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Induction of p53 dependent apoptosis upon overexpression of a nuclear protein tyrosine phosphatase

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Abstract Two ubiquitously expressed protein tyrosine phosphatases, PTP-S2 and PTP-S4 (also known as TC^{45} and TC^{48}, respectively), are alternately spliced products of the same gene. Overexpression of PTP-S2 by transient transfection induced chromatin condensation and nuclear fragmentation, typical of apoptosis. Expression of PTP-S4 resulted in a much lower number of cells with apoptotic phenotype. PTP-S2 induced apoptosis in MCF7 and A549 human tumor cell lines which are p53 positive but not in HeLa and SW620 cells which are p53 negative. Apoptosis induced by PTP-S2 in MCF7 cells was inhibited by cotransfection with mutant p53 (Arg-273 → His) but not by wild type p53. PTP-S2 induced apoptosis was inhibited by antiapoptotic protein Bcl2 and certain inhibitors of caspases. These results suggest that the nuclear tyrosine phosphatase PTP-S2 induces p53 dependent, serum starvation independent and caspase mediated apoptosis.

Key words: Apoptosis; Nuclear phosphatase; Protein tyrosine phosphatase

1. Introduction

Programmed cell death or apoptosis, as well as cell proliferation, is required for the normal development and function of multicellular organisms [1]. Protein tyrosine phosphorylation mediated by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) has been implicated in cell survival and cell death [2,3]. Specific tyrosine kinases like c-abl and AATYK have been implicated in apoptosis and the tyrosine kinase activity of some growth factor receptors is essential for survival of cells [4–6]. Using PTP inhibitors it has been shown that activity of PTPs is required for apoptosis to take place but in these studies the role of individual enzymes is not defined [2]. Recently two tyrosine phosphatases have been shown to be involved in the control of apoptosis. One of these, termed FAP-1, associates with Fas receptor and blocks Fas mediated apoptosis [7]. The other enzyme, LAR, is a transmembrane protein that induces apoptosis upon overexpression [8]. Dual specificity phosphatase Cdc25A is involved in the induction of apoptosis by c-Myc overexpression [9]. Another dual specificity phosphatase, MKP-1, is involved in the protection of cells against apoptosis induced by UV irradiation [10].

Four different forms of a non-receptor type PTP are generated by alternative splicing; two of these forms, PTP-S2 and PTP-S4 (also known as TC^{45} and TC^{48}, respectively), are major forms which are expressed in rat as well as human cells [11]. PTP-S2, which is a polypeptide of 382 amino acids, binds to non-specific DNA in vitro and localizes in the nucleus [12–16]. The last six amino acids of PTP-S2 are replaced by 34 amino acids (mostly hydrophobic) in PTP-S4. PTP-S4 does not bind to non-specific DNA and shows perinuclear and cytoplasmic localization [16]. A knock out mouse which does not express PTP-S has been created and the homozygous null animals are unable to survive beyond 3–5 weeks [17]. These mice exhibit specific defects in bone marrow, B cell lymphopoiesis and erythropoiesis and also impaired T and B cell functions. In the knock out mice all the forms of PTP-S were knocked out and, therefore, the observed effects cannot be attributed to any particular isoform.

The level of PTP-S mRNA is transiently increased in G1 phase upon mitogenic stimulation in a variety of cells, such as T lymphocytes, HeLa cells, F111 fibroblasts, liver cells, etc. [15,18,19]. In most of these studies individual isoforms were not analyzed. Recently it has been shown in our laboratory that PTP-S2 as well as PTP-S4 mRNA levels increase several fold in the G1 phase during rat liver regeneration [16]. Overexpression of PTP-S2 in HeLa cells at moderate levels leads to an increase in the rate of cell division, a loss of contact inhibition, an alteration in the cellular morphology and a reduction in the requirement of serum [20]. These observations suggest that PTP-S2 has a role in the regulation of cell proliferation.

Some of the proteins involved in regulating cell proliferation are also known to play a role in the induction or inhibition of apoptosis, such as p53, pRb, c-Myc, Cdc25A, etc. [21,22]. Therefore it is possible that PTP-S2 may have a role in the induction or inhibition of apoptosis. Here we have analyzed the effect of overexpression of PTP-S2 on the induction of apoptosis in various cells as determined by chromatin condensation and nuclear fragmentation. Our results suggest that the nuclear isoform PTP-S2 but not PTP-S4 induces a significant level of apoptosis.

