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Downregulation of phospho-tyrosine phosphatases in a macrophage tumor

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We provide evidence for the downregulation of phospho-tyrosine phosphatases (PTPases) in malignancy. AK-5, a rat macrophage tumor, shows the downregulation of the transcripts of two non-receptor-type PTPases, PTP-1 and PTP-S. Though downregulated fourfold, the genomic organization of PTP-S is unaltered. There is no gross alteration of the PTPase activity in AK-5 as compared to macrophages. Immunoblot analysis reveals no significant change in the total phospho-tyrosine levels in AK-5, but there is a qualitative difference in the pattern between AK-5 and macrophages. Our results lend credence to the conjecture that PTPases also might be involved in malignancy.

Phospho-tyrosine phosphatase; Downregulation; Malignancy; Rat macrophage cell line

1. INTRODUCTION

There is compelling evidence that dominant oncogenes play an important role in malignant transformation [1]. Of the implicated genes, proto-oncogenes, a surprisingly large proportion code for proteins with tyrosine kinase activity [2]. The evidence implicating protein-tyrosine kinases (PTKs) as critical regulators of cell proliferation has come mainly from two lines of enquiry. (i) Analysis of the genomes of transforming retroviruses has shown that in many, the 'transforming gene' codes for a protein with tyrosine kinase activity [3]. (ii) Studies on growth factor receptors have revealed that the effector domains of a number of these proteins (platelet derived growth factor receptor, epidermal growth factor receptor, nerve growth factor receptor) have intrinsic tyrosine kinase activity [4]. Thus the participation of tyrosine kinases in malignancy is certain.

Since activated tyrosine kinases act as dominant oncogenes in many systems, it is logical to presume that phospho-tyrosine phosphatases (PTPases) might act as 'recessive oncogenes' [5]. Yet, to date there is no published report substantiating this a priori logic. We hence decided to investigate the levels of PTPases in a spontaneous rat macrophage tumor cell line, AK-5, established in our laboratory.

In this report we present evidence for the downregulation of two PTPases in a malignant system.

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2. MATERIALS AND METHODS

2.1. *Cell lines*

AK-5 is a rat macrophage tumor cell line established in our laboratory [6]. It is maintained as ascites by passaging it serially in the peritoneal cavity of Wistar rats from a syngenic strain.

2.2. *Harvesting of peritoneal macrophages*

Peritoneal macrophages from inbred 6-week-old Wistar rats were collected by injection of phosphate-buffered saline into the peritoneal cavity, followed by aspiration, as previously described [7]. The cells were pelleted and resuspended in culture medium containing 2% fetal calf serum (Sigma). The cell suspension was enriched for macrophages by plating it in plastic Petri dishes (Nunc) and incubating for 2 h at 37°C in a humidified, 5% CO_2-containing environment, and collecting the adherent cells.

2.3. *RNA isolation*

Total cellular RNA was isolated by lysing the cells in guanidium isothiocyanate and extracting the lysate with water-saturated phenol [8].

2.4. *Gel electrophoresis and Northern blot analysis*

RNA samples were electrophoresed on 1% agarose/2.2 M formaldehyde denaturing gels. After electrophoresis the gels were photographed. RNA was transferred to nitrocellulose (Schleicher and Schuell), and crosslinked by baking at 80°C for 2 h. Blots were hybridized to the labeled probe, the specific activity of which was approximately $2–5 \times 10^8$ cpm/μg. Hybridizations were carried out at 50°C with 5 × SSPE (0.9 M NaCl, 50 mM sodium phosphate pH 7.4, 5 mM EDTA), 5 × Denhardt's (0.1% Ficoll, 0.1% Polyvinylpyrrolidone, 0.1% bovine serum albumin) 0.1% SDS and 100 μg/ml denatured salmon sperm DNA. The final wash was performed at 50°C in 0.1 × SSPE containing 0.1% SDS. Blots were autoradiographed by exposing to X-ray film with intensifying screens (DuPont) at −70°C.

2.5. *Southern blots*

High molecular weight genomic DNA was isolated using standard methods [9]. A 10-μg aliquot of each sample was digested with restriction endonuclease and separated on 0.7% agarose gels. After staining the gel with ethidium bromide the DNA was transferred to Hybond N^+ nylon membranes (Amersham) and fixed by treating the blot with

0.4 M NaOH. The blots were hybridized to a labeled probe with specific activity 5×10^8 cpm/μg. Hybridisations were performed in 0.5 M phosphate buffer (pH 7.5) containing 7% SDS at 65°C for 16 h. The final wash was done at 65°C with 0.1 × SSPE containing 0.1% SDS. Blots were autoradiographed by exposing to X-ray film at −70°C with intensifying screens (DuPont).

