The Role of Estradiol Receptor in the Proliferative Activity of Vanadate on MCF-7 Cells

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Abstract
Vanadate stimulates growth of the estradiol-responsive MCF-7 cells in the absence of estrogens through a mechanism requiring tyrosine kinase activity. The proliferative effect of vanadate is mediated by estradiol receptor, and it is inhibited by three antiestrogens, hydroxytamoxifen, ICI 164,384, and ICI 182,780. Estradiol abolishes the inhibitory effect of ICI 164,384 or ICI 182,780. Before stimulating cell proliferation, vanadate induces accumulation of tyrosine phosphorylation in several proteins including estradiol receptor and epidermal growth factor receptor. In addition, vanadate increases the binding activity of the estradiol receptor for its ligand. This is the first evidence of in vivo association between estradiol receptor tyrosine phosphorylation and its hormone-binding activation. Antiestrogens abolish the vanadate effect on estradiol receptor and epidermal growth factor receptor phosphorylation and reduce it on general protein tyrosine phosphorylation. These findings show that vanadate, apparently through estradiol receptor tyrosine phosphorylation, triggers activity of this receptor, which in turn stimulates protein tyrosine phosphorylation and induces cell proliferation.

Introduction
Protein tyrosine phosphorylation is a crucial step in cellular signal transduction, a process actively regulated by PTKs and PTP-ases. The turnover of phosphate in phosphotyrosine is rapid (1, 2). Therefore, the balance between phosphorylation and dephosphorylation can be strongly affected by PTP-ase inhibitors. One of the compounds able to increase the phosphotyrosine content of proteins is vanadate, which inhibits PTP-ases in vitro as well as in vivo (3–5). Vanadate is widely distributed in normal biological systems at concentrations typically less than 1 μM (6) and has different properties which include induction of cell transformation (7).

Nuclear receptors form a large family of ligand-regulated transcriptional factors likely deriving from an ancestor gene and sharing common structural organization (for reviews see Refs. 8–11). Most of them have been reported to be phosphoproteins (for reviews see Refs. 9 and 11–14). These receptors are phosphorylated on serine. Different properties have been attributed to steroid receptor phosphorylation, ranging from hormone binding to DNA binding, transcriptional activation, and receptor turnover. Four nuclear receptors, the 17β-ER (15) glucocorticoid receptor (16), thyroid hormone-receptor β (17), and retinoic acid receptor β (18) have been found to be phosphorylated on tyrosine in vivo. Moreover, an estradiol-stimulated uterus PTK has been isolated from calf uterus which phosphorylates tyrosine 537 on the hormone-binding domain of ER, and because of this phosphorylation confers hormone binding to either in vitro-synthesized (19) or phosphatase-treated ER (20). This kinase also phosphorylates estradiol receptors unable to bind the hormone that have been found in some hormone-independent mouse mammary tumors (21). It has been observed recently that estradiol treatment of estradiol-responsive MCF-7 cells, a human mammary cancer-derived cell line, immediately induces reversible activation of tyrosine kinase and phosphorylation of several proteins (22). Taken together these data show that different nuclear receptors including ER are physiological substrates for PTKs and suggest that estradiol receptor may act on proliferation by stimulating tyrosine phosphorylation pathways.

We have now analyzed the effect of vanadate on MCF-7 cells. The level of protein tyrosine phosphorylation under basal conditions appears to be strongly down-regulated by the activity of PTP-ases. Vanadate causes accumulation of tyrosine phosphorylated ER and EGFR, as well as other proteins. Furthermore, the ability of the estradiol receptor to bind hormone is stimulated in the presence of vanadate. All these effects are followed by a significant stimulation of cell growth which is dependent on the accumulation of tyrosine phosphorylation since it is abolished by genistein, a tyrosine kinase inhibitor. The vanadate activity on MCF-7 cells is dependent on the ability of vanadate to trigger the ER activity since it is inhibited by antiestrogens. The data presented here, showing that ER acts upstream to PTK pathways regulating cell growth, support findings that ER stimulates tyrosine phosphorylation in MCF-7 cells (22), and PTK inhibition prevents estradiol-induced proliferation (23). This points to an important role of ER in regulating proliferative tyrosine phosphorylation pathways.

