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The role of protein tyrosine phosphatases in density-dependent growth control of normal rat kidney cells

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In normal rat kidney cells protein tyrosine phosphatases (PTPases) play a role in attaining density-dependent growth arrest after stimulation with mitogens. The PTPase inhibitor sodium orthovanadate prevents density-dependent growth inhibition of EGF-treated cells and mimicks in that respect the action of TGFβ and retinoic acid. However, enhanced PTPase activity is not obligatory for maintaining cells in a density-arrested state. In contrast to TGFβ and retinoic acid, vanadate is unable to restimulate density-inhibited cells, indicating that different mechanisms are operating. Yet, vanadate is strongly potentiating the effect of low concentrations of TGFβ but not of retinoic acid, implicating that tyrosine phosphorylation is linked to TGFβ action and that PTPase may represent a negative control element in the TGFβ signaling pathway.

Density-dependent growth inhibition; Normal rat kidney cell; Protein tyrosine phosphatase; Retinoic acid; Transforming growth factor-β; Vanadate

1. INTRODUCTION

Normal rat kidney (NRK) cells have been widely used as a model system to study the role of polypeptide growth factors in the process of oncogenic transformation [1,2]. Stimulation of confluent NRK cells, made quiescent following serum-deprivation, by EGF induces cells to undergo one additional round of cell division. After completion of this cell cycle proliferation stops as a result of density-dependent growth inhibition. Such density-dependent growth arrest can be prevented by adding modulating agents like TGFβ or retinoic acid (RA). Moreover, these agents are able to restimulate density-inhibited EGF-treated cells, thereby inducing phenotypical characteristics of transformation [2]. Neither TGFβ nor RA by itself has any growth-stimulatory effect on NRK cells and it is believed that the action of TGFβ and RA involves the modulation of EGF receptor levels by transcriptional activation of the gene [3–6]. In several cell lines including NRK, receptor levels have been shown to decrease with increasing cell densities and a causal relationship with density-dependent growth inhibition has been suggested [7–9]. Indeed we could show that the ability of EGF to induce proliferation in NRK cells is a direct function of the EGF receptor density and that in density-arrested cultures EGF induced proliferation is limited by the level of EGF receptor expression (van Zoelen et al., submitted).

Recently we investigated the influence of the protein tyrosine phosphatase (PTPase) inhibitor orthovanadate on the mitogenic stimulation of serum-deprived quiescent NRK cells and its effect on density-dependent growth arrest [10]. It was shown that in contrast to TGFβ, vanadate hardly increased the proportion of serum-deprived quiescent cells which entered S-phase after stimulation with an optimal dosis of EGF. However, the effect of low doses of the growth factor was strongly potentiated by vanadate, indicating that it is able to optimize the growth factor signal in those cells that are capable of generating a signal sufficient to induce proliferation. Moreover, in EGF-stimulated cells the density-dependent inhibition of growth was found to be prevented by the addition of vanadate. In the latter respect the action of vanadate mimicked the effect of TGFβ and RA. Obviously NRK cells possess PTPase activity which can regulate signals transmitted by tyrosine kinase associated receptors and thus potentially plays a role in the process of phenotypical transformation. In agreement with this suggestion membrane PTPase activity of dense contact-inhibited Swiss 3T3 cells was shown to be increased 8-fold on average when compared to low-density proliferating cells [11].

Based on these observations it was hypothesized that, whereas TGFβ and RA upregulate EGF receptor densities and thus enhance incoming signals, vanadate in contrast does not amplify the initial signal, but prevents its down regulation resulting in prolonged receptor activation. This model predicts that after down regulation of EGF receptor densities, i.e. in density-inhibited cells,

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Abbreviations: EGF, epidermal growth factor; NRK, normal rat kidney; PDGF, platelet-derived growth factor; PTPase, protein tyrosine phosphatase; RA, retinoic acid; TGFβ, transforming growth factor-β.