2. Materials and methods

2.1. Cell culture and transfection

HeLa, Cos-1, MCF-7, SW620 and A549 cells were maintained and grown in DMEM containing 10% fetal calf serum at 37°C and 5% CO_2. For transfection, cells were plated on coverslips and transfected with the expression plasmids using Lipofectamine as described earlier [16]. Total concentration of DNA used was 1 μg/ml in all experiments.

2.2. Expression plasmids

In order to express PTP-S2, PTP-S4 and ΔPTP-S4 in mammalian

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Abbreviations: PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; GFP, green fluorescent protein

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cells, these cDNAs cloned in pCB6 vector were used as described earlier [16]. The murine Bcl2 gene in pMEP4 was provided by Dr. Yusuf A. Hannun [23], and green fluorescent protein (GFP) plasmid was from Clontech. Mutant p53 in which Arg-273 is replaced by His has been described previously [24]. Poxvirus gene CrmA cloned in pcDNA3 has been described previously [25]. In cotransfection experiments, PTP-S2 along with either Bcl2 or CrmA plasmids was used. Control vectors were used to maintain uniform DNA concentrations.

2.3. Immunostaining and quantitative analysis of apoptotic cells

After transfection, cells were fixed in 3.7% formaldehyde and permeabilized in 0.5% Triton X-100 and 0.05% Tween 20 as described earlier [13,16]. After blocking in 2% BSA, cells were incubated with monoclonal antibody (G11) that recognizes all the rat PTP-S isoforms but not human or monkey PTP-S proteins [13,16]. This monoclonal antibody recognizes an epitope in the C-terminal 40 amino acids of PTP-S2 which is also present in PTP-S4 and ΔPTP-S4. Anti-mouse FITC conjugated secondary antibodies were used to detect the cells expressing exogenously transfected PTP-S proteins. Cells were mounted in 90% glycerol with 1 mg/ml paraphenylenediamine and 0.5 μg/ml DAPI to visualize nuclear morphology. Cells were observed using an Olympus microscope. Cells showing FITC staining were counted and those that showed condensed chromatin or fragmented nuclei were scored as apoptotic. Cells not showing PTP-S expression in the same coverslips were also scored for the number of apoptotic cells. An average of 200 expressing cells and 1000 non-expressing cells were counted in each coverslip. The data shown in various figures represent mean ± S.D. from at least three independent experiments. Confocal microscopy was carried out using the Ultima Confocal system from Meridian equipped with an inverted Olympus microscope and an argon ion laser. Cells were analyzed by optical sectioning and in order to get an overall view of apoptotic cells, optical sections were combined.

2.4. Immunoblotting

Immunoblotting of transfected cells was carried out as described previously using G11 monoclonal antibody [13,16]. Cos-1 cells transiently transfected with plasmid vectors were harvested after 48 h of transfection and suspended in SDS sample buffer for electrophoresis. Cell lysates were fractionated by electrophoresis in 10% polyacrylamide gel in the presence of SDS. Proteins were then transferred to nitrocellulose membrane electrophoretically. The membrane was blocked for at least 1 h with 1% bovine serum albumin in 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20 and 0.2% gelatin. The blot was then incubated with monoclonal antibody G11 (hybridoma supernatant diluted 1:10) for 2 h. After washing, the blot was incubated with alkaline phosphatase conjugated anti-mouse IgG for 1 h. Immunoreactive bands were detected using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

3. Results and discussion

In order to analyze the effect of overexpression of PTP-S2 and PTP-S4 on the induction of apoptosis, Cos-1 cells were transiently transfected with the plasmid vectors expressing these proteins. After 24–72 h of transfection, cells were fixed and stained for PTP-S and DNA. It was found that about 30% of PTP-S2 overexpressing cells showed apoptosis (after 48 h of transfection) as determined by nuclear fragmentation and chromatin condensation (Figs. 1 and 2). Only about 3% of cells that did not express PTP-S2 in the same coverslips showed apoptosis. Cell death can occur by either apoptosis or necrosis which are biochemically and morphologically distinct processes. At the morphological level apoptosis is characterized by chromatin condensation and margination along the inner nuclear membrane, cytoplasmic condensation and membrane blebbing without disintegration of the cellular membrane. At a later stage the cell disintegrates into apoptotic bodies. During necrotic cell death these nuclear and other changes do not occur and instead, cytoplasmic swelling, lysis