2.6. *Hybridisation probes*

The PTP-S probe is a 1.5-kb full-length cDNA, cloned into pGEM-3Z [10]. PTP-1 is a cDNA clone from a rat brain cDNA library, and is the rat homolog of the human placental phosphatase 1b [11]. This was kindly provided by Dr. J.E. Dixon of Purdue University, USA.

2.7. *Nick translation*

Nick translation was carried out essentially according to the procedure of Rigby et al. [12], using a nick translation kit (Amersham).

2.8. *Immunoblotting*

SDS-PAGE and electrotransfer of the proteins to nitrocellulose (Schleicher and Schuell) were done using standard procedures [9]. Second antibodies conjugated to horseradish peroxidase (HRP) were used (Boehringer Mannheim). The enhanced chemiluminiscence (ECL) kit (Amersham) was used to detect antibody conjugated to HRP.

2.9. *Preparation of tissue fractions*

Particulate and soluble fractions were prepared as described previously [13]. Whole-cell lysates were prepared by lysing 10^7 cells in ice-cold 10 mM phosphate buffer containing 1% NP-40, 1 mM EDTA, 0.15 M NaCl, and 1 μg/ml aprotinin. The tubes were stored on ice for 20 min, and the lysates were clarified by centrifugation at 10,000 rpm in a refrigerated microfuge for 15 min. The supernatant was aliquoted and stored at −70°C.

2.10. *Assay for phosphotyrosine phosphatase activity*

PTPase activity was measured by estimating the phosphate released from [^{32}P]poly(Glu,Tyr) 4:1 as described previously [14].

2.11. *Densitometric scanning*

Molecular Dynamics laser densitometer using software from Microsoft Excel (Image Quant Release Version 3) was used to scan autoradiograms.

3. RESULTS AND DISCUSSION

3.1. *PTP-1 and PTP-S, two PTPases, are downregulated*

Our present understanding of gene regulation indicates that the major control of gene expression is at the initiation of transcription [15]. Hence as a first step we decided to check the amounts of two PTPase transcripts. PTP-S is a PTPase cloned from a rat spleen cDNA library, and is an alternatively spliced homologue of the human T cell phosphatase [10]. PTP-1 is a non-receptor-type PTPase cloned from a rat brain cDNA library and shares 97% identity with the human placental PTPase (EC 3.1.3.48) [11]. Blots of total cellular RNA from AK-5 and normal macrophages were probed with PTP-S and PTP-1. The transcripts of both these genes were normal in size but were present at lower levels in AK-5 when compared to macrophages (Fig. 1). The lower panels show that the quality of the RNA was good and the loading was quite equal. These results demonstrate downregulation of PTPases in a malignant system; similar observations have been made in certain patients with acute lymphoblastic leukemia where CD45 PTPase is not expressed in T and B-lymphoma cell populations [16]. The PTP-S, which is a ubiquitously expressed PTPase, is 4-fold downregulated compared to normal macrophages where it has the highest level of expression [10]. Recently, Zheng et al. have reported overexpression of a protein tyrosine phosphatase PTP-α with concomitant cell transformation and tumorigenesis [17]. Their results suggest the dephosphorylation of the Tyr-527 site at the carboxy-terminal and its involvement in the regulation of cell proliferation and oncogenic capability.

PTP-S has been shown to exhibit DNA binding activity in vitro [18], and the terminal region of PTP-S has clusters of basic amino acid residues homologous to the transcription factors *fos* and *jun* [10]. In light of the recent observation that p140$^{c\text{-}abl}$ a tyrosine kinase, has sequence-specific DNA binding activity and functions as a transcription factor [19], it is quite possible that PTP-S also might be involved in the regulation of transcription. Phosphorylation is a versatile and often used post-translational mechanism for the regulation of protein activity. Many critical nuclear proteins like p53 and p105-RB exist in two states, a phosphorylated and a dephosphorylated state [20,21]. The phosphorylation status of p53 and p105-RB is altered as a function of the stage of the cell cycle [20,22,23]. It is not far-fetched to speculate that PTP-S might be involved in regulating the

