Regulation of vitamin D receptor expression via estrogen-induced activation of the ERK 1/2 signaling pathway in colon and breast cancer cells

Liat Abovich Gilad, Tali Bresler, Julia Gnainsky, Patricia Smirnoff and **Betty Schwartz**

Institute of Biochemistry, Food Science and Nutrition, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel

(Requests for offprints should be addressed to B Schwartz; Email: bschwart@agri.huji.ac.il)

Abstract

We previously demonstrated that 17β-estradiol (E2) regulates the transcription and expression of the vitamin D receptor (VDR) in rat colonocytes and duodenocytes *in vivo*. The aim of the present study was to assess whether the extracellular signal-regulated kinase (ERK) induced by E2 is involved in regulating VDR expression. We compared E2-associated signaling activity in HT29 colon cancer cells, a non-classical E2-target, with that in MCF-7 breast cancer cells, the natural targets of the hormone. E2 did not affect proliferation of HT29 cells, but enhanced proliferation of MCF-7 cells. Vitamin D inhibited proliferation of both cell lines and the combined treatment induced potentiation of vitamin D activity. E2 upregulated VDR transcription and protein expression concomitantly with ERK 1/2 phosphorylation in both cell lines. PD98059, a specific mitogen-activated protein kinase (MAPK) inhibitor, prevented E2-mediated activation of ERK 1/2, with concomitant inhibition of VDR expression. ICI182780 inhibited VDR expression in HT29 and MCF-7 cell lines. A conjugate of E2 and bovine serum albumin upregulated phosphorylation of ERK 1/2 and concomitantly enhanced VDR expression in a similar fashion as the nonconjugated hormone. Expression of ERα and ERβ was detected in MCF-7 and HT29 cell lines respectively, which localized to the nuclei, cytosol and caveolar membrane rather than non-caveolar membrane. Disruption of lipid rafts/caveolae by depleting cellular cholesterol with the cholesterol-binding reagent β-methylcyclodextrin blocked ERK 1/2 phosphorylation concomitantly with VDR upregulation. The tyrosine phosphorylation inhibitor suramin and src kinase inhibitor PP2 inhibited both ERK 1/2 phosphorylation and VDR expression. E2 induced phosphorylation of Raf and Jun in a time-dependent manner. The Ras/Raf dependent inhibitor of transactivation sulindac sulfide also blocked E2 effects. The specific c-Jun phosphorylation inhibitor SP600125 dose dependently inhibited c-Jun phosphorylation and VDR expression. The MAPK/ERK kinase inhibitor PD 98059 downregulated both c-Jun phosphorylation and VDR expression indicating that upstream and downstream events in the signaling cascade are all related to the control of VDR expression. Taken together, our data suggest that E2 binds to receptors compartmentalized to membranal caveolar domains in HT29 and MCF-7 cells, inducing ERK 1/2 activation and transcriptional activity, which finally results in upregulation of expression of the VDR gene.

Journal of Endocrinology (2005) **185,** 577–592

Introduction

Estrogen receptor (ER) is a member of a superfamily of nuclear transcription factors. The cellular actions of estrogen are thought to be mediated through transcriptional regulation of target genes (Halachmi *et al.* 1994, Glass *et al.* 1997). This process occurs mainly when 17β-estradiol (E2) binds to nuclear ER. The resultant complex then binds directly to response elements present on various genes (Nilsson *et al.* 2001) or modifies transcription through protein–protein interactions prior to DNA binding (Pfahl 1993). When ER binds to E2, it undergoes a conformational change that results in dimerization, binding to specific DNA elements, and transcriptional regulation of target genes (Nahmias & Strosberg 1995, Csikos *et al.* 1998). It is becoming increasingly clear, however that multiple ligands for the steroid receptor superfamily can modulate cell function through nongenomic actions mediated through plasma-membrane proteins (Blackmore *et al.* 1991, Nemere *et al.* 1994, Wehling 1995). For example there is evidence that E2 can trigger a variety of signal-transduction events within seconds to a few minutes. These events include stimulation of adenylate cyclase (Aronica *et al.* 1994), activation of phospholipase C

DOI: 10.1677/joe.1.05770
0022–0795/05/0185–577
Online version via http://www.endocrinology-journals.org

(Le Mellay *et al.* 1997), and triggering of an intracellular calcium spike (Tesarik & Menoza 1995). Additionally, a putative cell-membrane ER, the existence of which was first reported more than 20 years ago (Pietras & Szego 1977, 1980), appears capable of activating signal-transduction pathways according to more recent investigations (Berthois *et al.* 1986, Pappas *et al.* 1995).

There is also evidence that E2 may be directly involved in determining intestinal calcium absorption. Because intestinal calcium absorption decline with age in humans (Avioli *et al.* 1965, Bullamore *et al.* 1970) and rats (Russell *et al.* 1986), a commonly held hypothesis suggests that this decreased absorption results from a sequence of events initiated by low estrogen levels, which result in bone resorption. According to this hypothesis, the released calcium increases the calcium concentration in extracellular spaces, thus suppressing parathyroid hormone secretion, a decrease in $1,25(OH)_2D_3$ production and plasma concentration follows, finally resulting in decreased intestinal calcium absorption (Gallagher *et al.* 1979).

E2 receptors (Francavilla *et al.* 1987, Meggouh *et al.* 1991, Hendrickse *et al.* 1993), as well as the ER-associated proteins pS2 antigen (Theisinger *et al.* 1993, Luqmani *et al.* 1992, Welter *et al.* 1994) and ER-D5 (Takeda *et al.* 1992), are consistently found in the mucosa along the alimentary tract, suggesting a specific physiologic role for E2 in the intestine. In this regard, available data indicate that decreased basal levels of $1,25(OH)_2D_3$ cannot solely account for the decrease in calcium absorption (Francis *et al.* 1984), suggesting that the intestines of estrogen-depleted elderly or ovariectomized women are resistant to $1,25(OH)_2D_3$ (Gennari *et al.* 1990). In addition, E2 administration has been shown to effectively restore the normal responsiveness of the intestine to $1,25(OH)_2D_3$ in ovariectomized pre- (Gennari *et al.* 1990) and postmenopausal (Civitelli *et al.* 1988, Heaney *et al.* 1978) women.

Previous studies have also indicated an age-related decrease in the number of VDR (Horst *et al.* 1990, Takamoto *et al.* 1990). The number of VDR is a primary determinant of the biological response to $1,25(OH)_2D_3$, as previously shown in osteoblastic cell lines (Dokoh *et al.* 1984, Liel *et al.* 1992) and in human populations in association with VDR-gene polymorphism (Gross *et al.* 1996, Arai *et al.* 1997, Harris *et al.* 1997). E2 has been shown to increase the number of VDR in the osteoblast-like cell line ROS 17/2·8, an increase associated with enhanced responsiveness of the cells to $1,25(OH)_2D_3$ (Liel *et al.* 1992). Increased VDR expression following E2 treatment has also been noted in other tissues and cell types, such as the uterus (Walters 1981, Levy *et al.* 1984), liver (Duncan *et al.* 1991), and human breast cancer cells (Escaleira *et al.* 1993).

Rapid activation of mitogen-activated protein kinase (MAPK) by E2 in ROS 17/2·8 cells has provided the first evidence of MAPK activation by E2 through phosphorylation, indicating the involvement of putative plasma-membrane receptors (Migliaccio *et al.* 1996, Endoh *et al.* 1997). Rapid effects exerted by E2 on growth-factor-related signaling pathways have also been demonstrated in neuronal cells, suggesting a potential mechanism by which E2 might affect the expression of genes with promoters that do not contain strictly estrogen-responsive elements but are responsive to factors acting through other response elements, such as activation protein-1 (AP-1) and serum response elements (Watters *et al.* 1997).

Detailed mechanisms whereby MAPK activation mediates E2 gene regulation remain speculative. The present study was designed to investigate the nature of the interactions between E2, the MAPK signaling pathway, and VDR in cells representative of various organs: namely HT29 cells, considered to be classic non-E2 targets, and MCF-7 cells, considered to be typical E2 targets. Herein we present molecular evidence that E2 regulates VDR gene expression via binding to caveolar membranal ER, and activates downstream signaling involving the MAPK pathway.

