Involvement of a phosphotyrosine protein phosphatase in the suppression of platelet-derived growth factor receptor autophosphorylation in *ras*-transformed cells

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The nature of the suppression of platelet-derived growth factor (PDGF) receptor autophosphorylation in *ras*-transformed NIH 3T3 fibroblasts was investigated. The PDGF receptor from *ras*-transformed cells that had been purified by wheatgerm-lectin affinity chromatography displayed normal PDGF-induced autophosphorylation, indicating that the receptor is not irreversibly modified. Various phosphotyrosine-protein-phosphatase inhibitors did not reverse the inhibition of PDGF-receptor kinase in crude membrane preparations from *ras*-transformed cells. However, treatment of intact *ras*-transformed cells both with 2 mM sodium orthovanadate and with 20 μ M phenylarsine oxide

restored PDGF-receptor tyrosine-kinase activity to a level similar to that observed in normal cells. Direct measurement of the phosphatase activities in crude cellular fractions revealed a 2.5-fold higher membrane-associated phosphotyrosine-protein-phosphatase activity in *ras*-transformed cells, whereas phosphoserine-protein-phosphatase activity remained unchanged between the cell lines. These data suggest that the suppression of the PDGF-receptor tyrosine-kinase activity in *ras*-transformed cells is mediated via an inhibitory component, distinct from the receptor, that may be positively regulated by the dephosphorylation of tyrosine residue(s).

INTRODUCTION

Platelet-derived growth factor (PDGF) belongs to the large family of peptide growth factors that induce a cascade of intracellular events upon binding to their cell-surface receptors. These include the mobilization of calcium ions, transcription of growth-related genes, Na^+ influx, stimulation of phospholipase C and phospholipase A_2 activities and stimulation of DNA synthesis [1–4]. The earliest steps within the PDGF-stimulated signal-transduction pathway require receptor oligomerization, receptor autophosphorylation on several tyrosine residues and association with various proteins that include the regulatory subunit of phosphatidylinositol 3-kinase [5,6], phospholipase $\text{C-}\gamma 1$ [7–10], the c-Raf serine/threonine kinase [11,12], the c-Src, c-Fyn, and c-Yes protein kinases [13], the cytoskeletal protein, tensin [14] and the p21^{ras} -GTPase-activating protein (GAP) [15–17]. All of these interactions require the phosphorylation of specific tyrosine residues in the receptor, implying a critical role for receptor autophosphorylation for full mitogenic activity [18–20].

One of the key intermediates within the PDGF-mediated signal transduction pathway is p21^{ras} , since treatment of cells with PDGF leads to increased GTP binding by p21^{ras} [21,22]. p21^{ras} proteins associate with the plasma membrane, bind guanine nucleotides and exhibit weak GTPase activity that is dramatically stimulated by their GAPs [23–26]. The requirement for p21^{ras} in mitogenesis has been directly demonstrated [27], but the exact mechanism of its action and its effector molecule(s) have yet to be identified. However, there is a great deal of evidence suggesting that p21^{ras} -GAP can have a dual function both as a negative regulator and as a downstream effector [28–30]. Oncogenic

variants of p21^{ras} are constitutively active (because of their inability to hydrolyse GTP) and stimulate a variety of events, including DNA synthesis, that lead ultimately to cell transformation. However, transformation of cells by oncogenic *ras* results in the desensitization of some cellular processes, including those stimulated by PDGF [31–37]. This was shown to be the result of the suppression of PDGF-receptor autophosphorylation [38].

The role of protein phosphatases in mitogenesis has been recognized, both as positive and as negative regulators of the signal-transduction pathways from the membrane to the nucleus [39,40]. For example, genetic and biochemical studies have indicated that the protein tyrosine phosphatase (PTPase), *cdc25*, mediates the dephosphorylation of the serine/threonine kinase, *cdc2*, triggering the initiation of mitosis [41–45]. The involvement of PTPase in the p21^{ras} signal-transduction pathway was suggested by a recent study of Medema et al., which indicated the presence of a phenylarsine oxide (PAO)-sensitive protein in the insulin-mediated activation of p21^{ras} [46].

