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Negative growth control by a novel low M_r phosphotyrosine protein phosphatase in normal and transformed cells

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Having determined the complete amino acid sequence of a cytosolic phosphatase purified from bovine liver, we studied the role of this enzyme (referred to as 'PTPase') in the control of cell proliferation. We used NIH/3T3 fibroblasts, both normal and transformed by the oncogenes v-*erb*B, v-*src*, and v-*raf*: a synthetic gene coding for PTPase was transfected into, and overexpressed in, normal and transformed NIH/3T3 cells with resulting inhibition of cell growth. Inhibition of proliferation correlated with the level of foreign PTPase; growth in soft agar was also inhibited in transformants overexpressing the enzyme. However, PTPase overexpression did not inhibit the rapid turnover of inositol lipids stimulated by platelet-derived growth factor. We conclude that this novel PTPase is active on cell type-specific signalling substrates that control normal and transformed fibroblast proliferation.

Phosphatase; Oncogene; Neoplasia; Inositol lipid

1. INTRODUCTION

Several growth factor receptors and oncoproteins have tyrosine kinase domains, and activation of tyrosine kinases is a necessary pre-requisite for the stimulation of cell growth. The phosphorylated state of any given protein is the result of a finely regulated balance of kinase and phosphatase activities, and phosphotyrosine protein phosphatases have been proposed as key regulators of cell proliferation [1]. A recent study indicated that expression of rat PTP1 phosphatase in mouse 3T3 fibroblasts transformed by the v-*src* oncogene, negatively affected some transformed features of these cells [2]. Rat PTP1 is an ubiquitous, single domain, phosphatase associated with cell membranes.

We determined the complete primary structure of a different cytosolic phosphatase purified from bovine liver (originally termed low M_r acid phosphatase; in this study defined as 'PTPase') [3]. The reaction mechanism of this enzyme involves the formation of a cysteinyl-phosphate intermediate [4] which is peculiar of enzymes of the PTPase family [5,6]. Furthermore, this low M_r PTPase shows in its active site the signature motif (sequence: XCXXXXXRXX) which is a main feature of all the members of this family [5]. With site-directed mutagenesis [7], and with kinetic studies [3], we demonstrated that C and R residues are both essential for the enzyme catalytic mechanism. The enzyme actively dephosphorylated phosphotyrosine-containing proteins without affecting phosphoserine or phosphothreonine [8,9]. We also demonstrated that PTPase dephosphorylated the tyrosine-autophosphorylated epidermal growth factor receptor in vitro [8], and inhibited the growth of v-*erb*B-transformed NIH/3T3 cells [10]. In order to ascertain whether the inhibitory effect of PTPase was directly associated with growth factor receptor tyrosine phosphorylation, we decided to extend our study to cells transformed by the v-*src* and v-*raf* oncogenes. In fact, these oncogenes code for non-receptorial transforming proteins having distinct functions both within (*src*), and outside (*raf*) the tyrosine kinase signalling cascade; v-*src* encodes a membrane-associated non-receptor tyrosine kinases which is constitutively active; v-*raf* codes for a cytosolic serine/threonine kinase significantly homologous to protein kinase C (for review see [11]).

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2. MATERIALS AND METHODS

2.1. *Determination of PTPase level*

PTPase determination was carried out by a non-competitive sandwich ELISA. Antisera to the enzyme purified from bovine liver were raised in rabbits. From mixture of equal volumes of three rabbit antisera, specific antibodies were purified by affinity chromatography using the antigen linked to Sepharose 4B as immunoadsorbent. Antibodies were then conjugated to horseradish peroxidase [12]. Microtitre polystirene wells were coated with affinity-purified antibodies. After washing cycles, standards and samples, diluted in the assay buffer (TBS-T), were added to the wells, and incubated overnight at 4°C. After washing, the appropriate dilution of conjugate was added to each well for three hours. The quantity of formed immunocomplex

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was then determined by measuring the peroxidase activity using *o*-phenylendiamine as substrate. Standard solutions of PTPase purified from rat [13] and bovine liver were used to assess the basal level of the native enzyme and its overexpression. The lower limit of quantitation was 0.1 ng/ml for overexpressed PTPase, and 0.75 ng/ml for the native one, with linear ranges of 0.1 to 2 ng/ml, and 0.75 to 20 ng/ml, respectively. Lysates of different cell lines, depleted of PTPase by repeated incubation in the presence of antibodies linked to Sepharose-4B and supplemented with the enzyme standards, showed linear and reproducible concentration/absorbance relationship. The responsiveness of lysates, as treated above, were very similar to the assay buffer supplemented with the same PTPase standards. In the used range there were negligible effects of lysate dilutions on the assays. Quantitative values determined by ELISA were consistent with the increase of hydrolytic activity assayed in selected clone lysates using *p*-nitrophenylphosphate as substrate.

