Journal of Cellular Biochemistry 93:384–397 (2004)

Inhibition of Serum-Stimulated Mitogen Activated Protein Kinase by 1α,25(OH)$_2$-Vitamin D$_3$ in MCF-7 Breast Cancer Cells

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Abstract 1α,25-Dihydroxyvitamin D$_3$ [1α,25(OH)$_2$D$_3$], the hormonally active form of vitamin D$_3$, has been shown to be a potent negative growth regulator of breast cancer cells both in vitro and in vivo. 1α,25(OH)$_2$D$_3$ acts through two different mechanisms. In addition to regulating gene transcription via its specific intracellular receptor (vitamin D receptor, VDR), 1α,25(OH)$_2$D$_3$ induces rapid, non-transcriptional responses involving activation of transmembrane signal transduction pathways, like growth factors and peptide hormones. The mechanisms that mediate the antiproliferative effects of 1α,25(OH)$_2$D$_3$ in breast cancer cells are not fully understood. Particularly, there is no information about the early non-genomic signal transduction effectors modulated by the hormone. The present study shows that 1α,25(OH)$_2$D$_3$ rapidly inhibits serum induced activation of ERK-1 and ERK-2 MAP kinases. The tyrosine kinase Src is involved in the pathway leading to activation of ERK 1/2 by serum. Furthermore, 1α,25(OH)$_2$D$_3$ increases the tyrosine-phosphorylated state of Src and inhibits its kinase activity, while induces the association of the VDR with Src, either in the presence or absence of serum. In parallel, the hormone rapidly increases the amounts of VDR associated to plasma membranes (PM). Pretreatment with the tyrosine phosphatase inhibitors orthovanadate or bpV (phen) prevented mitogen-activated protein kinase (MAPK) inhibition by 1α,25(OH)$_2$D$_3$. These data altogether suggest that 1α,25(OH)$_2$D$_3$ inhibits the MAPK cascade by inactivating Src tyrosine kinase through a mechanism mediated by the VDR and tyrosine phosphatases. J. Cell. Biochem. 93: 384–397, 2004.

Key words: 1α,25(OH)$_2$D$_3$; breast cancer cells; MAP kinase; Src; VDR; tyrosine phosphatases

It is well established that 1α,25-dihydroxyvitamin D$_3$ [1α,25(OH)$_2$D$_3$], the active form of vitamin D$_3$, in addition to its crucial role in the regulation of calcium homeostasis and bone mineralization [DeLuca, 1988; Reichel et al., 1989], acts as a potent negative growth regulator of a wide array of cancer cells [Van Weelden et al., 1998; Colston and Hansen, 2002; Banerjee and Chatterjee, 2003 and references therein], including breast cancer cells, both in vitro and in vivo.

1α,25(OH)$_2$D$_3$ acts through two different mechanisms [Walters, 1992]. In addition to regulating gene transcription via its specific

Abbreviations used: 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D$_3$; VDR, vitamin D receptor; MAPK, mitogen-activated protein kinase; FBS, fetal bovine serum; BSA, bovine seroalbumin; PM, plasma membrane; ER, endoplasmic reticulum; RTK, receptor protein tyrosine kinase; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; Csk, Src specific protein kinase; PBS, phosphate-buffered saline; PMSF, phenylmethansulphonyl fluoride; DTT, dithiothreitol; PVDF, polyvinylidene difluoride.

Daniela A. Capiati and Ana M. Rossi have equally contributed to this work.

Daniela A. Capiati and Ricardo L. Boland are members of the Investigator Career of CONICET.

Grant sponsor: Fundación Antorchas and the Agencia Nacional de Promoción Científica y Tecnológica, Argentina; Grant sponsor: Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

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Received 9 December 2003; Accepted 12 April 2004
DOI 10.1002/jcb.20165

intracellular receptor (vitamin D receptor, VDR) as other steroid hormones [Minghetti and Norman, 1988; Perlman et al., 1990], $1\alpha,25(OH)_2D_3$ induces rapid, non-transcriptional responses involving activation of transmembrane signal transduction pathways like growth factors and peptide hormones [De Boland and Nemere, 1992; Nemere and Farach-Carson, 1998].

Regardless of the attempt of many research groups, the mechanisms that mediate the antiproliferative effects of $1\alpha,25(OH)_2D_3$ in breast cancer cells are not fully understood. While there is a wealth of information existing on nuclear events regulated by the steroid, not all actions of vitamin D can be explained by the genomic mechanism [Mehta and Mehta, 2002]. In fact, there are no data concerning early non-genomic signal transduction effectors modulated by $1\alpha,25(OH)_2D_3$ in breast cancer cells.

Mitogen-activated protein kinases (MAPK) are ubiquitous serine/threonine kinases that are phosphorylated and thereby activated in response to a wide variety of stimuli. The ERK 1/2 (p42/p44 MAPK) members of the MAP kinases are involved in transduction of externally derived signals regulating cell growth and differentiation [Schaeffer and Weber, 1999]. Upon activation, MAPK kinases translocate to the nucleus where they phosphorylate and activate nuclear transcription factors involved in DNA synthesis and cell division [Luttrell et al., 2001]. It is generally accepted that an unregulated activation of this cascade can result in oncogenesis [Davis, 1994; Cano and Mahadevan, 1995].

Serum growth factors bind to cell membrane receptors, activate receptor tyrosine kinases, cause isoprenylation of Ras, and activate the ERK 1/2 MAP kinase pathway [Gomperts et al., 2002]. A mechanism by which vitamin D and its derivatives may influence breast cancer cell growth and viability could be through a blockade of the mitogenic effects of growth factors. Src, a cytosolic tyrosine kinase which may also be activated by serum, can trigger the Ras/Raf/MAPK cascade through the recruitment of the Shc-Grb2-Sos adaptor complex [Hall et al., 1999]. The phosphorylation state of two tyrosine residues, Tyr-527 and Tyr-416, play an important role in the regulation of Src catalytic activity [Nada et al., 1991; Superti-Furga and Courtneidge, 1995].

The activation of intracellular kinases by $1\alpha,25(OH)_2D_3$ supports the hypothesis on the existence of a receptor located at the cell surface. There is controversial data on this regard, as a membrane-bound novel $1\alpha,25(OH)_2D_3$ binding protein [Nemere et al., 1994], annexin II [Baran et al., 2000], or the VDR itself [Kim et al., 1996; Capiati et al., 2002] have been implicated in the fast actions of the hormone. $1\alpha,25(OH)_2D_3$ rapidly induces translocation of the VDR from the nucleus to the plasma membrane (PM) in myoblasts [Capiati et al., 2002]. Moreover, localization of the VDR in a caveolae enrichment fraction from chick intestinal tissue has been recently reported [Norman et al., 2002]. Therefore, it is of interest to establish whether the VDR participates in the non-genomic effects of $1\alpha,25(OH)_2D_3$ in breast cancer cells.

Specifically, the aims of the present work were to characterize the rapid effects of $1,25(OH)_2D_3$ on MAP kinase activity in MCF-7 breast cancer cells and to investigate the involvement of Src and the VDR in the modulation of this key mitogenic signaling pathway.

