Effects of Protein Tyrosine Phosphatase Inhibitors on EGF- and Insulin-Dependent Mammary Epithelial Cell Growth (44221)

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Abstract. **Epidermal growth factor (EGF)- and insulin-dependent mammary epithelial cell mitogenesis is mediated by specific tyrosine kinase receptors. Receptor tyrosine kinase activity is highly regulated in normal cells, whereas amplification of intracellular protein tyrosine phosphorylation is associated with abnormal growth and/or neoplastic transformation. Since protein tyrosine phosphatases (PTPs) are involved in regulating receptor tyrosine kinase signaling, studies were conducted to determine the effects of the PTP inhibitors, vanadate and pervanadate, on mitogen-receptor signal transduction and cell growth. Mammary epithelial cells isolated from midpregnant BALB/c mice were grown within collagen gels and maintained on serum-free media. Treatment with 2–8 μ*M* vanadate or pervanadate greatly increased intracellular protein tyrosine phosphorylation. However, in the presence of optimal mitogenic stimulation (10 ng/ml EGF and 10 μg/ml insulin), these treatments induced a slight, but significant decrease in cell growth. In contrast, these treatments significantly increased mammary epithelial cell growth, albeit less than optimally, under submitogenic culture conditions (500 pg/ml EGF and 10 μg/ml insulin). Neither vanadate nor pervanadate was found to mimic the mitogenic actions of EGF and/or insulin in these cells. The growth-stimulatory effects of PTP inhibitors in submitogenic conditions appear to result from enhanced receptor tyrosine kinase mitogenic signaling, whereas PTP inhibitor attenuation of optimal cell growth may be due to the suppression of PTP activity associated with cell cycle progression. In addition, treatment with PTP inhibitors was not found to stimulate anchorage-independent growth, as determined by the inability of single cells to form colonies in soft agarose. In conclusion, these data demonstrate that optimal mitogen-dependent mammary epithelial cell growth requires both receptor tyrosine kinase and PTP activity. Furthermore, PTP inhibitor-induced amplification of receptor tyrosine kinase mitogenic signaling is not in itself sufficient to induce enhanced cell growth or phenotypic expression of neoplastic transformation.** [P.S.E.B.M. 1998, Vol 217]

Receptor tyrosine kinase mitogenic signaling has become an area of intense investigation since many oncogene products have been identified as variants of growth factor receptors that display constitutively high levels of tyrosine kinase activity (1–3). In normal cells, tyrosine kinase activity is highly regulated and accounts for only a small percentage of total intracellular protein phosphorylation, whereas elevations in intracellular protein tyrosine phosphorylation are associated with neoplastic transformation (1–3). Protein tyrosine phosphatase (PTP) enzymes dephosphorylate receptor autophosphorylation sites and substrates and function as an important regulatory mechanism for receptor tyrosine kinase activity and signaling (4–6). In some cell types, treatments that inhibit PTP activity result in tyrosine hyperphosphorylation of a large number of intracellular proteins and phenotypic expression of neoplastic transformation (4, 7–10). PTP enzymes have also been implicated in modulating mitogenesis by regulating tyrosine phosphorylation and activity of various cyclin-

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dependent kinases during the different phases of the cell cycle (11, 12).

Vanadate and pervanadate are potent inhibitors of PTPs and have been shown to greatly enhance receptor tyrosine kinase activity and signaling (5, 13–16). Vanadate is less potent than pervanadate in suppressing PTP activity primarily because vanadate is a competitive inhibitor, whereas pervanadate is an irreversible PTP inhibitor (17). Vanadate has also been shown to be more sensitive than pervanadate to inactivation by intracellular reduction and/or protein chelation (18, 19). Despite differences in potency, vanadate and pervanadate induce essentially identical cellular responses. Nevertheless, different cell types display marked differences in sensitivity and response to treatment with PTP inhibitors. In some cell types, vanadate and pervanadate display insulin-mimetic properties (14, 20–23) and/or stimulate cell proliferation (8, 24, 25), while in other cell types these compounds inhibit mitogenesis (11, 25).

Primary culture of normal mouse mammary epithelial cells provides an excellent experimental model system for investigating the interrelationship between receptor tyrosine kinases and PTPs in modulating mitogenesis. EGF and insulin are potent co-mitogens for these cells, and their actions are mediated through specific membrane-bound tyrosine kinase receptors (26, 27). Mammary epithelial cell proliferation *in vitro* requires the presence of both EGF and insulin (26, 27). Cells cultured in serum-free media containing only one of these co-mitogens remain viable, but do not actively divide (26, 27). Since various oncogenic products associated with mammary tumorigenesis, such as *erb* B-2, are members of the EGF-receptor superfamily and display constitutively high levels of tyrosine kinase activity (1–3, 28–30), it was of interest to determine if PTP inhibitor-induced amplification of mitogen-receptor tyrosine kinase signaling is associated with alterations in normal mammary epithelial cell growth characteristics and/or phenotypic expression of neoplastic transformation as evidenced by anchorage independent growth. Studies were also conducted to determine if vanadate and pervanadate mimic the mitogenic actions of EGF and/or insulin in these cells.