This paper demonstrates the role of PTPase activity in the inhibition of PDGF-receptor autophosphorylation in *ras*-transformed cells. Treatment of *ras*-transformed NIH 3T3 cells with vanadate and PAO, known tyrosine-phosphatase inhibitors [47,48], enhanced the PDGF-dependent autophosphorylation of the receptor significantly and differentially, when compared with normal cells. In contrast these inhibitors had no effect on autophosphorylation *in vitro*. Furthermore *ras*-transformed cells had about 2.5-fold higher membrane-associated PTPase activity than had parental NIH 3T3 fibroblasts. We show also that *ras*-transformed cells may contain an inhibitor of the PDGF-receptor kinase that can be dissociated from the receptor by partial

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; GAP, GTPase-activating protein; PAO, phenylarsine oxide; PDGF, platelet-derived growth factor; PTPase, protein tyrosine phosphatase.

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purification using wheatgerm-lectin affinity chromatography. We suggest that this factor is positively regulated by the dephosphorylation of tyrosine residue(s).

MATERIALS AND METHODS

Cell lines

NIH 3T3, v-Ha-*ras* transfectants (568) and c-Ha-*ras* transfectants (RS-485) were obtained from R. Bassin (NIH).

Reagents

PDGF-BB homodimer was purchased from GIBCO BRL, [γ - 32 P]ATP was from DuPont/New England Nuclear, 125 I-Protein A was from ICN and wheatgerm-lectin-Sepharose 6 MB was obtained from Pharmacia LKB. Sodium orthovanadate was purchased from Fisher, PAO and rabbit anti-mouse IgG were from Sigma and the monoclonal anti-phosphotyrosine antibody FB2 was a generous gift from Dr. F. McCormick (Onyx Pharmaceuticals, Richmond, CA). The polyclonal anti-human PDGF-type A/type B-receptor antibody was obtained from UBI. Chicken c-Src kinase was purified from baculovirus-infected insect cells as described [49] and the catalytic subunit of cyclic AMP-dependent protein kinase was the generous gift from Dr. E. Fisher (University of Washington).

Tissue culture

Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; 4.5 g/litre glucose; GIBCO), supplemented with 10 mM Hepes buffer, pH 7.4, gentamycin (40 μ g/ml; GIBCO) and 10% (v/v) newborn-calf serum (GIBCO), which is designated as 'complete medium'. For experiments, cells were seeded on to appropriately sized culture dishes (Falcon) at a density of 1×10^4 – 1.5×10^4 cells per cm^2 of complete medium and were grown to confluency (3–4 days) in a humidified atmosphere of 5% CO_2 /95% air at 37 °C.

Membrane preparation

Membranes from confluent cells were prepared by the method of Williams et al. [50], with the modifications that were used by Rake et al. [38].

Determination of protein concentration

Protein determinations were by the method of Bradford [51].

Solubilization of membranes

Membranes (5 mg/ml protein) were mixed with an equal volume of 20 mM Hepes buffer, pH 7.4, 2% (v/v) Triton X-100, 150 mM NaCl, and then were vortexed and were incubated on ice for 30 min. The suspension was centrifuged at 100 000 *g*, for 45 min, at 4 °C; the supernatant was aliquoted and was stored at –70 °C.

Partial purification of the PDGF receptor

Confluent cells were scraped from the dishes in to ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl,

4 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4) containing 0.3 mM EDTA. The suspended cells were washed twice with ice-cold PBS and were solubilized in 50 mM Tris/HCl buffer, pH 8, 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 2 mM phenylmethanesulphonyl fluoride (Sigma), 10 μ g/ml leupeptin (Chemicon) and 20 μ g/ml aprotinin (FBA Pharmaceuticals), using 2 ml per 150-mm dish. The suspension was vortexed and was incubated for 30 min on ice. The particulate material was removed by centrifugation at 100 000 *g*, for 30 min, at 4 °C and the supernatant was incubated with wheatgerm-lectin-Sepharose 6 MB (0.1 ml of packed gel per 150-mm culture dish) for 2 h, at 4 °C, with end-over-end rotation. The gel was loaded on to a column and was washed with 50 vol. of 1 M NaCl, 10 mM Tris/HCl buffer, pH 8, 0.1% (v/v) Triton X-100, 0.1 mM EDTA, 0.1 mM vanadate, followed by 50 vol. of the same solution without NaCl. Bound proteins were eluted with 1 M NaCl, 10 mM Tris/HCl buffer, pH 8, 0.1% (v/v) Triton X-100 and 0.3 M *N*-acetylglucosamine (Sigma). Fractions of 0.5 ml were collected and were stored at –70 °C.