MATERIALS AND METHODS

Chemicals

$1\alpha,25(OH)_2D_3$ was provided by Dr. P. Weber and Dr. E.-M. Gutknecht from Hoffmann-La Roche (Basel, Switzerland). Protein A-Sepharose CL4B, enolase, myelin basic protein, bovine pancreas trypsin, leupeptin, aprotinin, sodium orthovanadate, phenylmethansulphonyl fluoride (PMSF), dithiothreitol (DTT), and Immobilon P (polyvinylidene difluoride, PVDF) membranes were from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Gen S.A. (Buenos Aires, Argentina). RPMI-1640 medium was from Hyclone (Milan, Italy). PP1 and PP2 were from Biomol Research Lab., Inc. (Plymouth Meeting, PA) and Calbiochem-Novabiochem Corp. (San Diego, CA), respectively. bpV (phen) was from Calbiochem-Novabiochem Corp. Western blot chemiluminescence reagents (Renaissance) and [γ^{32}P]-ATP (3,000 Ci/mmol) were provided by New England Nuclear (Chicago, IL). All other reagents used were of analytical grade. Anti-VDR rat monoclonal antibody (clone 9A7) was from Affinity Bioreagents (Golden, CO). Anti-ERα mouse monoclonal antibody (Ab10, clone TE111.5D11, directed against the ligand-binding domain of ER) was obtained from

NeoMarkers (Fremont, CA). Anti-MAP kinase antibody and anti-phospho-MAP kinase, an antibody to the active phosphorylated form of MAP kinase (reactive against p42 and p44 isoforms), were from Promega (Madison, WI). Anti-Src mouse monoclonal, anti-lamin B goat polyclonal, anti-rat, anti-mouse, anti-rabbit, and anti-goat IgG horse radish peroxidase-conjugated antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine rabbit monoclonal antibody was from Promega. Rabbit polyclonal, highly specific anti-Gαs antibody was a gift from Dr. G. Schultz (Pharmakologisches Institut, Freie Universität Berlin).

Cell Culture

MCF-7 cells (a human breast cancer epithelial cell line) were obtained from the American Type Culture Collection (Rockville, MD) and kindly provided by Dr. A. Baldi (Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina). Cells were cultured in serum-supplemented media composed of RPMI-1640, 10% FBS, penicillin and streptomycin. Confluent monolayers were harvested by trypsin (0.1%) treatment. Cultures were established and maintained at 37°C in a humid air atmosphere (5.5% CO_2). The medium was replaced every 2 days and cultures were passaged every 4–5 days. Prior to treatments, serum was removed and cells were incubated for 24 h in RPMI-1640 medium (without phenol red) containing 0.1% bovine seroalbumin (BSA). FBS (20%) and/or 1α,25$(OH)_2D_3$ (1–100 nM) dissolved in isopropanol were added and the cells incubated for 1–5 min. The vehicle was applied in an equivalent concentration to control cells (less than 0.1% in all cases).

Thymidine Incorporation

Cell monolayers were treated with serum (20% FBS) plus vehicle (isopropanol <0.1%) or serum plus 100 nM 1α,25$(OH)_2D_3$ for 24 h. After treatment, the rate of thymidine incorporation into DNA was determined by adding 0.5 μCi [^{3}H]thymidine/ml followed by incubation for 4 h at 37°C. After washing the cells three times with phosphate-buffered saline (PBS) solution, DNA and proteins were precipitated with ice-cold 12% trichloroacetic acid, resuspended in 1 N NaOH and the radioactivity counted in a liquid scintillation counter.

Western Blot Analysis

Protein samples were subjected to SDS–PAGE according to the method of Laemmli [1970]. The separated proteins were electrophoretically transferred to PVDF membranes using a semidry transfer unit. Non-specific sites were blocked by 5% non-fat dry milk in TBST (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) overnight at 4°C. The membranes were incubated with the primary antibody 1.5 h at room temperature in TBST containing 5% (w/v) non-fat dry milk, washed three times with TBST for 10 min and incubated 1 h with the respective secondary antibody in TBST 5% non-fat dry milk (antibodies were diluted according to manufacturers' instructions). After washing, bands were visualized by chemiluminescence detection. To strip the membrane for reprobing with a different antibody, the membrane was washed with TBS, 1% Tween-20 for 10 min and then incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 50 mM 2-mercaptoethanol) for 30 min at 55°C. The membrane was again blocked and blotted as describe above. Images were obtained with a model GS-700 Imaging Densitomer (Bio-Rad, Hercules, CA) by scanning at 600 dpi and printing at the same resolution. Densitometric analysis of the bands was performed with Scion Image software (Scion Corp., Frederick, MD).

Immunoprecipitation

Immunoprecipitation assays were performed under dissociating conditions in order to disrupt protein–protein interactions. After treatments, MCF-7 cells were homogenized using a teflon-glass hand homogenizer in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1mM EDTA, 0.5% Nonidet P40, 1% Tritón X-100, 1 mM PMSF, 8 μg/ml aprotinin, 6 μg/ml leupeptin, 1 mM Na_3VO_4, 1 mM NaF). Lysates were clarified by centrifugation at 14,000*g* for 5 min. Immunoprecipitation assays were performed by incubating the samples (200 μg protein) with the corresponding antibodies (overnight at 4°C), followed by precipitation of the immunocomplexes with protein A-sepharose or protein G-sepharose (2 h at 4°C). The precipitated complexes were washed four times with cold immunoprecipitation buffer and subjected to kinase activity assays or, alternatively, immunoblot blot analysis (after boiling for 5 min in non-reducing Laemmli). In each

experiment, controls were performed using protein A-sepharose or protein G-sepharose alone (without antibody), to ensure that no non-specific precipitation would have taken place.

MAP Kinase Activity

Lysates (200 μg) from conveniently treated MCF-7 cells were immunoprecipitated with anti-MAP kinase antibody as indicated above. The immune complexes were washed four times with immunoprecipitation buffer and incubated at 37°C for 15 min in 35 μl of buffer containing 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 0.5 mg/ml myelin basic protein as an exogenous substrate for MAPK, 40 μM ATP, and [γ ^{32}-P]ATP (2 μCi/assay). The reaction was stopped by separating the phosphorylated product from labeled ATP on ion-exchange phosphocellulose filters (Whatman P-81). Papers were immersed immediately into ice-cold 75 mM H_3PO_4, washed three times with the same solution for 20 min and the radioactivity was determined in a scintillation counter.

Src Kinase Activity

Immunoprecipitation of Src was performed in lysates (200 μg protein) from conveniently treated MCF-7 cells as indicated above. The pellets were washed four times with immunoprecipitation buffer and incubated at 37°C for 15 min in 35 μl of buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 0.15 mg/ml enolase as an exogenous substrate for Src kinase, 40 μM ATP, and [^{32}-P]ATP (5 μCi/assay). The reaction was stopped with sample buffer containing SDS. The samples were subjected to electrophoresis, transferred to PVDF membranes as described and imaged by autoradiography.

Co-immunoprecipitation

Co-immunoprecipitation assays were performed under non-dissociating conditions in order to preserve protein–protein associations. After treatment, MCF-7 cells were homogenized in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 3 mM KCl, 0.5 mM EDTA, 1% Tween 20, containing protease inhibitors (1 mM PMSF, 6 μg/ml leupeptin, 8 μg/ml aprotinin) and phosphatase inhibitors (1 mM sodium fluoride, 1 mM sodium orthovanadate), using a teflon-glass hand homogenizer. Lysates were clarified by centrifugation at 14,000*g* for 5 min. Proteins (200 μg) from supernatants were incubated with the corresponding antibodies overnight at 4°C, followed by precipitation of the immunocomplexes with protein A-sepharose or protein G-sepharose (2 h at 4°C). After four sequential washes with coimmunoprecipitation buffer, the pellets were boiled for 5 min in non-reducing Laemmli buffer. Proteins were resolved by SDS–PAGE, transferred to PVDF membranes, and Western blot analysis was performed with the antibodies indicated in the figure legends. In each experiment, controls were performed using protein A-sepharose or protein G-sepharose alone (without antibody), to ensure that no non-specific precipitation would have taken place.

