose of comparison, lysate of rat fibroblast cell line F111 was also included, which showed a doublet; the lower band of about 45 kDa corresponds to PTP-S2. Immunofluorescence analysis showed that PTP-S2 is present in the nucleus (Fig. 3A) as was shown previously in transiently transfected HeLa cells and fibroblasts. This suggests that PTP-S2 protein compartmentalizes properly in the stably transfected cells. Overexpressed PTP-S2 in D3 and B5 cells constitutes about 0.04% of the total PTPase activity as determined by estimating PTPase activity in the PTP-S2 immunoprecipitates. The overall PTPase activity of D3 and B5 cell extracts showed no significant change as compared to HeLa cells (data not shown).

The PTP-S2 overexpressing clones D3 and B5, and control cell lines were studied for comparison of morphology and growth rates. In subconfluent cultures D3 and B5 cells looked similar to HeLa cells (Fig. 3B). However in confluent cultures, unlike control cells, D3 and B5 cells were not growth arrested due to contact inhibition and grew in multilayers forming foci containing refractile and disordered cells (Fig. 3C). For measuring the growth in liquid culture, cells were plated in duplicate 35 mm dishes in DMEM with 10% FCS and viable cells counted on consecutive days. As seen from Fig. 4, while HeLa and n^r HeLa cells showed similar growth rate, the two PTP-S2