In vitro kinase assay

Triton X-100 solubilized membranes (10 μ g) or PDGF receptor that had been partially purified by wheatgerm-lectin affinity chromatography (1 μ g) were assayed for PDGF-dependent phosphorylation in a final volume of 50 μ l, containing 20 mM Hepes buffer, pH 7.4, 3 mM MnCl_2 and 0.1% (v/v) Triton X-100. Samples were pre-incubated with PDGF-BB (200 ng/ml) for 15 min on ice and then the kinase reaction was started by adding 20 μ M [γ - 32 P]ATP (10 000–15 000 c.p.m./pmol). After 10 min of incubation on ice, the reactions were stopped by adding 12.5 μ l of 5 $\times$ Laemmli SDS/PAGE sample buffer. The samples were boiled for 5 min and electrophoresis was carried out using 10% acrylamide gels in the buffer system of Laemmli [52]. The gels were stained with Coomassie Blue, dried and exposed to Kodak XAR-5 film at –70 °C.

To measure the PDGF-dependent receptor-kinase activity in crude lysates, 150 μ g of protein was treated with PDGF-BB (200 ng/ml) in a final volume of 0.5 ml, containing 20 mM Hepes buffer, pH 7.4, 3 mM MnCl_2 , 0.1% (v/v) Triton X-100, 1 mM vanadate, 0.1 mM ZnCl_2 and 20 μ g/ml aprotinin, for 15 min. The kinase reaction was started by adding 20 μ M [γ - 32 P]ATP (15 000 c.p.m./pmol) for 10 min and was stopped by adding 10 mM EDTA. Samples were added to 20 μ l of wheatgerm-lectin-Sepharose 6 MB, were incubated at 4 °C with gentle continuous agitation for 2 h and were washed twice with 1 M NaCl, 10 mM Tris/HCl buffer, pH 8, 0.1% (v/v) Triton X-100 and 1 mM vanadate and twice with the same solution without NaCl. The final pellet was resuspended in 100 μ l of 1 $\times$ Laemmli SDS/PAGE sample buffer and electrophoresis was carried out, as described above.

In vivo assay for PDGF-dependent tyrosine-kinase activity

Confluent cells, grown in 60-mm culture dishes, were starved for 16 h in DMEM containing 0.5% newborn-calf serum. The starved cells were washed, were covered with 2 ml of serum-free DMEM and were treated as indicated in the legend to Figure 2. PDGF-BB (50 ng/ml) was added for 5 min, then the dishes were placed immediately on ice, the medium was aspirated and the cells were washed twice with ice-cold PBS and were solubilized with the solubilization buffer [50 mM Tris/HCl buffer, pH 8, 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 2 mM vanadate, 0.1 mM ZnCl_2 , 10 μ g/ml leupeptin and 20 μ g/ml aprotinin]. The suspensions were transferred to cen-

trifuge tubes, vortexed and incubated on ice for 30 min. After the particulate material had been removed by centrifugation at 100000 *g*, for 30 min, at 4 °C, an equal amount of protein was mixed with wheatgerm-lectin-Sepharose 6 MB (15 μ l per sample) that had been prewashed with the solubilization buffer. The samples were incubated at 4 °C with gentle continuous agitation for 2 h and were washed twice with 0.5 ml of 1 M NaCl, 10 mM Tris/HCl buffer, pH 8, 0.1% (v/v) Triton X-100, 1 mM EDTA and 1 mM vanadate and then twice with the same solution without NaCl. The final pellet was resuspended in 70 μ l of 1 $\times$ Laemmli SDS/PAGE sample buffer. Samples were boiled for 5 min at 95 °C and were analysed by 6% SDS/PAGE. Resolved proteins were transferred to nitrocellulose filters and were immunoblotted, as described below.