Plasma Isolation (PM) Isolation

Purified PM preparations were obtained by a method previously reported with some modifications [Suzuki et al., 1989; Kim et al., 1996; Capiati et al., 2002]. MCF-7 cells were collected in TEDK buffer solution (10 mM Tris-HCl, pH 7.4, 0.3 M KCl, 1 mM EDTA, 5 mM DTT) containing protease inhibitors (1 mM PMSF, 40 μ/ml leupeptin and 40 μ/ml aprotinin) and phosphatase inhibitors (1 mM Na_3VO_3, 1 mM NaF). The cell suspension was homogenized in a teflon-glass hand homogenizer (50 stokes). The homogenized solution was centrifuged at 14,000*g* for 20 min. The supernatant fluid was centrifuged at 100,000*g* for 30 min. The pellet (microsomes) was resuspended in 1.5 ml of 15% sucrose in TEDK and layered on a discontinuous sucrose density gradient composed of 2 ml of 30% sucrose and 1.5 ml of 45% sucrose in TEDK. This gradient was centrifuged at 76,000*g* for 3 h. The 15–30% (PM fraction) and 30–45% interfaces and the pellet were collected, diluted with TEDK as necessary, and centrifuged at 100,000*g* for 1 h. The resulting pellets were suspended in TEDK plus protease inhibitors. The purity of the PM fraction was assessed by Western blotting detection of Gαs (PM specific) and lamin B (nuclei specific) [Rossi et al., 2002], and by determination of glucose-6-phosphatase (endoplasmic reticulum (ER) specific). Glucose-6-phosphatase was measured by determining the rate of release of inorganic phosphate from glucose-6-phosphate. To begin the reaction, 100 μg of PM protein in a volume of 0.05 ml were added to 0.45 ml of assay mixture containing 22 mM glucose-6-phosphate, 20 mM histidine, and 1 mM EDTA. The reactants were

incubated at 37°C for 30 min in a shaking water bath. The reactions were stopped by adding 2.5 ml of 8% TCA to each tube, centrifuged 15 min at 1,000*g* and inorganic phosphate was determined in 2 ml aliquots of the supernatant according to the method described by Chen et al. [1956].

Nuclei Isolation

MCF-7 cells were collected in TEDK buffer solution (10 mM Tris-HCl, pH 7.4, 0.3 M KCl, 1 mM EDTA, 5 mM DTT) containing protease inhibitors (1 mM PMSF, 40 μ/ml leupeptin, 40 μ/ml aprotinin) and phosphatase inhibitors (1 mM Na_3VO_3, 1 mM NaF). The cell suspension was homogenized in a teflon-glass hand homogenizer (50 strokes). The lysates were centrifuged first at 200*g* 10 min to eliminate cell debris, followed by 1,000*g* for 10 min. The resulting pellet (nuclei) was washed twice with TEDK buffer containing protease and phosphatase inhibitors and resuspended in the same buffer [Rossi et al., 2002].

RESULTS

Inhibition of MCF-7 Cell Proliferation by 1α,25(OH)$_2$D$_3$

Treatment of MCF-7 cells in the presence of serum with 100 nM 1α,25(OH)$_2$D$_3$ for 24 h significantly diminished serum-dependent [^{3}H]thymidine incorporation into DNA ($-27.7 \pm 3.1\%$; $P < 0.001$, $n = 7$), validating the antiproliferative action of the hormone in MCF-7 cells reported in previous studies [Bratland et al., 2000; Verlinden et al., 2000].

1α,25(OH)$_2$D$_3$ Inhibits Serum-Induced MAPK Activation in MCF-7 Cells

In order to establish whether 1α,25(OH)$_2$D$_3$ inhibits ERK 1/2 MAP kinase as an early effect that leads to inhibition of cell proliferation, MCF-7 cells were treated with 20% FBS or 20% FBS plus different doses of 1α,25(OH)$_2$D$_3$ (1–100 nM) for 3 min. The hormone decreased the amounts of the phosphorylated (and thereby active) form of ERK1 and ERK2 MAP kinases, without affecting the total quantity of the enzymes, in a dose-dependent manner (Fig. 1A). Time-response experiments indicated that FBS and 1α,25(OH)$_2$D$_3$ maximal effects occur at 3 min, and were less noticeable after 1 and 5 min (not shown). These results were confirmed determining MAP kinase activity by phosphorylation of myelin basic protein as exogenous substrate. The hormone significantly decreased serum-induced MAPK activity after 3 min treatment ($-46.15 \pm 3.79\%$; $P < 0.05$, $n = 3$; for 1 nM); moreover, 1α,25(OH)$_2$D$_3$ inhibited the serum-induced increase in the nuclear levels of ERK1/2 (Fig. 1B). This is most likely a result from a decrease in serum-induced ERK phosphorylation and/or activation. No effects of 1α,25(OH)$_2$D$_3$ on MAPK were observed under basal conditions (after 24 h of serum deprivation) when the levels of active enzyme are almost undetectable (Fig. 1C).

Serum-Induced MAPK Activation Is Diminished by the Src Inhibitors PP1 and PP2

As Src is an upstream positive modulator of ERK 1/2 [Hall et al., 1999], the participation of Src in serum-induced MAPK activation was investigated using the Src inhibitors PP1 and PP2. Pretreatment of MCF-7 cells with PP1 or PP2 (10 μM) for 30 min decreased serum-induced MAPK activation (Fig. 2A), indicating the upstream participation of Src in the signaling pathway that leads to MAPK stimulation. PP1 and PP2 used at the same concentration and incubation time showed to be potent inhibitors of Src kinase activity in vitro using enolase as exogenous substrate (Fig. 2B).

1α,25(OH)$_2$D$_3$ Increases Tyrosine-Phosphorylated State of Src and Inhibits Its Activity

Experiments were carried out to determine whether 1α,25(OH)$_2$D$_3$ inhibits Src activity. It is well established that phosphorylation of a conserved tyrosine residue (Tyr-527) at the carboxy-terminal tail of the Src molecule negatively modulates its kinase activity by a mechanism which implies interaction of the carboxy-terminal phosphorylated tyrosine with the SH2 domain of Src [Piwnica-Worms et al., 1987; Brown and Cooper, 1996]. Activation of Src requires removal of this C-terminal phosphate. Once activated, autophosphorylation in a second regulatory site in Tyr-416 is required to achieve maximal stimulation [Nada et al., 1991; Superti-Furga and Courtneidge, 1995]. MCF-7 cells were treated with 20% FBS or 20% FBS plus 1α,25(OH)$_2$D$_3$ (10 nM) for 3 min. As illustrated in Figure 3A, the hormone increased tyrosine phosphorylation of Src as evidenced by immunoprecipitation under dissociating conditions of lysates with anti-Src antibody followed