Materials and Methods

Materials

Suramin, sulindac sulfide, β-methylcyclodextrin and an E2–6-(O-carboxylmethyloxime-BSA)-conjugate were purchased from Sigma Chemical Co. (St Louis, MO, USA). PP2 was purchased from Calbiochem (La Jolla, CA, USA). Tissue culture media and antibiotic antimycotic solution supplements were obtained from Biological Industries Beit Haemek LTD, Israel. The PhosphoPlus p42/44 MAPK antibody kit was from New England Biolabs, Inc. (Beverly, MA, USA). Monoclonal human anti-VDR antibody and rabbit anti-Raf-1 antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit anti-ERβ polyclonal antibody was from Chemicon (Temecula, CA, USA), and ERα monoclonal antibody was from Cell Signaling Technology, Inc. (Beverly, MA, USA). Polyclonal antiserum to caveolin-1 was from Transduction Laboratories, Inc. (Lexington, KY, USA). Phospho-Raf, Phospho-c-Jun and c-Jun antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The enhanced chemiluminescence kit was from Amersham Biosciences (Buckinghamshire, UK). The protein determination kit, based on bicinchoninic acid, was obtained from Pierce (Rockford, IL, USA). PD98059, an inhibitor of MEK activation was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). ICI182780 was purchased from Tocris (Bristol, UK). All other biochemicals were purchased from Sigma Chemical Co.

Cell lines and culture conditions

HT29, a human colon adenocarcinoma cell line, was cultured in DMEM supplemented with 10% (w/v) FCS,

1% (w/v) L-glutamine, and 0·2% (w/v) antibiotic antimycotic solution 1 and was maintained under a humidified atmosphere and 5% carbon dioxide at 37 °C. MCF-7, a human breast cancer cell line, was cultured similarly, but the medium was also supplemented with 2% (w/v) insulin solution. MCF-7 cells were grown to 80–90% confluence and the medium was replaced every other day.

Treatment of cells

Cells were harvested, washed twice by centrifugation in PBS and then cultured in 50 ml flasks or into 6-well plates in DMEM with phenol red (PR) or DMEM without PR supplemented with 10% charcoal-stripped FCS, 1% L-glutamine and 0·2% antibiotic solution. Cells were treated with different concentrations of E2 (from 10^{-11} to 10^{-7} M) dissolved in ethanol. Control cultures included ethanol at a final concentration of 0·0067% in the medium. For proliferation analyses, cells were exposed to a medium containing the designated treatment for 6 days, which was found to be the optimal time period in preliminary experiments (data not shown). Treatment and medium were replaced every other day. To study the involvement of the MAPK signal-transduction pathway, cells were treated with different concentrations of E2 in the presence or absence of 5×10^{-5} M PD98059 or IGF-I (100 ng/ml). To study the role of ER, cells were treated with 10^{-6} M ICI182780. To study the role of membrane-bound tyrosine phosphorylation activity, cells were treated with suramin at concentrations between $0{\cdot}05 \times 10^{-3}$ M and 4×10^{-3} M and concomitantly incubated with E2 to determine ERK 1/2 activation analyses or VDR expression. In companion studies cells were treated with different concentrations of PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) which was purchased from Calbiochem and dissolved in dimethyl sulfoxide (DMSO). Sulindac sulfide was also dissolved in DMSO and added to cells at a concentration of 100 μM. The potent inhibitor of c-Jun N-terminal kinase (SP600125) was obtained from Calbiochem and dissolved in DMSO. Drugs were freshly diluted in culture media for each experiment.

Subcellular fractionations

Subcellular fractions were prepared from HT29 or MCF-7 cells. Cells were washed three times with PBS, and then homogenized at 1000 ***g*** in 50 mM Tris–HCl (pH 7·5) containing general-purpose protease inhibitor cocktail basically as recently described by Chaban *et al* (2004). Nuclear pellets were collected through low-speed centrifugation. The remaining supernatant was centrifuged at 49 000 ***g*** for 15 min at 4 °C. The supernatant, containing nonmembrane proteins, i.e. the cytosol, was collected. The pellet was washed with 300 μl of 50 mM Tris pH 7·5 containing protease inhibitors and centrifuged at 49 000 ***g*** for 15 min at 4 °C to pellet the membranes. Different subcellular protein fractions were separated on SDS-PAGE gels, blotted and probed with antibodies against ERα and ERβ.

Isolation of caveolae

HT29 and MCF-7 cells were grown to confluence in 150 mm dishes and non-treated or treated with E2 (10–7 M). We prepared caveolin-enriched membrane fractions, without the use of detergent and the use of sodium carbonate buffer, following the detergent-free protocol developed by Song *et al*. (1995). After two washes with ice-cold PBS, HT29 and MCF-7 (two confluent 150 mm dishes) were scraped into 2 ml of 500 mM sodium carbonate, pH 11·0. Homogenization was carried out sequentially in the following order using a loose-fitting Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10 s bursts; Kinematica GmbH, Brinkmann Instruments, Westbury, NY, USA), and a sonicator (three 20 s bursts; Branson Sonifier 250, Branson Ultrasonic Corp., Danbury, CT, USA). The homogenate was then adjusted to 45% sucrose by the addition of 2 ml of 90% sucrose prepared in MES-buffered saline (MBS) (25 mM MES, pH 6·5, 0·15 M NaCl) and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient was formed above (4 ml of 5% sucrose/4 ml of 35% sucrose; both in MBS containing 250 mM sodium carbonate) and centrifuged at 200 000 ***g*** for 16–20 h in an SW41 rotor (Beckman Instruments, Palo Alto, CA, USA). A light-scattering band confined to the 5–35% sucrose interface was observed that contained caveolin but excluded most other cellular proteins.

Successful separation of caveolae from non-caveolae plasma membrane was confirmed by immunoblot analyses for the caveolae marker protein caveolin-1 (Chang *et al*. 1994).

Western blot analysis

Cells were lysed, electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gels, transferred to nylon-transfer membranes (Amersham Biosciences, Buckinghamshire, UK), blocked in 10^{-3} M Tris-base and 0·1 M sodium chloride, containing 5% dry nonfat milk, incubated with monoclonal human anti-VDR antibody, and subsequently incubated with a secondary antibody coupled to horseradish peroxidase. Proteins were visualized using an ECL kit (Amersham Biosciences, Buckinghamshire, UK). Signal intensities were analyzed by a BAS1000 Bio-Image Analyzer (Fuji, Tokyo, Japan) and the densities were quantified with the NIH computer program ImageJ 1·19 (NIH, Bethesda, MD, USA).

To determine ERK 1/2 phosphorylation, HT29 and MCF-7 cells were plated in six-well plates in DMEM-PR and gradually deprived of FCS as follows: cells were

exposed for 2 days to 0·5% charcoal-stripped FCS–DMEM-PR, and then to media devoid of FCS for 24 h including different concentrations of E2 in the presence or absence of 5×10^{-5} M PD98059. Western blot was performed on cell lysates using a rabbit polyclonal phospho-p42/44 MAPK (Thr202/Tyr204) antibody.

Effect of the E2-BSA conjugate on ERK 1/2 phosphorylation

Before each experiment, stock solutions of BSA conjugates were submitted to charcoal dextran (0·05 mg/ml) and charcoal (50 mg/ml) for 30 min, centrifuged at 3000 ***g*** for 10 min and filtered through a 0·22 m filter to obtain E2-BSA free of unbound E2. E2-BSA was dissolved in phenol-free growth medium at 0·2 mg/ml. The concentration of BSA conjugate was adjusted to the values of the free hormone.

