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Inhibition of cellular response to platelet-derived growth factor by low M_r phosphotyrosine protein phosphatase overexpression

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Abstract

The role of low M_r phosphotyrosine protein phosphatase (PTPase) in the control of cell proliferation was studied. A synthetic gene coding for PTPase was transfected and expressed in NIH/3T3 fibroblasts. The effects of the enzyme were particularly evident when cells were stimulated by platelet-derived growth factor (PDGF). The mitogenic response to PDGF was decreased and the inhibition reached 90%. This effect was more pronounced with respect to fetal calf serum stimulation. Hormone-dependent autophosphorylation of the PDGF receptor was significantly reduced. These results demonstrate that low M_r PTPase, a cytosolic enzyme, not only affects cellular response to PDGF but also reduces the membrane receptor autophosphorylation.

Key words: Phosphotyrosine protein phosphatase; PTPase; Platelet-derived growth factor

1. Introduction

The phosphorylation of specific tyrosine residues of cellular proteins is a crucial event in the regulation of the cell cycle and proliferation [1,2]. An important family of growth factor receptors and oncogene products possesses a tyrosine kinase activity leading to the phosphorylation of receptors themselves as well as of several intracellular proteins. The phosphotyrosine level is determined by a finely regulated equilibrium between tyrosine kinase and phosphatase activity and several different phosphotyrosine protein phosphatases (PTPases) seem to have a role in the modulation and/or in the counter regulation of growth factor-dependent tyrosine kinase activity during signal transduction [3]. Often PTPases may inhibit cell growth functioning as potential antioncogenes [4].

Molecular analysis of the PTPases has resulted in the identification of a common homology domain containing a cysteine residue that is located in the active site and absolutely required for enzyme activity. This motif is common to the non-receptor cytosolic as well as to the receptor-like transmembrane PTPases [5–7].

In spite of numerous studies, a very limited number of cellular substrates of PTPases has been clearly identified and few data confirm the in vivo interaction between PTPases and phosphoproteins implicated in signalling pathways. For example, studies have indicated that the PTPase cdc25 causes the dephosphorylation of $p34^{cdc2}$, a serine/threonine kinase, triggering the onset of cell mitosis [8].

Indirect confirmations of the hypothesis about the role of PTPase in affecting cell growth arise from the effects of molecules such as vanadate and phenylarsine oxide, which are specific inhibitors of phosphotyrosine phosphatases [9–11]. Inhibitors are able to amplify receptor-dependent tyrosine phosphorylation and to produce all the events linked to this kinase activity.

Growth factors, like platelet-derived growth factor (PDGF), induce different events upon binding to their surface receptor. Some of these, as mentioned above, are connected to receptor autophosphorylation on several tyrosine residues. A function of the autophosphorylation, at least in the case of PDGF receptor, seems to be the creation of binding sites available for several proteins characterised in their sequence by Src homology domains. Among these we can include the phospholipase Cγ [12,13], the 85 kDa polypeptide subunit (p85) of phosphatidylinositol-3-kinase [14,15], the c-*raf* serine/threonine kinase [16], members of the *src* family [17] and GTPase activating protein of *ras* [18]. Receptor autophosphorylation is a main event for this protein recruitment and probably for developing total mitogenic activity.

Results obtained by the expression of a T cell-derived intracellular PTPase in baby hamster cells [19], suggest that a cytosolic PTPase may not directly interact with growth factor tyrosine kinases. More recently it has been demonstrated that transfection of the transmembrane PTPase CD45 into C127 cells decreases PDGF and IGF-1-dependent receptor phosphorylation [20,21] and inhibits hormone-dependent phosphatidylinositol-3-kinase

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Abbreviations: PTPase, phosphotyrosine protein phosphatase; PDGF, platelet-derived growth factor; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; IGF-1, insulin-like growth factor; PAO, phenylarsine oxide.

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activation, tyrosine phosphorylation of protein substrates and their association to the receptor, but it does not affect the tyrosine phosphorylation of cytosolic proteins that are not substrates of the growth factor receptors. These data support the hypothesis that cellular localization makes more logical the interaction between autophosphorylated receptors and those PTPases which possess a domain closely associated with cellular membrane. This type of PTPases could possess a specific role in the regulation of receptor signal transduction.