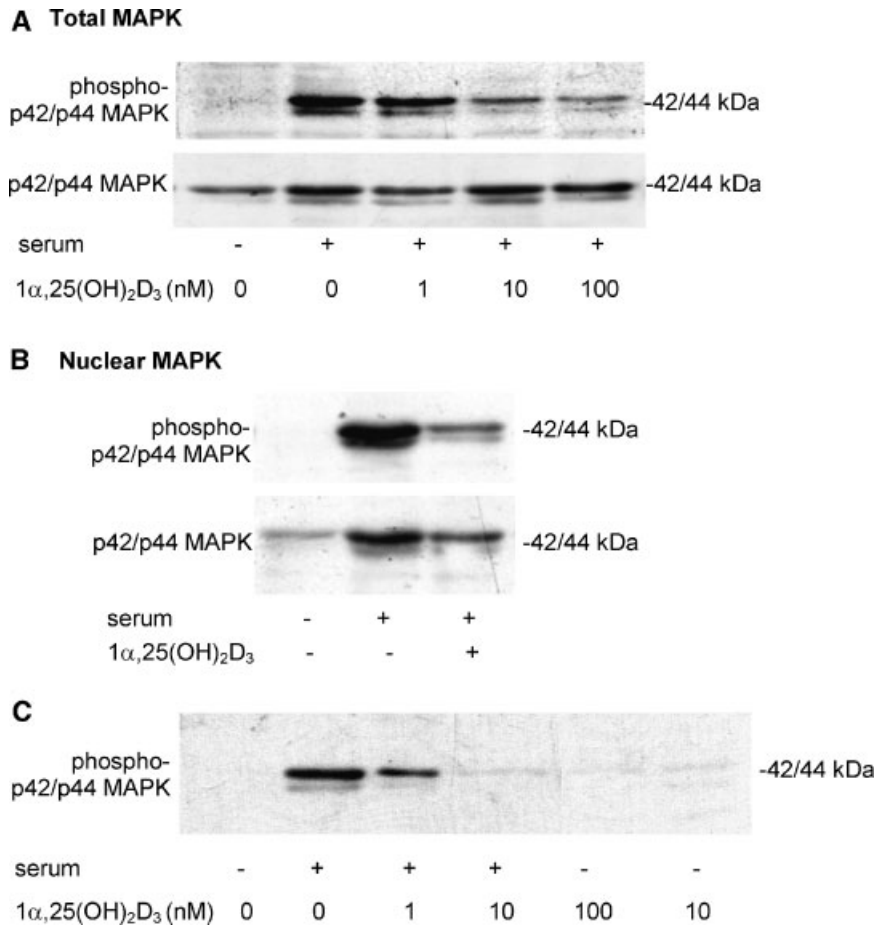

Fig. 1. 1α,25(OH)$_2$D$_3$ inhibits serum-induced MAPK activation and translocation into the nucleus in MCF-7 cells. **A**: Total active MAPK. MCF-7 cells were treated with serum (20% FBS) plus vehicle (isopropanol <0.1%) or serum plus different doses of 1α,25(OH)$_2$D$_3$ (1–100 nM) for 3 min. Controls were treated with vehicle alone. Cells were incubated for 24 h in the absence of serum for 24 h prior to treatment. Cells were homogenized and subjected to Western blot analysis using anti-(phospho)-active p42/p44 MAPK antibody followed by reblotting with anti-p42/p44 MAPK antibody. **B**: Nuclear active MAPK. MCF-7 cells were treated with serum plus vehicle (isopropanol < 0.1%) or serum plus 1α,25(OH)$_2$D$_3$ (10 nM) for 3 min. Controls were treated with vehicle alone. Cells were homogenized, the nuclear fraction was isolated and subjected to Western blot analysis using anti-(phospho)-active p42/p44 MAPK antibody followed by reblotting with anti-p42/p44 MAPK antibody. **C**: MCF-7 cells were treated as in (A) with serum, serum plus 1α,25(OH)$_2$D$_3$, or 1α,25(OH)$_2$D$_3$ alone for 3 min. Western blot analysis was performed using anti-(phospho)-active p42/p44 MAPK antibody. Representative blots from at least four independent experiments are shown.

by blotting with anti-P-tyrosine antibody and vice versa.

It was investigated whether this increase in Src tyrosine phosphorylation state correlates with a decrease in its activity. After immunoprecipitation of Src, under dissociating conditions, enzyme assays were performed using enolase as exogenous substrate and [γ-^{32}P]ATP. As expected, two bands were observed after autoradiography, one corresponding to phosphoenolase (p-enolase) and another corresponding to phosphorylated Src (p-Src) (Fig. 3B). In agreement with the results described above, it was observed that 1α,25(OH)$_2$D$_3$ decreases Src kinase activity. MCF-7 cells exhibit elevated basal Src activity; this may be possibly related to high levels of phosphorylation at Tyr-416, as evidenced in the p-Src band. This may account for the fact that the stimulatory effects of serum on Src phosphorylation and activity are not very pronounced. The 1α,25(OH)$_2$D$_3$-induced increase in tyrosine phosphorylation of Src (Fig. 3A) may be ascribed to Tyr-527 which should prevail over that of Tyr-416 explaining thereby the clear decrease of Src activity evidenced after 3 min treatment with the

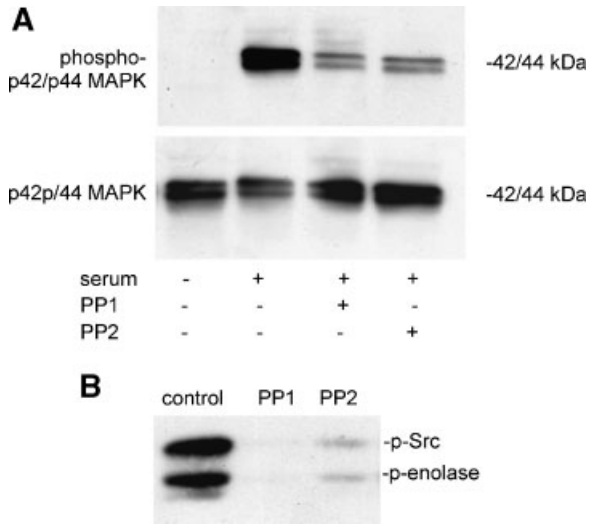

Fig. 2. Serum-induced MAPK activation is blocked by the Src inhibitors PP1 and PP2. **A**: MCF-7 cells were pretreated with PP1 or PP2 (10 μM) for 30 min before incubation with serum (20% FBS) for 3 min. Controls were treated with vehicle alone (DMSO <0.1%). Cells were homogenized and subjected to Western blot analysis using anti-(phospho)-active p42/44 MAPK antibody followed by reblotting with anti-p42/p44 MAPK antibody. Representative blots from three independent experiments are shown. **B**: Inhibition of in vitro Src activity by PP1 or PP2. MCF-7 cell lysates were assayed for Src tyrosine kinase activity as described in Materials and Methods in the absence or presence of 10 μM PP1 or PP2.

hormone (Fig. 3B, Top). As Src activity is lowered by $1\alpha,25(OH)_2D_3$, a lesser extent of autophosphorylation of Tyr-416 may tentatively be the explanation why decreased phosphorylation of the Src band is observed in enzymatic assays. The effects of the hormone on Src activity were less pronounced at 1 and 5 min (Fig. 3B, Bottom).

$1\alpha,25(OH)_2D_3$ Induces Association of Src With VDR

The way by which $1\alpha,25(OH)_2D_3$ modulates the MAP kinase pathway in MCF-7 cells remains unknown. It is possible that the VDR could be involved in the hormone mechanism of action by interacting with Src. The human VDR primary sequence contains a putative tyrosine phosphorylation site corresponding to amino-acids 141–147 (KTYDPTY), which could be recognized by the SH2 domain of Src. In order to evaluate this possibility, VDR-Src association in response to $1\alpha,25(OH)_2D_3$ was investigated. MCF-7 cells were treated with 20% FBS or 20% FBS plus $1\alpha,25(OH)_2D_3$ for 3 min. Cell lysates were immunoprecipitated with anti-Src antibody under non-dissociating conditions, followed by Western blot analysis of VDR, and vice versa. As illustrated in Figure 4 (top and bottom panels), 10 nM $1\alpha,25(OH)_2D_3$ increased the association between Src and VDR, suggesting the involvement of the VDR in the inhibition of Src activity. These effects were also evident at 100 nM $1\alpha,25(OH)_2D_3$ when immunoprecipitation with anti-Src antibody and immunoblotting with anti-VDR antibody were performed (Fig. 4, top panel). Appropriate negative controls in which the immunoprecipitation was performed with non-immune IgGs showed the absence of inmunoreactive bands (−IgG).