Materials and Methods

Isolation and Culture of Mammary Epithelial Cells. Mammary epithelial cells were isolated and prepared for culture, as previously described (31). All materials were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise stated. Briefly, mammary glands from midpregnant BALB/c mice were removed, minced, and subjected to collagenase followed by pronase E (Type XIV bacterial from *Streptomyces griseus*) digestion. Mammary epithelial cells isolated from midpregnant mice were selected for experimentation because these cells are highly responsive to EGF-induced mitogenesis (27). The animal experimentation protocols presented in this study were approved by IACUC. Mammary epithelial cell organoids were isolated by a series of filtrations through sterile 250 μm and 48 μm nitex filters (Tetko Inc., Briarcliff Manor, NY), plated within rat tail collagen gels (32) at a density 2.2×10^7 cells/8 ml of collagen in 100-mm plastic plates, fed 10 ml growth media (DMEM/F12) 1:1, insulin 10 μg/ml, penicillin 100 U/ml, streptomycin 100 μg/ml, 10% bovine calf serum (Hyclone, Logan, UT), and placed in a humidified incubator at 37°C in an environment of 95% air and 5% CO_2. After a 2-day incubation period, mammary epithelial cell organoids were again isolated by collagenase digestion and filtration. This double isolation procedure provides isolated mammary epithelial cell organoids ready for experimentation that are devoid of fibroblast and adipocyte contaminants (33). Mammary epithelial cells were then plated within collagen gels at an initial density of 2×10^5 cells/well in 15 mm 24-well tissue culture plates and maintained on serum-free treatment media. Treatment media consisted of DMEM/F12 containing 5 mg/ml BSA, 10 μg/ml transferrin, 100 U/ml soybean trypsin inhibitor, 1 μg/ml d-α-tocopherol, 10 ng/ml or 500 pg/ml EGF, 0 or 10 μg/ml insulin, and 0–8 μ*M* vanadate or pervanadate. Cells were fed fresh media daily. Vanadate and pervanadate were prepared for experimentation as previously described by Evans *et al.* (16).

Measurement of Viable Cell Number. Mammary epithelial cell number was determined by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described previously (31, 34). On the day of assay, treatment medium was replaced with fresh growth medium containing 0.83 mg/ml MTT, and the cells were then returned to the incubator for 4 hr of incubation. Afterwards, gels were removed from the culture plates, placed 12 × 75-mm test tubes, and dissolved in 0.5 ml of 25% acetic acid. MTT crystals were pelleted by centrifugation, and the MTT crystals were dissolved in 1 ml of 2-propanol. Optical density of each sample was read on a microplate reader (model 7520, Cambridge Technology, Inc., Watertown, MA) at 570 nm against a blank prepared from cell-free collagen gels. The number of cells/well was calculated against a standard curve prepared by plating various concentrations of cells, as determined by hemocytometer, at the start of each experiment (31). In separate control studies, various doses (0–8 μ*M*) of vanadate and pervanadate were not found to affect the specific activity of the MTT colorimetric assay.

Discontinuous Electrophoresis and Western Blot Analysis. Sample preparation was previously described in detail (34). Isolated mammary epithelial cells were plated in 100-mm culture plates (1×10^7 cells/8 ml of collagen), divided into different groups, and cultured in their respective treatment media. Cells were isolated from collagen gels with collagenase on Day 5 of culture, pelleted and washed, and then immediately lysed with Laemmli buffer. Cell lysates were boiled for 5 min, sonicated, and centrifuged at 8300*g* for 7.5 min. The supernatant from each sample was removed and stored at −70°C. Protein concen-

trations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's directions. Samples from each treatment group (10 μg/lane) were loaded on polyacrylamide minigels and electrophoresed through a 5%–10% gradient gel. Proteins were transblotted (25V for 12–16 hr) to PVDF membranes (Dupont, Boston, MA) according to the method of Towbin *et al.* (35). To identify tyrosine-phosphorylated proteins, membranes were blocked with 2% BSA in 10 m*M* Tris-HCl containing 50 m*M* NaCl and 0.1% Tween 20 pH 7.4 (TBST), then incubated for 2 hr with antiphosphotyrosine monoclonal antibody (PY20) (Transduction Laboratories, Lexington, KY) diluted 1:5000 in TBST with 2% BSA. Membranes were then rinsed four times with TBST, and incubated for 1 hr with horseradish peroxidase-conjugated goat antimouse antibody (Transduction Laboratories, Lexington, KY), diluted 1:5000 in TBST with 2% BSA (34). Afterwards, blots were rinsed with TBST, incubated with chemiluminescent reagents according to the manufacture's instructions (Pierce, Rockford, IL), and bands visualized on film (Kodak X-OMAT AR, Rochester, NY).

Anchorage Independent Growth Assays. The potential of mono-dispersed mammary epithelial cells to form colonies in soft agarose was determined as previously described (36). Briefly, mammary epithelial cell organoids were isolated as described above and then cultured in T25 tissue culture flasks with growth media for 24 hr. Cells from the resulting monolayer were then isolated by trypsin digestion. Single cells were obtained by filtration through a 48-μm nitex filter (36). Cell viability ranged from 90%–95% as determined by trypan blue exclusion. A base layer of 250 μl of 0.5% agarose in growth media was heated and added to 24-well tissue culture plates. After solidification, mammary epithelial cells (1×10^3/250 μl) were plated in triplicate within a solution of 0.3% agarose in growth media. Cells were fed fresh control or treatment media daily. Treatment media consisted of 2–8 μ*M* vanadate, 2–8 μ*M* pervanadate, or 30 n*M* phorbol 12-myristate 13-acetate (PMA) in growth media. Within 12 hr after plating, aggregates of cells (2 or more cells in each well) were scored and subtracted from the number of colonies counted in their respective well on Day 14. After 14 days in culture, cells were fed fresh growth media containing 0.83g/ml MTT and incubated for 4 hr to verify cell viability (31). Afterwards, cells were rinsed and fixed in 5% formaldehyde in phosphate buffered saline. Viable mammary epithelial cell masses consisting of eight or more cells were scored as colonies (36). Viable mammary epithelial cells were photographed with a Nikon inverted scope (Nikon, Garden City, NY) with Sony digital camera (Sony, Montvale, NJ) using Metamorph software (Inner Media, Hollis, NH). Photomicrographs were constructed using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

Statistical Analysis. Differences among the various treatment groups were determined by analysis of variance, followed by Duncan's multiple range test. A difference of $P < 0.05$ was considered significant, as compared to controls or as defined in the figure legends.