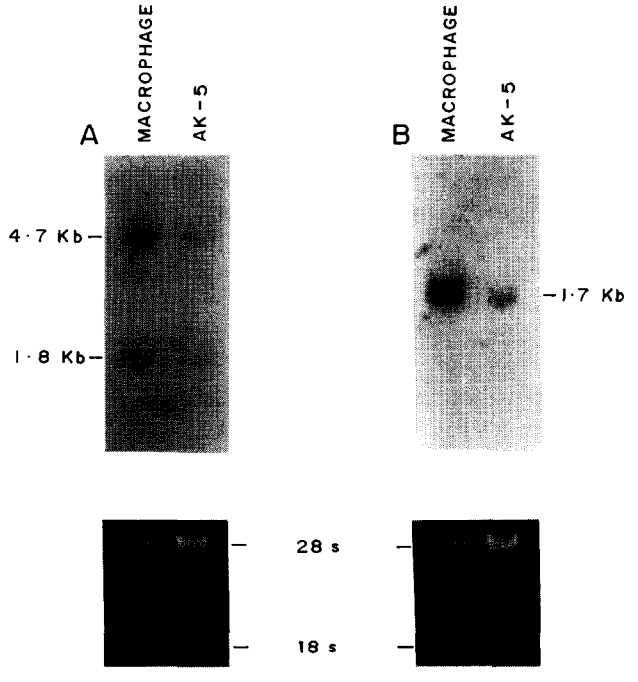

Fig. 1. Expression of PTP-1 and PTP-S. 10 μg of total cellular RNA from AK-5 and normal peritoneal macrophages was Northern blotted. Autoradiogram of the blot probed with (A) a full-length rat PTP-1 cDNA clone, and (B) a full-length rat PTP-S cDNA clone. The lower panels show the ethidium bromide stained gel.

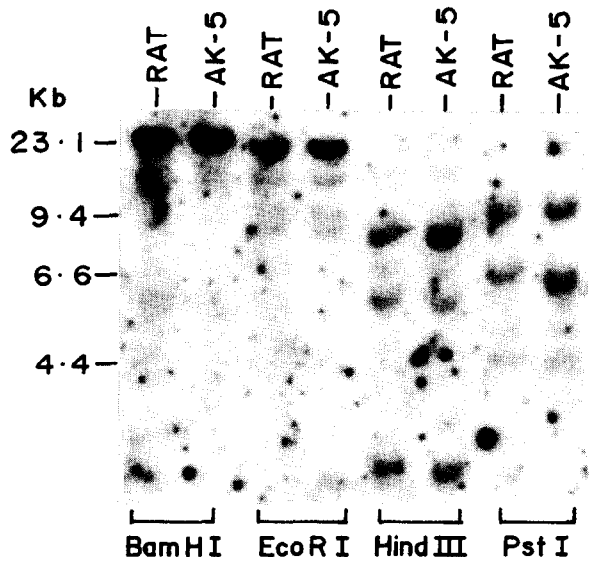

Fig. 2. Genomic organization of PTP-S. Southern blot of Wistar rat and AK-5 genomic DNA was probed with a full-length PTP-S cDNA clone.

phosphorylation of nuclear proteins which have critical regulatory roles. If indeed PTP-S is involved in such crucial regulatory activities, then the downregulation of PTP-S might have profound implications for the regulation of proliferation. Yet, since we do not know the physiological substrates of either of these PTPases, it is hard to ascertain whether these alterations have a causal link to the transformation of AK-5 or are merely amongst the unrelated perturbations of malignant transformation.

3.2. *The organization of the PTP-S gene is normal*

To check whether the downregulation of PTP-S was due to a rearrangement of this locus, rat genomic DNA and AK-5 DNA were digested with four different restriction endonucleases, Southern blotted and probed with the full-length rat PTP-S cDNA. No rearrangement was discernable (Fig. 2). The lack of gene rearrangement does not preclude it from behaving as a tumor suppressor, for loss of function might arise from either direct damage to the gene or from mechanisms that act in *trans* to repress gene expression. A good example of this latter mechanism is the transcriptional suppression in neuroblastomas of CD44, the transmembrane receptor for hyaluronate [24].

3.3. *The PTPase activity in AK-5 is not altered*

The turnover number of PTPases is one order of magnitude higher than that of PTKs [5], and hence it is presumed that PTPases are expressed constitutively and keep in check the activity of PTKs. To determine whether the transcriptional down-regulation of these two PTPases had affected the total PTPase level in AK-5, a comparison of the PTPase activities of AK-5 and macrophages was carried out using the artificial substrate [^{32}P]poly (Glu,Tyr) 4:1. In these experiments tissue fractions of the rat spleen were used as a positive control because it has been reported that of a variety of rat tissues examined for their total PTPase activity the rat spleen had the highest [14]. We found no gross alteration in the PTPase activity of whole cell lysates of AK-5 as compared to macrophages (Table I). The general pattern of all the samples resembled that of spleen, with the specific activity of the particulate fraction considerably higher than that of the soluble fraction (Table I). Since all the tissues examined belong to the hematopoietic class the overall similarity is not surprising. Thus the downregulation of the two non-receptor-type PTPases is not reflected by the activity levels. The other