RT-PCR analyses

RNA isolation was preformed using Tri Reagent solution (MRC, Cincinnati, OH, USA). RT-PCR assay was performed using the Promega kit assay (Madison, WI, USA). The specific selected VDR primers were 5′-ATGCCATCTGCATCGTCTC-3′ and 5′-GCACCG CACAGGCTGTCCTA-3′. The PCR protocol was 5 min at 94 °C, then 31 cycles (1 min at 94 °C, 1 min at 54 °C and 1 min at 72 °C), and finally 10 min at 72 °C.

Statistical analyses

Presented data represent means ± S.E Differences between the control and treatments were evaluated by Student's *t*-test.

Results

Expression of ERα and ERβ in HT29 and MCF-7 cells

HT29 and MCF-7 cell lines were chosen for the present studies because HT29 cells are considered classic non-E2 targets, whereas MCF-7 cells are considered typical E2 targets. Western blot analyses were performed with specific anti-ERα and -ERβ antibodies. MCF-7 cells expressed both ERα and ERβ, but in HT29 cells, we detected only ERβ (Fig. 1). Equal loading was verified by Ponceau red staining (not shown).

Effect of E2, vitamin D and combination of E2 and vitamin D on cell growth

We analyzed whether E2 can directly affect the proliferation of HT29 and MCF-7 cells. E2 induced enhanced proliferation of MCF-7 cells at a concentration range

Figure 1 Expression of ERα and ERβ in HT29 and MCF-7 cells. Western blot of HT29 and MCF-7 cell lysates with specific ER antibodies.

of 10^{-7}–10^{-11} M (Fig. 2B). In contrast, E2 at the same concentrations did not exert any significant effect on the HT29 proliferation rate after 3, 6 or 9 days at any of these hormone concentrations (Fig. 2A). This difference may be due to the differential repertoires of expression of ERα and ERβ in these cell lines (see Fig. 1). We next analyzed whether E2 and vitamin D (D), as single treatments or in combination, can affect HT29 and MCF-7 cell proliferation. The treatment lasted 6 days. Figs 3A and B show that E2 at 10^{-8} M did not inhibit proliferation in HT29 or MCF-7 cells, but, a significant anti-proliferative effect was

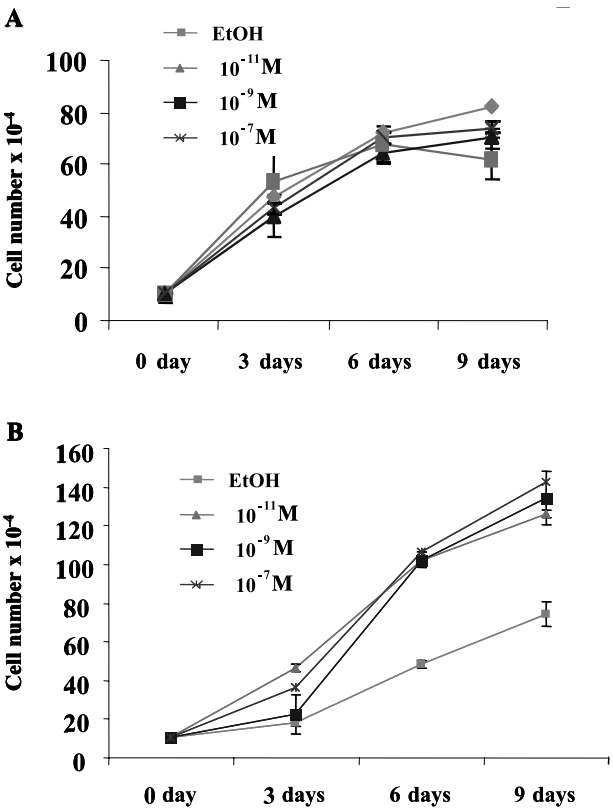

Figure 2 Effect of E2 on HT29 and MCF-7 cancer cell growth. HT29 (A) and MCF-7 (B) cells were treated with different E2 concentrations for 3, 6, or 9 days. Medium was replaced every other day. Control cells were treated with the E2 vehicle (ethanol) at the maximum concentration used in the experiment, 0·0067%. EtOH, ethanol.

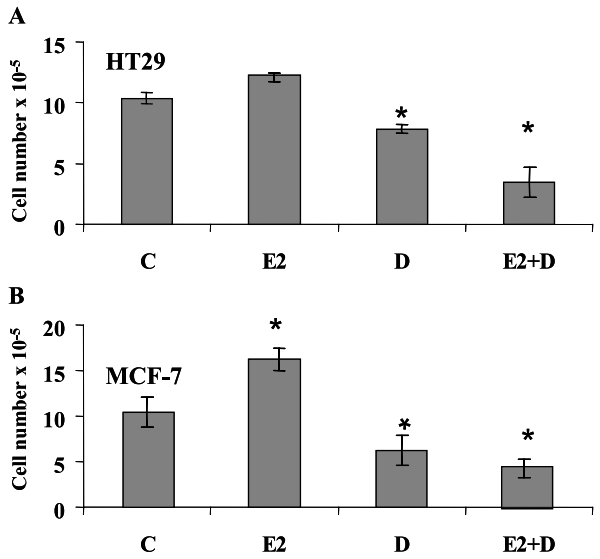

Figure 3 Effects of E2, D and combined E2+D treatment on HT29 and MCF-7 cancer cell growth. Cells were exposed for 6 days to individual or combined treatments of 10^{-8} M estradiol (E2), 10^{-8} M Vitamin D (D). Control cells (C) were treated with vehicle control (0·0067% ethanol). *P<0·005.

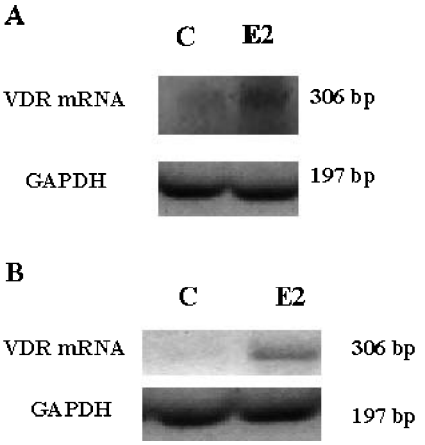

Figure 4 VDR mRNA expression. HT29 (A) and MCF-7 (B) cells were treated with 10^{-7} M E2, and RNA extracts were analyzed for VDR mRNA expression by RT-PCR, as compared to GAPDH expression.

observed when both cell lines were exposed to 10^{-8} M D (P<0·005), effect that was significantly potentiated following combined 10^{-8} M E2 and 10^{-8} M D treatments (P<0·001).

Effect of E2 on VDR mRNA and protein-expression levels in HT29 and MCF-7 cells

Previously we demonstrated that E2 regulates the transcription and expression of VDR in rat colonocytes and duodenocytes *in vivo* (Liel *et al.* 1999, Schwartz *et al.* 2000). Here, we analyzed the effect of E2 on VDR mRNA and protein expression in HT29 and MCF-7 cells. E2 upregulated VDR transcription in both cell lines; mRNA expression was enhanced in HT29 (Fig. 4A) and MCF-7 (Fig. 4B) following treatment with E2.

The effect of E2 on VDR protein expression was assessed by western blot analysis in both HT29 (Fig. 5A) and MCF-7 cells (Fig. 5B). Densitometric analyses showed that E2 at all concentrations tested (10^{-11}–10^{-7} M) significantly upregulated VDR expression in both cell lines. Both HT29 and MCF-7 cells were exposed to a medium containing the hormonal treatment for 6 days, which was found to be the optimal time period in preliminary experiments (data not shown). In HT29 cells, the increase in VDR expression was dependent on E2 dose, reaching highest significance (P<0·01) at 10^{-7} M (Fig. 5A). In MCF-7 cells, up-regulation of VDR expression followed a similar pattern as in HT-29 cells, and reached highest significance at 10^{-9} M. The specific ER inhibitor ICI182780 blocked E2-mediated VDR upregulation in both cell lines, suggesting that E2 mediates VDR expression via a process involving ER's (Fig. 5C). In these western analyses equal loading was verified by Ponceau red staining (not shown).