Tyrosine Phosphatase Inhibitors Abolish $1\alpha,25(OH)_2D_3$ Effects on MAPK

Tyrosine protein phosphatases play an important role in the generation of signals which may lead to either deactivation or activation of the MAP kinase family [Gomperts et al., 2002]. To obtain additional information regarding the mechanism by which $1\alpha,25(OH)_2D_3$ inhibits serum-induced MAPK activity in MCF-7 cells, experiments were carried out using the cell-permeable tyrosine phosphatase inhibitor orthovanadate or a more potent and specific inhibitor, bpV (phen). Pretreatment (30 min) with 1 mM orthovanadate or 1 μM bpV (phen) prevented MAPK inhibition by $1\alpha,25(OH)_2D_3$ (Fig. 5). These results indicate that tyrosine phosphatases are involved in $1\alpha,25(OH)_2D_3$-inhibition of MAPK activity. Further experiments are required to identify the specific phosphatases mediating this event.

$1\alpha,25(OH)_2D_3$ Inhibits Src Activity and Induces Src-VDR Association in Non-Stimulated MCF-7 Cells

The effects of $1\alpha,25(OH)_2D_3$ on Src activity and VDR-Src interaction were also studied in MCF-7 cells not stimulated with serum. In these conditions, MAP kinase activity levels are undetectable, although Src activity levels are high, as previously reported for various breast cancer cells [Verbeek et al., 1996; Frame, 2002].

MCF-7 cells were treated with $1\alpha,25(OH)_2D_3$ and the tyrosine-phosphorylation state of Src, Src kinase activity and Src-VDR interaction were evaluated. Appropriate hormone dose and time treatments were established by measuring Src kinase activity at different conditions (not shown). Incubation of cells with 1 nM $1\alpha,25(OH)_2D_3$ for 5 min markedly increased

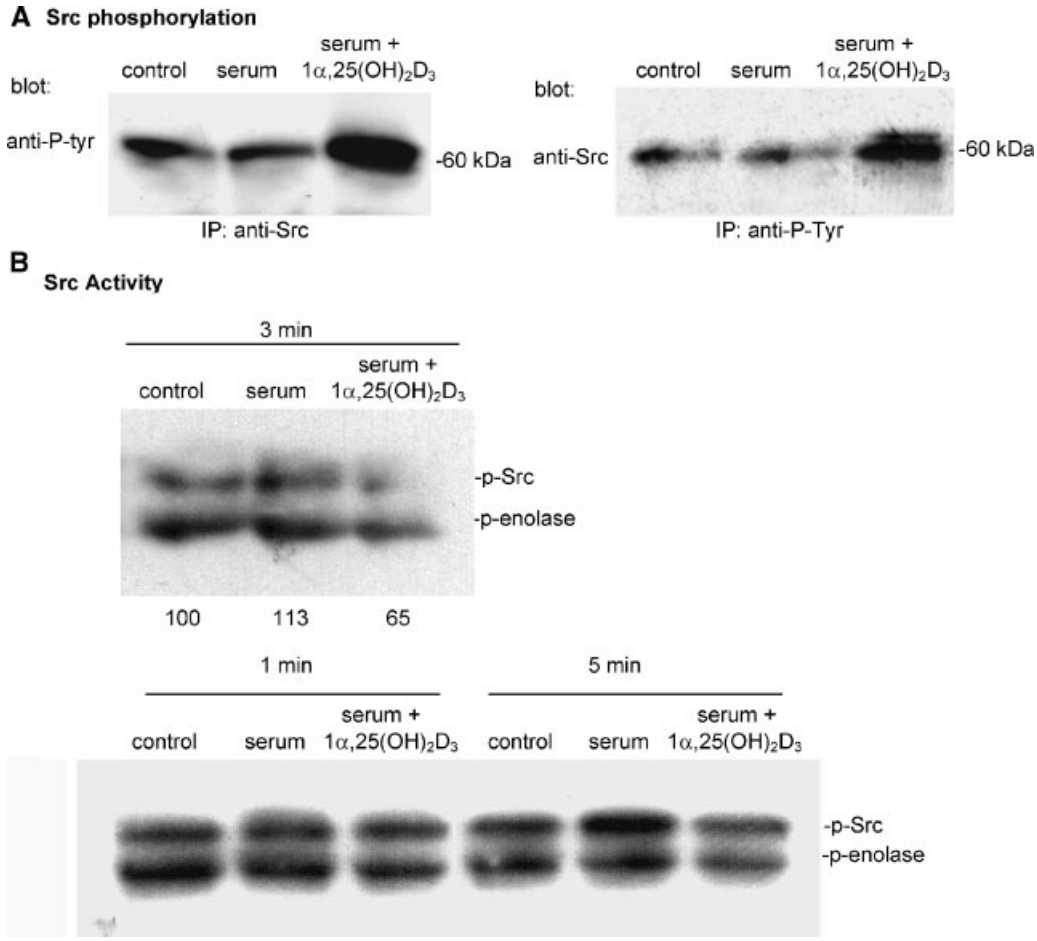

Fig. 3. $1\alpha,25(OH)_2D_3$ increases tyrosine-phosphorylated state of Src and inhibits its activity. **A**: MCF-7 cells were treated with serum (20% FBS) plus vehicle (isopropanol <0.1%) or serum plus $1\alpha,25(OH)_2D_3$ (10 nM) for 3 min. Controls were treated with vehicle alone. Cell lysates were obtained, Src was immunoprecipitated with anti-Src antibody under dissociating conditions, resolved by SDS–PAGE, and then immunoblotted with anti-phosphotyrosine antibody (anti-P-tyr). Immunoprecipitation with anti-P-tyr antibody followed by immunoblotting with anti-Src antibody was also performed. **B**: **Top**: Alternatively, Src tyrosine kinase activity was determined by phosphorylation of enolase with [^{32}P]ATP as described in Materials and Methods. The samples were subjected to electrophoresis, transferred to PVDF membranes followed by autoradiography. The numbers under the lanes represent the intensity of the p-enolase band quantified using the ScionImage program. **Bottom**: Src tyrosine kinase activity was also determined in MCF-7 cells treated with serum (20% FBS) plus vehicle (isopropanol <0.1%) or serum plus $1\alpha,25(OH)_2D_3$ (10 nM) for 1 or 5 min (Bottom). Representative blots (A) and autoradiograms (B) from three independent experiments are shown.

tyrosine phosphorylation of Src; less significant changes were detected after 1 and 3 min treatment intervals (Fig. 6A). The hormone (1 nM, 5 min) inhibited Src activity (Fig. 6B) and induced Src-VDR association (Fig. 6C). Decreased labeling of enolase in Src activity assays (Fig. 6B) should not be ascribed to a general inhibitory effect of $1\alpha,25(OH)_2D_3$ on the incorporation of [γ-^{32}P]ATP to all proteins in MCF-7 cells. It has been shown for other cell types that the hormone does not induce non-specific changes in protein ^{32}P-labeling [Santillán and Boland, 1998; Santillán et al., 1999].

$1\alpha,25(OH)_2D_3$ Induces Translocation of VDR From the Nucleus to the PM

The modulation of second messenger systems by $1\alpha,25(OH)_2D_3$ in MCF-7 cells supports the hypothesis that a membrane located receptor exists. We have recently shown that $1\alpha,25(OH)_2D_3$ increases association of the VDR to the PM of avian muscle cells after 5 min of treatment [Capiati et al., 2002]. In order to find out whether this event also occurs in breast cancer cells, nuclear and PM fractions were purified from MCF-7 cells treated with 20% FBS

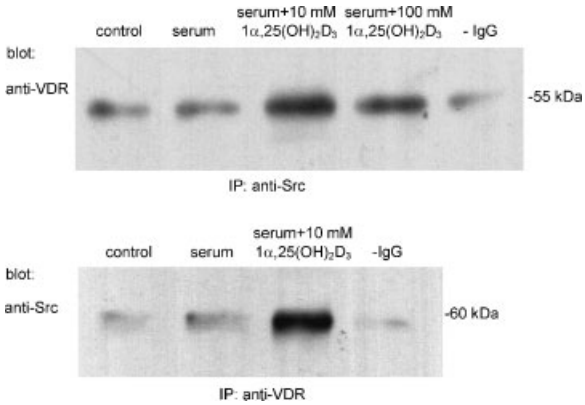

Fig. 4. $1\alpha,25(OH)_2D_3$ induces association of VDR with Src. **A**,**B**: MCF-7 cells were treated with serum (20% FBS) plus vehicle (isopropanol <0.1%) or serum plus $1\alpha,25(OH)_2D_3$ (10 or 100 nM) for 3 min. Controls were treated with vehicle alone. Cell lysates were obtained, Src was immunoprecipitated with anti-Src antibody under native conditions, resolved by SDS–PAGE, and then immunoblotted with anti-VDR antibody as described in Materials and Methods. Immunoprecipitation with anti-VDR antibody followed by immunoblotting with anti-Src was also performed. In each case, controls were performed using sepharose beads alone, without antibody (−IgG). Representative blots from four independent experiments are shown.