vanadate is unable to restimulate proliferation in contrast to TGFβ and RA. In the present study we put this hypothesis to the test by comparing the effects of vanadate, TGFβ and RA on density-inhibited NRK cells.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Cell culture procedures and measurement of [^{3}H]thymidine incorporation were performed as described earlier [1,10,12]. Normal rat kidney cells, clone 49F, were plated at a density of 2.5×10^4 /1.8 cm^2 in 24 well plates (Costar, Cambridge MA, USA) in bicarbonate-buffered Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, Irvine, Scotland), supplemented with 10% newborn calf serum (Hyclone, Logan). After incubation for 4 days cells had reached confluency, and the medium was exchanged for 1 ml of a 1:1 bicarbonate-buffered mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Flow Laboratories), supplemented with 30 nM Na_2SeO_3 (ICN, Plainview NY, USA) and 10 μg/ml human transferrin (Sigma, St. Louis MO, USA). After 72 hr in this serum-free medium, the cells were stimulated with EGF (5 ng/ml) and insulin (5 μg/ml) in 0.1 ml of binding buffer (DMEM containing 50 mM N, *N*-bis(2-hydroxyethyl)-2-amino-ethane sulphonic acid (Sigma) and 0.1% bovine serum albumin (Sigma), pH 6.8). Fourty-eight hours after mitogenic stimulation, when cell growth had stopped because of density-dependent growth inhibition, cells were treated again with growth factors and/or vanadate. Unless otherwise indicated modulating agents were routinely added in triplicate in the following concentrations: TGFβ$_1$, 2 ng/ml, RA 50 ng/ml. [^{3}H]Thymidine ([^{3}H]TdR) incorporation was measured between 4 and 17 hrs after restimulation. [^{3}H]TdR (0.5 μCi, 43 Ci/mmol, Amersham, Buckinghamshire, UK) was added in 0.1 ml Ham's F12 medium. After the indicated time interval cells were washed four times with ice-cold phosphate-buffered saline and incubated for 15 min with 0.5 ml methanol at room temperature. The methanol was removed and the wells were allowed to air dry. The cells were then solubilized in 1 ml 0.5 N NaOH and [^{3}H]TdR incorporation was measured by liquid scintillation counting. [^{3}H]TdR incorporation was measured in triplicate. Sample standard deviation was always less than 10%.

2.2. *Vanadate solutions and growth factors*

Stock solutions of 25 mM sodium orthovanadate (Na_3VO_4, Sigma) were either prepared fresh in distilled water without any further precautions or were adjusted to pH 10, heated ad readjusted to pH 10 as described [13]. The latter preparation could be stored at 4°C for several months without any loss in activity. Transforming growth factor-β$_1$ was purified from outdated human blood platelets as described [14]. Receptor grade EGF was obtained from Collaborative Research (Waltham MA, USA), retinoic acid and insulin (bovine pancreas) were obtained from Sigma (St. Louis, MO).

3. RESULTS

3.1. *Influence of vanadate on density-inhibited NRK cells*

We compared the effects of the PTPase inhibitor vanadate and modulating factors like TGFβ and RA on NRK cells after reaching the density-inhibited state. Confluent NRK cells were kept quiescent by serum deprivation for three days and consecutively stimulated by EGF and insulin. A fraction of the cells (approx. 40% [1,2]) respond by completing one round of cell division, after which (48 h) proliferation stops because of density-dependent growth inhibition [1,10], inspite of the presence of EGF and insulin. Cells are restimulated then by adding TGFβ, RA or vanadate (Fig. 1). In accordance with earlier studies [1,2] TGFβ and RA are able to induce DNA synthesis in density-inhibited cells. However, in contrast to these effectors vanadate by itself increases [^{3}H]TdR incorporation only to a very limited extent. This slight stimulatory effect is reached at a vanadate concentration as low as 10 μM (Fig. 2), and no further stimulation can be observed at higher concentrations.

3.2. *Influence of vanadate on restimulation of density-inhibited cells by TGFβ and RA*

In the next series of experiments we investigated whether vanadate is able to modulate the growth inducing activities of TGFβ and RA. Initially we only used optimal concentrations of these effectors (Fig. 2). Under these conditions there appeared to be a slight stimulatory effect of vanadate on cumulative [^{3}H]TdR incorporation, which was to be largely additive. The effect was maximal at concentrations varying between 10–25 μM. In contrast, at higher concentrations an inhibitory effect was observed. The latter effect is in accordance with earlier findings showing similar inhibitory effects of higher concentrations of vanadate on mitogenic stimulation of NRK cells, prevention of density-dependent growth inhibition induced by several growth factors and induction of anchorage independent growth [10].