Phosphorylation and activation of ERK 1/2 by E2 via the MAPK pathway

To assess whether E2 can induce rapid cellular signaling effects, we measured ERK 1/2 phosphorylation. E2 activated ERK 1/2 phosphorylation in HT29 cells within 5 min after exposure to a 10^{-11} M concentration (Fig. 6), and reached maximum at 10 min. Higher concentrations of E2 induced similar ERK 1/2 phosphorylation, but within shorter time periods (data not shown). Therefore, for technical convenience, we used exposure to 10^{-11} M E2 in these experiments. The extent of ERK 1/2 phosphorylation by E2 was comparable to the effect of IGF-I, a potent and well-documented membranal ERK 1/2 activator, except that the effect of IGF-1 was still significant at 15 min of exposure, whereas the effect of E2 fell off after reaching its peak at 10 min. Similar time-dependent ERK 1/2 phosphorylation was obtained in MCF-7 cells (data not shown).

We then compared the phosphorylation patterns of ERK 1/2 in HT29 and MCF-7 cells following 10 min exposure to different concentrations of E2 (Fig. 7). In HT29 cells, E2 increased the level of ERK 1/2 phosphorylation at concentrations of 10^{-9} M and 10^{-7} M (Fig. 7A). In MCF-7 cells, activation of ERK 1/2 phosphorylation was readily measurable at all E2 concentrations tested, even at 10^{-11} M (Fig. 7B). The differences could be explained by the different estrogen receptors repertoire expressed in MCF-7 versus HT29 cells.

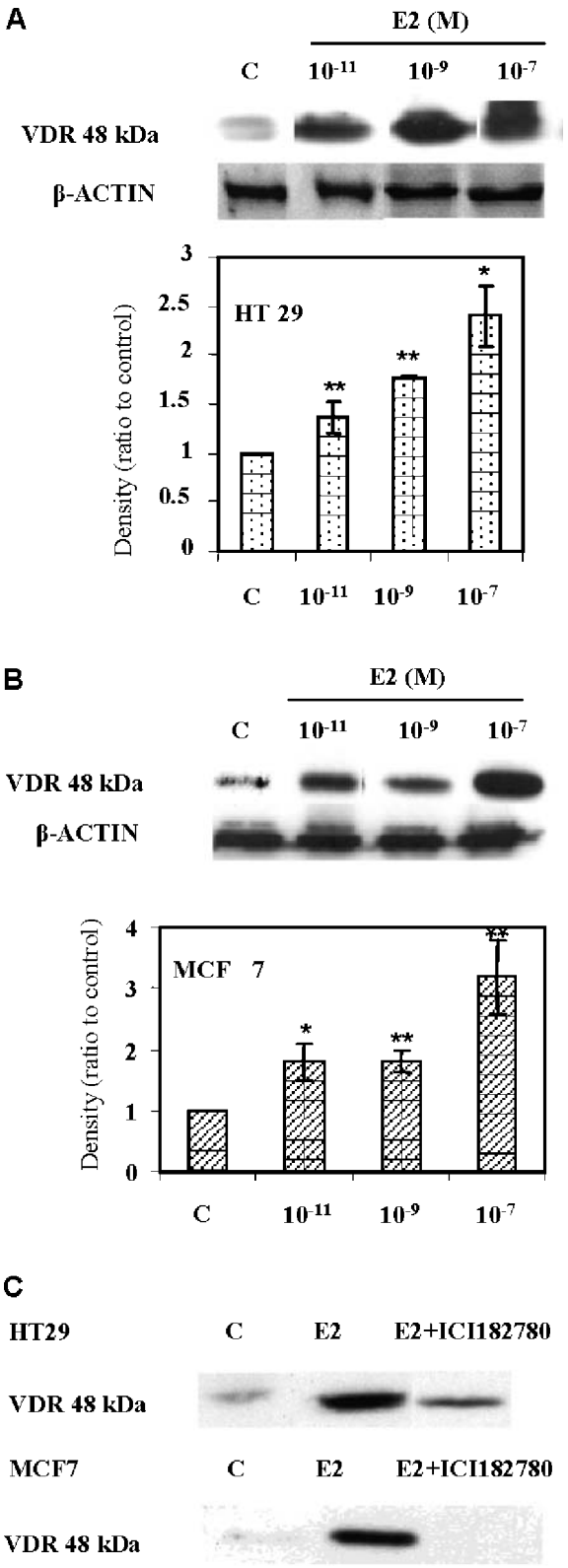

Figure 5 VDR protein expression. HT29 (A) and MCF-7 (B) cells were treated with different E2 concentrations for 6 days. Medium and treatment were replaced every other day. Cell extracts were analyzed by SDS-PAGE and immunoblotting using anti-VDR antibody. One representative experiment from three identical ones is shown. Densitometric analyses of VDR western blots for both cell lines are expressed in arbitrary units relative to each cell line control treated with 0·0067% EtOH. (C) Cells treated with 10^{-7} M E2 in the absence or presence of the ER inhibitor ICI182780 (10^{-6} M). *P<0·01, **P<0·03.

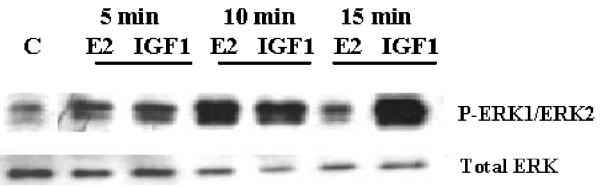

Figure 6 Effect of E2 and IGF-I on ERK 1/2 phosphorylation in HT29 cells. Cells were treated with 10^{-11} M E2 or 100 ng/ml IGF-I and harvested at the time periods indicated. Total ERK 1/2 was detected with anti-ERK1 and anti-ERK2 antibodies or with anti-phospho-ERK 1/2 antibodies to detect the phosphorylated proteins. Control cells (C) were treated with E2 vehicle (ethanol-0·0067% EtOH) or with no treatment at all (C). The figure is a representative example of four similar, independent experiments.

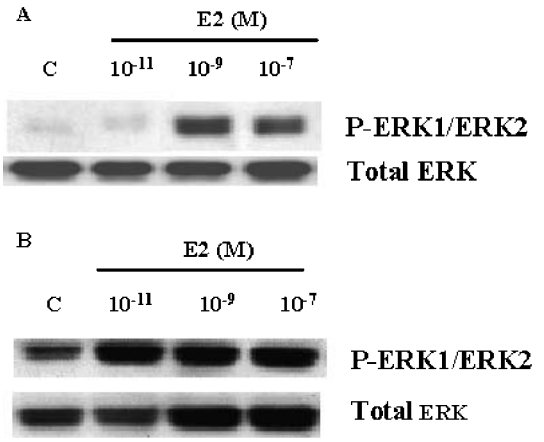

Figure 7 E2-mediated ERK 1/2 phosphorylation in HT29 and MCF-7 cells. ERK 1/2 phosphorylation was measured in HT29 cells (A) and MCF-7 cells (B). The cells were cultured in PR-free DMEM without FCS (HT29) or with 0·5% FCS (MCF-7) for 24 h and then treated with different E2 concentrations for 10 min. Control cells (C) were treated with 0·0067% ethanol. Cell extracts were analyzed as described in the legend to Fig. 6. One representative experiment of three similar ones is shown.