2.2. *Transfection and overexpression of the synthetic PTPase gene*

In order to study the effect of PTPase overexpression on cell behaviour, a synthetic gene coding for the bovine isoform of the enzyme was transfected into normal and oncogene-transformed NIH/3T3 fibroblasts (obtained from the Laboratory of Cellular and Molecular Biology of the National Cancer Institute, NIH, Bethesda, USA). The same cell lines were also transfected with a gene coding only for resistance to neomycin in order to obtain clones that had undergone the same treatment but were not overexpressing the PTPase. Restriction endonucleases, *E. coli* DNA polymerase I (Klenow fragment), and DNA ligase were obtained from Promega and used under standard conditions. DNA sequence analysis was carried out using the UBS-Sequenase kit. Neomycin (G418) was from Sigma Chem. Co. Plasmid preparation was as follows. pSVPTP: pExPAIII [14] was cut with *Hin*dIII, treated with Klenow polymerase, and then partially digested with *Nco*I to obtain the 477 bp DNA fragment coding for PTPase; this band was cloned in pGEM5Zf(+) vector (Promega) cut with *Sal*I, treated with Klenow polymerase and cut with *Nco*I in order to obtain the pGEMPTP construct. The *Sph*I–*Hin*dIII band obtained from pGEMPTP, containing the synthetic cDNA, was cloned in the blunt-ended *Eco*RI site of the pSVT7 vector [15] in order to obtain the pSVPTP plasmid. DNA sequence analysis confirmed that the expected sequence (containing an ATG start codon followed by the coding sequence for PTPase and a TGA stop codon) had been inserted in the right orientation downstream of the SV40 early promoter of the pSVT7 plasmid. Transfection experiments were performed as follows; 10 μg of pSVPTP and 0.5 μg of pK0neo (i.e. the function coding for resistance to neomycin, used to select transfection-positive clones, and, when transfected alone, as a control) were used with the calcium phosphate coprecipitation technique [16–18] for transfecting 1.5 million cells on a 100-mm plate. Selection of stable neomycin (G-418)-resistant clones was performed supplementing the medium with 500 μg/ml of antibiotic. mRNA analysis was performed using the [^{32}P]-labelled PTPase synthetic cDNA as a probe, in high stringency conditions (2 × SET at 65°C). Total RNA preparation and Northern blot analysis were performed according to Solito et al. [19]. Both normal and transformed cells were also transfected with pK0neo alone. Stable overexpression of PTPase was assessed by ELISA, and the level of the enzyme remained approximately the same for up to two months.

2.3. *Determination of thymidine incorporation and growth kinetics*

Normal NIH/3T3 v-*erb*B, v-*src*, and v-*raf* transformants have been previously described [20,21]. The kinetics of growth, and the incorporation of [^{3}H]thymidine (from New England Nuclear) were performed as previously described [21,22]. Data obtained measuring [^{3}H]thymidine incorporation were normalized for the number of cells per dish.

2.4. *Determination of inositol lipid metabolism*

Cells prelabelled to equilibrium with [^{3}H]*myo*-inositol (New England Nuclear) were stimulated with 100 ng/ml of platelet-derived growth factor, BB chain homodimer (PDGF-BB) (Pepro Tech Inc.) in the presence of 20 mM LiCl as described [21,22]. In some experiments, tyrphostin (Calbiochem; 100 μM) was added 6 h before stimulation with PDGF-BB. In a first series of experiments, calcium-mobilizing inositol (1,4,5)-trisphosphate was separed by high-pressure liquid chromatography; as expected from previously published results, the compound was rapidly formed and degraded, reaching its peak about 30 s after stimulation with PDGF-BB both in normal cells and in transfectants. Thus, we decided to routinely measure the formation of total inositol polyphosphates which were extracted and separated by ion-exchange chromatography (with Dowex resin from Bio-Rad) as described [22].