Results

The effects of various doses of vanadate on EGF-and insulin-dependent mammary epithelial cell proliferation are shown in Figure 1. After 5 days in culture, control media (10 ng/ml EGF and 10 μg/ml insulin) induced a 2.5-fold increase in viable cell number (optimal cell growth), as compared to Day 1 of culture (Fig. 1A). Treatment with 1–8 μ*M* vanadate induced a slight, but significant dose-dependent decrease in cell growth, as compared to controls (Fig. 1A). Since vanadate displays insulin-mimetic effects in some cell types (21–23), additional studies were conducted to determine if vanadate could substitute for insulin as the co-mitogen. In the absence of insulin, combined treatment of 1–8 μ*M* vanadate and 10 ng/ml EGF did not induce mammary epithelial cell mitogenesis (Fig. 1B). In the absence of EGF, treatment with various doses of vanadate in combination with 10 μg/ml insulin was also not found to be mitogenic (data not shown). Cells maintained in media containing 500 pg/ml EGF and 10 μg/ml insulin (submitogenic growth conditions) remained viable, but did not display a significant change in cell number after 5 days in culture (Fig. 1C). Treatment with 1–8 μ*M* vanadate resulted in a slight, but significant dose-dependent increase in cell growth as compared to corresponding controls (Fig. 1C), but this increased growth did not reach the magnitude displayed by controls grown in optimal mitogenic conditions (Fig. 1A). In the absence of insulin, treatment with 1–8 μ*M* vanadate in combination with 500 pg/ml EGF did not induce mammary epithelial cell mitogenesis (Fig. 1D). In the absence of EGF, treatments with various doses of vanadate and 10 μg/ml insulin were not mitogenic (data not shown). Regardless of mitogenic conditions, vanadate treatment had no apparent effect on mammary epithelial organoid morphology throughout the 5-day culture period. Mammary epithelial cell duct formation in each treatment group was directly related to the magnitude of cell proliferation.

The effects of various doses of pervanadate on EGF- and insulin-dependent mammary epithelial cell growth are shown in Figure 2. Under optimal mitogenic conditions (10 ng/ml EGF and 10 μg/ml insulin), treatment with 1–8 μ*M* pervanadate induced a significant dose-dependent decrease in cell proliferation, as compared to controls (Fig. 2A). In the absence of insulin, treatment with 1–8 μ*M* pervanadate and 10 ng/ml EGF was not found to be mitogenic (Fig. 2B). In the absence of EGF, treatment with 1–8 μ*M* pervanadate and 10 μg/ml insulin was also found not to be mitogenic (data not shown). In submitogenic culture conditions (500 pg/ml EGF and 10 μg/ml insulin), treatment with 1–8 μ*M* pervanadate induced a slight but significant dose-dependent increase in cell number, as compared to corresponding controls (Fig. 2C). However, this increased growth was less than that displayed by controls cultured under optimal mi-

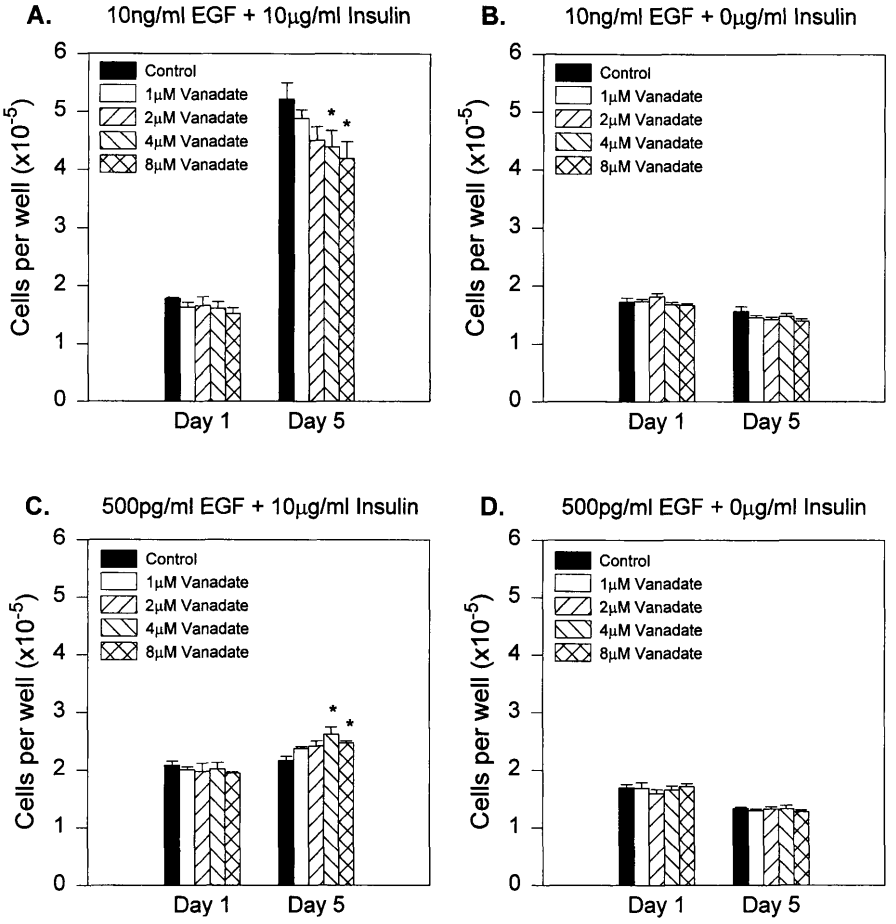

Figure 1. Effects of vanadate on mammary epithelial cell growth in serum-free media containing various doses of EGF and insulin. Viable cell number was determined by MTT colorimetric assay on Days 1 and 5 of culture. Vertical bars represent mean cell number ± SEM for six wells in each treatment group. $^*P < 0.05$, as compared to controls within each respective treatment group.

togenic conditions (Fig. 2A). In the absence of insulin, treatment with 1–8 μ*M* pervanadate and 500 pg/ml EGF was not found to be mitogenic (Fig. 2D). Likewise, in the absence of EGF, treatment with 1–8 μ*M* pervanadate and 10 μg/ml insulin did not stimulate cell proliferation (data not shown). Mammary epithelial cell duct formation in each treatment group was directly related to the magnitude of cell proliferation.