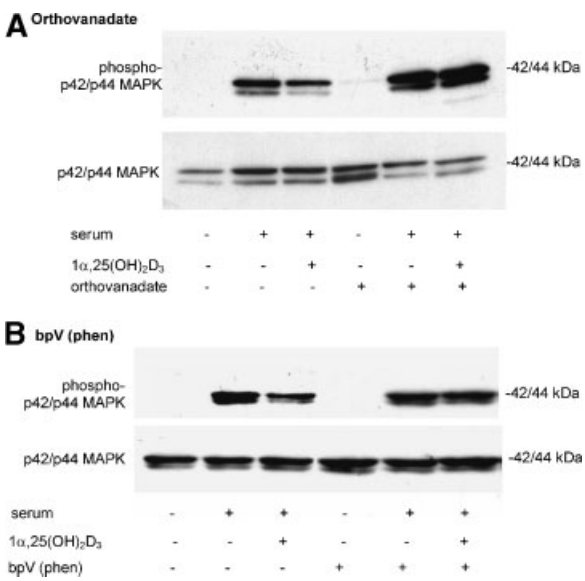

Fig. 5. Tyrosine phosphatase inhibitors abolish $1\alpha,25(OH)_2D_3$ inhibition of MAPK. MCF-7 cells were pretreated with 1 mM orthovanadate (**A**) or 1 μM bpV (phen) (**B**) for 30 min followed by incubation with serum (20% FBS) plus vehicle (isopropanol <0.1%) or serum plus $1\alpha,25(OH)_2D_3$ (10 nM) for 3 min. Controls were treated with vehicle alone. Cells were homogenized and subjected to Western blot analysis using anti-(phospho)-active p42/p44 MAPK antibody followed by reblotting with anti-p42/p44 MAPK antibody. Representative blots from four independent experiments are shown.

or 20% FBS plus $1\alpha,25(OH)_2D_3$ for 3 min, followed by Western blot analysis of the VDR. The purity of the PM fraction was established by immunoblot analysis revealing the absence of lamin B, a nuclear specific protein, and the presence of Gαs, a PM-associated protein; also, PMs were not significantly contaminated with ER as determined by its specific marker glucose-6-phosphatase (Fig. 7A). As shown in Figure 7B, $1\alpha,25(OH)_2D_3$ decreased VDR levels in the nuclear fraction, whereas it increased the amount of VDR associated to the PM in serum-stimulated cells. Similar results were obtained when MCF-7 cells were treated with $1\alpha,25(OH)_2D_3$ alone, without serum (Fig. 7C). However, as shown in Figure 7D, $1\alpha,25(OH)_2D_3$ did not change the amounts of PM-bound estrogen receptor α (ERα), a non-relevant protein previously shown to be localized both in the nucleus and surface of MCF-7 cells [Monje et al., 2001].

DISCUSSION

It is well known that many growth factors contained in serum have a stimulating influence on cell proliferation. They bind to receptor protein tyrosine kinases (RTK), inducing the phosphorylation of tyrosine residues on the cytoplasmic domain of the RTK which can interact with SH2 or PTB domains of a diversity of effector molecules, among them, the tyrosine kinases of the Src family and the adaptor protein Grb2 of Ras signal transduction. Of relevance in the control of cell proliferation is the modulation of the Ras-MAP kinase cascade by RTK. After its activation, the adaptor protein Grb2, in association with the guanine exchange factor Sos, attaches to the tyrosine phosphorylated receptor through its SH2 domains. The Grb2/Sos complex catalyzes the guanine nucleotide exchange in Ras. The activated Ras associates with the serine/threonine protein kinase Raf-1 which phosphorylates and thereby activates the dual specificity kinase MEK. MEK phosphorylates the ERK (p42/p44 MAPK) on both tyrosine and threonine residues. As a result of the double phosphorylation, MAPK becomes active and translocates to the nucleus. This is followed by activation of early response genes, such as c-Fos, c-myc, and c-jun, which is mediated via phosphorylation of transcription factors such as Elk-1 [Gomperts et al., 2002].

Within the described conceptual framework, we observed that serum activates MAPK and

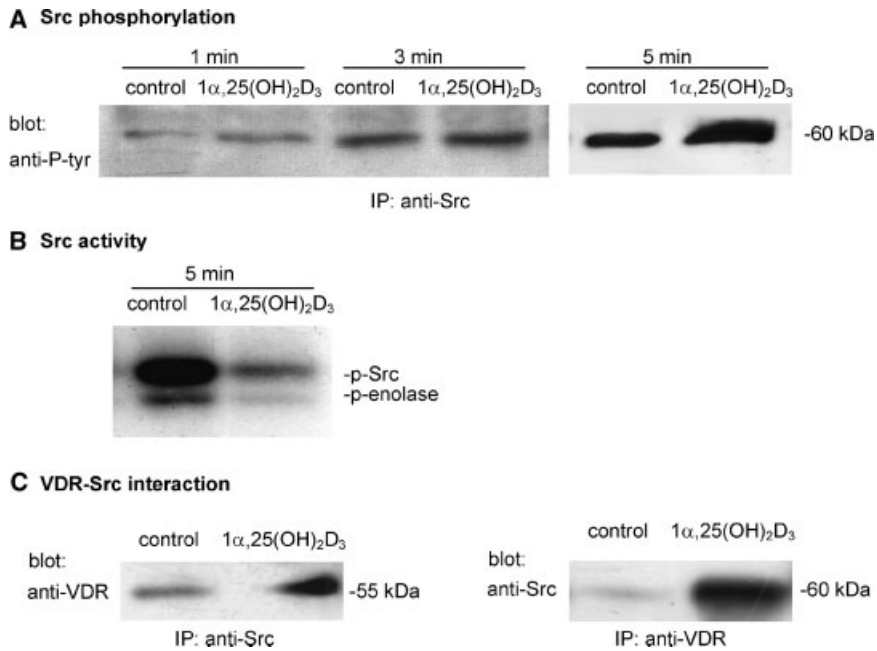

Fig. 6. 1α,25(OH)$_2$D$_3$ inhibits Src activity and induces Src-VDR association in non-stimulated MCF-7 cells. **A**: MCF-7 cells were treated with 1α,25(OH)$_2$D$_3$ (1 nM) for 1, 3, or 5 min. Controls were treated with vehicle alone (isopropanol <0.1%). Cell lysates were obtained, Src was immunoprecipitated with anti-Src antibody under dissociating conditions, resolved by SDS–PAGE and then immunoblotted with anti-phosphotyrosine antibody. **B**: Alternatively, Src tyrosine kinase activity was determined by phosphorylation of enolase in cells treated for 5 min as described in the legend to Figure 3. **C**: MCF-7 cells were treated with 1α,25(OH)$_2$D$_3$ (1 nM) for 5 min. Controls were treated with vehicle alone (isopropanol <0.1%). Cell lysates were obtained, Src was immunoprecipitated with anti-Src antibody under native conditions, resolved by SDS–PAGE and then immunoblotted with anti-VDR antibody as described. Immunoprecipitation with anti-VDR antibody followed by immunoblotting with anti-Src was also performed. Representative blots (A,C) and autoradiogram (B) from three independent experiments are shown.