E2 regulates VDR expression via phosphorylation of ERK 1/2

To determine whether a direct relationship exists between ERK 1/2 activation and VDR expression in HT29 and MCF-7 cells, we used the specific ERK 1/2 phosphorylation inhibitor PD98059 at the previously determined most effective dose (5×10^{-5} M). ERK 1/2 phosphorylation and VDR protein expression were evaluated on western blots. We exposed the cells to different E2

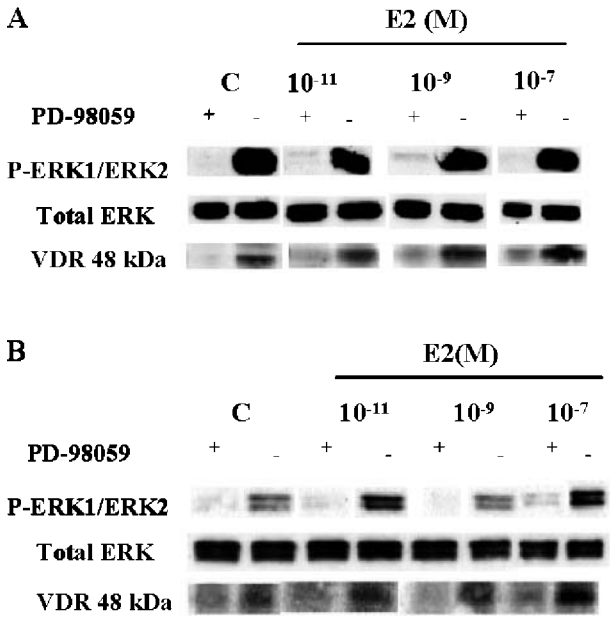

Figure 8 Inhibition of ERK 1/2 phosphorylation and its relation to VDR expression. Effect of PD98059 on ERK 1/2 phosphorylation and VDR expression in HT29 cells (A), and MCF-7 cells (B). Cells were treated with different E2 concentrations and pretreated or not with 5×10^{-5} M PD98059, as described in Materials and Methods. A control treated with 0·0067% ethanol was used. Cell extracts were analyzed by western blot using an anti-phospho-ERK 1/2 and anti-ERK 1/2 antibodies, or a VDR antibody. One representative experiment of four identical ones is shown.

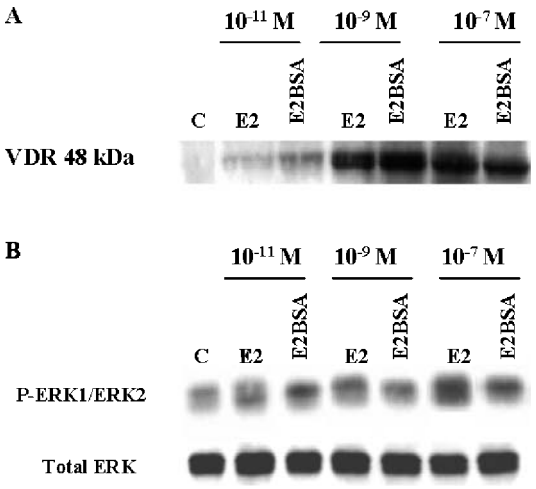

Figure 9 Effect of E2-BSA on ERK 1/2 phosphorylation and VDR expression. (A) HT29 cells were cultured in PR-free DMEM and treated with E2-BSA or E2 for 10 min at concentrations of 10^{-11} M, 10^{-9} M, and 10^{-7} M. Control cells (C) were exposed to 0·0067% ethanol. Cell extracts were analyzed by western blot using an anti-phospho-ERK 1/2 and anti-ERK 1/2 antibodies. One representative experiment of three identical ones is shown. (B) HT29 cells were cultured in PR-free DMEM and treated with E2-BSA or E2 for 10 min at concentrations of 10^{-11} M, 10^{-9} M, and 10^{-7} M. Control cells (C) were exposed to 0·0067% EtOH. Cell extracts were analyzed by SDS-PAGE and immunoblotting using anti-VDR antibody. One representative experiment of three identical ones is shown.

concentrations and PD98059 for 6 days, the time period previously shown to be required for E2 to induce effective upregulation of VDR expression. In HT29 cells PD98059 completely blocked ERK 1/2 phosphorylation at all E2 concentrations (Fig. 8A), and also prevented the activation of ERK 1/2 in the absence of E2. Basal phosphorylation of ERK 1/2 in the control cells (treated with E2 vehicle, ethanol) was observed in these experiments most likely because cells were exposed to culture media containing FCS. In MCF-7 cells, PD98059 prevented the phosphorylation of ERK 1/2 in the absence of E2 and also blocked phosphorylation at all E2 concentrations (Fig. 8B). In MCF-7 cells, PD98059 abrogated the upregulatory effect exerted by E2 on VDR expression (Fig. 8B). In HT29 cells (Fig. 8A), PD98059 more effectively inhibited the effect exerted by E2 on VDR expression. These differences again, may be due to the different receptor repertoire expressed in these cell lines.

Effect of E2-BSA on VDR expression and ERK 1/2 phosphorylation

To determine whether E2-induced VDR regulation is mediated by the activity of the hormone at the cell membrane, HT29 cells were treated with an E2-BSA conjugate that could not traverse the plasma membrane. The effect of E2-BSA mimicked the effect of E2 on VDR protein expression and ERK 1/2 phosphorylation: E2-BSA, at all concentrations tested (10^{-11}–10^{-7} M), significantly upregulated VDR expression in HT29 cells (Fig. 9A). Following exposure to E2-BSA for 10 min, significant ERK 1/2 phosphorylation was detected, similar in pattern and intensity to the nonconjugated hormone (Fig. 9B). E2-BSA exerted similar effects on MCF-7 cells (data not shown). We conclude from these experiments that E2 activates MAPK and enhances VDR expression acting at the cellular membrane domain.

Localization of ERα and ERβ to the plasma membrane and caveolae

Immunoblot analyses detected ERα protein (67 kDa) and ERβ protein (54 kDa) in whole-cell lysates of HT29 (Fig. 1A) and ERα protein (67 kDa) in MCF-7 cells (Fig. 1B). Subfractionation of the cancer cells detected ERβ in HT29 cells in cytosol, nuclei and plasma membranes (Fig. 10A), and in MCF-7 cells ERα was detected in cytosol, nuclei and plasma membranes (Fig. 10A). ERα and ERβ were expressed most distinctively in the plasma membrane of HT29 and MCF-7 cells following E2 stimulation (not shown).

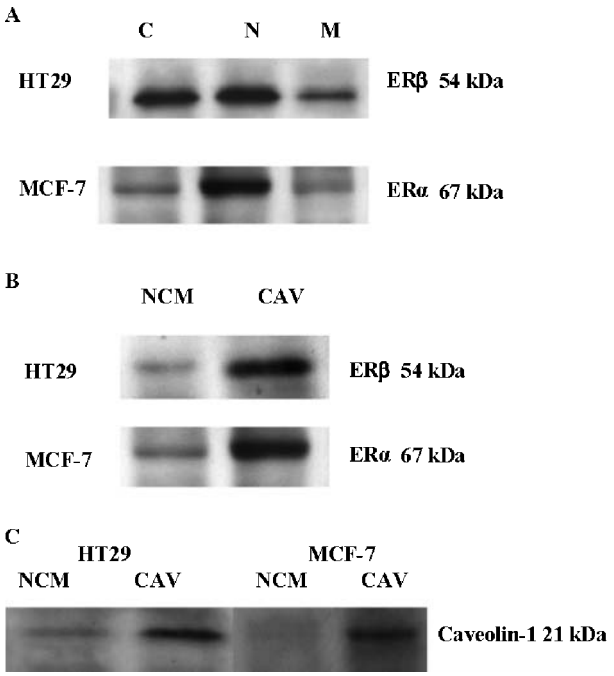

Figure 10 Cellular localization of ERα and ERβ in HT29 and MCF-7 cells. (A) Immunoblot analyses for ERα and ERβ in MCF-7 and HT29 cancer cells respectively in cytosol (C), nucleus (N) and plasma membrane (M). (B) Localization of ERα and ERβ to caveolae in E2-treated MCF-7 and HT29 cells. Immunoblot analyses for ERα, ERβ in noncaveolae membranes (NCM) and caveolae membranes (CAV) obtained from MCF-7 and HT29 cell's whole plasma membranes. Signals were obtained at the expected 54 kDa (for ERβ) and 67 kDa (for ERα) in HT29 and MCF-7 cells respectively. (C) Immunoblot analyses for caveolin-1 to confirm caveolae membrane subfractionation. Results are representative of three independent experiments.