Immunoblotting of nitrocellulose immobilized proteins

Nitrocellulose filters were blocked for 2 h at room temperature with blocking solution [20 mM Tris/HCl buffer, pH 7.5, 150 mM NaCl and 2% (w/v) nonfat milk]. For the anti-phosphotyrosine immunoblots, the filters were incubated overnight at 4 °C with the blocking solution containing anti-phosphotyrosine monoclonal antibody (2 μ g/ml), were washed five times with washing buffer [20 mM Tris/HCl buffer, pH 7.5, 150 mM NaCl and 0.05% (v/v) Tween-20] and were incubated for 2 h at room temperature with blocking solution, containing rabbit anti-mouse IgG (14 μ g/ml). After five washes with the washing buffer, the immunoblots were developed with the blocking solution containing 125 I-Protein A (1 μ Ci/ml, $\geq$ 30 μ Ci/ μ g), were washed five times with washing buffer, dried and exposed to Kodak XAR-5 film at -70 °C. The relative levels of phosphotyrosine were determined by cutting the corresponding bands from the filter and counting them in a γ counter.

Fractionation of cells to measure the protein-phosphatase activity

Confluent cells were scraped from 150-mm culture dishes into ice-cold PBS, were washed twice and were lysed using a glass-on-glass homogenizer in five pellet vol. of ice-cold hypotonic lysis buffer [20 mM Hepes buffer, pH 7.4, 0.1 mM EDTA, 1 mM MgCl₂, 1 mM dithiothreitol (DTT), 10 μ g/ml leupeptin, 20 μ g/ml aprotinin and 2 mM phenylmethanesulphonyl fluoride]. After low-speed fractionation (300 *g* for 10 min), the intact cells and debris were discarded and the supernatant was centrifuged at 12000 *g* for 15 min to remove the mitochondria. The resulting supernatant was centrifuged at 100000 *g*; the pellet (plasma membranes) was resuspended in 20 mM Hepes buffer, pH 7.4, and 250 mM sucrose. The protein concentration was adjusted to 1 mg/ml with 20 mM Hepes buffer, pH 7.4, and 250 mM sucrose and the protein suspension was aliquoted and stored at -70 °C.

In vitro assay for protein-phosphatase activity

Poly(Glu₄Tyr₁) (Sigma) was phosphorylated by c-Src kinase in a reaction mixture containing 20 mM Tris/HCl buffer, pH 7.4, 10 mM MgCl₂, 1 mM DTT and 20 μ M [γ - 32 P]ATP (10000 c.p.m./pmol) for 16 h at 22 °C. Poly(Arg₃Ser₁) (Sigma) was phosphorylated by the catalytic subunit of the cyclic AMP-dependent protein kinase in a reaction mixture containing 20 mM Hepes buffer, pH 6.5, 4 mM MgCl₂, 20 mM DTT and 250 μ M [γ - 32 P]ATP (2500 c.p.m./pmol) for 16 h at 22 °C. The phosphoryl-

ated polymers were placed on ice, ice-cold trichloroacetic acid was added to a final concentration of 10% (v/v) and the mixture was incubated for 30 min. The precipitated proteins were centrifuged at 12000 *g* in a Microfuge for 30 min, were washed three times with 10% (v/v) trichloroacetic acid and 10 mM pyrophosphate and then three times with ethanol/ether (1:1, v/v), dried and resuspended in 0.2 M Hepes buffer, pH 7.4 and were stored at -20 °C.

The phosphoprotein-phosphatase activity of the cell fractions was then assayed, in a final volume of 50 μ l that contained 20 mM Hepes buffer, pH 7.4, 10 mM MgCl₂, 0.1% (v/v) Triton X-100, 1 mM DTT, 1 μ g of the corresponding phosphorylated synthetic polymer and 5 μ g of the fractionated protein. After incubation for 3 min at 37 °C, the samples were transferred to tubes containing 4 ml of 1.25 M HClO₄, 1.25 mM NaH₂PO₄ and 5 ml of a isobutanol/benzene (1:1, v/v) mixture and 1 ml of 5% (w/v) ammonium molybdate was then added. The samples were vortexed for 45 s and aliquots of the upper organic phase containing the phosphate released were counted in a Beckman scintillation counter.