Recently, we have shown the effects of the overexpression of the low M_r PTPase in tumoral cell lines [22,23]. This enzyme actively dephosphorylates phosphotyrosine-containing proteins and peptides [24], and belongs to the non-receptor-like subfamily. The reaction mechanism involves the formation of a cysteinylphosphate intermediate and the enzyme has the signature [25] of members of the PTPase family in its active site.

The low M_r PTPase dephosphorylates the tyrosine-autophosphorylated epidermal growth factor receptor in vitro [26] and inhibits the growth of NIH/3T3 cells transformed by v-*erb*B [22] and by other different oncogenes [23].

In this study, the effects of the overexpression of the low M_r PTPase in murine NIH/3T3 fibroblasts on cell growth and tyrosine phosphorylation are investigated. Particularly we demonstrate that the low M_r PTPase, a cytosolic PTPase, inhibits the cellular response to PDGF-BB and greatly affects PDGF receptor autophosphorylation.

2. Experimental

2.1. Materials

Human recombinant PDGF-BB was from Calbiochem. RC20-peroxidase conjugated antibodies to phosphotyrosine were from Transduction Laboratories (Lexington, KY). All other reagents were from Sigma en were of the highest quality available.

2.2. Cell culture and transfection

Parent NIH/3T3 murine fibroblasts were routinely cultured at low density in a humidified atmosphere of 5% CO_2/95% oxygen at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (complete medium). Cells were transfected with pSVPTP plasmid, containing a synthetic DNA sequence coding for low M_r PTPase [27] under the control of the SV40 early region promoter, together with pK0neo, expressing neomycin (G418) resistance, using the calcium phosphate precipitation method as previously described [22]. Selection of stable G418-resistant clones was performed by supplementing the medium with 400 μg/ml of antibiotic. Neomycin resistant subclones were isolated and periodically grown in selective medium to maintain a stable expression.

2.3. Evaluation of PTPase overexpression

Northern blot analysis was done on total RNA under standard conditions, according to Ramponi et al. [22]. Hybridization, using the ^{32}P-labelled PTPase synthetic gene as a probe, was performed under high stringency conditions (2 × SET at 65°C).

The total content of PTPase in transfected cells was determined by a non-competitive immunoenzymatic assay. Antibodies to the recombinant enzyme were purified by affinity chromatography from rabbit antisera and conjugated to horseradish peroxidase. Microtiter polystyrene wells coated with purified antibodies were chosen as solid phase. Using a standard solution of recombinant PTPase, the lower limit of quantitation was 0.1 ng/ml and linearity was kept up to 2 ng/ml. In this range there were negligible effects of lysate dilutions on the assay, observed using lysates depleted of the PTPase by immunoadsorption.

The activity of the overexpressed enzyme in cell lysates was checked using *p*-nitrophenyl-phosphate as substrate. We measured the phosphatase activity in the presence of vanadate to evaluate its inhibited portion. The rise of substrate hydrolysis was compared to the quantitative increase of the enzyme by assuming the specific activity of the purified enzyme [28].

The level of enzyme expression remained approximately the same for up to two months.

2.4. Hormone stimulation of cells

Subconfluent 35-mm dishes of control and PTPase overexpressing cells were starved for 24 h in serum-free medium. Medium was then changed and a serum-free medium containing PDGF-BB was added to the cells. After a 5 min hormone treatment, dishes were placed on ice, cells were washed twice with ice-cold phosphate-buffered saline (PBS) at pH 7.4, then they were harvested in PBS/EDTA 5 mM, centrifuged and the pellet was boiled in 100 μl of 1 × Laemmli sample buffer.

2.5. Gel electrophoresis and immunoblotting

Whole cells extracts were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% gel and then electrotransferred to nitrocellulose membrane (Sartorius). The membrane was blocked with 1% bovine serum albumin in Tris-buffered saline (TBS) (25 mM Tris, pH 7.4, 137 mM NaCl, 0.1% Tween) for 60 min and incubated with RC20 anti-phosphotyrosine antibody HRP-conjugated for 70 min at 25°C. The membrane was then extensively washed with TBS and the immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) (Amersham) detection following the manufacturer's instructions.

2.6. Mitogenic response in PTPase-positive cells and controls

PTPase-positive cells and controls were serum starved for 24 h. Fetal calf serum (FCS) (10% final), or PDGF-BB, were added with a change of media and the cells were allowed to grow for an additional 20 h. They were then labelled with 1 μCi/ml of [^{3}H]thymidine for4 h, washed once with 10% trichloroacetic acid, twice with PBS and solubilized in 0.2 N NaOH. This lysate was used to quantitate protein concentration and incorporation of label into DNA by liquid scintillation counting.