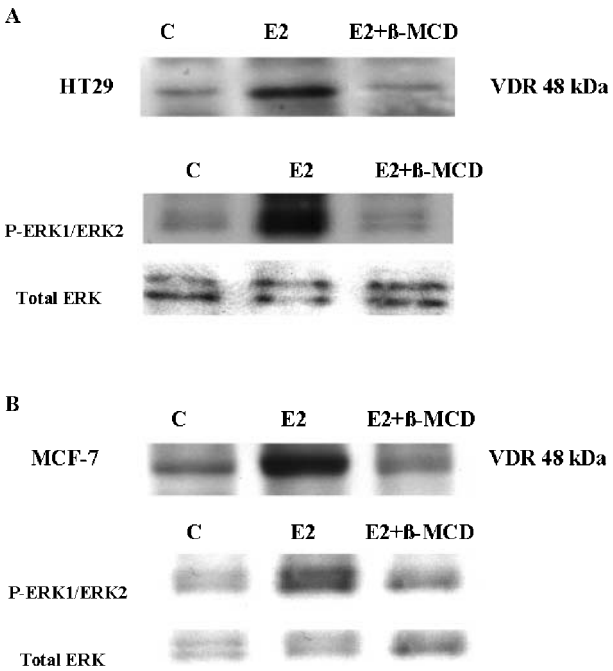

Figure 11 Confirmation of association of ERα and ERβ in HT29 and MCF-7 cells with lipid rafts/caveolae. HT29 (A) and MCF-7 (B) cells were treated with E2 (10^{-7} M), or combined E2 (10^{-7} M) and β-methylcyclodextrin (β-MCD, 10 mM) treatment. Cell extracts were analyzed by western blot using VDR and anti-phospho-ERK 1/2 and anti-ERK 1/2 antibodies in HT29 (A) and MCF-7 (B) cells.

Thereafter, additional fractionation experiments were conducted in cells treated with E2. We determined whether ERβ and ERα protein (54 and 67 kDa respectively) are associated with caveolae subfraction of HT29 and MCF-7 cells' cell plasma membranes. We demonstrate that ERβ localizes to the plasmalemmal caveolae in HT29 cells treated with E2 (10^{-7} M), (Fig. 10B) and ERα localized to plasmalemmal caveolae in MCF-7 cells treated with E2 (10^{-7} M) (Fig. 10B). In contrast, no such association was detected under basal unstimulated conditions (not shown). Immunoblots of caveolae and non-caveolae fractions with anti-caveolin-1 confirmed that the caveolae sub-fractionation technique was satisfactory (Fig. 10C).

Blockade of E2-mediated VDR up-regulation by caveolae disruption

To analyze the function of caveolae in E2-mediated signaling, the cholesterol binding reagent, methylcyclodextrin was used to deplete the cellular cholesterol, which is essential for the structural integrity of lipid rafts/caveolae. We based the experiments on the report by Fielding & Fielding (2000), and used 10 mM β-methylcyclodextrin (β-MCD) to treat HT29 and MCF-7 cells. After combined E2 (10^{-7} M) and β-MCD (10 mM) treatment, lipid rafts/caveolae were significantly disrupted, as indicated by dislocation of caveolin in density gradient separation (not shown), and inhibition of downstream MAPK signaling and VDR expression in HT29 (Fig. 11A) and MCF-7 (Fig. 11B) cells.

Suramin and PP2 blocks ERK 1/2 phosphorylation induced by E2 and inhibits E2-mediated VDR expression

To investigate the upstream signals mediating E2 action on MAPK, we questioned whether the effects of E2 on MAPK involved Src or other tyrosine kinases. To that end, we used suramin a polysulfonated naphthylurea compound that interferes with the signal exerted by the binding of several polypeptides known as activators of membrane-associated signaling pathways involved in tyrosine phosphorylation. HT29 cells were exposed to different suramin concentrations for 4 h and then triggered with E2. Assessment of ERK 1/2 phosphorylation indicated that the phosphorylation activity was significantly

and dose-dependently downregulated in response to suramin. At low suramin concentrations, the inhibitory effect on ERK 1/2 activation was minimal whereas at the 4×10^{-3} M concentration, HT29 ERK 1/2 phosphorylation was completely abolished (Fig. 12A). In parallel experiments cells were treated for 4 days with E2 and then 2 days with both E2 and 1 or 4×10^{-3} M suramin. Suramin dose-dependently inhibited the enhanced VDR expression induced by E2 (Fig. 12B). Similar results were obtained with MCF-7 cells (data not shown). We next used a pan Src inhibitor PP2, which has been recently identified as a potent and selective inhibitor of the Src-family tyrosine kinases, and demonstrated that it blocked E2-induced MAPK phosphorylation, implying that Src family members are required for this step. PP2 in the presence of E2 inhibited dose dependently E2-mediated MAPK phosphorylation in HT-29 cells (Fig. 12C). We next wanted to determine whether Src tyrosine phosphorylation is involved in regulating VDR expression mediated by E2. Exactly as for Suramin, PP2 dose-dependently inhibited the enhanced VDR expression induced by E2 in HT-29 cells (Fig. 12D). Similar results were obtained with MCF-7 cells (data not shown).

We conclude from these experiments that Src tyrosine phosphorylation of membrane-associated proteins is involved in E2-mediated VDR expression.

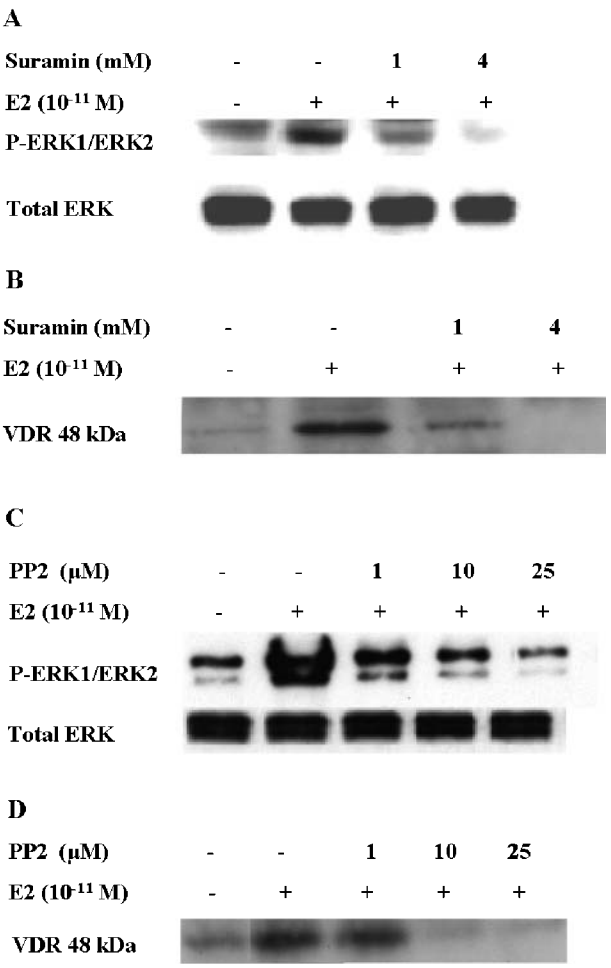

Figure 12 Effect of suramin and PP2 on ERK 1/2 phosphorylation and VDR expression. (A) HT29 cells were cultured in PR-free DMEM and treated with different suramin concentrations for 4 h and then exposed to 10^{-11} M E2 for 10 min. Control cells were exposed to 0·0067% ethanol. Cell extracts were analyzed by western blot using an anti-phospho-ERK 1/2 and anti-ERK 1/2 antibodies. One representative experiment of three identical ones is shown. (B) HT29 cells were treated for 4 days with 10^{-11} M E2 and then for 2 days with both 10^{-11} M E2 and 1 or 4×10^{-3} M suramin. Cell extracts were analyzed by western blot using anti-VDR antibody. (C) Effect of the Src tyrosine kinase inhibitor PP2 on E2-mediated ERK 1/2 activation on HT29 cells. Cells grown in serum-free PR-free medium were treated for 60 min with vehicle (DMSO) or PP2 (1, 10 and 25 μM) and then exposed to 10^{-11} M E2 for 10 min. Cell lysates were analyzed by western blot for ERK 1/2 phosphorylation with anti-phospho-ERK 1/2 antibodies. (D) HT29 cells were treated for 5 days with 10^{-11} M E2 and then for 1 day with both 10^{-11} M E2 and PP2 (1, 10 and 25 μM). Cell extracts were analyzed by western blot using anti-VDR antibody. One representative experiment of three or four identical ones is shown.