promotes its translocation into the nucleus in MCF-7 cells, indicative of the mitogenic action of serum growth factors. More important, the present study provides new information on the non-genomic mechanism by which 1α,25(OH)$_2$D$_3$ may exert antiproliferative effects on breast cancer cells. Thus, it was shown that the hormone rapidly (within 3 min) opposes serum-induced activation and translocation of MAP kinase, using a concentration range (1–100 nM) at which 1α,25(OH)$_2$D$_3$ has been shown to cause growth inhibition of breast cancer cells both in vitro and in vivo [Van Weelden et al., 1998; Colston and Hansen, 2002; Welsh et al., 2002, and references therein]. To our knowledge, these are the first data demonstrating that the hormone antagonizes the mitogenic action of serum, or the growth factors therein, on the MAPK cascade of tumorigenic breast cells. The inhibitory effects of 1α,25(OH)$_2$D$_3$ on ERK 1/2 were revealed by a marked decrease of both serum-induced phosphorylation of the p42 and p44 MAPK isoforms and MAPK activity, and the inhibition of serum-induced MAPK translocation into the nucleus.

It is important to take into consideration that the effect of the hormone on MAP kinase activity seems to be dependent on the cell type. In addition to the inhibitory effect reported here, it has been shown that in NB4 and HL60 leukemia cells, 1α,25(OH)$_2$D$_3$ stimulates MAPK [Song et al., 1998; Wang and Studzinski, 2001].

Evidence was obtained indicating that Src is involved in the activation of the mitogenic MAP kinase cascade by serum growth factors and the counter effects of 1α,25(OH)$_2$D$_3$ thereupon. Src belongs to a family of closely related cytoplasmic tyrosine-specific protein kinases involved in the regulation of cell division, cell differentiation, and cell aggregation. Src itself is involved in signal transduction via growth factor receptors. Thus, functional interactions have been described with Tyr-P residues of PDGF and EGF receptors [Anderson et al., 1990]. The Src kinases family share a similar structure. An unique domain at the N-terminus is followed by SH3 and SH2 domains. Following this, there is a kinase domain and finally a short C-terminal tail. The N-terminal domain of Src

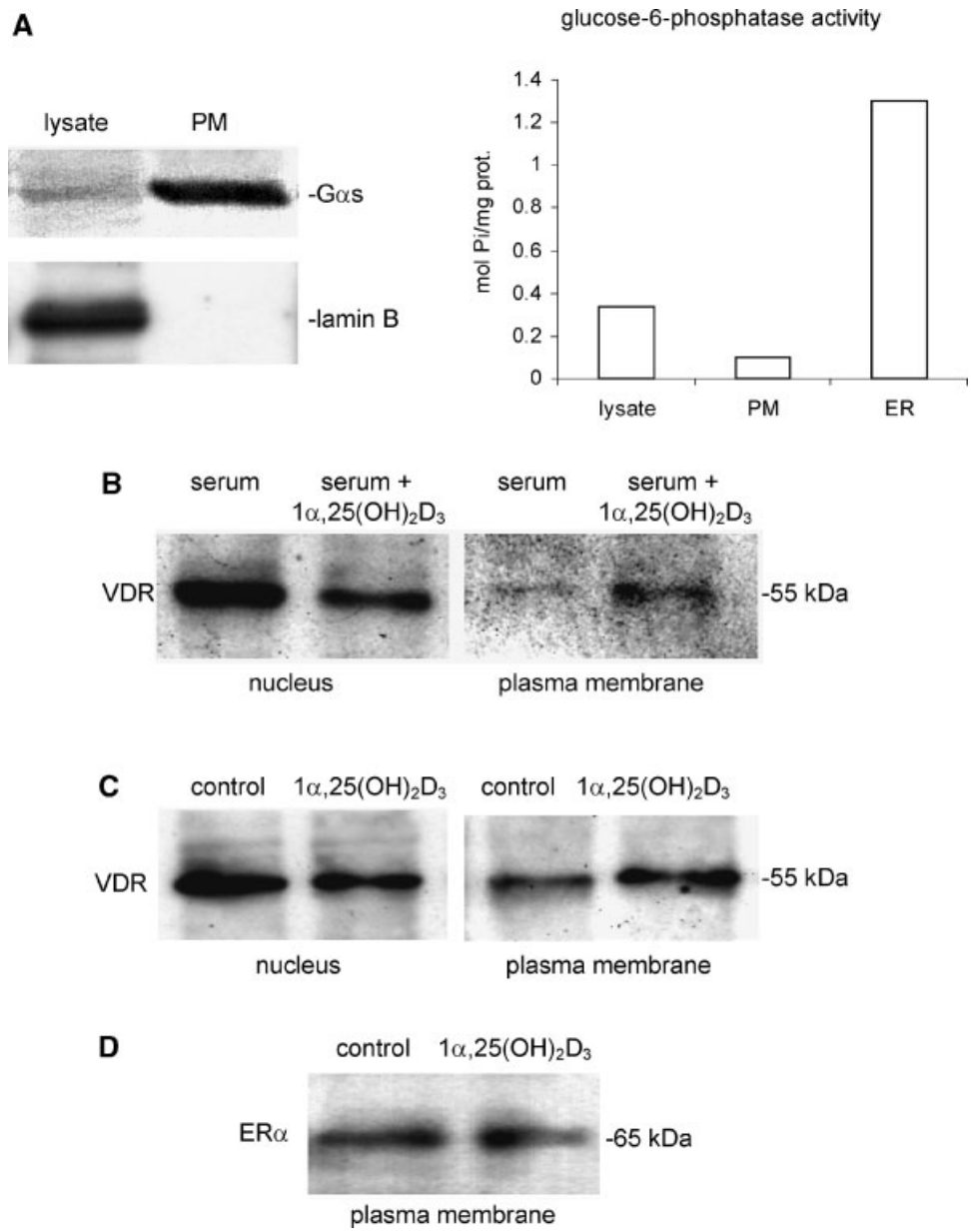

Fig. 7. $1\alpha,25(OH)_2D_3$ increases VDR association to the plasma membrane. **A**: Characterization of the plasma membrane (PM) fraction isolated from MCF-7 cells as described in Materials and Methods. Proteins from whole cell lysates and PM fractions were subjected to Western blot analysis for Gαs protein and lamin B. Glucose-6-phophatase activity was assayed in whole cell lysates, plasma membrane and endoplasmic reticulum (ER) fractions. **B**: MCF-7 cells were treated with serum (20% FBS) plus vehicle (isopropanol <0.1%) or serum plus $1\alpha,25(OH)_2D_3$ (1 nM) for 3 min. **C**: Alternatively, MCF-7 cells were treated with $1\alpha,25(OH)_2D_3$ (1 nM) or vehicle (control, isopropanol <0.1%) for 3 min. Cells were homogenized followed by isolation of nuclear and plasma membrane fractions and Western blot analysis of VDR. **D**: MCF-7 cells were treated as in (C) followed by Western blot analysis of estrogen receptor α(ERα). Representative blots from three independent experiments are shown.

can be myristoylated to anchor to the PM, which promotes association of the kinase with macromolecular signaling complexes assembled at membranes sites. Phosphorylation at Tyr-527, which is catalyzed by a Src specific protein kinase (Csk), and phosphorylation at Tyr-416, are key elements of regulation [Sicheri and Kuriyan, 1997; Xu et al., 1997]. The phosphorylation at Tyr-527, located in the C-terminus, inhibits the Src kinase by intramolecular binding with the SH2 domain forming a loop which distorts the catalytic domain. Activation requires removal of the C-terminal phosphate, and then phosphorylation at Tyr-416.