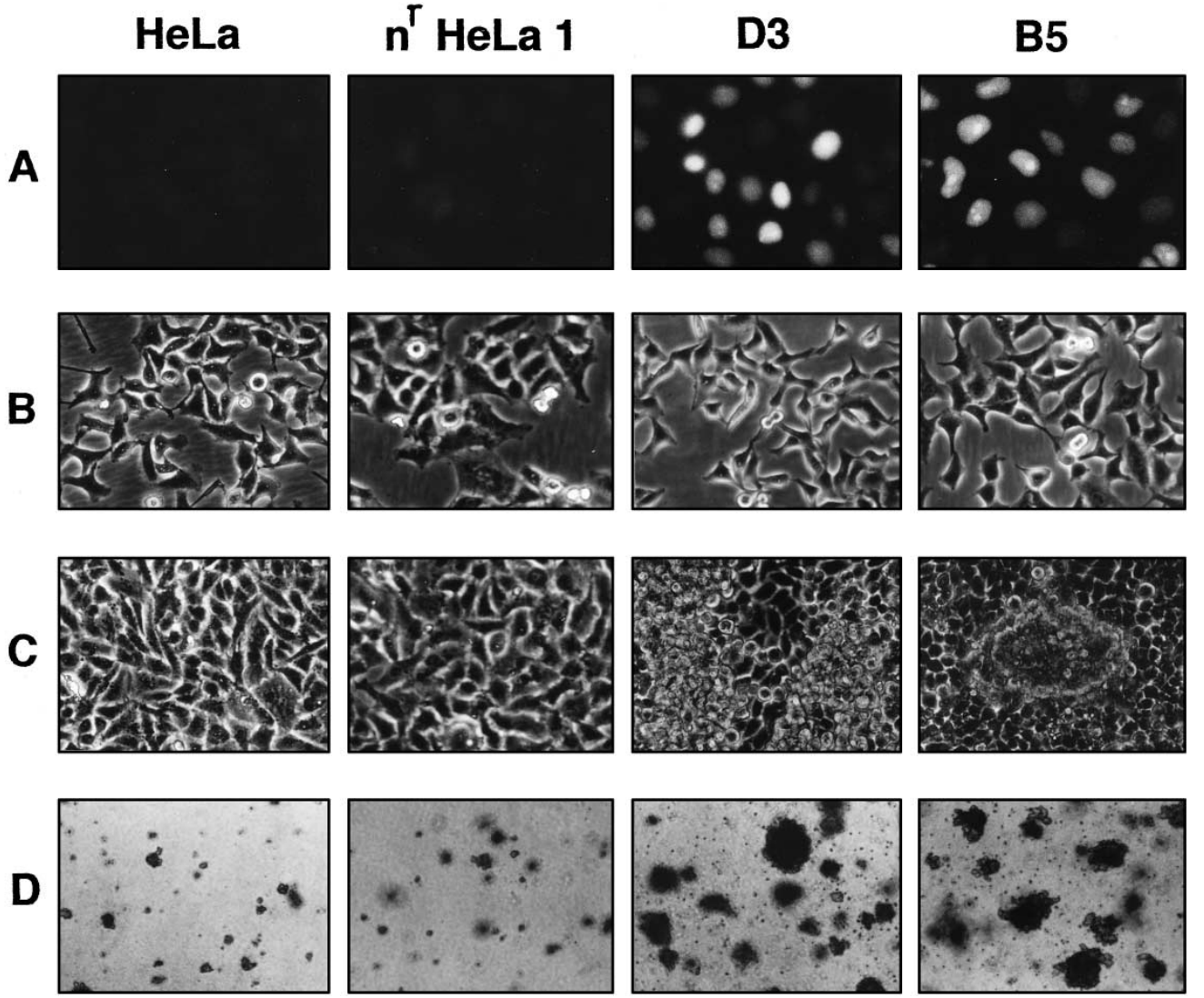

Fig. 3. Morphology of control and PTP-S2 overexpressing cells. A: Immunofluorescent staining of cells grown on coverslips. B: Subconfluent culture (×50). C: Three-day post-confluence cells (×50). D: Colonies in soft agar (X 50).

expressing clones had accelerated growth rates, reaching 2–3 times greater cell numbers by the 5th day after seeding. We determined the saturation density by measuring the number of cells present in 4-day post-confluence cultures and it was found to be $1.7\times10^5/cm^2$ for the control clones and $4.9\times10^5/cm^2$ for PTP-S expressing clones.

We next looked for differences in anchorage-independent growth of the PTP-S2 overexpressing clones and found that after 2 weeks, D3 and B5 cells formed colonies in soft agar which were much larger (0.05–0.5 mm) than colonies formed by HeLa or n^r HeLa cells (0.01–0.1 mm) (Fig. 3D).

We also investigated the growth of PTP-S2 overexpressing clones in low serum culture medium. Cells were plated in duplicate 35 mm dishes in 10% FCS and after 24 h, cells were washed with PBS and medium containing 0%, 1%, 2%, 4% or 10% serum was added. Four days later, viable cells were counted. As can be seen from Fig. 5, growth of control cells and HeLa cells was greatly reduced in low serum medium, while PTP-S2 overexpressing cells grew in low serum at nearly the same rate as in 10% serum. The control cells were unable to proliferate and survive in serum-free medium, whereas D3 and B5 cells could grow, though at a lower rate compared to serum-containing medium.

4. Discussion

Accumulating evidence has shown that PTPases counteract the action of tyrosine kinases by dephosphorylating their substrates; in addition they also modulate the activities of Ser, Thr and Tyr kinases and regulate intermolecular interactions [11–13]. The cellular function of most of the PTPases are not yet understood. 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. Thus in the oncogene transformed cells, PTP1B and PTP1, have been shown to suppress the transformation phenotype [14,15]. Yet, the human homolog of PTP-S4, TCPTP, when transfected along with *v-fms* oncogene shows no effect on cell growth; however, a truncated enzyme lacking the C-terminal non-catalytic domain is found to suppress the oncogenic potential of *v-fms* [16]. When introduced into non-transformed cells, the full-length TCPTP exhibits no effect in terms

of growth and morphology, while overexpression of the truncated enzyme results in multinuclear cells [17]. On the other hand, overexpression of PTP-MEG, a membrane and cytoskeletal phosphatase, in COS-7 cells inhibits cell proliferation and blocks the ability of anchorage-independent growth [18]. Overexpression of the low molecular mass tyrosine phosphatase (acid phosphatase) in normal and v-erbB-transformed cells results in inhibition of growth and reduced ability of colony formation in soft agar [19].