Results
Cell Growth. MCF-7 cells were maintained for a week in the absence of phenol red, a substance with weak estrogenic activity (24), and in presence of charcoal-treated serum. The cell growth under these conditions was slow (Fig. 1A). Two μM of vanadate significantly stimulated cell growth (Fig. 1A). The study of dose dependence showed that the proliferative effect of vanadate reached a plateau at 1 μM, declined at 3 μM, and disappeared at 5 μM (Fig. 1B).
In Fig. 2B the effect of 2 μM vanadate can be compared with that of 10 nM estradiol on cell growth. The efficiency of vanadate in stimulating cell growth was 30–50% that of estradiol in different experiments. Interestingly, when vanadate was added simultaneously to either hydroxytamoxifen (Fig. 2A) or ICI 164,384 (Fig. 2, B and C), its effect was strongly reduced (Fig. 2, B and C) or abolished (Fig. 2A). Addition of ICI 164,384 to the medium in the absence of vanadate did not reduce the slow basal growth rate of MCF-7 cells (Fig. 2B). The inhibitory effect of ICI 164,384 on vanadate-induced cell growth was removed by estradiol (Fig. 2C). Ten μM ICI 182,780 also inhibited the proliferative effect of vanadate. Twenty μM of estradiol in the cell medium removed this inhibition (not shown). The proliferative effect of vanadate was abolished by 10 μM of genistein (not shown).

Fig. 1. Vanadate dose-dependent effect on MCF-7 cell growth. (A) Subconfluent cells were grown in the absence (□) and in the presence (●) of 2 μM vanadate. The values of each point are averaged from seven different experiments. The statistical significance of these results was evaluated by paired t test. P values were ≤ 0.001 at third and fourth days and ≤ 0.005 at eighth day. Points, mean; bars, SD. (B) Cells were added with increasing amounts of vanadate and counted after 4 days.

Fig. 2. Stimulation by vanadate of MCF-7 cell growth. (A) Cells were grown in the absence (□) and in the presence of 2 μM vanadate either alone (●) or with 1 μM hydroxytamoxifen (■). (B) Cells were grown in the absence (□) and in the presence of 2 μM vanadate either alone (●) or with 10 μM ICI 164,384. Cells were also grown in the presence of either ICI 164,384 (A) or 10 nM 17β-estradiol (◇); (C) Cells were grown in the absence (□) and in presence of 2 μM vanadate either without (●) or with (■) 10 μM ICI 164,384. Cells were also grown in the presence of vanadate and ICI 164,384 and subsequently (see the arrow) treated with 1.25 μM estradiol (◇).

Total Protein Tyrosine Phosphorylation. Twelve h of vanadate treatment stimulated tyrosine phosphorylation of MCF-7 cells in a dose-dependent manner (Fig. 3A), which is related to the growth-stimulatory effect of this compound (Fig. 1B). The effect on both growth and phosphorylation was minimal at 0.2 μM vanadate and increased at 0.5 μM vanadate. The maximal effect on cell growth was observed at 1–2 μM vanadate (Fig. 1B); 2 μM vanadate also strongly stimulated tyrosine phosphorylation (Fig. 3A). In the ab-
In conclusion, vanadate induces tyrosine phosphorylation and cell growth of MCF-7 cells. Both are inhibited by antiestrogen treatment, and estradiol removes this inhibition. This shows that estradiol receptor plays a key role in stimulating tyrosine phosphorylation and proliferation induced by the PTPase inhibitor.