2.7. Membrane preparation

Cells were washed in PBS, scraped from culture dishes, and collected by centrifugation. Cells were then resuspended in lysis buffer (20 mM HEPES, pH 7.4, 0.1 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM benzamidine and 2 mM phenylmethanesulphonyl fluoride) and disrupted by Dounce homogenization. The homogenate was clarified at 300 × *g* for 10 min and centrifuged at 2000 × *g* for 15 min to remove nuclei and then the supernatant was newly centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatant fraction (S100) was adjusted to 1% Triton. The pellet (P100) was rinsed in 1 ml of phosphate-buffered saline and then solubilized in lysis buffer containing 1% Triton. Protein concentration and PTPase content were determined respectively by Bradford's method [29] and ELISA in S100 and P100 fractions.

2.8. PTPase inhibitors

The stock solutions of sodium orthovanadate were prepared as described previously [30]. Phenylarsine oxide (PAO) was dissolved in dimethylsulphoxide. Cells were treated with 20 μM PAO for 30 min and with 2 mM vanadate for 10 min before growth factor stimulation.

3. Results and discussion

cDNAs coding for low M_r PTPase and neomycin-resistance were transfected in NIH/3T3 murine fibroblasts. From resistant cells we isolated many clones some of which were selected for the analysis of low M_r PTPase expression at both mRNA and protein level. The levels of PTPase mRNA were determined by Northern blot

analysis of total cellular RNA. A non-competitive ELISA using affinity-purified antibodies was used to quantitatively determine the low M_r PTPase protein expression in whole cell extracts. In low M_r PTPase transfected subclones, the level of the expressed enzyme was in agreement with the Northern blot analysis. The increase in vanadate-sensitive phosphatase activity, measured in cell extracts using *p*-nitrophenyl-phosphate as substrate, gave results completely comparable to the quantitative determination of the enzyme by ELISA, by relating experimental data on the basis of the specific activity of the purified PTPase. Therefore the transfected gene product was demonstrated to be the active PTPase. The subclones, which maintained a stable low M_r PTPase expression by periodical exposure to G418, were used for our studies. These cells expressed the enzyme about 10-fold over the levels of controls. Interestingly, no subclone showing an overexpression of the enzyme higher than this was found, contrary to what happened using NIH/3T3 fibroblasts transformed by various oncogenes in transfection experiments [20,21]. This observation suggests that in our case the absence of an oncogene product such as, for instance, a highly active tyrosine kinase, might not have allowed PTPase overexpression at levels that were cytotoxic to the NIH/3T3 normal cells.

The constitutive expression of the low M_r PTPase in neomycin-resistant control clones depended on the rate of proliferation. We observed that the level of the enzyme increased progressively as the cells reached confluence and arrested their growth (Fig. 1). Its quantity ranged approximately from 12 to 77 ng/mg of proteins. After the confluence, the PTPase level became lower, probably as a consequence of cell suffering. This behaviour is common to other PTPases [31] and it is peculiar to enzymes which counteract the mitogenic signal.

The overexpression of the low M_r PTPase affected the mitogenic potential of PDGF. This growth factor was an effective mitogen in the NIH/3T3 cell line, giving rise to a response, calculated as [^{3}H]thymidine incorporation, similar to 10% FCS. When the cells were grown in a complete medium permanently supplemented with 10% FCS, the effects of PTPase overexpression were mild. The clones grew somewhat more slowly than comparable cells transfected only with the pK0neo plasmid. However, the ability of PTPase transfected cells to recover from serum starvation was significantly lower (73% inhibition), as appeared from the [^{3}H]thymidine incorporation under serum stimulation after 24 h serum starvation (Fig. 2). Moreover, when cells were starved and then stimulated with PDGF-BB, the effect, due to the presence of higher levels of expressed enzyme, was extremely significant. The inhibition of PDGF-BB induced [^{3}H]thymidine incorporation in cells overexpressing PTPase compared to the untransfected ones reached 90% (Fig. 2).