Preparation of stock solutions of PTPase inhibitors

The stock solutions of vanadate were prepared as described previously [47]. PAO was dissolved in 70% (v/v) ethanol.

Reproducibility of data

Each experiment described in this paper was repeated at least twice, with similar results.

RESULTS AND DISCUSSION

A number of previous reports have shown that *ras*-transformed cells fail to respond to PDGF [31-37]. In those studies it was shown that the suppression of PDGF-dependent events in *ras*-transformed cells is not due to the failure of the PDGF-receptor to bind to PDGF. In particular the two cell lines compared in the present studies [NIH 3T3 and NIH 3T3 transformed by the v-Ha-*ras* oncogene (clone 568)] have similar capacities to bind to 125 I-PDGF, as shown previously by Parries et al. [31]. Furthermore we have confirmed by immunoblot assay, using anti-PDGF receptor antibody, that essentially equal quantities of PDGF receptors are present in both cell lines (results not shown).

The activity of the PDGF receptor from *ras*-transformed fibroblasts can be restored by partial purification

As reported recently by Rake et al. [38], the blockade of PDGF-stimulated cellular events in *ras*-transformed cells is due to the loss of PDGF-receptor autophosphorylation. The authors suggested the presence of a dissociable factor in *ras*-transformed cells that is capable of inhibiting the PDGF-receptor-kinase activity from normal cells when tested *in vitro*. To confirm that the suppression in *ras*-transformed cells is actually caused by a distinct component and not by the modification of the PDGF receptor, we partially purified the PDGF receptors from normal and from transformed 3T3 fibroblasts, using wheatgerm-lectin affinity chromatography. As shown in Figure 1, although there is no PDGF-stimulated receptor autophosphorylation in the crude lysates from *ras*-transformed cells, when compared with normal cells (lanes A, B, E, F), the extent of the PDGF-stimulated receptor autophosphorylation is similar for wheatgerm lectin-purified receptor from normal and from *ras*-transformed cells

Figure 1 Partial purification of the PDGF receptor from *ras*-transformed cells using wheatgerm-lectin affinity chromatography

The PDGF receptor in crude lysates (lanes A, B, E, F) or partially purified by wheatgerm-lectin affinity chromatography (lanes C, D, G, H) from *ras*-transformed (lanes A–D) and from normal cells (lanes E–H) was assayed *in vitro* for PDGF-dependent autophosphorylation, as described in the Materials and methods section. The samples were analysed by SDS/PAGE (10% gel) and ^{32}P incorporation was visualized by autoradiography.

Figure 2 Effect of vanadate and of PAO on PDGF-receptor tyrosine-kinase activity *in vivo* in *ras*-transformed and in normal cells

Starved *ras*-transformed (lanes A–H) and normal (lanes I–P) cells were incubated in serum-free media without any treatment (lanes A, B, I, J), in the presence of 2 mM vanadate for 10 min (lanes C, D, K, L), in the presence of 20 μM PAO for 30 min (lanes E, F, M, N) or in the presence of both inhibitors (lanes G, H, O, P). PDGF-dependent receptor autophosphorylation was assayed as described in the Materials and methods section. The samples were subjected to 6% SDS/PAGE and were analysed for their phosphotyrosine content by immunoblotting with anti-phosphotyrosine antibody and ^{125}I -Protein A and the results were visualized by autoradiography.

(lanes C, D, G, H). The elution profile of the active fractions was also similar for the cell lines. Purification of the PDGF receptor by wheatgerm-lectin chromatography from the cell line RS 485, transformed by the overexpression of c-Ha-*ras*, yielded similar results (results not shown).