The effects of vanadate and pervanadate on EGF-and insulin-receptor tyrosine kinase signaling in mammary epithelial cells are shown in Figure 3. Western blot analysis of intracellular protein phosphotyrosine intensity showed that 2–8 μ*M* vanadate treatment in combination with either 500 pg/ml or 10 ng/ml EGF and 10 μg/ml insulin caused a dose-dependent increase in phosphotyrosine content of numerous intracellular proteins (Fig. 3A). Treatment with 2–8 μ*M* pervanadate induced similar increases in tyrosine phosphorylation of intracellular proteins (Fig. 3B). In the absence of EGF and insulin, treatment with vanadate and pervanadate had no effect on intracellular protein tyrosine phosphorylation intensity (data not shown).

The effects of PTP inhibitors and phorbol ester on normal mammary epithelial cell anchorage-independent growth are summarized in Figure 4. Isolated single cells grown in control media displayed little or no anchorage-independent growth, as determined by a lack of colony formation in soft agarose (Fig. 4A, B and C). Similarly, various doses of vanadate (Fig. 4A) and pervanadate (Fig. 4B) did not affect colony formation. In contrast, treatment with 30 n*M* PMA, a potent tumor promoter, significantly stimulated anchorage-independent mammary epithelial cell growth (Fig. 4C).

Photomicrographs comparing the effects of vanadate, pervanadate, and PMA on mammary epithelial cell colony formation in soft agarose are presented in Figure 5. Monodispersed cells cultured in control media primarily remained as single cells and did not form colonies in agarose (Fig. 5A and B). Treatment with 8 μ*M* vanadate (Fig. 5C and D), or 8 μ*M* pervanadate (Fig. 5E and F), also had no effect on colony formation. However, treatment with 30 n*M* PMA (positive control) was found to greatly stimulate colony formation (Fig. 5G and H).

Discussion

These studies demonstrate that treatment of normal mouse mammary epithelial cells with the PTP inhibitors, vanadate and pervanadate, results in a dose-dependent tyrosine hyperphosphorylation of specific intracellular proteins associated with mitogen-receptor signal transduction. However, amplification of receptor tyrosine kinase mitogenic signaling was not associated with a corresponding increase in cell proliferation, alterations in cellular morphology, or anchorage-independent growth. Rather, it was ob-

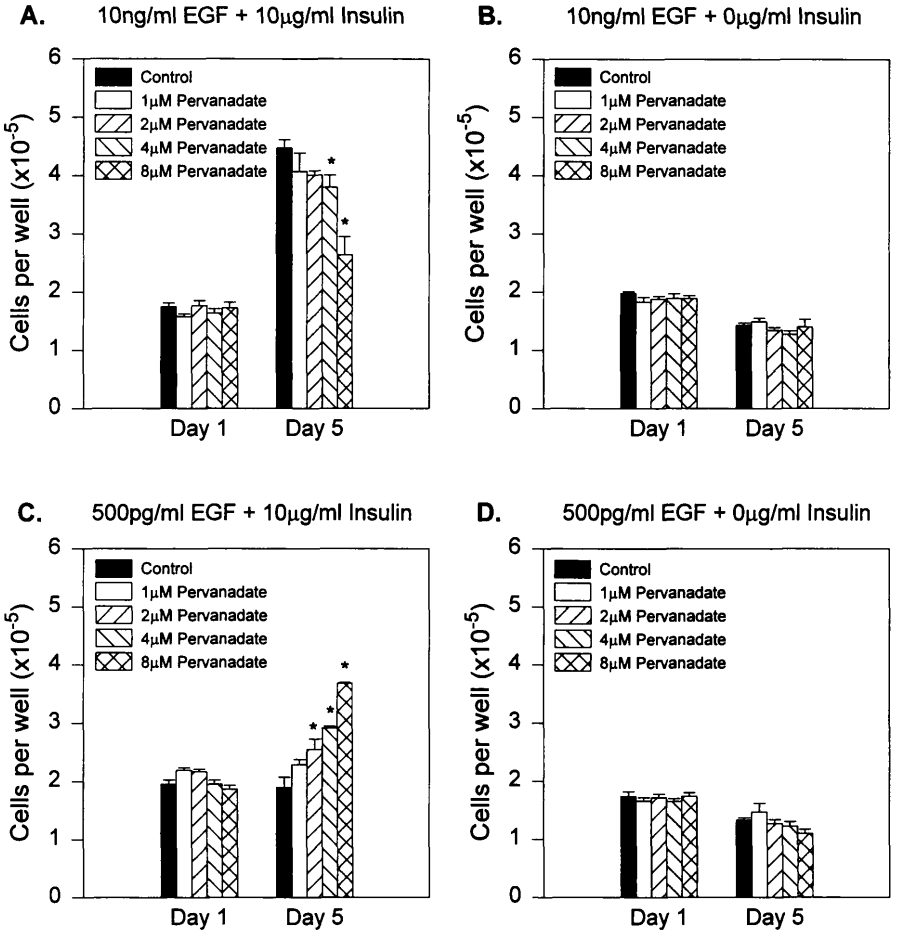

Figure 2. Effects of pervanadate on mammary epithelial cell growth in serum-free media containing various doses of EGF and insulin. Viable cell number was determined by MTT colorimetric assay on Days 1 and 5 of culture. Vertical bars represent mean cell number ± SEM for six wells in each treatment group. *P < 0.05, as compared to controls within each respective treatment group.