In accordance with the above information, it was shown that the Src inhibitors PP1 and PP2 diminished serum-induced MAP kinase activation, indicating that Src mediates the stimulation of MAPK by serum growth factors upstream in the mitogenic signaling pathway. Increased Src tyrosine phosphorylation and decreased Src kinase activity observed after treatment with $1\alpha,25(OH)_2D_3$ suggests that the hormone exerts its inhibitory effect on MAPK, at least in part, at the level of Src.

In the search for evidence on the possible basis underlying the mechanism by which $1\alpha,25(OH)_2D_3$ modulates Src activity, we obtained data indicating that the hormone increases VDR-Src association. Previous studies with avian muscle cells have shown $1\alpha,25(OH)_2D_3$-induced VDR-Src interaction correlated with tyrosine phosphorylation of the VDR, in agreement with the fact that the SH2 domain of Src specifically recognizes phosphotyrosine residues [Buitrago et al., 2000]. However, the significance of this interaction on the inhibition of Src by the hormone in MCF-7 cells should be further investigated.

The present work shows for the first time in a mammalian cell system reverse trafficking of the VDR, that is, from the nucleus to the PM, in response to $1\alpha,25(OH)_2D_3$. Once located in the membrane, the VDR may associate to Src through P-tyr-SH2 domain interactions as mentioned above. Additional studies are required to demonstrate that is the membrane-bound VDR which interacts with Src.

Noteworthy, the fact that treatment of MCF-7 cells with $1\alpha,25(OH)_2D_3$ under basal conditions, that is, without serum, also resulted in increased tyrosine phosphorylation of Src, inhibition of Src activity and augmented interaction VDR-Src, strengthens the concept that an intrinsic cell mechanism is involved. Under basal conditions it is quite difficult to detect P-MAPK; therefore, the experiments described before were carried out in the presence of serum to upregulate the cascade and be able to measure P-MAPK.

The tyrosine phosphatase inhibitors orthovanadate or bpV (phen) prevented MAPK inhibition by $1\alpha,25(OH)_2D_3$, suggesting that tyrosine phosphatases are involved somehow in the mechanism by which $1\alpha,25(OH)_2D_3$ inhibits MAPK activity. Protein tyrosine phosphatases comprise a large family of multidomain proteins having an exceptional diversity and which play an important role in the generation of signals for both deactivation and

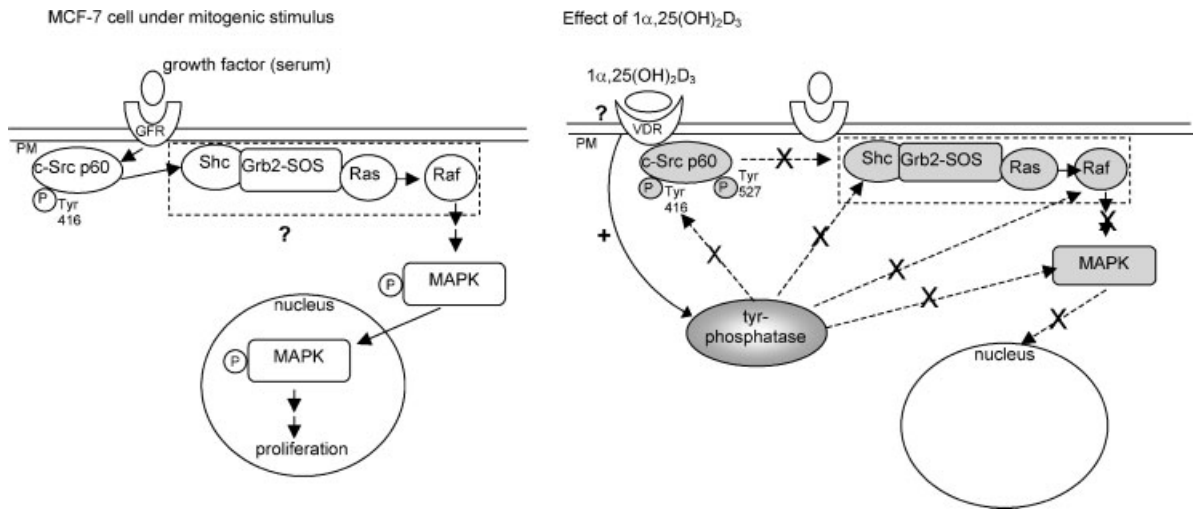

Fig. 8. Proposed model for the sequence of molecular events based on these studies. **Left**: Mitogenic factors contained in serum stimulate the MAPK pathway and thus promote proliferation. Growth factors can bind to cell membrane receptors (GFR) and activate Src, a cytosolic tyrosine kinase which can trigger the Ras/Raf/MAPK cascade through the recruitment of the Shc-Grb2-Sos adaptor complex. Upon activation, MAPK kinases translocate to the nucleus where they activate nuclear transcription factors involved in proliferation. **Right**: A mechanism by which $1\alpha,25(OH)_2D_3$ inhibits breast cancer cell proliferation could be through a blockade of the mitogenic effects of growth factors. The hormone may bind to a yet unidentified plasma membrane-located receptor (VDR) to trigger signaling events that lead to the inactivation of MAPK. $1\alpha,25(OH)_2D_3$ induces Src-VDR interaction and Src tyrosine kinase inactivation, and consequently turns off the Ras/Raf/MAPK cascade. On the other hand, the hormone may activate tyrosine phosphatase(s) which can dephosphorylate the positive regulatory tyrosine (Tyr-416) in Src kinase domain contributing to its inactivation or act over other components of the cascade with phosphorylated tyrosine residues, for example, Shc, Raf, or MAPK itself.

activation [Fisher et al., 1991]. Of potential relevance for our work, it has been shown in other cell types that Csk binds with high affinity to a family of cytoplasmic protein tyrosine phosphatases which can dephosphorylate the Src family kinases [Wang et al., 2001]. In a complex together, the functional effects of both the kinase Csk and the phosphatases are augmented, suggesting that these enzymes act synergistically to inactive Src kinases. However, the possibility that other upstream components of the cascade with phosphorylated tyrosine residues, for example, Shc, are involved, cannot be excluded. In addition, the subclass of dual-specific protein phosphatase, for example, MAP kinase phosphatase-1 (MKP-1), whose substrates are predominantly members of the MAP kinase family [Sun et al., 1993; Assoian, 1997], may also participate in $1\alpha,25(OH)_2D_3$ inhibition of the MAPK kinase pathway. Clearly, it is necessary to identify the phosphatase(s) which mediate the hormone effects.

In addition to the non-genomic mechanism that may lead to the antiproliferative response to $1\alpha,25(OH)_2D_3$ in MCF-7 cells, it is possible that in this effect the classical genotropic action could also be involved. Thus, in cells of the hematopoietic lineage, the antiproliferative effects of the steroid have been shown to be mediated by the genotropic mechanism involving VDR-dependent regulation of cyclin kinase inhibitors [Liu et al., 1996]. Moreover, it has been observed that $1\alpha,25(OH)_2D_3$ inhibited cell proliferation of MCF-7 cells through G1 arrest due to the upregulaition of p21 and p27 cyclin kinase inhibitors [Verlinden et al., 1998].

In conclusion, the data presented suggest that $1\alpha,25(OH)_2D_3$ exerts inhibitory effects on the MAPK cascade by a non-genomic mechanism which involves at least the inactivation of Src and the stimulation of protein tyrosine phosphatases. This hypothetical mechanism is depicted in the diagram of Figure 8. The relative roles of these events and their interrelationships as well as the exact function of the VDR need to be investigated to understand the hormone mode of action. Such studies may provide clues on how $1\alpha,25(OH)_2D_3$ negatively regulates growth of breast cancer cells.

ACKNOWLEDGMENTS

Ana Rossi is a recipient of a graduate research fellowship from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

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