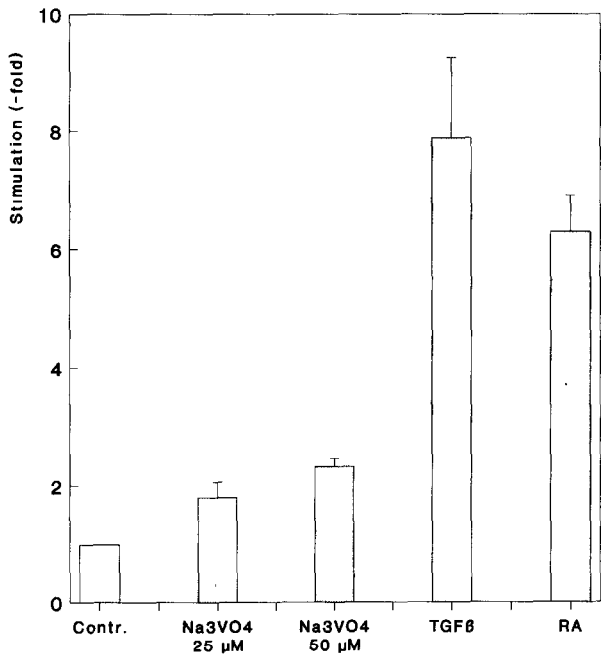

Fig. 1. Stimulation of density-inhibited NRK cells by vanadate and growth factors. Quiescent cells are stimulated with insulin and EGF. After 48 h proliferation has stopped because of density-dependent growth inhibition. Cells are restimulated then with vanadate and growth factors. Cumulative [^{3}H]TdR incorporation is measured 5–17 h after the latter additions. Stimulation is expressed as -fold increase over the control to which only binding buffer is added. The bars represent the mean±standard error of five independent experiments.

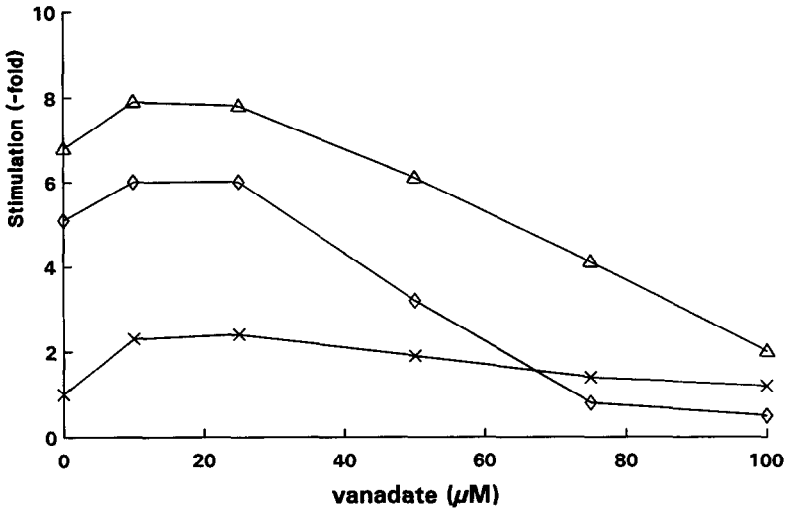

Fig. 2. Concentration dependence of the influence of vanadate on the stimulation of density-inhibited cells by growth factors. Cells are restimulated with increasing concentrations of vanadate in the presence of saturating concentrations of TGFβ (◇) or RA (△). In the control experiment (×) no growth factor is added. Conditions are as described in the legend to Fig. 1. A representative experiment is shown. The optimal concentration of vanadate varied from experiment to experiment between 10–25 μM. S.D.'s of triplicate determinations were always < 10%.

Remarkably, in contrast to the absence of any appreciable effects of vanadate under optimal growth factor conditions, vanadate strongly potentiated the effect of TGFβ when present at suboptimal concentrations. The dose–response curve for the effect of the modulating agent was shifted to much lower concentrations in the presence of 25 μM vanadate. In the absence of vanadate half-maximal stimulation occurred at approx. 0.6 ng/ml TGFβ, whereas vanadate lowered this concentration approx. 10 times (Fig. 3A). In contrast, no potentiating effects were found in case of restimulation with RA (Fig. 3B).

4. DISCUSSION

The phosphorylation of tyrosyl residues plays a key role in the regulation of growth and differentiation. Also in the regulation of density-dependent growth inhibition of NRK cells, which is used as a model for phenotypical transformation, the action of receptor tyrosine kinases has been shown to be of primary importance [2]. Since a regulatory role for PTPases have been suggested in counteracting the effects of tyrosine kinases [15,16], we decided to study the role of PTPase activities in the transformation of NRK cells by using the inhibitor sodium orthovanadate. In accordance with an earlier report of Klarlund [17], we recently demonstrated that vanadate was able to transform NRK cells: It mimicked the action of TGFβ and RA in the prevention of density-dependent growth inhibition [10]. Similarly to TGFβ and RA, vanadate by itself hardly influenced cell growth, but required the additional presence of EGF. Also, anchorage-independent growth in presence of EGF and insulin could be induced by TGFβ, RA as well as vanadate. These results suggest that modulating agents such as TGFβ and RA as well as PTPase inhibitors such as vanadate influence the EGF signaling pathway similarly and that PTPase(s) play a regulatory role in density-dependent growth inhibition. In agreement with the latter suggestion membrane PTPase activity of dense contact-inhibited Swiss 3T3 cells was shown to be increased 8-fold on average when compared to low-density proliferating cells [11].