Possible involvement of PTPase in the downregulation of the PDGF-receptor-kinase activity in *ras*-transformed cells

From the work of Rake et al. [38] it seemed clear that the suppression of receptor activity in the membranes from *ras*-transformed cells was not due to an enhanced PTPase activity, since 1 mM vanadate did not reverse the inhibition of PDGF-receptor autophosphorylation. In the event that *ras*-transformed cells express a vanadate-insensitive PTPase, we tested different PTPase inhibitors (100 μM ZnCl_2 and 20 μM PAO) on a membrane preparation that had been solubilized with 1% (v/v) Triton X-100. We observed no significant changes in the level of autophosphorylation, excluding a direct role of phosphatase in the downregulation of the PDGF receptor (results not shown). In contrast when intact cells were treated with vanadate and/or PAO, followed by a challenge with PDGF, the effects of these inhibitors on PDGF-receptor activity in normal and in *ras*-

transformed cells differed dramatically (Figure 2 and Table 1). In the absence of any inhibitor, *ras*-transformed cells showed approx. 10% of the PDGF-stimulated autophosphorylation of the PDGF receptor compared with normal cells. When cells were pretreated for 10 min with 2 mM vanadate, the tyrosine-kinase activity of the receptor in *ras*-transformed cells increased 4-fold as opposed to the 2-fold increase seen in normal cells. When 20 μM PAO was present for 30 min before adding PDGF, the autophosphorylation of the PDGF receptor increased about 4-fold in the *ras*-transformed cells, whereas in normal cells this treatment resulted in a 50% inhibition. When the inhibitors were combined, the receptor-tyrosine-kinase activity in *ras*-transformed cells increased 13.5-fold, which was about 80% of the activity observed in normal cells under these conditions. The concomitant exposure of normal cells to PAO and to vanadate had no effect on the PDGF-stimulated autophosphorylation of the PDGF receptor compared with cells treated only with vanadate, suggesting either that the PDGF receptor has reached maximum activity already or that the PAO-sensitive component is not active in normal cells. However prolonged treatment (up to 90 min) of normal cells with vanadate in the absence or in the presence of PAO, increased the basal, PDGF-independent phosphorylation of the PDGF receptor, while the total activity remained unchanged, which was probably due to the saturation of the phosphorylation sites on the receptor (results not shown).

To investigate further the role of phosphatase(s) in the inhibition of the PDGF-receptor autophosphorylation, the intact *ras*-transformed cells were treated for 20 min with 1 μM okadaic acid, a potent inhibitor of the phosphoserine-specific phosphatases 1 and 2A *in vivo* [53,54]. This treatment had no effect on the PDGF-dependent receptor-tyrosine C-kinase activity, which argues against the involvement of these two protein phosphatases in the inhibition of the PDGF receptor in *ras*-transformed cells, although this does not rule out the involvement of other phosphoserine/phosphothreonine protein phosphatases (results not shown).

Quinones et al. [55] reported previously that the loss of PDGF-mediated activation of phospholipase C and the expression of the growth-related genes *c-myc*, *c-fos* and *JE* in *ras*-transformed cells could be restored by cyclic AMP or by the introduction of the tumour suppressor gene *Krev-1*. However the PDGF-dependent receptor autophosphorylation remained inhibited. Price et al. [36] demonstrated that scrape-loading of the cells with oncogenic p21^{ras} resulted in the inhibition of PDGF-dependent phospholipase C activity, which was probably due to the activation of protein kinase C, since chronic treatment of these cells with phorbol 12-myristate 13-acetate recovered the PDGF-stimulated inositol-phosphate turnover. Thus we investigated the role of protein kinase C in the suppression of PDGF-receptor tyrosine-kinase activity in *ras*-transformed cells. After exposure to 400 nM phorbol 12-myristate 13-acetate for 18 h, no detectable increase in PDGF-receptor tyrosine-kinase activity was observed (results not shown). Together with the results of Quinones et al. [55], this result indicates that the PDGF receptor participates both in kinase-dependent and in kinase-independent pathways.