3. RESULTS AND DISCUSSION

We determined the effect of PTPase overexpression on DNA synthesis in normal and transformed NIH/3T3 fibroblasts which had been serum-starved for 24 h. Fig. 1A shows that cells overexpressing the PTPase gene incorporated about half as much [^{3}H]thymidine as their untransfected counterpart. We also monitored transformed cell division by following growth kinetics over extended periods of time in the presence of complete culture medium (serum-containing Dulbecco's modified Eagle medium (DMEM). Fig. 1B shows that PTPase overexpression significantly inhibited cell proliferation under these conditions, thus demonstrating that inhibition of growth was independent of culture conditions.

To determine whether inhibition of proliferation was related to the level of PTPase expression, we performed the same experiment described in Fig. 1, using different transfected clones overexpressing PTPase at different levels. Fig. 2 shows a correlation between PTPase expression and inhibition of cell proliferation in v-*src* (A) and v-*raf* (B) transformants. Although inhibition of cell growth correlated with PTPase levels, it was not linearly proportional to the level of PTPase overexpression. We also could not establish a 'threshold' of PTPase that invariably inhibited cell proliferation to the same extent. Identical results were obtained in v-*erb*B transformants, and in normal NIH/3T3 cells overexpressing the PTPase gene (data not shown). Normal cells required lower expression of the PTPase to be growth-inhibited; these results suggest that PTPase inhibited with more efficiency normal cells that were growing at a lower rate if compared with transformants. This might be reason why we could not select normally growing clones harboring higher level of PTPase, just because they would not be able to proliferate.

The effect of PTPase overexpression on another assay of transformation, growth in semi-solid medium, was studied in oncogene-transformed cells that were capable of forming colonies in soft agar. Fig. 3 shows that PTPase-overexpressing transformants formed a significantly reduced number of colonies as compared with their untransfected counterpart. These results indicate that PTPase exert multifaceted effects on different parameters of transformation. However, PTPase-overexpressing cells did not show appreciable changes of their

transformed phenotype, as monitored by light microscope observation.

PTPase inhibition of cell proliferation driven by oncogenes whose products differed greatly in structure and function, suggested that PTPase did not directly interact with transforming proteins, but rather inhibited downstream signalling pathways. In an attempt to determine whether a known second messenger system was affected by PTPase expression, we studied the metabolism of inositol lipids [23] induced by PDGB-BB and/or