 Estradiol Receptor Tyrosine Phosphorylation. Preliminary, we have verified whether the receptor in MCF-7 cells is phosphorylated on tyrosine under basal conditions. The receptor was purified by either antireceptor H222 antibodies or control antibodies from cell lyse, and blotted with either H222 antibodies or antiphosphotyrosine antibodies. Fig. 4A shows that the M, 67,000 receptor, specifically precipitated by H222 antibodies, is phosphorylated on tyrosine. The specificity of the interaction of the receptor with antiphosphotyrosine antibodies was indicated by the absence of this interaction when the blot was performed in the presence of an excess of phosphotyrosine (not shown). We then studied the effect of 12-h vanadate treatment on the tyrosine phosphorylation of ER (Fig. 4B), which was significantly stimulated despite the amount of ER was decreased. We also verified the effect of hydroxytamoxifen and ICI 164,384 on the receptor phosphorylation induced by vanadate. Purification of the receptor from lyse by either antibodies directed against ER or control antibodies followed by blotting with antireceptor antibodies showed that a minor protein band also interacting with antireceptor antibody and migrating at about M, 68,000 appeared after vanadate treatment (Fig. 5A). Hydroxytamoxifen and ICI 164,384 (Fig. 5A), as well as ICI 182,780 (not shown), abolished the M, 68,000 protein. The doublet seems to be the consequence of receptor phosphorylation. In fact, Fig. 5B shows that vanadate had a heavy influence on the tyrosine phosphorylation of the M, 67,000 receptor and brought the tyrosine phosphorylated M, 68,000 protein to light. Previous work has shown that ER phosphorylation produces a doublet detectable by SDS-PAGE (25). The identification of the two tyrosine phosphorylated proteins with the ER resulted from the specificity of their immunoprecipitation by H222 antibodies from MCF-7 cell lyse and interaction with antireceptor antibodies in blot (Fig. 5, A and B). Hydroxytamoxifen abolished the vanadate effect on tyrosine phosphorylation of the M, 67,000 and 68,000 proteins. ICI 164,384 had a similar effect.

 The discrepancy between the experiment presented in Fig. 4B and the experiment in Fig. 5, A and B, showing that the phosphatase inhibitor induces a M, 68,000 protein interacting with H222 antibodies is probably due to the difficulty in consistently separating two very similar molecular weight proteins under our experimental conditions. Despite the high amount of nuclear receptor from cells not treated with orthovanadate and stimulated by 10 nM 17β-estradiol utilized in the experiment presented in Fig. 5C, no tyrosine phosphorylation of this receptor was seen.

 [¹H]Estradiol Binding to Estradiol Receptor. In three different experiments, either nontreated or 12 h vanadate-treated MCF-7 cells were homogenized, and specific estradiol binding of cytosols was analyzed by Scatchard plots. The data are summarized in Fig. 6A. In each experiment vanadate slightly increased the affinity of ER for its ligand and significantly raised the maximal number of specific estradiol-binding sites. Fig. 6 also shows the Scatchard plots from experiments 2 (Fig. 6B) and 3 (Fig. 6C) of Fig. 6A. The increase in maximal binding induced by vanadate treatment is smaller in experiment 2 and larger in experiment 3.
It should be highlighted that experiment 3 shows a number of sites in the cytosol from nontreated cells (Fig. 6, A and C) lower than that in cytosol assays from the other two experiments. Cells used in experiment 3 were grown for 1 week in phenol red-free DMEM and charcoal stripped serum before being treated with or without vanadate, whereas cells from the other two experiments were incubated under the same conditions for only 4 days. Whatever the reason for the difference of hormone binding under basal conditions, vanadate treatment increased the maximal estradiol binding to similar values in all 3 experiments. The amount of cytosol receptor in the binding assays of experiment 3 (Fig. 6C, insert) as well as in experiment 2 (Fig. 6B, insert) and experiment 1 (not shown) was similar in vanadate-treated and in control cells. This was verified by blotting with anti-ER antibodies. The present findings suggest that while the level of hormone-binding ER can fluctuate under different conditions, the total amount of ER is constant and vanadate treatment converts most of it to hormone-binding receptor. In regards to the effect of vanadate treatment on the amount of ER, our findings present an apparent discrepancy: while no difference (Fig. 6) or only small differences
in the level of ER from cytosols (not shown) were seen between control and vanadate-treated cells, in immunoprecipitated samples (Figs. 4 and 5) vanadate seems to significantly reduce the amount of ER. Comparison between the amounts of ER in cytosol before and after immunoprecipitation confirmed that the receptor was significantly reduced only after immunoprecipitation of vanadate-treated cells. It is likely that vanadate increased association between ER and other proteins and, under the non-denaturing conditions used during the immunoprecipitation procedure, reduced the interaction of ER with the antireceptor antibody.