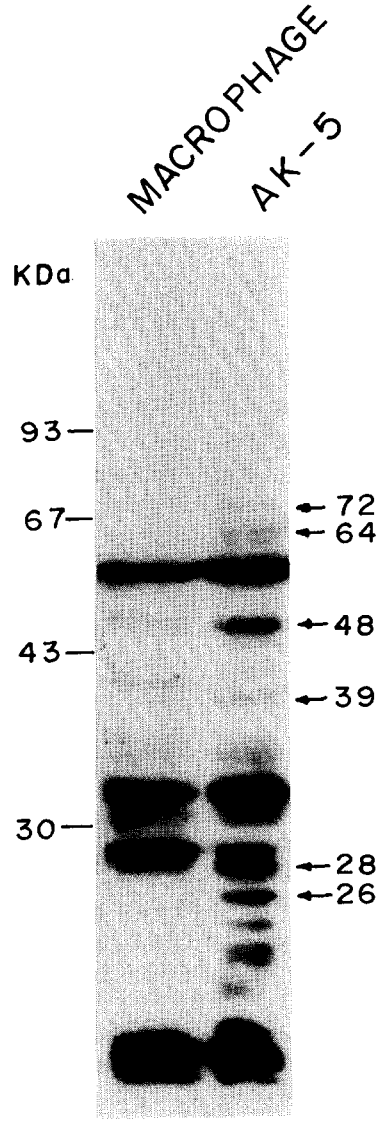

Fig. 3. Immunoblot analysis for p-tyr containing proteins. Autoradiogram of a whole-cell lysate blot. 100 μg protein from macrophages and AK-5 was loaded on each lane and the blot was probed with a monoclonal specific to p-tyr (Sigma, USA).

Table I

PTPase activity of AK-5, spleen and normal peritoneal macrophages

Tissue	Fraction	Enzyme activity (units)
AK-5	Whole cell lysate	1.92 ± 0.25
	Particulate	1.52 ± 0.06
	Soluble	1.17 ± 0.06
Macrophage	Whole cell lysate	1.71 ± 0.21
	Particulate	1.94 ± 0.22
	Soluble	0.62 ± 0.05
Spleen	Particulate	2.03 ± 0.07
	Soluble	1.17 ± 0.06

1 Unit is defined as 1 nmol of phosphate released from [^{32}P]poly-(Glu,Tyr) 4:1 per mg of protein in 1 min [14]. Values represent mean ± S.D. of three experiments.

possibility for which we have no experimental evidence is that there is increased translational activity of a low amount of PTPase mRNA in AK-5 cells. This in no way undermines our observation of the downregulation of the transcripts of PTP-S and PTP-1 because what has been assayed is the sum total activity of all the PTPases and it is not inexplicable that the specific downregulation of a couple of PTPases does not produce any gross changes. One other factor which has to be considered is the fact that we have used an artificial substrate and hence may not have been able to assay all the PTPases. There has been one report which has indicated that transformation can be brought about by decreasing the activity of PTPases. Culturing of normal rat kidney cells in the presence of sodium orthovanadate (Na_3VO_4) caused an increase in the total phospho-tyrosine (p-tyr) in protein, and the cells acquired certain traits of transformation [25]. Though it has not been rigorously proved this is thought to be due to the inhibitory activity of Na_3VO_4 towards PTPases, for Na_3VO_4 selectively inhibits PTPases in vitro and has no effect on serine/threonine phosphatases [26].

3.4. *Altered phospho-tyrosine profile in AK-5*

In the normal cell p-tyr is present in vanishingly small amounts and constitutes less than 0.1% of the total phosphate covalently linked to protein [27]. In cells transformed by retroviruses carrying tyrosine-kinase encoding oncogenes there is tremendous increase in the total p-tyr [28]; and this adventitious phosphorylation is believed to lead to malignant transformation. To check whether the downregulation of the two PTPases had produced any alteration in the p-tyr levels, an immunoblot analysis was done. AK-5 cells and macrophages were lysed in boiling sample buffer, Western blotted and probed with a monoclonal antibody specifically reactive to p-tyr. The relative proportion of p-tyr as determined by the densitometric scanning of the autoradiogram (Fig. 3) was, macrophage: 1.00; AK-5: 1.30, and there are qualitative differences between AK-5 and macrophage samples in that there was a prominent 48 kDa band and multiple bands of molecular mass less than 30 kDa in AK-5 which are absent in macrophages (Fig. 3). Our present observations do not permit us to make any guess as to whether the downregulation of the two PTPases has contributed to this effect, but recent experiments in our laboratory have indicated that these additional p-tyr containing proteins might be the effect of $p56^{lck}$ which is expressed ectopically in AK-5 (our unpublished results).

For some years now, it has been speculated that PTPases might function as tumor suppressors [29]. Our observation of the suppression of two PTPases in a malignant cell line offers an opportunity to probe this issue further. Our observations also indicate that kinases alone are not responsible for altered phosphorylation. Phosphatases also play an important role in regulating tyrosine phosphorylation.

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