Overexpression of the transmembrane tyrosine phosphatase, PTPα, enhances the activity of $pp60^{c-src}$ and leads to a transformed phenotype [20]. PRL-1, a mitogen induced 20 kDa PTPase, when overexpressed in NIH3T3 cells enhances cell growth [21]. PRL-1 does not show any sequence homology to other PTPases including PTP-S2. However, functionally PTP-S2 and PRL-1 share certain properties such as nuclear location and induction during liver regeneration, in addition to their effects on cell growth. Time course of induction suggests that *PRL-1* is an immediate early gene [21] whereas *PTP-S2* behaves as a delayed early gene [6,7]. Therefore PRL-1 and PTP-S2 are likely to have different cellular targets.

The effect of overexpression of a tyrosine phosphatase may not always be the same as the normal cellular function, particularly when the overexpression is very high and it alters the overall PTPase activity of the cell. In the present study, the overexpressed PTP-S2 represents only a very small fraction (0.04%) of the total PTPase activity of the cells and the level of overexpression is less than or comparable to that present in rat fibroblasts. Although further work is required to elucidate the role of PTP-S2 in cell proliferation, the results presented here suggest that PTP-S2 is involved in the regulation of cell proliferation.

In conclusion, overexpression of PTP-S2 in HeLa cells leads to (i) an increase in the rate of cell division, (ii) a loss of contact inhibition, (iii) an alteration in the cellular morphology and (iv) a reduction in the requirement of serum. These observations suggest that PTP-S2 is a positive regulator of cell growth.

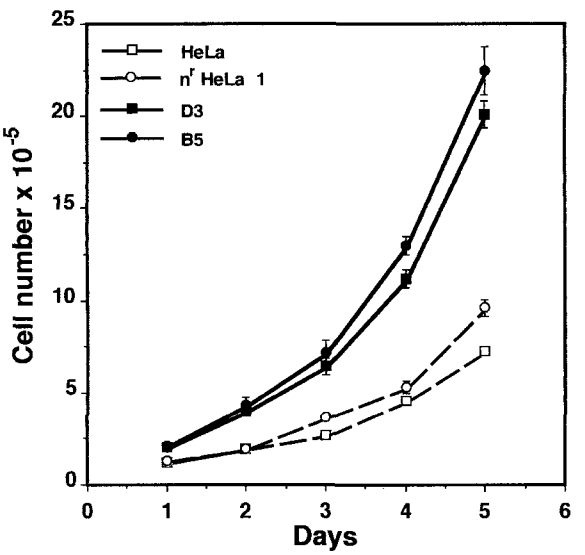

Fig. 4. Growth curves of PTP-S2 overexpressing and control cells. Details of the experiment are described in Section 2. The data represent mean ± SE of 3 experiments done with duplicate dishes.

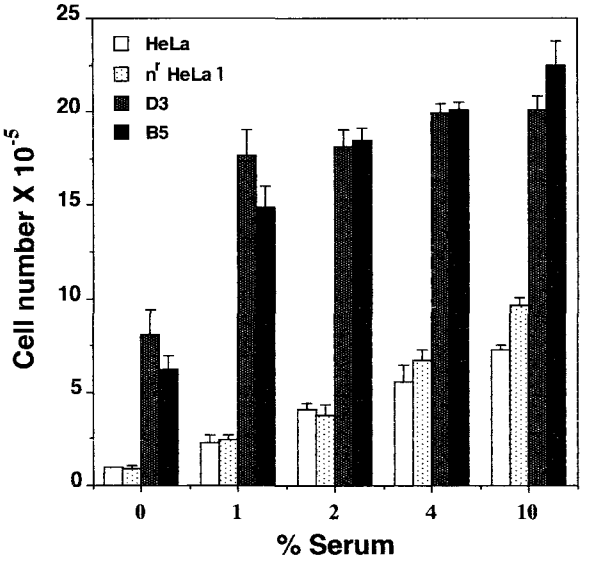

Fig. 5. Serum requirement of PTP-S2 overexpressing and control cells. The data represent mean ± SE of 3 experiments done with duplicate dishes.

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