**Protein Level and Tyrosine Phosphorylation of EGF Receptor.** MCF-7 cells treated with or without vanadate for 12 h were lysed, and lysates were submitted to immunoprecipitation with either control antibodies or antibodies against EGF as described in “Materials and Methods.” The precipitated proteins were submitted to SDS-PAGE and blotted with antibodies directed against EGF or anti-P-tyrosine antibodies (Fig. 7). Under basal conditions the EGFR was undetectable by both antibodies. This confirms that the level of EGFR expressed in these cells is very low (26). Vanadate treatment induced the specific appearance of a small but reproducible amount of the EGFR in the blots detectable by antireceptor as well as anti-P-tyrosine antibodies. This finding indicated that vanadate treatment induced an activated form of EGFR, since tyrosine phosphorylation of EGFR determines the affinity and/or the specificity of the receptor intrinsic kinase for its cellular substrates (27). Vanadate action on EGFR protein level was inhibited by the antiestrogen ICI 182,780, which also abolished tyrosine...
phosphorylation of this receptor. Therefore, in vanadate-treated MCF-7 cells ER plays an important role in the induction of tyrosine-phosphorylated EGFR.

Discussion
The estradiol-dependent MCF-7 cells used here were maintained for 1 week in an estrogen-free medium. The cells under these conditions either did not grow or grew at a very slow rate. The slow growth rate of MCF-7 cells in some experiments under basal conditions was not due to residual estradiol in charcoal-treated serum since antiestrogen ICI 164,384 did not affect their growth (Fig. 2B). The addition of vanadate induced reproducible and significant cell growth, which was abolished by the tyrosine kinase inhibitor genistein. Therefore, the mitogenic activity of vanadate was dependent on tyrosine phosphorylation.

Under basal conditions the amount of proteins phosphorylated on tyrosine was very low, suggesting that a high level of PTPase activity regulated tyrosine phosphorylation in MCF-7 cells. A major increase of tyrosine-phosphorylated proteins detected in the presence of vanadate corroborated this hypothesis. Addition of the nonsteroidal antiestrogen, hydroxytamoxifen, and two steroidal antiestrogens, ICI 164,384 and ICI 182,780 (28), reduced or abolished the vanadate-induced cell growth. The action of these antagonists is different. Tamoxifen promotes high affinity DNA binding of the receptor but fails to induce the formation of TAF-2 in the hormone-binding domain (29, 30); ICI 164,384 treatment reduces the half-life of estrogen receptor protein by decreasing the cellular content of the receptor (31). In addition, tamoxifen is a partial antagonist; ICI 164,384 and ICI 182,780 are “pure” antiestrogens (28, 32). Therefore, the inhibition of these antiestrogens on proliferation by vanadate pointed to a central role for estradiol receptor. The inhibitory effect of ICI 164,384 and ICI 182,780 on tyrosine phosphorylation was strong after 12 h of vanadate treatment. The effect of the antiestrogens on both phosphorylation and proliferation suggests a causal association between vanadate action on tyrosine phosphorylation and growth. Using estradiol to remove the inhibitory effect of ICI 182,780 on vanadate-induced cell growth and tyrosine phosphorylation suggests that the antiestrogens inhibit phosphorylation and proliferation through occupancy of ER.