Increased membrane-associated PTPase activity in *ras*-transformed fibroblasts

To rationalize the differential sensitivity of normal and *ras*-transformed fibroblasts to PAO and to vanadate *in vivo* (Figure 2 and Table 1), the PTPase activities in fractions from normal and from *ras*-transformed cells were compared by measuring the dephosphorylation of the phosphorylated synthetic polymer, glutamate/tyrosine (Table 2). Whereas the cytosolic and the

Table 1 Restoration of PDGF-receptor autophosphorylation in *ras*-transformed cells

The relative levels of phosphotyrosine were determined from Figure 2 by excising the corresponding bands from the nitrocellulose filter and counting them in a γ counter. A reference value of 1.00 was assigned to the PDGF-stimulated tyrosine phosphorylation of the PDGF receptor in *ras*-transformed cells (line 2, column 4). The last column represents the relative PDGF-receptor activity of *ras*-transformed cells, compared with normal NIH 3T3 fibroblasts under the different conditions shown.

Conditions	PDGF	Relative levels of tyrosine phosphorylation		
		NIH 3T3	v-Ha- <i>ras</i>	NIH 3T3
Control	—	0.25	0.00	—
	+	11.80	1.00	0.08
2 mM Vanadate	—	0.05	0.03	—
	+	19.00	4.00	0.21
20 μ M PAO	—	0.00	0.20	—
	+	5.30	3.00	0.57
2 mM Vanadate	—	4.20	0.20	0.05
and 20 μ M PAO	+	16.00	13.50	0.84

Table 2 Comparison of the phosphotyrosine- and phosphoserine-protein-phosphatase activities in fractions from normal and from *ras*-transformed cells

Normal and *ras*-transformed NIH 3T3 fibroblasts were analysed for phosphotyrosine- and phosphoserine-protein-phosphatase activities in the membrane and cytosolic fractions, as described in the Materials and methods section. Abbreviation used: PSPase, phosphoserine protein phosphatase.

Phosphatase	P_i released [$\text{pmol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$]	
	NIH 3T3	v-Ha- <i>ras</i>
PTPase		
Membranes	215.4 ± 67.2	477.7 ± 0.3
Cytosol	268.2 ± 20.5	343.6 ± 38.8
PSPase		
Membranes	312.5 ± 27.7	288.2 ± 29.8
Cytosol	1155.2 ± 11.2	1135.4 ± 62.2

membrane-associated phosphoserine-phosphatase activity was similar for both cell lines, the *ras*-transformed cells exhibited about a 2.5-fold increase in PTPase activity in crude plasma membranes, but not in the cytosolic fraction.

The membrane-associated PTPase activity from NIH 3T3 and from v-Ha-*ras*-transformed cells was analysed further for its sensitivity to vanadate and to PAO (Table 3). Since PAO interacts with vicinal sulphhydryl groups, membranes used in these experiments were isolated without DTT and then were tested in the absence and in the presence of DTT. Despite a 10–20-fold reduction in PTPase activity in the absence of DTT, *ras*-transformed cells still exhibited a 2.5-fold greater activity than their normal counterparts. PAO inhibited the PTPase activity in the membranes from *ras*-transformed cells by approx. 25%, but had no effect on the activity in membranes from normal cells. This differential effect is similar to the results that are observed in intact cells (Figure 2). Vanadate reduced the PTPase activity by approx. 90% in membranes from both cell lines. The concomitant addition of both inhibitors had no additional effect. Since crude membrane preparations contain a mixture of

Table 3 Inhibition of the membrane-associated PTPase activity by vanadate and by PAO

Membranes from normal and from *ras*-transformed NIH 3T3 fibroblasts were prepared as described in the Materials and methods section, except that DTT was omitted. PTPase activity was analysed as described, in the absence or in the presence of 1 mM vanadate, 20 μ M PAO and 1 mM DTT. Abbreviation used: N.D., not determined.

Conditions	P_i released [$\text{pmol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$]			
	NIH 3T3		v-Ha- <i>ras</i>	
	— DTT	+ DTT	— DTT	+ DTT
Control	10.2 ± 1.1	146.2 ± 9.6	24.5 ± 2.7	353.9 ± 40.3
1 mM Vanadate	1.8 ± 0.7	15.6 ± 2.1	1.9 ± 1.0	25.0 ± 3.9
20 μ M PAO	10.3 ± 1.0	N.D.	18.5 ± 2.1	N.D.
1 mM Vanadate and 20 μ M PAO	2.3 ± 0.9	N.D.	1.5 ± 0.5	N.D.