We now show, that although PTPase activity may be obligatory for attaining density-dependent growth inhibition, it certainly is not necessary for maintaining density arrest. In contrast to TGFβ and RA, vanadate by itself is unable to restimulate density-inhibited cells. Thus its mechanism of action in the prevention of density-dependent growth arrest appears to be different from that of TGFβ and RA. It can be hypothesized that, whereas TGFβ and RA are supposed to upregulate receptor densities and thus to enhance incoming signals, vanadate in contrast doesnot amplify the initial signal, but prevents its down regulation (Fig. 4). In EGF-stimulated cells vanadate may act by inhibiting a PTPase which is responsible for the dephosphorylation of autophosphorylated EGF receptors or tyrosylphosphorylated substrates downstream in the signal transduction pathway. As a consequence the feedback regulation of the signal is impaired, resulting in a prolonged stimulatory effect of EGF in sensitive cells. In density-inhibited cells the EGF receptor level is down- regulated to a very low level [8] and the EGF signaling pathway is no longer sufficient for growth stimulation, not even in the presence of vanadate.

Remarkably, although the effect of vanadate by itself on density-inhibited NRK cells was negligable, it poten-

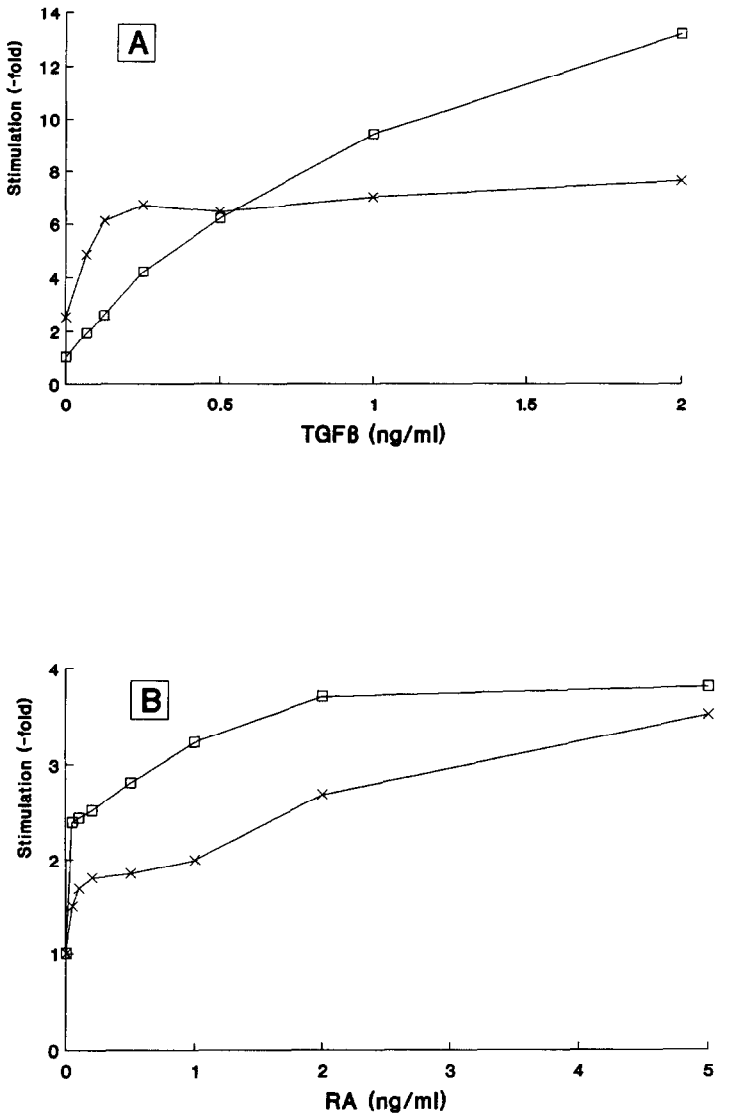

Fig. 3. Influence of vanadate on the dose–response curve of TGFβ and RA for the stimulation of density-inhibited cells. Cells are restimulated with increasing concentrations of TGFβ (A) and RA (B) in the presence of 0 (□) and 25 (×) μM vanadate. Conditions are as described in the legend to Fig. 1.