We have analyzed the role of ER tyrosine phosphorylation in the vanadate-induced cell proliferation. As for tyrosine phosphorylation of ER under basal conditions, we have observed previously that it is phosphorylated on tyrosine in whole uteri (15). The experiments presented here show that ER was slightly tyrosine phosphorylated under basal conditions in MCF-7 cells. Twelve h of 2 μM vanadate treatment strongly increased tyrosine phosphorylation of the receptor. These data taken together once more confirm that vanadate in the medium can be required to identify proteins phosphorylated on tyrosine (15, 33–39) and suggests that tyrosine phosphorylation of this receptor is strongly regulated by PTPase. In this regard, it is worth noting that in our previous experiments where estradiol receptor was phosphorylated on tyrosine in rat uteri, tissues were incubated with vanadate. We used this PTPase inhibitor since estrogen target tissues like the uterus and mammary gland contain a nuclear PTPase (40). This enzyme dephosphorylates ER phosphorylated on tyrosine (41), abolishes the interaction of estradiol receptor with antiphosphotyrosine antibody and its hormone binding, and is inhibited by vanadate (15). Our finding that in cells not treated with vanadate the nuclear estradiol receptor complex from MCF-7 cells either went unphosphorylated on tyrosine or was phosphorylated somewhere below detection is in agreement with findings by other authors who also used estradiol-occupied receptor from cells or tissues incubated without vanadate for the phosphorylation studies and did not find tyrosine phosphorylation of estradiol receptor (25, 42).

In addition to stimulating ER tyrosine phosphorylation, vanadate increased the hormone binding of ER. The in vivo association between tyrosine phosphorylation and the hormone-binding activity of ER in MCF-7 cells reinforces the physiological meaning of our previous in vitro findings, showing that the phosphorylation on tyrosine 537 of a synthetic ER by a purified estradiol-dependent tyrosine kinase confers hormone binding to a large portion of the receptor (19, 43).

These experiments show that the tyrosine phosphorylation of ER and EGFR, as well as other proteins, was stimulated by vanadate action on ER since this phosphorylation was strongly inhibited or abolished by antiestrogens. In some aspects the effects of vanadate are similar to those of estradiol. The finding that the vanadate-stimulated tyrosine phosphorylation of ER was inhibited by ICI 164,384 as well as by hydroxytamoxifen is reminiscent of our previous observation that in vitro ER once activated by estradiol stimulated its own tyrosine phosphorylation, and this stimulation was inhibited by antiestrogens (44). In addition stimulating the hormone binding to ER by vanadate in MCF-7 cells is also reminiscent of the in vitro ER hormone-binding activation by estradiol (43, 44). Vanadate induced EGFR and its tyrosine kinase activity. The same effect has been attributed to estradiol (26, 45–47). Furthermore, vanadate and estradiol (22) were both able to induce with similar patterns stimulation of protein tyrosine phosphorylation in MCF-7 cells, which is inhibited by antiestrogens. In addition, estradiol removed the inhibitory effect of ICI 182,780 on vanadate-stimulated tyrosine phosphorylation. The comparison between Lanes 2 and 6 of Fig. 3C shows that estrogen partially restored protein phosphorylation induced by vanadate alone. The proliferative effects of vanadate and estradiol (23) on MCF-7 cells are also similar in inhibition by tyrosine kinase inhibitors. The observation that the effects of vanadate and estradiol present some analogy suggests that vanadate treatment of MCF-7 cells induced a receptor modification necessary for cell proliferation which mimicked that induced by estradiol and was prevented by the binding to ER of antiestrogens. Tyrosine phosphorylation of ER appears to be the most likely putative candidate for this modification. Several pieces of evidence support the existence of cross-talk between membrane and steroid receptors. It has recently been reported that estradiol and progesterone receptors are transcriptionally activated by a membrane receptor agonist, dopamine, in the absence of a ligand-binding event (48). EGF causes estradiol receptor nuclear translocation (49) and transcriptional activation of the progesterone receptor (50). It appears that membrane receptors can activate steroid receptors in the absence of steroid ligands. This is further supported by the finding that antiestrogens inhibit the growth of MCF-7 cells induced by peptide growth factors in the absence of estradiol (51–53). Therefore, some authors propose that growth factors can stimulate proliferation of estradiol-dependent cells by...
acting on the estrogen receptor (54). The effect of vanadate on MCF-7 cell proliferation could be similarly explained. It could be hypothesized that vanadate activates ER by inducing its tyrosine phosphorylation through a process initiated by a yet to be identified growth factor receptor. The activated ER in turn becomes able to stimulate MCF-7 cell tyrosine kinases), initiating a process leading to cell growth. The finding that ER activated by estradiol stimulates tyrosine phosphorylation in MCF-7 cells (22) supports this model.