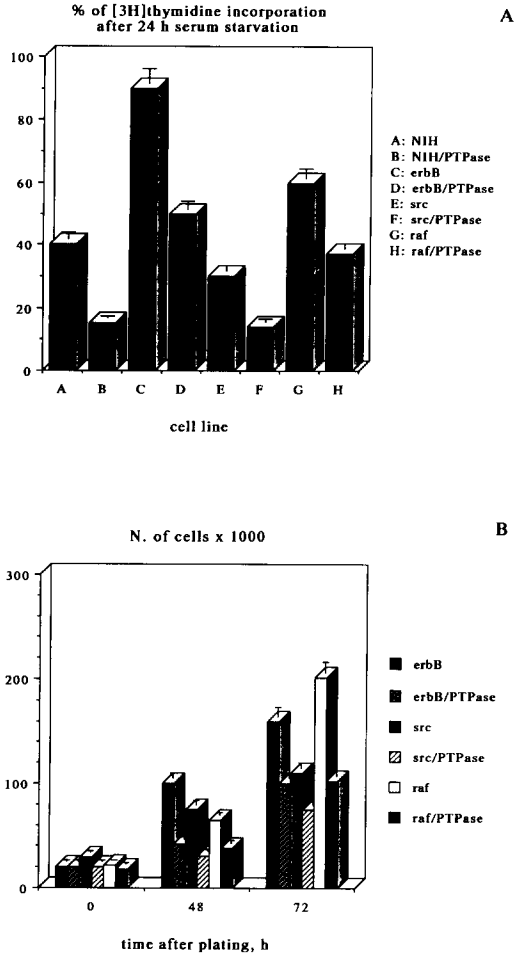

Fig. 1. Thymidine incorporation and growth kinetics of normal and transformed cells overexpressing the synthetic gene for PTPase. The kinetics of growth, and the incorporation of [^{3}H]thymidine were performed as described. Data obtained measuring [^{3}H]thymidine incorporation were normalized for the number of cells per dish. (A) Wild-type (or pK0neo-transfected) normal and transformed NIH/3T3 cells, and cells overexpressing the PTPase gene were serum-starved for 24 h, after which [^{3}H]thymidine incorporation was measured by pulse (4 h) labelling. Results are expressed as % of incorporated [^{3}H]thymidine before starvation (i.e. thymidine incorporation of growing cells was taken as 100%), and are means ± S.E. of quadruplicate samples from one experiment, representative of three others that gave qualitatively identical results. (B) Wild-type (or pK0neo-transfected) transformants, and transformants overexpressing the PTPase gene were plated at the same density. Cell number was determined every 24 h for 3 days thereafter. Data from one representative experiment are reported as means ± S.E. (n = 8).

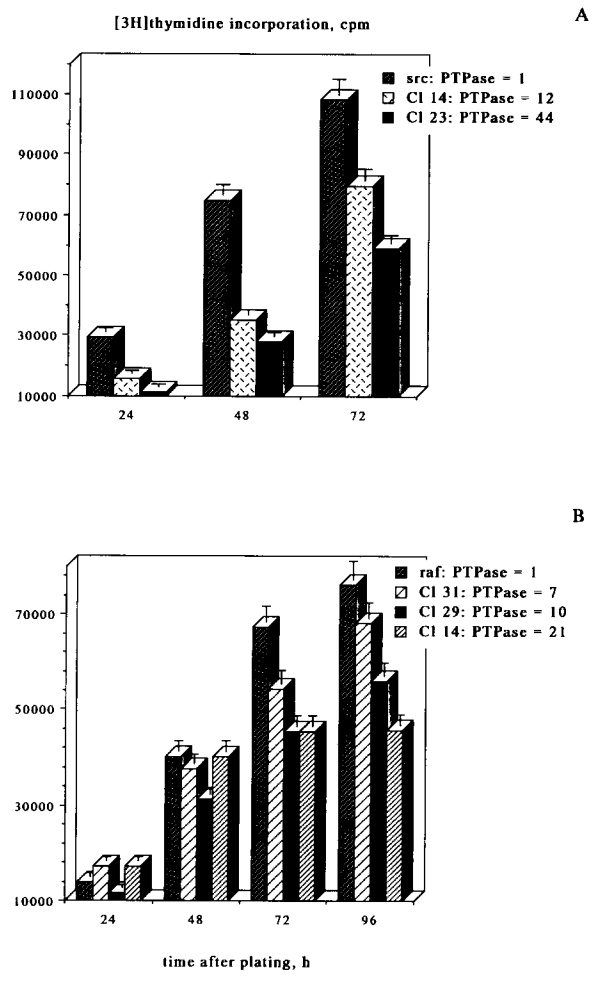

Fig. 2. Growth kinetics in transformed cells expressing different levels of PTPase. Clones of v-*src* (panel A), and v-*raf* (panel B) transformants expressing different levels of PTPase were assayed as described, and [^{3}H]thymidine incorporation was determined every 24 h after plating. On the side of the Figure is reported the relative fold increase of PTPase in each clone (Cl number). The level of PTPase in untransfected (or pK0neo-transfected) cells was normalized as 1, and the PTPase value referring to each clone indicates how many fold was the PTPase level increased above normal cells. Data from one representative experiment are reported as means ± S.E. (n = 8).

serum in normal and transformed fibroblasts harboring the PTPase gene. As expected, PTPase-NIH/3T3 fibroblasts showed impaired mitogenic response to PDGB-BB. Inhibition of PDGF-BB-induced [^{3}H]thymidine incorporation in PTPase-transfected cells was about 50% (NIH/3T3 stimulated with 100 ng/ml of PDGF-BB: 38,530 ± 1267. PTPase-NIH/3T3: 19,852 ± 859, cpm ± S.E. The experiment was performed in cells that had been serum-starved for 24 h, and then stimulated with PDGF-BB for 24 h). However, when we measured inositol lipid turnover we found that inositol phosphate formation in response to PDGF-BB in PTPase-transfected cells was almost identical to that of untransfected cells (Fig. 4). Tyrphostin, a known inhibitor of tyrosine

kinases [24], reduced inositol phosphate formation in response to PDGF-BB both in normal and PTPase-transfected cells. The inhibitory effect of tyrphostin was unaffected by PTPase overexpression. Qualitatively identical results (i.e. no effect of PTPase overexpression on inositol lipid metabolism) were obtained when v-*erb*B, v-*src*, and v-*raf* transformants overexpressing the PTPase gene were stimulated with serum (data not shown).