served that treatment with either vanadate or pervanadate caused a biphasic effect on mammary epithelial cell growth, which was dependent upon the level of EGF present in the culture media. Under optimal growth conditions (10 ng/ml EGF and 10 μg/ml insulin), treatment with PTP inhibitors caused a slight dose-dependent decrease in mammary epithelial cell growth. In contrast, similar treatments in submitogenic culture conditions (500 pg/ml EGF and 10 μg/ml insulin) significantly enhanced cell proliferation, although the magnitude of this enhanced cell growth was less than that of controls exposed to optimal levels of EGF and insulin. In addition, vanadate and pervanadate did not mimic the mitogenic effects of either EGF or insulin in mammary epithelial cells. Although elevations in intracellular protein tyrosine phosphorylation are often associated with abnormal growth characteristics and/or oncogenic transformation, these present data demonstrated that amplification of receptor tyrosine kinase mitogenic signaling is not solely responsible for phenotypic expression of neoplastic growth in mammary epithelial cells.

The exact mechanism(s) responsible for mediating the stimulatory effects of vanadate and pervanadate on mammary epithelial cell growth in the presence of submitogenic levels of EGF is unknown. Previous studies have correlated the insulin-mimetic and growth-promoting properties of vanadate and pervanadate with their indirect ability to enhance receptor tyrosine kinase signaling (7, 8, 21–25). Treatments that stimulate tyrosine kinase signaling enhanced cell proliferation, whereas treatments that inhibit tyrosine kinase activity decreased cell proliferation (23–25, 37). Therefore, it is possible that PTP inhibitor-induced amplification of EGF and insulin second-messenger signal transduction may be responsible for stimulating mammary epithelial cell growth under submitogenic conditions. This suggestion is supported by the finding that pervanadate was more potent than vanadate in stimulating both submitogenic cell growth and inducing the hyperphosphorylation of intracellular proteins. It is also possible that the growth stimulatory effects of PTP inhibitors under submitogenic conditions may be related to vanadate and pervanadate effects on gene transcription. Studies have shown that vanadate, in combination with either insulin or EGF, increased c-*fos* expression in 3T3-L1 cells (38). Reports have also demonstrated that vanadate treatment increased c-Ha-*ras* and c-*jun* levels, whereas pervanadate treatment greatly enhanced c-*jun* expression (39).

In contrast, vanadate- and pervanadate-induced suppression of mammary epithelial cell growth, under optimal growth conditions, may be due to the direct inhibitory effect of these compounds on PTP activity. In some cell lines, vanadate and pervanadate inhibit cell proliferation by causing a reversible blockade at the G2/M transition of the cell

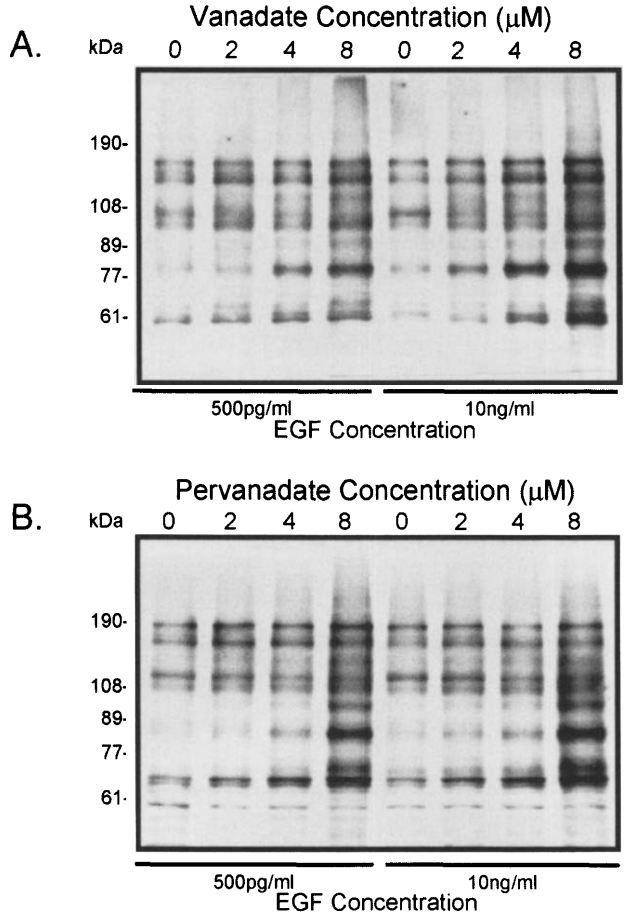

Figure 3. Effects of vanadate (A) and pervanadate (B) on total protein phosphotyrosine levels after 5 days in culture in mammary epithelial cells maintained in media containing various doses of EGF and insulin. Whole cell lysates (10 μg/lane) were fractionated by SDS-PAGE, and proteins were transferred to PVDF membranes for Western blot analysis for phosphotyrosine.