Materials and Methods
Materials. Colored protein molecular weight markers (Rainbow) and [3H]estradiol (91 Ci/mm) were from Amersham (Bucks, UK). Soluble or agarose-linked monoclonal Ig2bk antiphosphotyrosine antibodies and mouse anti-EGFR mAb (clone LA1) were from Upstate Biotechnology, Inc. (Lake Placid, NY). Acrlyamide, BIS, N,N,N',N'-tetramethylethylene diamine, ammonium persulfate, SDS, Tween 20, pre-stained molecular weight markers for protein electrophoresis, and protein assay kit were purchased from BioRad (Richmond, CA). BA-85 nitrocellulose was from Schleicher & Schuell (Dassel, Germany). Gelatine was purchased from Serva (Heidelberg, Germany). Anti-mouse and anti-rat IgG alkaline phosphatase conjugates were from Promega (Madison, WI). Anti-rat IgG (fractionated antisera- um), estradiol, BSA (fraction V), sodium orthovanadate, HEPES, Tris, glycine, EDTA, Triton X-100, phenylmethylsulfonyl fluoride, leupeptin, pepstatine, antipain, soybean trypsin inhibitor, and Norit A were from Sigma Chemical Co. (St. Louis, MO). Genistin was from ICN Biochemicals (Costa Mesa, CA). Hydroxyamoxifen, ICI 164,384, and ICI 182,780 were from ICI (Macclesfield, UK). Reagents for cell culture media including FCS were from Gibco (Gaithersburg, MD).

Cell Culture Conditions and Vanadate Treatment.
Human breast cancer MCF-7 cells were routinely grown in 10% CO2 in air using plastic Petri dishes (100 mm Ø) in DMEM supplemented with phenol red, l-glutamine (2 mm), penicillin (100 units/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), insulin (6 ng/ml), hydrocortisone (3.75 ng/ml), and 5% FCS. Cells were passaged every 7 days and media were changed every 2–3 days.

For the vanadate experiments, cells were plated in 100-mm diameter Petri dishes with 8 ml of phenol red-free DMEM containing 5% FCS at roughly 1.5 × 106 cells/dish. Serum was treated twice with charcoal-coated dextran prepared using a previously reported procedure (22). This treatment reduced the concentration of estradiol in the medium below the sensitivity of the assay method used (0.9 × 10^-12 M). After 4 days cells were plated in 35-mm diameter multicwells at roughly 100–300 × 10^3 cells/well to follow growth rate or in 100-mm diameter Petri dishes with either 3 or 8 ml of the same medium. Three days later vanadate was freshly dissolved in HEPES buffer at a concentration of 100 mM and diluted with cell medium at a final concentration of 2 µM. The cells were maintained in this medium for the indicated times. Control cells were added with HEPES buffer without vanadate and grown in parallel. The viable cell number was determined after trypsinization using a Burker counting chamber.