From these data it was evident that clones overex-

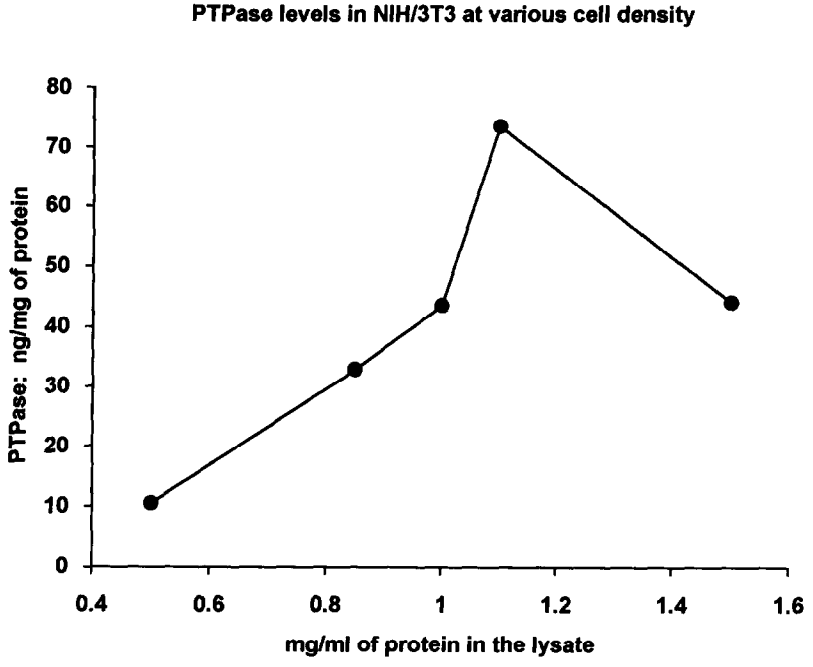

Fig. 1. Level of endogenous low M_r PTPase in NIH/3T3 fibroblasts. Cells were grown at various final density in complete medium in 60-mm dishes, washed twice with PBS and then harvested in 300 μl of lysis buffer (50 mM MES, 1 mM EDTA, pH 6.8, 1.5 mM phenylmethanesulphonyl fluoride, 0.1 mM benzamidine, 0.1 mM DTT). After sonication an aliquot was taken for total protein determination. PTPase levels were determined on the lysate by a sandwich ELISA using a polyclonal antibody raised against low M_r PTPase, then normalized for protein concentration of the lysate. The PTPase content is related to the total protein concentration of the lysate taken as a measure of cell density in the dish. Results are mean values of 3 independent experiments.

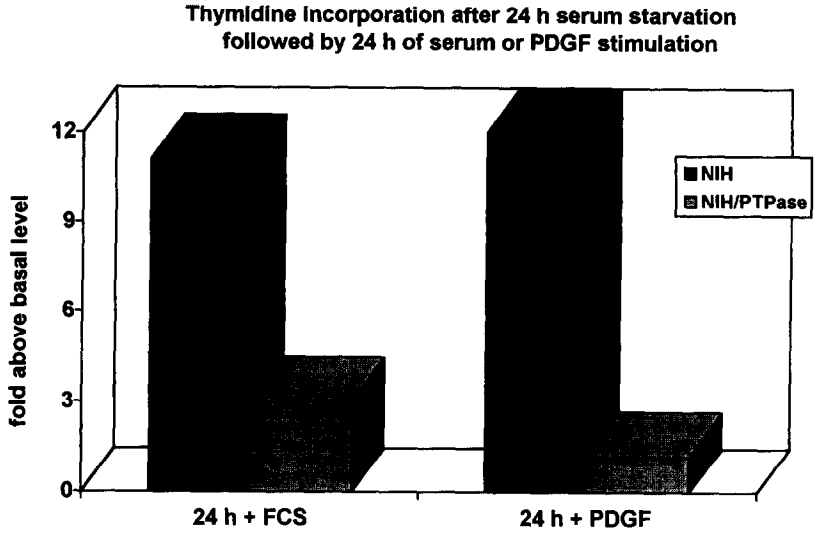

Fig. 2. [^{3}H]Thymidine incorporation in control and low M_r PTPase overexpressing NIH/3T3. Cells were serum-starved for 24 h, after which the basal level of [^{3}H]thymidine incorporation was measured by a pulse (4 h) labelling. Starved cells were then stimulated with 10% FCS or 20 ng/ml PDGF-BB for 24 h, and [^{3}H]thymidine incorporation was again measured. Values of incorporation are normalized for protein concentration and are reported as increase above the basal level. Data are obtained as means ± S.E.M. (n = 4) of one experiment representative of 3 that gave qualitatively identical results.

pressing the enzyme showed an impaired mitogenic response to PDGF-BB maximal stimulation. Such a drastic effect was not so evident when the cells were fed in the presence of FCS because in this case they were induced to proliferate by a lot of different stimuli. We could thus conclude that among the mitogenic factors present in FCS there are some whose action is not affected or less affected by the expression of the low M_r PTPase.