Sulindac sulfide inhibits E2-mediated VDR expression

The ability of sulindac sulfide to inhibit Ras (Herrmann *et al.* 1998) warrants examination whether sulindac sulfide can inhibit other pathways dependent on Ras. To that end, we incubated HT29 and MCF-7 cells with sulindac sulfide (100 μM) in the presence of E2 and tested the effect on VDR expression (Fig. 13). This concentration was chosen following preliminary experiments (not shown). This was accomplished by incubating HT29 and MCF-7 cells in the presence of increasing concentrations of sulindac sulfide and measuring their effect on VDR expression (not shown). Sulindac sulfide at a concentration of 100 μM caused the most impressive effect. We therefore chose a concentration of 100 μM for all subsequent experiments since this concentration is also consistent with that used in previous studies (Herrmann *et al.* 1998). We found that sulindac sulfide significantly inhibited E2-mediated VDR upregulation in both HT29 and MCF-7 cells, indicating that Ras signaling is involved in E2-mediated VDR transcriptional control.

Phosphorylation of Raf and c-Jun by E2

To investigate additional upstream and downstream signals to MAPK mediating E2 action, we questioned whether the effect of E2 involves Raf and c-Jun phosphorylations. Raf and c-Jun were avidly phosphorylated in response to E2 treatment (10^{-7} M). Fig. 14 shows that in HT29 cells, Raf was significantly phosphorylated following 5 and 10 min of exposure to 10^{-7} M E2, and in MCF-7 cells significant phosphorylation was achieved following at least 10 min of exposure to 10-^{7} M E2. c-Jun was significantly

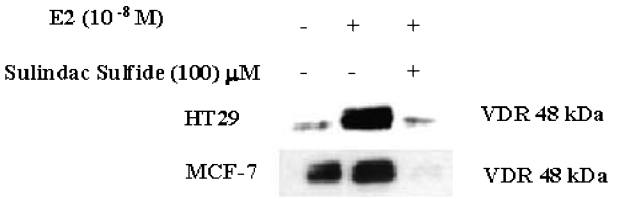

Figure 13 Effect of sulindac sulfide on VDR expression. HT29 and MCF-7 cells were treated with 10^{-8} M E2 or combination of 10^{-8} M E2 and 100 μM sulindac sulfide for 6 days. Medium and treatment were replaced every other day. Cell extracts were analyzed by SDS-PAGE and immunoblotting using anti-VDR antibody. One representative experiment from three identical ones is shown.

phosphorylated following 15 min exposure to E2 in HT29 cells, and in MCF-7 cells the phoshorylation process of c-Jun was faster (Fig. 14).

E2 regulates VDR expression via phosphorylation of c-Jun

To determine whether a direct relationship exists between c-Jun phosphorylation and VDR expression in HT29 and MCF-7 cells, we used the specific c-Jun phosphorylation inhibitor SP600125 at a range of doses previously demonstrated to be non-cytotoxic to the cells. Concentrations higher than 10 μM was cytotoxic to both cell lines (data not shown). C-Jun phosphorylation and VDR protein expression were evaluated on western blots. We exposed the cells to E2 (10^{-8} M) and various concentrations of SP600125 (1, 2·5, 5 and 10 μM) for 6 days (the exact protocol as for PD 98059), the time period previously shown to be required for E2 to induce effective upregulation of VDR expression. In HT29 cells SP600125 dose dependently inhibited c-Jun phosphorylation and VDR expression (Fig. 15A). Basal phosphorylation of c-Jun in the control cells was observed in these experiments most likely because cells were exposed to culture media containing FCS. Similarly to HT-29 cells, in MCF-7 cells, SP600125 prevented the phosphorylation of c-Jun and concomitantly dose-dependently abrogated the upregulatory effect exerted by E2 on VDR expression (Fig. 15B).

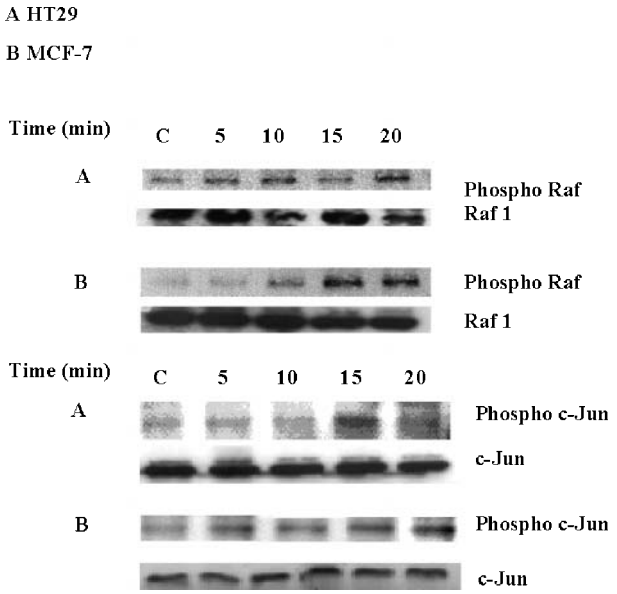

Figure 14 Effect of E2 on Raf and c-Jun phosphorylation in HT29 and MCF-7 cells. HT29 cells (A) and MCF-7 cells (B) were treated with 10^{-7} M E2 and harvested at the time periods indicated. Total Raf and c-Jun were detected with anti-Raf-1 and anti-c-Jun antibodies. Phosphorylation of the above-mentioned proteins was detected with anti-phospho-Raf and anti-phospho-c-Jun antibodies. Control cells (C) were treated with E2 vehicle (ethanol-0·0067%). The figure is a representative example of four similar, independent experiments.

PD98059 blocks c-Jun phosphorylation induced by E2 and inhibits E2-mediated VDR expression

In order to link MAPK activation with downstream signals we tested the effect of the PD 98059 on c-Jun phosphorylation and concomitantly on VDR expression. E2, as previously described, induced c-Jun phosphorylation concomitantly with VDR enhanced expression in both cell lines (HT-29 and MCF-7; Fig. 16A and B). We exposed the cells to E2 (10^{-8} M) concomitantly with 50 μM of the MAPK/ERK kinase inhibitor PD 98059. PD 98059 downregulated both c-Jun phosphorylation and VDR expression indicating that upstream and downstream events in this signaling cascade are all related to the control of VDR expression. The MEK1 and MEK2 inhibitor UO 126 at a concentration of 10 μM exerted similar effects to those exerted by PD 98059, both on c-Jun phosphorylation and E2-mediated VDR expression (not shown), supporting the view that E2-mediated MAPK activation is linked to c-Jun phosphorylation and VDR expression.

Discussion

The present report adds to our previous *in vivo* studies (Liel *et al.* 1999, Schwartz *et al.* 2000) by establishing that E2 induces VDR expression in cells originating from several organs. Specifically, we addressed here the question of whether this interaction between E2 and VDR also takes place *in vitro* in HT29 colon cancer cells and MCF-7 breast cancer cells.

MCF-7 is a breast-cancer-derived cell line representing a typical model of estrogen dependent cell growth and we demonstrate in this study that E2 upregulates MCF-7 proliferation (Tada *et al.* 1986). Conversely, in HT29 colon cancer cells, estrogen had no effect on cell proliferation in the E2 concentration range used, which represents physiological E2 concentrations. These results are consistent with similar previously reported effects of E2 in other colon cancer cell types (Fiorelli *et al.* 1999).