Analysis of Tyrosine Phosphorylation and Immunopurification by Anti-P-Tyrosine Antibodies of Proteins from Vanadate-treated MCF-7 Cells. Cells from 3 Petri dishes (about 1.5 × 10^6 cells/dish) untreated or treated with vanadate for different times were washed 3 times with ice-cold PBS, pH 7.4, and scraped. Cells were lysed in 350 µl of ice-cold lysis buffer [50 mM Tris-HCl 4 mM EDTA-150 mM KCI-1 mM phenylmethylsulfonyl fluoride-1 µg/ml each of antipain, leupeptin, and pepstatin (LAP)-10 µg/ml of soybean trypsin inhibitor-1 mM sodium orthovanadate-1% Triton X-100 (pH 7.4)]. Lysate was shaken at 4°C, centrifuged at 15,000 × g for 30 min, and the protein concentration of the clear supernatant was assayed. An aliquot of this fraction containing about 30 µg of proteins was mixed with Laemml SDS-PAGE sample buffer, submitted to SDS-PAGE on minigel, followed by Western blot with anti-P-tyrosine antibodies. In the experiments of immunopurification by anti-P-tyrosine antibodies, 0.3 ml of cell lysate containing 0.9 mg proteins was added with 25 µl of anti-P-tyrosine antibodies linked to agarose and incubated for 3 h at 4°C under gentle shaking. At the end of incubation the suspension was centrifuged at 15,000 × g for 10 min at 4°C. The anti-P-tyrosine antibodies-agarose beads were collected and washed twice with lysis buffer, and proteins were specifically eluted by incubating the beads with 75 µl of lysis buffer containing 5 mM phosphotyrosine for 10 min at 4°C. The eluate was mixed with Laemml sample buffer, boiled, and submitted to SDS-PAGE and Western blot with anti-P-tyrosine antibodies.

Scatchard Plot Analysis of Hormone Binding to Estradiol Receptor from Untreated or Vanadate-treated MCF-7 Cells. Subconfluent cells in 145-mm diameter Petri dishes were maintained for 7 or 4 days in phenol red-free DMEM added to 5% charcoal-treated FCS. Cells of 20 Petri dishes were then treated for 12 h with 2 µM vanadate. Control cells from 20 Petri dishes were analyzed in parallel. Cells were washed three times with ice-cold PBS (pH 7.4) and scraped. They were washed in 10 ml of ice-cold TGD-sucrose buffer [50 mM Tris-HCl (pH 7.4) containing 0.2 mM EGTA, 1 mM DTT, and 250 mM sucrose] and finally homogenized in 4 ml of TGD-buffer containing 1% aprotinin, 1 mM PMSF, and 10 µg/ml LAP. Cells were homogenized with 40 strokes of a pestle B using a Dounce homogenizer and then centrifuged at 40,000 rpm for 30 min in a Beckman ultracentrifuge using a Ti 75 rotor. The supernatant (cytosol) was collected and proteins were assayed. Aliquots (300 µl) of cytosols from untreated or vanadate-treated cells containing the same amount of proteins were equilibrated at 2°C for 18 h with different concentrations of [3H]estradiol (from 0.01 to 6 nM) in the absence or presence of a 200-fold excess of radioinert estradiol. The estradiol-specific binding sites were assayed by dextran-coated charcoal treatment as reported previously (21), and 300-µl aliquots were counted in a Beckman LS 1801 β-spectrometer. One hundred-µl aliquots of cytosol from untreated or vanadate-treated cells were reduced in Laemml sample buffer, resolved on 8% SDS-PAGE, and immunoblotted with anti-estrogen receptor mAbs (H222).

Immunopurification of Estradiol Receptor. Cells from 10–30 Petri dishes were washed three times with ice-cold PBS (pH 7.4) and then scraped. One ml of lysis buffer was added to cells. Lysate was shaken at 4°C, centrifuged at 15,000 × g for 30 min, and protein concentration of the clear supernatant was assayed. The supernatant was diluted eight times with the lysis buffer lacking Triton X-100, divided into two aliquots, and separately loaded on a micro-column containing 35 µl of protein C-Sepharose-packed
beads incubated previously with 10 μg of anti-rat antibody and 8 μg of either anti-estradiol receptor H222 antibody or control rat IgG. Microcolumns were washed with 0.5 ml of lysis buffer and eluted with 150 μl of 20 mM NaOH, pH 11.6. The eluate was mixed with Laemmli sample buffer, boiled, and submitted to SDS-PAGE.