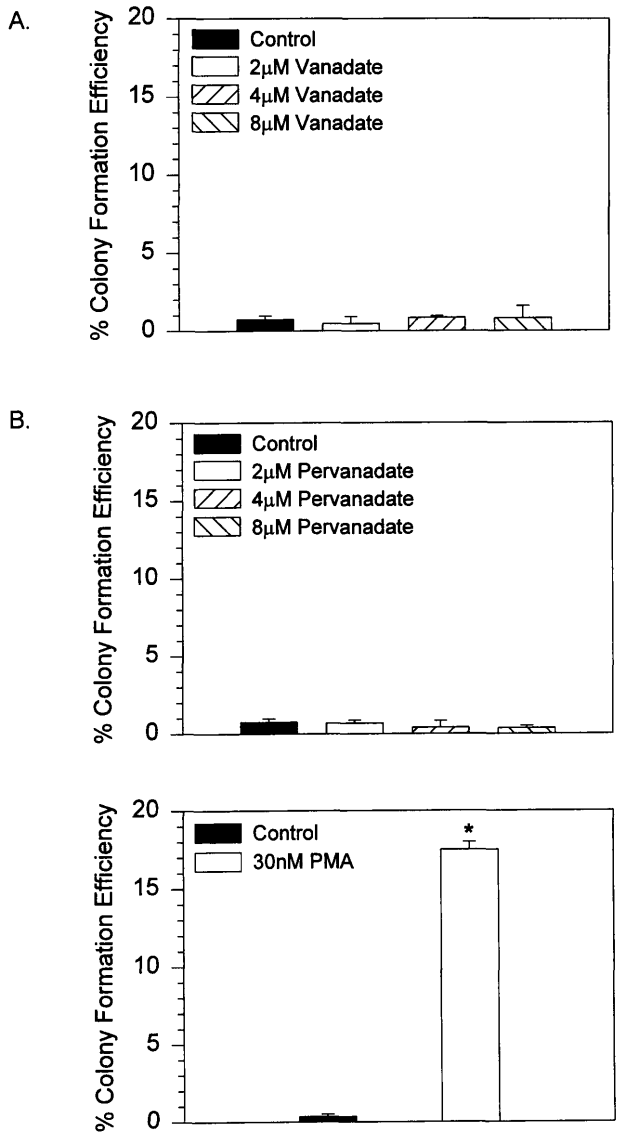

Figure 4. Effects of various doses of vanadate (A), pervanadate (B), and 30 n*M* PMA (C) on isolated single mammary epithelial cell colony formation in soft agarose after 14 days of culture. Vertical bars represent the mean ± SEM of colony formation efficiency of triplicate cultures for each treatment group. $^*P < 0.05$, as compared to controls within each respective treatment group.

cycle (11, 12). These studies also showed that tyrosine dephosphorylation of cdc2, a cyclin dependent kinase, is an obligatory step for cell-cycle progression (11, 12). Vanadate, and to a greater extent pervanadate, have been shown to inhibit cdc25 activity, a PTP involved in tyrosine dephosphorylation of cdc2 (11, 12). Suppression of cdc25 activity results in the hyperphosphorylation of cdc2 and subsequent arrest of the cell cycle (11, 12). Since optimal mammary epithelial cell proliferation was attenuated by PTP inhibitor treatment, these results suggest that PTP may also be involved in regulating normal mammary epithelial cell mitogenesis and cell-cycle progression. Nevertheless, different cell types display a wide range of sensitivity and response to PTP inhibitors, and in some cell types, these compounds do not block cell-cycle progression (8, 24–25). Studies are currently underway in our laboratory to determine if vanadate and pervanadate inhibition of optimal mammary epithelial cell proliferation results from a blockade in cell-cycle progression. It is also possible that vanadate and pervanadate inhibit cell growth through a cytotoxic mechanism. However, this suggestion is not supported by experimental findings, which demonstrated that these compounds did not significantly decrease viable cell number.

An important characteristic of many tumor cells is enhanced tyrosine phosphorylation of intracellular proteins (40, 41). Additionally, anchorage-independent growth is an established method for evaluating phenotypic expression of neoplastic transformation (42). However, experimental results showed that vanadate- and pervanadate-induced amplification of intracellular protein tyrosine phosphorylation did not stimulate anchorage-independent mammary epithelial cell growth, as evidenced by an absence of colony formation in soft agarose.

In conclusion, PTP inhibitor-induced amplification of receptor tyrosine kinase signaling in mammary epithelial