In order to assess the level of the signal cascade at which the antagonism to the mitogenic potential of the growth factor was effective, we examined the growth factor-dependent tyrosine phosphorylation pattern in low M_r PTPase transfected and normal cells. The overexpression of the low M_r PTPase actually modified the PDGF-BB-dependent tyrosine phosphorylation in intact cells. In NIH/3T3 control cells the PDGF-BB produced increased phosphorylation of many proteins, the major one being the 180 kDa PDGF receptor. Other proteins appeared to be phosphorylated on tyrosine residues in response to PDGF-BB in a directly proportional measure to the receptor itself. In low M_r PTPase overexpressing cells, the phosphorylation of the receptor band was significantly diminished at all the PDGF-BB concentrations used (see Fig. 3). Beyond this, other proteins that were phosphorylated in response to PDGF stimulation seemed to diminish their phosphotyrosine content. Considering the molecular weight, one could connect them with the PDGF receptor substrates presently known and argue their potential identity. So a 140-kDa protein was similar in size to phospholipase Cγ and a 85-kDa one was probably the phosphatidylinositol-3-kinase major subunit. All these proteins possess phosphotyrosine residues in their sequence and are known to form a complex with the autophosphorylated PDGF receptor by their *src* homology domains. More detailed studies will be needed to ascertain the actuality of these observations which point to a downstream effect of the diminished PDGF receptor phosphorylation.

The observed decrease in tyrosine phosphorylation in response to PDGF was not produced by a smaller

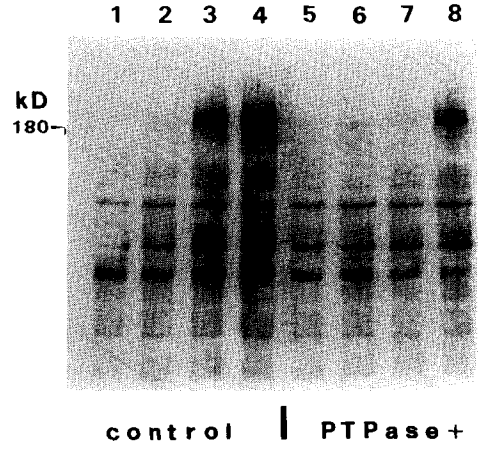

Fig. 3. Tyrosine phosphorylation pattern in control and low M_r PTPase overexpressing NIH/3T3 under PDGF-BB stimulation. After SDS-polyacrylamide gel electrophoresis, cell lysates were blotted, incubated with RC20-peroxidase conjugated antibodies to phosphotyrosine and detected by ECL. Cells were grown to subconfluence in 35-mm dishes and then serum-starved for 24 h. At the end of this period they were stimulated with different doses of PDGF-BB for 5 min. Lanes 1–4 = control NIH/3T3; lanes 5–8 = PTPase NIH/3T3. Lanes 1 and 5 = unstimulated; lanes 2 and 6 = 2 ng/ml PDGF; lanes 3 and 7 = 10 ng/ml; lanes 4 and 8 = 40 ng/ml.

amount of specific receptor present in PTPase-positive cells or by a decrease in its constitutive kinase activity. PTPase inhibitors, like vanadate and phenylarsine oxide, were used to verify the integrity of the receptor in PTPase-positive and control cells. By inhibiting the phosphotyrosine phosphatase activity, we found an extremely high tyrosine kinase activity and a similar extent of protein phosphorylation in PTPase-positive and control cell lysates (Fig. 4). When cells were pretreated for 10 min with 2 mM vanadate and 20 μM PAO for 30 min, the phosphotyrosine content in many unidentified proteins increased several-fold over the level seen in untreated cells. This effect was evident, with very few differences, both in PDGF-stimulated and unstimulated cells. The basal level of PDGF receptor autophosphorylation, excluding the direct role of phosphatases in the down-regulation, seemed to reach the saturation of available sites [20,21,32]. In addition, the PDGF receptor has been partially purified using wheat germ agglutinin linked to a matrix and autophosphorylated by stimulating with PDGF-BB in vitro. The level of autophosphorylation was almost the same in the receptor isolated from PTPase-positive and control cells (results not shown), thus confirming the presence of an equal amount of active PDGF receptors in both cases.