To analyze the nuclear receptor, cells from 10 Petri dishes were treated with 10 nm estradiol in 0.1% ethanol for 60 min and then scraped and homogenized in 1 ml of TGD buffer with 20 strokes of a pestle B using a Dounce homogenizer (type 357452; Wheaton, Melville, NJ). The homogenate was repeatedly passed through a 21-gauge needle containing sucrose at a final concentration of 250 mM, then centrifuged at 750 × g for 30 min. The nuclear pellet was washed twice with TGD buffer containing 250 mM sucrose, suspended in 300 μl of TGD buffer supplemented with 0.8 mM NaCl, incubated under gentle shaking for 30 min at 4°C, and centrifuged at 15,000 × g to obtain the nuclear extract. The extract was diluted with 4 volumes of TGD buffer lacking salt and proteins were assayed. One ml fraction of nuclear extract was submitted to immunopurification as follows. The sample was incubated with 1 μg of H222 antibodies for 2 h at 4°C. It was then loaded on a 30-μl microcolumn of protein-G-Sepharose. The microcolumn was washed with 1 ml of 150 mM NaCl and 0.5 ml of 500 mM NaCl in TGD buffer, and finally eluted with 50 μl of 20 mM NaOH (pH 11.6). The eluate was mixed with Laemmli sample buffer, boiled, and submitted to electrophoresis and Western blotting.

**Immunopurification of EGF Receptor.** Cells from 12 Petri dishes that were either treated or not with vanadate in the absence or presence of 10 μM ICI 182,780 were washed three times with ice-cold PBS (pH 7.4) and scraped. One ml of lysis buffer was then added to the cells. The lysate was shaken at 4°C, centrifuged at 15,000 × g for 30 min, diluted to a final concentration of 1 mg/ml of proteins with the lysis buffer (final volume 2.2 ml), and incubated with 80 μl of a 50% suspension of protein G-Sepharose for 90 min. At the end of the incubation each sample was divided in two aliquots and centrifuged, and the supernatant was incubated overnight with either 5 μg of mouse immunoglobulins or 5 μg of anti-EGF-receptor mouse mAbs. The day after, 5 μg of goat anti-mouse IgG antibodies were added to each sample, and samples were incubated for 60 min at 4°C. Eighty μl of a 50% suspension of protein G-Sepharose were added and incubation was continued for an additional hour. The samples were centrifuged, and pellets were washed with 1 ml of lysis buffer four times. Proteins were eluted by boiling in 100 μl of Laemmli sample buffer, and were submitted to SDS-PAGE and immunoblotting with anti-EGF receptor antibodies or anti-P-tyro sine antibodies.

**Electrophoresis and Immunoblotting.** One hundred μl samples on 10 × 15 cm gel or 30-μl samples on minigel were submitted to SDS-PAGE (10% acrylamide; acrylamide:BIS ratio, 37.5:1). At the end of the run proteins from gel slabs were electrophoretically blotted onto nitrocellulose filters at 25 V overnight at room temperature by using a transfer buffer containing 50 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol (55). The nitrocellulose filters were soaked in 10 mM Tris-HCl-150 mM NaCl-0.05% Tween 20, pH 8.0 (TBST buffer) containing 3% BSA to block nonspecific binding sites (blocking solution) and incubated for about 2 h. They were then incubated for another 2–3 h with either H222 rat anti-estrogen receptor mAbs or with mouse anti-EGF receptor mAbs (both anti-bodies at 1 μg/ml in blocking solution) or mouse anti-P-tyrosine mAbs (1 μg/ml in 10 mM Tris-HCl, 150 mM NaCl, added with 0.01% Tween 20 and 1% BSA) in the absence or presence of 5 mM phosphotyrosine, and thereafter washed at least 3 times for 10 min with TBST buffer. After washing the filters treated with H222 antibodies were incubated with alkaline phosphatase-linked anti-rat IgG antibodies (1:2500 dilution in TBST buffer); the filters treated with anti-P-tyrosine or anti-EGFR antibodies were incubated with alkaline phosphatase-linked anti-mouse IgG antibodies (1:4000 dilution in TBST) for 45 min at room temperature. Finally, they were washed again as described above and protein-antibody complexes revealed according the manufacturer instructions.

**Protein Assay.** Proteins were assayed using a BioRad assay kit.

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