Recently, attention has focused on tyrosine phosphatases as putative counterregulators of mitogenic tyrosine kinases. Receptor-like and membrane-associated phosphatases with *src* homology (SH_2) domains and PEST (proline-glutamic acid/serine/threonine) sequences have been characterized, thus leading to suggestion that phosphatase activity might be regulated in a manner similar to that of tyrosine kinases (for review see [1]). The PTPase we described in this study is peculiar among other enzymes of its class in that, with the exception of the active site, it does not share general sequence homology with other known phosphatases, neither does it contain SH_2 domains or PEST sequences [3]. Our results demonstrate that PTPase expression was inversely related to cell proliferation, either in normal or oncogene-transformed fibroblasts. The effect of PTPase overexpression on cells transformed by the *raf* oncogene (i.e. by a non tyrosine kinase-encoding oncogene), together with the lack of effect on inositol lipid metabolism, suggested that PTPase acts on downstream phosphorylated substrates that are activated by growth factor-dependent mitogenic stimulation as well as by oncogenic transformation. PTPase inhibition of cell proliferation in a rather nonspecific manner led us to suspect that PTPase overexpression was causing some type of damage to cell structure or was acting 'pharmacologically'. However, PTPase overexpression did not affect the proliferation of either normal [25] or v-*erb*B-transformed [26,27] 32D hematopoietic cells (not shown), thus suggesting that the inhibitory effect observed in NIH/3T3 fibroblasts was not due to nonspecific cell damage. Thus, comparative study of protein substrates dephosphorylated by PTPase may help to identify the critical regulators of cell proliferation in response to normal and aberrant growth stimuli.

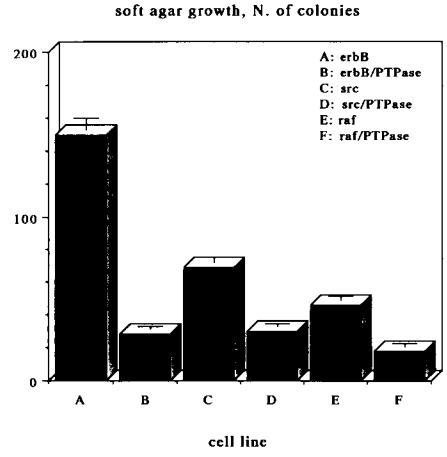

Fig. 3. Soft agar growth of v-*erb*B, v-*src*, and v-*raf* transformants overexpressing the synthetic gene for PTPase. Plates were previously coated with 1.2% sea plaque agarose medium, in order to prevent cell adhesion. Single cell suspensions (2000 cells/ml) were plated in 0.27% agarose medium plus 10% calf serum. Visible colonies were scored at 14 days. The results represent the mean values of three independent experiments.

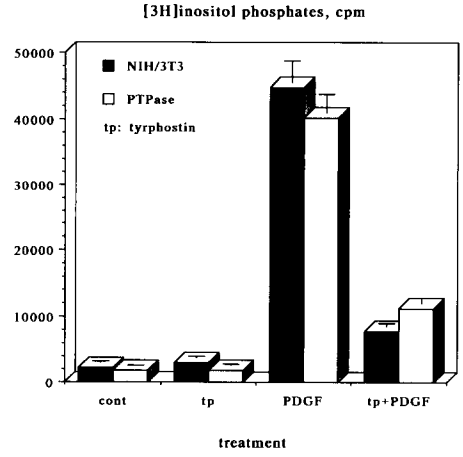

Fig. 4. Inositol lipid metabolism in PTPase-overexpressing fibroblasts. Cells prelabelled to equilibrium with [^{3}H]*myo*-inositol were stimulated with 100 ng/ml of PDGF-BB for 1 h. In some experiments, tyrphostin (100 μM) was added 6 h before stimulation with PDGF-BB. Total inositol phosphates were extracted and separated by ion-exchange chromatography. Data from one representative experiment are reported as means ± S.E. (n = 3).

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