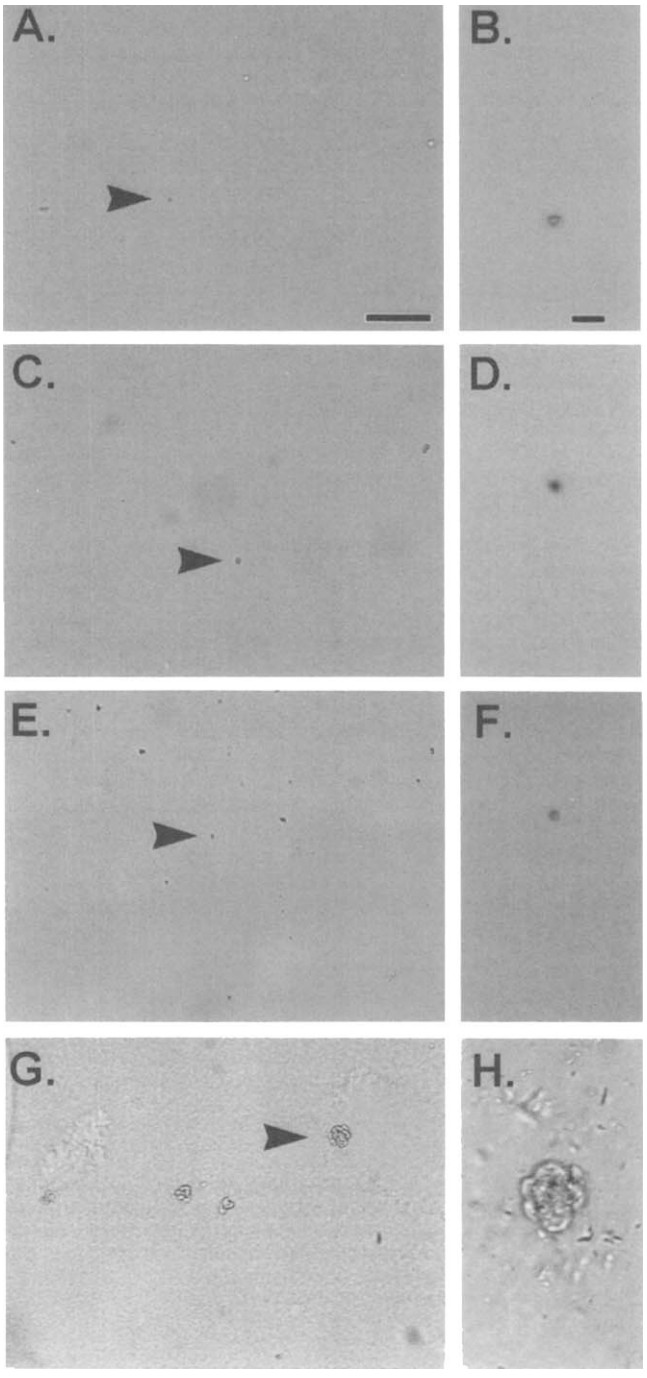

Figure 5. Effects of control medium alone (A and B) or in combination with 8 μ*M* vanadate (C and D), 8 μ*M* pervanadate (E and F) or 30 n*M* PMA (G and H) on isolated single mammary epithelial cell colony formation in soft agarose. Mono-dispersed mammary epithelial cells in the various treatment groups were cultured for 14 days in soft agarose. Magnification is 40× for photomicrographs A, C, E, and G, and the dark bar in A represents 100 μm in length. Magnification is 200× for photomicrographs B, D, F, and H, and the dark bar in B represents 10 μm in length. Arrows in A, C, E, and G point to the objects that are magnified in B, D, F, and H.

cells cultured in optimal mitogenic conditions does not result in enhanced, abnormal, or anchorage-independent growth. Optimal mitogen-dependent mammary epithelial cell proliferation appears to require a balance between receptor tyrosine kinase and PTP activity. Treatment with PTP inhibitors appears to disrupt this balance and inhibits optimal mitogen-dependent cell growth. Although vanadate and pervanadate stimulate cell growth in the presence of submitogenic levels of EGF, the magnitude of growth displayed by these cells was less than optimal. Finally, neither vanadate nor pervanadate mimics the mitogenic actions of EGF and/or insulin in normal mammary epithelial cells.

1. Jove R, Hanafusa H. Cell transformation by the viral *src* oncogene. Ann Rev Cell Dev Biol **3:**31–56, 1987.
2. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. Cell **61:**203–212, 1990.
3. Hunter T. Protein modification: Phosphorylation on tyrosine residues. Curr Opin Cell Biol **1:**1168–1181, 1989.
4. Fischer EH, Charbonneau H, Tonks NK. Protein tyrosine phosphatases: A diverse family of intracellular and transmembrane enzymes. Science **253:**401–406, 1991.
5. Walton KM, Dixon JE. Protein tyrosine phosphatases. Annu Rev Biochem **62:**101–120, 1993.
6. Zhang Z-Y, Dixon JE. Protein tyrosine phosphatases: Mechanism of catalysis and substrate specificity. Adv Enzymol Relat Areas Mol Biol **68:**1–36, 1994.
7. Klarlund JK. Transformation of cells by an inhibitor of phosphatases acting on phosphotyrosine in proteins. Cell **41:**707–717, 1985.
8. Maher PA. Stimulation of endothelial cell proliferation by vanadate is specific for microvascular endothelial cells. J Cell Physiol **151:**549–554, 1992.
9. Sabbioni E, Pozzi G, Pintar A, Casella L, Garattini S. Cellular retention, cytotoxicity and morphological transformation by vanadium (IV) and vanadium (V) in BALB/3T3 cell lines. Carcinogenesis **12:**47–52, 1991.
10. Kowalski TE, Tsang S-S, Davison AJ. Vanadate enhances transformation of bovine papillomavirus DNA-transfected C3H/10T1/2 cells. Cancer Lett **64:**83–90, 1992.
11. Faure R, Vincent M, Dufour M, Shaver A, Posner BI. Arrest at the G2/M transition of the cell cycle by protein-tyrosine phosphatase inhibition: Studies on a neuronal and a glial cell line. J Cell Biochem **59:**389–401, 1995.
12. Morla AO, Draetta G, Beach D, Wang JYJ. Reversible tyrosine phosphorylation of cdc2: Dephosphorylation accompanies activation during entry into mitosis. Cell **58:**193–203, 1989.
13. Stern A, Yin X, Tsang S-S, Davison A, Moon J. Vanadium as a modulator of cellular regulatory cascades and oncogene expression. Biochem Cell Biol **71:**103–112, 1993.
14. Kadota S, Fantus IG, Deragon G, Guyda HJ, Hersh B, Posner BI. Peroxide(s) of vanadium: A novel and potent insulin-mimetic agent which activates the insulin receptor kinase. Biochem Biophys Res Commun **147:**259–266, 1987.
15. Faure R, Baquiran G, Bergeron JJ, Posner BI. The dephosphorylation of insulin and epidermal growth factor receptors. J Biol Chem **267:**11215–11221, 1992.
16. Evans GA, Garcia GG, Erwin R, Howard OM, Farrar WL. Pervanadate stimulates the effects of interleukin-2 (IL-2) in human T-cells and provides evidence for the activation of two distinct tyrosine kinase pathways by IL-2. J Biol Chem **269:**23407–23412, 1994.
17. Huyer G, Lui S, Kelly J, Moffat J, Payette P, Kennedy B, Tsaprailis G, Gresser MJ, Ramachandran C. Mechanism of inhibition of protein-tyrosine phosphatases by vanadate and pervanadate. J Biol Chem **272:**843–851, 1997.
18. Cantley LC Jr., Aisen P. The fate of cytoplasmic vanadium: Implications on (Na,K)-ATPase inhibition. J Biol Chem **254:**1781–1784, 1979.
19. Crans DC, Bunch R, Theisen LA. Interaction of trace levels of vana-