Logical inferences and several authors [19–21], who suggested that cytosolic PTPases may not interact with growth factor receptors, prompted us to consider more critically these results showing the possibility of an interaction between the enzyme and the cellular membrane. The analysis of the molecular structure of the enzyme [33] had not revealed a C-terminus hydrophobic domain and no chemical derivatizations allowing association with the particulate fractions of the cells were found. Nevertheless, cell lysates from control and overexpressing cells were prepared to examine the subcellular distribution of the overexpressed, as well as the endogenous PTPase. Cytosolic (S100) and membrane fractions (P100), obtained by high speed centrifugation, were checked for enzyme content by ELISA. More than 93% of the PTPase was present in the S100 of control and overexpressing cells. Similar results were obtained when cells were stimulated with PDGF-BB before the subcellular fractionation. Therefore, the localization of PTPase was not altered by the level of expression, and the distribution was not changed by growth factor stimulation. The demonstrated activity of this PTPase on the autophosphorylated EGF receptor in vitro [26], together with the specific dephosphorylation of the PDGF receptor in vivo, point to a direct action of this cytosolic PTPase on a membrane substrate.

Our results demonstrate that the overexpression of the low M_r PTPase in NIH/3T3 fibroblasts produces a decrease of cellular response to PDGF with regard to mitogenic activity and protein phosphorylation. Contrary to the findings of studies on other intracellular PTPases

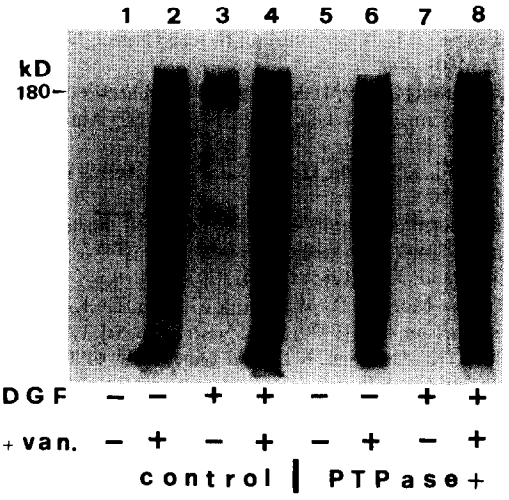

Fig. 4. Tyrosine phosphorylation pattern in control and low M_r PTPase overexpressing NIH/3T3 under PDGF-BB stimulation in the presence of PTPase inhibitors. Immunoblot of tyrosine-phosphorylated proteins separated by SDS-PAGE. Cells were treated as in Fig. 3 except that the stimulation with 10 ng/ml of PDGF-BB was preceded by incubation for 10 min with 2 mM vanadate and for 30 min with 20 μM PAO. Lanes 1–4 = control NIH/3T3; lanes 5–8: PTPase NIH/3T3. Lanes 1 and 5 = unstimulated; lanes 2 and 6 = unstimulated, treated with PAO and vanadate; lanes 3 and 7 = 10 ng/ml PDGF; lanes 4 and 8 = 10 ng/ml PDGF, treated with PAO and vanadate.

[34], this enzyme, a cytosolic low M_r PTPase, not only inhibits cell proliferation but also affects tyrosine autophosphorylation of a transmembrane kinase, the PDGF receptor. The interaction between the PTPase and the receptor does not proceed by *src* homology domains and the enzyme is not phosphorylated by the receptor kinase activity. Cellular compartmentalization as a major factor in defining activity and function seems to be a rather necessary limitation to particulate enzymes but not to soluble ones. The capability of the low M_r PTPase for dephosphorylating the autophosphorylated EGF receptor in vitro paves the way for a possible action on other phosphorylated receptors that will be the object of further investigations. In conclusion, the study of this enzyme, and in general of PTPases, could offer new possibilities in the understanding of the role of PTPases as a crucial part of signal transduction.

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