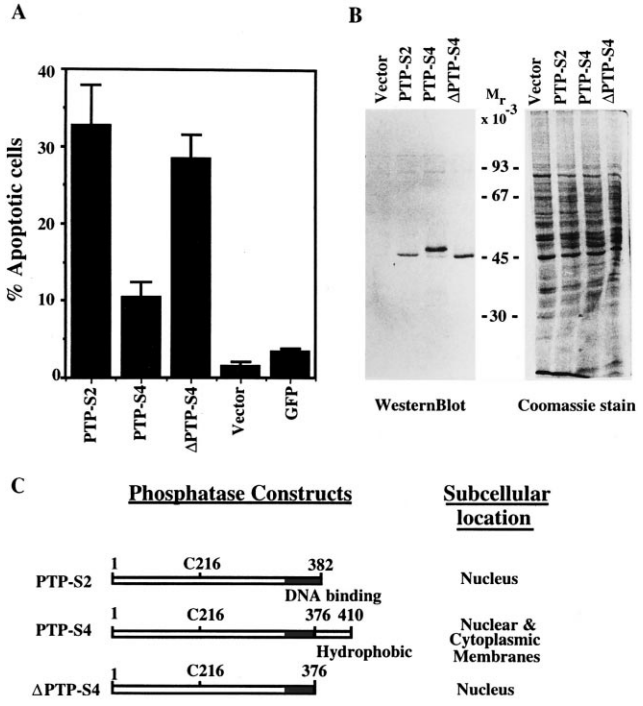

Fig. 1. A: Quantitative representation of cell death induced by PTP-S2, PTP-S4 and ΔPTP-S4. Cos-1 cells were transiently transfected with the indicated constructs and stained for PTP-S expression and DNA after 48 h. The data represent mean ± S.D. of percentage of FITC stained cells with apoptotic phenotype relative to the total number of FITC stained cells from at least three independent experiments. B: Western blot showing expression of PTP-S2, PTP-S4 and ΔPTP-S4 in transiently transfected Cos-1 cells and Coomassie blue stained gel of the same samples. C: Schematic presentation of PTP-S2, PTP-S4 and ΔPTP-S4. Numbers indicate amino acid position.

of cell membrane takes place. In this study, for the purpose of quantitation we have used direct visual quantitation of apoptotic nuclei which is considered to be one of the most accurate measures of apoptosis [25–27]. Non-apoptotic PTP-S2 expressing cells showed staining only in the nucleus but in apoptotic cells PTP-S2 was distributed throughout the cell (Fig. 2).

About 10% of the PTP-S4 expressing cells showed apoptosis (Figs. 1 and 2). Cells transfected with control plasmid showed apoptosis in about 3% of the cells. As an additional control effect of overexpression of GFP was determined and it was found to induce apoptosis only in about 3–4% of expressing cells. The difference in the induction of apoptosis by PTP-S2 and PTP-S4 is not due to the levels of expression since immunofluorescence staining and immunoblot analysis (Fig. 1B) showed that PTP-S2 and PTP-S4 are expressed at nearly the same level. Time course of PTP-S2 and PTP-S4 induced apoptosis at 24, 48 and 72 h of transfection showed that PTP-S2 induced apoptosis was highest at 48 h and lower at 24 or 72 h (data not shown). PTP-S4 induced apoptosis was same at about 10% after 24 or 48 h of transfection and decreased thereafter.

PTP-S2 and PTP-S4 differ in many properties including subcellular location, interaction with non-specific DNA, substrate specificity, etc. These differences are largely due to the presence of a hydrophobic tail of 34 amino acids in PTP-S4 [16]. Therefore the effect of deletion of C-terminal 34 amino acids of PTP-S4 on the induction of apoptosis was analyzed.