tiated significantly the effect of low concentrations of TGFβ: half-maximal stimulation of DNA synthesis occurred at 10-fold lower concentrations of TGFβ in the presence of vanadate. This result suggests that PTPase activity acts as a negative control element in the TGFβ signaling pathway and conversely that tyrosine phosphorylation plays a positive role somewhere in the transduction of the signal. The intracellular signaling pathway triggered by TGFβ is almost completely unknown [18] and no tyrosine phosphorylation has been linked to TGFβ action up to now [18,19]. Recent cloning of one of the primary TGFβ binding proteins functioning as a transmembrane signaling receptor, the TGFβ type II receptor, revealed evidence for the presence of a functional intracellular protein kinase domain [20]. However, its sequence fitted better with the serine/threonine kinase consensus than with the tyrosine kinase concensus [18,20], making a direct regulatory mechanism through dephosphorylation of the receptor itself or its substrates by PTPase activity less likely. Consequently, the involvement of tyrosine phosphorylation in the TGFβ action, which is suggested in the present study, may be localized more downstream in the signal transduction pathway. On the other hand the activin receptor, which is related to the TGFβ receptor, has been shown to have a dual specificity as it phosphorylates both Ser/Thr and Tyr residues [21].

By comparing the effect of vanadate on TGFβ and RA action, it can be concluded that it is specific for the signal transduction pathway triggered by TGFβ and

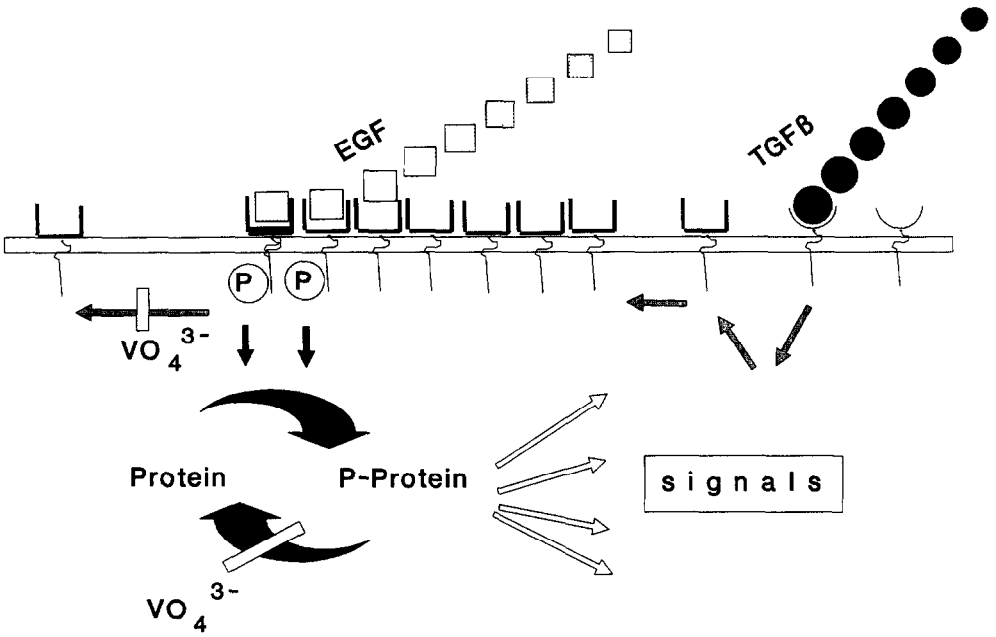

Fig. 4. Signal amplification by TGFβ and vanadate. In this model TGFβ acts by increasing the level of EGF receptor and thus amplifies the incoming signal. Vanadate, on the other hand, inhibits the PTPase(s) responsible for the dephosphorylation of autophosphorylated EGF receptor and receptor substrates and thus prevents downregulation of the signal.

not an aspecific effect associated with weak growth stimulatory signals in general. Although TGFβ and RA have comparable effects on density-inhibited NRK cells and both act by increasing the EGF receptor density in this cell line, no shift of the dose–response curve of RA could be observed. To our knowledge this is the first biological difference observed in the action of TGFβ and RA in the NRK model system.

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