dium(IV) and vanadium(V) in biological systems. Journal of the American Chemical Society **111:**7597–7607, 1989.
20. Leighton B, Cooper GJ, Da Costa C, Foot EA. Peroxovanadates have full insulin-like effects on glycogen synthesis in normal and insulin-resistant skeletal muscle. Biochem J **276:**289–292, 1991.
21. Heffetz D, Rutter WJ, Zick Y. The insulinomimetic agents H_2O_2 and vanadate stimulate tyrosine phosphorylation of potential targets for the insulin receptor kinase in intact cells. Biochem J **288:**631–635, 1992.
22. Shisheva A, Shechter Y. Mechanism of pervanadate stimulation and potentiation of insulin-activated glucose transport in rat adipocytes: Dissociation from the vanadate effect. Endocrinology **133:**1562–1568, 1993.
23. Fantus IG, Kadota S, Deragon G, Foster B, Posner BI. Pervanadate [peroxide(s) of vanadate] mimics insulin action in rat adipocytes *via* activation of the insulin receptor tyrosine kinase. Biochemistry **28:**8864–8871, 1989.
24. Cortizo AM, Etcheverry SB. Vanadium derivatives act as growth factor-mimetic compounds upon differentiation and proliferation of osteoblast-like UMR106 cells. Mol Cell Biochem **145:**97–102, 1995.
25. Raid A, Oliver B, Abdelrehman A, Sha'afi RI, Hajjar J-J. Role of tyrosine kinase and phosphotyrosine phosphatase in growth of intestinal crypt cell (IEC-6) line. Proc Soc Exp Biol Med **202:**435–439, 1993.
26. Imagawa W, Tomooka Y, Nandi S. Serum-free growth of normal and tumor mouse mammary epithelial cells in primary culture. Proc Natl Acad Sci USA **79:**4074–4077, 1982.
27. Imagawa W, Bandopadhyay GK, Nandi S. Regulation of mammary epithelial cell growth in mice and rats. Endocrine Rev **11:**494–523, 1990.
28. Nicholson RI, McClelland, Finlay P, Eaton CL, Gullick WJ, Dixon AR, Robertson JFR, Ellis IO, Blamey RW. Relationship between EGF-R, c-erbB-2 protein expression and Ki67 immunostaining in breast cancer and hormone sensitivity. Eur J Cancer **29:**1018–1023, 1993.
29. Dougall WC, Qian X, Greene MI. Interaction of the neu/p185 and EGF receptor tyrosine kinases: Implications for cellular transformation and tumor therapy. J Cell Biochem **53:**61–73, 1993
30. Peles E, Yarden Y. Neu and its ligands: From an oncogene to neural factors. Bioessays **15:**815–824, 1993.
31. Sylvester PW, Birkenfeld HP, Hosick HL, Briski KP. Fatty acid modulation of epidermal growth factor-induced mouse mammary epithelial cell proliferation *in vitro.* Exp Cell Res **214:**145–153, 1994.
32. Yang J, Richards J, Bowman P, Guzman R, Enami J, McCormick K, Hamamoto S, Pitelka D, Nandi S. Sustained growth and three-dimensional organization of primary mammary tumor epithelial cells embedded in collagen gels. Proc Natl Acad Sci USA **76:**3401–3405, 1979.
33. Jones W, Hallowes RC, Choongkittaworn N, Hosick HL, Dils R. Isolation of the epithelial subcomponents of the mouse mammary gland for tissue-level culture studies. Journal of Tissue Culture Methods **8:**17–25, 1983.
34. McIntyre BS, Birkenfeld HP, Sylvester PW. Relationship between epidermal growth factor receptor levels, autophosphorylation and mitogenic -responsiveness in normal mouse mammary epithelial cells *in vitro.* Cell Prolif **28:**45–56, 1995.
35. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA **76:**4350–4354, 1979.
36. Kanazawa T, Hosick HL. Transformed growth phenotype of mouse mammary epithelium in primary culture induced by specific fetal mesenchymes. J Cell Physiol **153:**381–391, 1992.
37. Burke TR. Protein-tyrosine kinase inhibitors. Drugs of the Future **17:**119–131, 1992.
38. Chen Y, Chan TM. Orthovanadate and 2,3,-dimethoxy-1,4,naphthoquinone augment growth factor-induced cell proliferation and *c-fos* gene expression in 3T3-L1 cells. Arch Biochem Biophys **305:**9–16, 1993.
39. Yin X, Davison AJ, Tsang S-S. Vanadate-induced gene expression in mouse C127 cells: Roles of oxygen derived active species. Mol Cell Biochem **115:**85–96, 1992.
40. Weinberg-RA. Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. Cancer Res **49:**3713–3721, 1989.
41. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science **244:**707–712, 1989.
42. McGeady ML, Kerby S, Shankar V, Ciardiello F, Salomon DS, Seidman M. Infection with TGF-α retroviral vector transforms normal mouse mammary epithelial cells but not normal rat fibroblasts. Oncogene **4:**1375–1382, 1989.