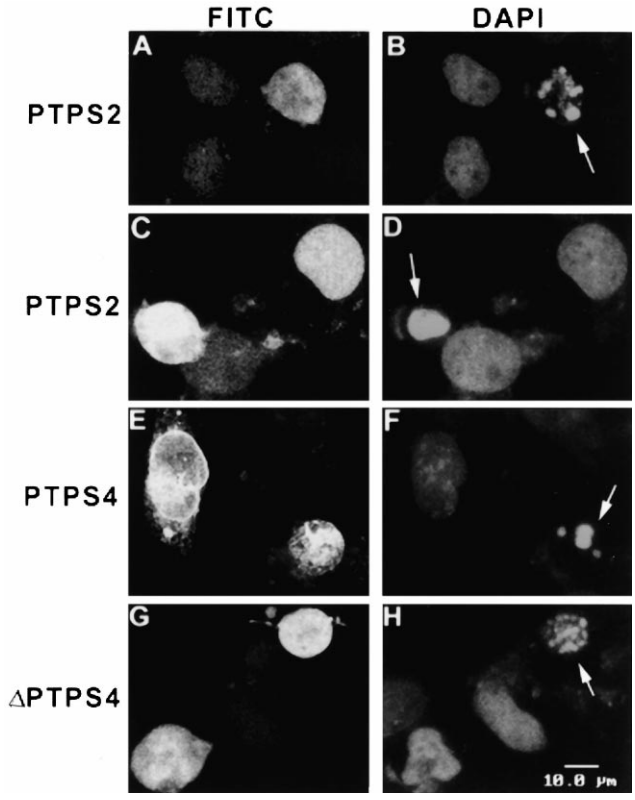

Fig. 2. Nuclear fragmentation and chromatin condensation induced by overexpression of PTP-S2. Cos-1 cells were transfected with indicated PTP-S plasmid, stained for PTP-S expression with a monoclonal antibody and for DNA with DAPI. Cells were visualized using a confocal microscope. Arrows indicate cells showing apoptosis.

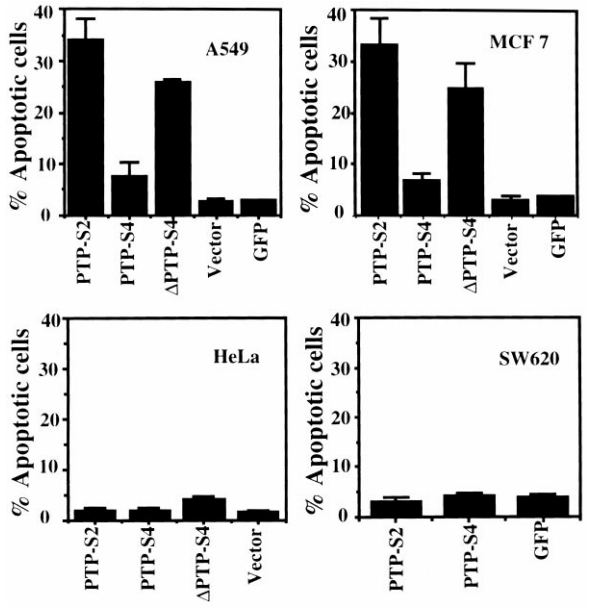

Fig. 3. Induction of apoptosis in human tumor cell lines by PTP-S2 and PTP-S4. MCF-7, A549, HeLa and SW620 cells were transfected with indicated plasmids and PTP-S expressing cells with apoptotic morphology were quantitated.

Overexpression of this truncated protein ΔPTP-S4 (which showed nuclear localization like PTP-S2) resulted in apoptosis in about 28% of expressing cells (Figs. 1A and 2).

To investigate whether PTP-S2 induced apoptosis in a cell type specific manner, four other cell lines, HeLa, MCF7, SW620 and A549, were used in similar transient transfection assays. It was found that PTP-S2 induced apoptosis in MCF7 (human breast tumor) and A549 (lung carcinoma) cell lines in about 30% of expressing cells after 48 h of transfection but not in HeLa (cervical tumor) and SW620 (colon carcinoma) cells (Figs. 3 and 4). MCF-7 and A549 are p53 positive cell lines whereas HeLa and SW620 are p53 negative [28,29]. PTP-S4 induced very little apoptosis in MCF-7 and A549 cells (Fig. 3). ΔPTP-S4 induced apoptosis in MCF-7 and A549 cells but not in HeLa cells (Fig. 3).

Since PTP-S2 did not induce apoptosis in p53 negative human tumor cell lines, it was possible that PTP-S2 induced apoptosis may require a functional p53 protein. Therefore we tested this possibility by cotransfecting PTP-S2 with a mutant p53 which had His in place of Arg-273. Expression of mutant p53 inhibited PTP-S2 induced apoptosis in MCF-7 cells whereas wild type p53 had no significant effect (Fig. 5). Since mutant p53 carried a mutation in the region of p53 involved in sequence specific DNA binding, it seems likely that PTP-S2 induced apoptosis requires the transcriptional activation function of p53 which is dependent on sequence specific DNA binding. The role of p53 in the induction of apoptosis is quite complex and cell type dependent [30,31]. In some cells transactivation function of p53 which is dependent on sequence specific DNA binding is required for induction of apoptosis, whereas in others it is not required [30,31]. In Cos-1 cells which are SV40 transformed, the large T antigen would bind to p53 and functionally inactivate it. Therefore it is likely that in Cos-1 cells PTP-S2 induced apoptosis does not require functional p53. However, from the data on Cos-1 cells it is not possible to draw a conclusion about the role of p53 in PTP-S2 induced apoptosis, since SV-40 large T antigen is known to induce apoptosis or cause a decrease in apoptosis depending on the system used [32–35].