Figure 3 Alternative models for the suppression of PDGF-receptor autophosphorylation in *ras*-transformed cells

An inhibitory component (X) is activated by the dephosphorylation of a tyrosine residue (Y-P) by a PTPase or is inhibited by the phosphorylation of a tyrosine residue (Y-P) by a protein tyrosine kinase (PTK). The suppression of the PDGF-receptor (R) autophosphorylation would be a result either of (a) the direct inhibition by X or of (b) the generation of an inhibitory product (Pr) from its substrate (S).

PTPases, it is difficult to evaluate their individual or collective roles in the suppression of the PDGF-receptor activity.

The results presented in this paper suggest that the suppression of the PDGF-receptor-kinase activity in *ras*-transformed cells may occur as a result of an altered PTPase activity. This is supported by two observations. Firstly, the exposure of *ras*-transformed cells to PTPase inhibitors increased the PDGF receptor autophosphorylation differentially, when compared with the parental cell line, and secondly, *ras*-transformed cells contain elevated levels of PTPase activity. The most obvious explanation would be the direct dephosphorylation of the receptor, although this seems less likely since PTPase inhibitors cannot restore PDGF receptor autophosphorylation *in vitro*. An alternative possibility is the presence of an inhibitory component in *ras*-transformed cells that is activated by dephosphorylation. This factor may inhibit the PDGF receptor, either directly (Figure 3a) or indirectly, by generating an inhibitory product (Figure 3b). Both mechanisms of PDGF-receptor inhibition

could explain the discrepancy between the results obtained from experiments using phosphatase inhibitors *in vitro* and *in vivo*. In intact cells there is a balance between kinase and phosphatase activities and inhibiting the latter leads to phosphorylation (and deactivation) of the inhibitor, restoring the tyrosine-kinase activity of the PDGF receptor. When membranes are prepared from *ras*-transformed cells, the inhibitor is already in the dephosphorylated (and active) state and thus phosphatase inhibitors have no effect.

Evidence in support of the role of PTPases in signal transduction and in oncogenesis has been accumulated over the past decade. For example, chicken fibroblasts transformed with Rous sarcoma virus exhibit about 30–50% higher PTPase activity in the soluble fraction [56]; the differentiation of HL-60 cells towards granulocytic and monocytic phenotypes is accompanied by a 7–11-fold increase in PTPase activity, respectively [57], and the level of *cdc25B* PTPase is dramatically increased in primary human fibroblasts that are transformed either with simian virus-40 (SV40) or with human papillomavirus type 16 or type 18 [58]. Recently, increasing numbers of membrane-associated PTPases have been identified. They either belong to the family of receptor-like PTPases such as CD45, a leukocyte common antigen-related phosphatase (LAR) and different isoforms of the human PTPase (HPTP) [39,40], or they have the potential to associate with the membrane through their interaction with other proteins. A PTPase (PTPIC) possessing two copies of the Src-homology region 2 (SH2 domain) and able to form high-affinity complexes with the activated epidermal-growth-factor receptor has been cloned from a human-breast-carcinoma cDNA library [59]. The *Drosophila* gene product *corkscrew*, a putative PTPase possessing two N-terminal Src-homology 2 domains and similar to the mammalian PTPIC, was shown to be involved in the signal-transduction pathway from the receptor tyrosine kinase *torso* [60]. Recently a PAO-sensitive protein that is critical for *p21^{ras}* activation has been identified in the insulin-dependent signal-transduction pathway, implying a role for PTPase in *p21^{ras}* regulation [46].

In conclusion, our data imply that the suppression of the PDGF-receptor autophosphorylation in *ras*-transformed cells is due to an inhibitory component that is activated by the dephosphorylation of tyrosine residue(s) as a result of increased PTPase activity associated with the plasma membrane. The eventual identification of the inhibitory component, as well as the phosphatase and kinase that are responsible for its regulation, will broaden our understanding of the cascade of events initiated by PDGF, both in normal cells and in cells transformed by the *ras* oncogene.

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