Expression of Bcl2 has been shown to inhibit cell death triggered by diverse pathways [36]. We therefore performed transient transfection of PTP-S2 in the presence or absence of Bcl2 expression plasmid and found that cotransfection with Bcl2 reduced the number of apoptotic cells with PTP-S2 expression in a dose dependent manner (Table 1). No difference

Table 1
Inhibition of PTP-S2 induced apoptosis by Bcl2 and Crm A

Expression plasmid		% apoptosis in expressing cells	% apoptosis in non-expressing cells
1. PTP-S2: Bcl2	1:0	32.9 ± 1.7 (n = 6)	3.5
	1:1	20.3 ± 6.5 (n = 6)	3.7
	1:2	10.2 ± 2.9 (n = 6)	3.3
2. PTP-S2: Crm A	1:0	29.5 ± 1.3 (n = 5)	2.4
	1:1	20.5 ± 2.8 (n = 5)	2.5
	1:2	12.8 ± 1.0 (n = 6)	2.6

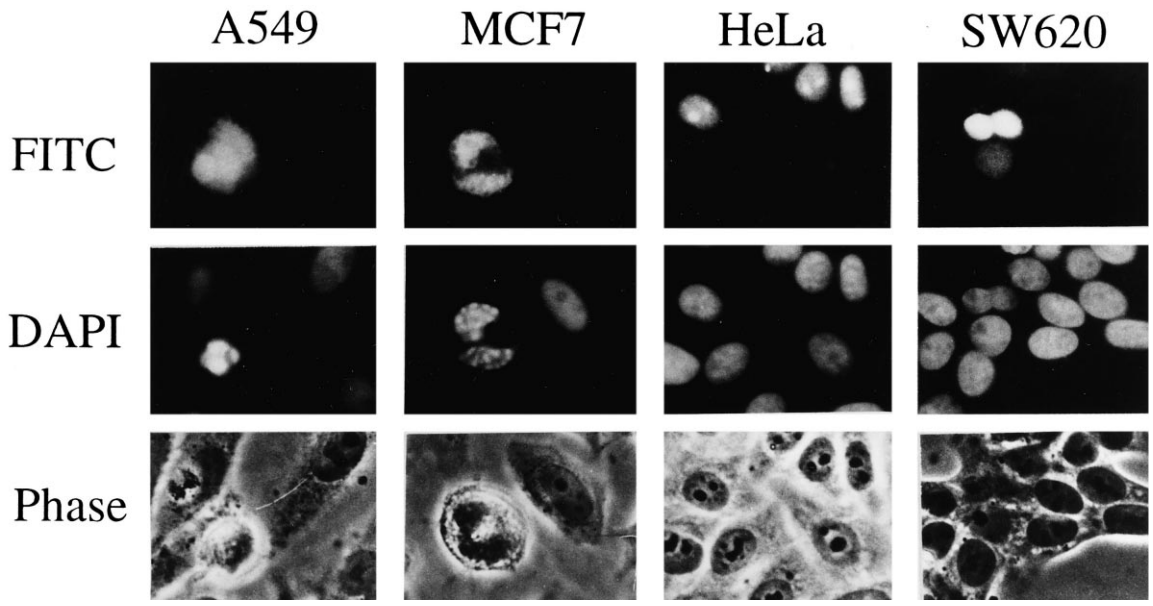

Fig. 4. Effect of overexpression of PTP-S2 in human tumor cell lines. Representative immunofluorescence photomicrographs of cells transfected with PTP-S2 plasmid are shown. After 48 h, transfected cells were detected by staining for PTP-S2 expression (FITC) and apoptotic cells by DAPI staining. In the bottom row, phase contrast pictures show morphology of the stained cells.

was observed in the number of expressing cells or level of PTP-S2 expression in the presence of Bcl2 plasmid.

Overexpression of certain genes involved in cell proliferation induces apoptosis only when cells are grown in medium lacking growth factors [21]. In our experiments cells were fed with serum containing medium 6 h after transfection and maintained in 10% serum for the rest of the experiment. Therefore serum starvation is not required for PTP-S2 induced apoptosis. When the cells were kept in medium containing 0.5% serum up to 48 h after transfection there was some reduction in the percentage of apoptotic cells in low serum (data not shown).

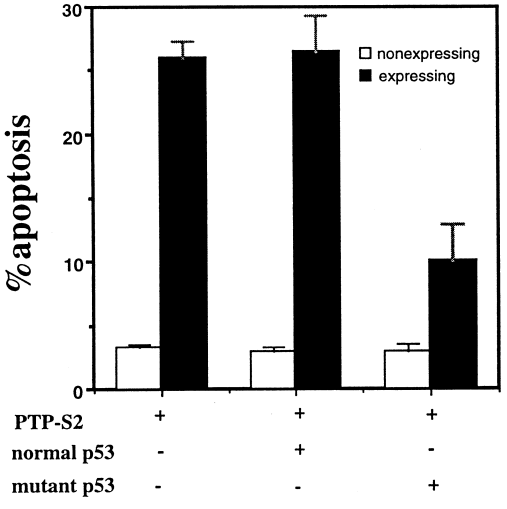

Fig. 5. Inhibition of PTP-S2 induced apoptosis by mutant p53. MCF-7 cells were cotransfected with PTP-S2 and mutant p53 or normal p53 expression plasmids keeping the plasmid concentration constant by using control plasmid. After 48 h of transfection, cells were processed for immunostaining and percentage of apoptotic cells was determined in the PTP-S2 expressing and non-expressing cells.

Poxvirus gene product Crm A is a potent inhibitor of certain caspases in vitro [37]. When a plasmid encoding Crm A was cotransfected with PTP-S2, there was inhibition of PTP-S2 induced apoptosis in a dose dependent manner (Table 1). This suggests that caspase activity is required for PTP-S2 induced apoptosis. It was also found that addition of the tetrapeptide Z-YVAD-cmk, a potent inhibitor of caspase 1, inhibited PTP-S2 induced apoptosis whereas Z-DEVD-cmk, a caspase 3 inhibitor, showed much less effect (data not shown). These results raise the possibility of involvement of caspase 1 in PTP-S2 induced apoptosis. Caspase 1 has been implicated in apoptosis induced by various treatments [38–40]. Caspase 3 is not required for PTP-S2 induced apoptosis since it could induce apoptosis in MCF-7 cells (Figs. 3 and 4) which lack active caspase 3 due to a deletion in the gene [41].

The results presented here show that the nuclear tyrosine phosphatase PTP-S2 upon overexpression induces apoptosis as observed by morphological changes such as chromatin condensation, nuclear fragmentation and formation of apoptotic bodies which are characteristic features of cells undergoing apoptosis. The extent of apoptosis seen upon transient transfection of PTP-S2 in Cos-1 cells is comparable to that induced under similar conditions by other signalling molecules such as LAR and MEKK1 [8,42]. Bcl2 prevents many, but not all forms of cell death [36]. Cotransfection with Bcl2 reduced the number of PTP-S2 overexpressing apoptotic cells indicating that PTP-S2 triggers a pathway directly connected to the cell death machinery. This is supported by the observation that cowpox virus protein CrmA and the peptide YVAD-cmk inhibit PTP-S2 induced apoptosis.

Earlier, we have shown that stable cell lines derived from HeLa cells overexpressing PTP-S2 have a growth advantage over control cells [20]. In these clones, the level of PTP-S2 expression was very moderate compared to levels of overexpression achieved in transient transfection assays. Several proteins like Cdc25A, c-Myc, p53 and E1A regulate pathways leading to both cell proliferation and apoptosis [9,21]. Some

of these proteins like c-Myc and Cdc25A induce apoptosis only under conditions of growth factor deprivation. But our experiments showed that PTP-S2 can activate apoptotic pathways even in 10% serum supplemented medium. These results suggest that PTP-S2 induced apoptosis does not take place due to a conflict created by signals to enhance cell proliferation. Maintenance of appropriate levels of this ubiquitously expressed nuclear enzyme appears to be essential, as deregulated expression disrupts cellular homeostasis leading to cell death. Overexpression of this phosphatase at high level may result in dephosphorylation of those proteins which play a critical role in cell survival, leading to activation of a caspase mediated pathway of cell death. Activation of this apoptotic pathway requires sequence specific DNA binding function of p53 protein. To our knowledge this work is the first example of involvement of a protein tyrosine phosphatase in the induction of p53 dependent apoptosis.

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