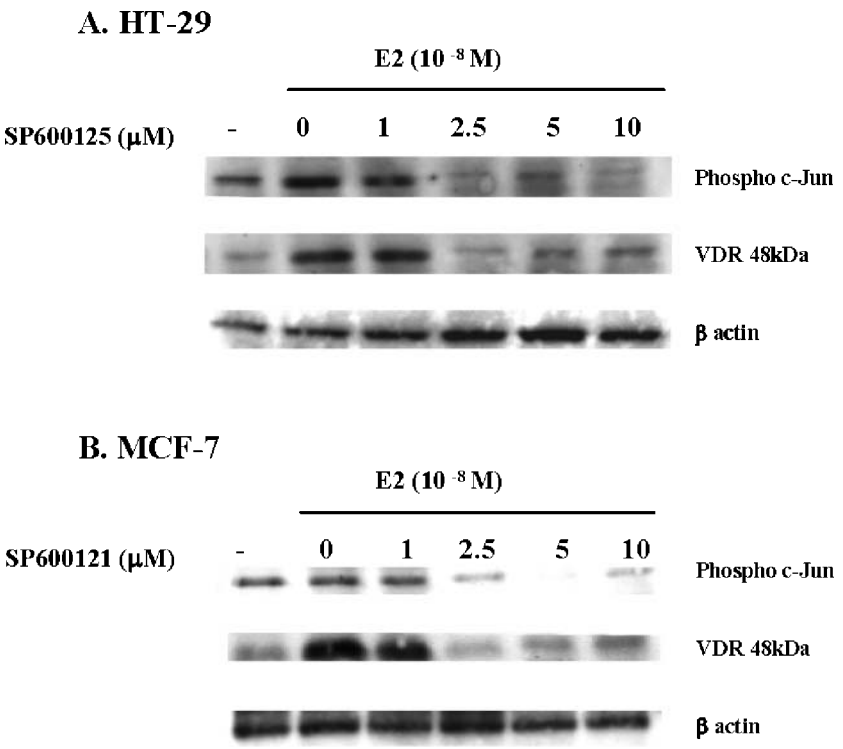

Figure 15 Effect of SP600125 on c-Jun phosphorylation and VDR expression in HT29 cells (A), and MCF-7 cells (B). Cells were treated with E2 (10^{-8} M) and with different concentrations of SP600125 (1–10 μM), as described in Materials and Methods. Cell extracts were analyzed by western blot using an anti-phospho-jun, or a VDR antibody. One representative experiment of three identical ones is shown.

The reason for the different mitogenic effects exerted by E2 in colon cancer and breast cancer cells may reside in the expression of different estrogen receptors in the two cell lines. In this regard, we demonstrated that HT29 colonocytes express mainly ERβ, supporting previous observations (Arai *et al.* 2000), whereas MCF-7 cells express mainly ERα, as in previously reported findings (Cullen *et al.* 2000). Further, ERα and ERβ have different roles in the transcription regulation of various genes. In support of these hypotheses, different responses to AP1-activation sites exerted by ERα and ERβ have been described by Paech *et al.* (1997). Moreover, gene expression of ERβ in colorectal mucosa has been described (Arai *et al.* 2000, Campbell-Thompson *et al.* 2001), but ERβ is detected at very low levels in breast cancer tissues, according to our and others findings (Cullen *et al.* 2000). These observations led us to suggest a functional implication for ERβ in estrogen's mediation of VDR expression in colonic cells. Possible heterodimer or homodimer formation between the different receptors could be responsible for activation of a MAPK signal-transduction pathway (Pettersson *et al.* 1997).

We demonstrate that E2 affects VDR transcription in both breast and colon cancer cells. We showed that the effect on VDR transcription and protein expression exerted by E2 is mediated by ER, because the ER-specific inhibitor ICI182780 was extremely effective in abrogating E2-mediated VDR upregulation.

E2 may control gene expression by direct nuclear effects following binding to specific DNA sequences. However, no evidence has been accrued demonstrating the existence of estrogen-responsive elements within the VDR promoter. Therefore, we adopted the alternative view that E2 may regulate VDR transcription and expression through signaling pathways. This approach seems feasible because earlier studies have described a TATA-containing promoter immediately upstream of exon 1*c* of the human VDR gene. This region was shown to be responsive to E2 treatment, resulting in human VDR promoter action in a MCF-7-transfected cell line (Byrne *et al.* 2000). Therefore, we hypothesized that E2 may stimulate VDR promoter activation by activating a signaling pathway, such as the Raf/MEK (MAPK or ERK kinase)/ERK module of the MAPK cascade since this is a major intracellular mediator of signaling that regulates numerous biological processes.

Treatment with E2 in both HT29 and MCF-7 cell lines significantly induced MAPK activity. Activation of the

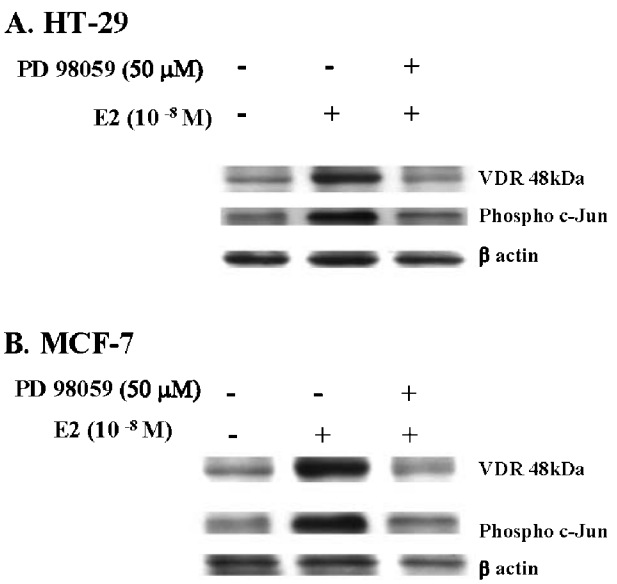

Figure 16 Effect of PD 98059 on c-Jun phosphorylation and VDR expression in HT29 and MCF-7 cells. HT29 cells (A), and MCF-7 cells (B) cells were treated E2 (10^{-8} M) and with PD 98059 (50 μM), as described in Materials and Methods. Cell extracts were analyzed by western blot using an anti-phospho-jun, or a VDR antibody. One representative experiment of three identical ones is shown.

ERK pathway in MCF-7 cells has already been shown (Migliaccio *et al.*1996), and a similar effect of MAPK activation in osteoblast-like cells has been previously reported to be exerted by E2 through signaling pathways (Endoh *et al.* 1997). We showed that ERK 1/2 is activated within short time periods following exposure to E2.

The inhibition of VDR protein expression with the specific ERK 1/2 phosphorylation inhibitor PD98059 in HT29 and MCF-7 cells supports the notion that E2 activation, through ERK 1/2, modulates VDR expression. An interesting observation was that in control cells not exposed to E2 but treated with PD98059, there was complete abrogation of basal MAPK phosphorylation and a concomitant decrease in basal VDR expression (Fig. 8). These results further support the concept that MAPK activation plays a central role in the regulation of VDR expression by E2.

Tyrosine phosphorylation specially src-mediated tyrosine phosphorylation is a well-documented event that occurs following interaction with membrane receptors and signaling. Suramin is a polysulfonated naphthylurea compound that interferes with the signal exerted by the binding of several polypeptides, such as epidermal growth factor (Fujiuchi *et al.* 1997), IGF1 (Vincent *et al.* 1996), interleukins (Leland *et al.* 1995) and tumor necrosis α (LaPushin *et al.* 1994), which are known activators of membrane-associated signaling pathways involved in tyrosine phosphorylation. In the present study, suramin treatment markedly blocked, in a dose-dependent manner, E2-mediated ERK 1/2 activation in HT29 cells. A similar effect was measured in MCF-7 cells (data not shown). In addition, suramin was shown to inhibit, dose-dependently, VDR expression. Supportive data on the role of src phosphorylation on VDR regulation exerted by E2 was obtained from experiments performed with PP2. Our data show that PP2, a specific Src tyrosine kinase inhibitor, significantly and dose-dependently inhibited ERK activation and subsequently inhibited VDR expression. These findings strongly support the notion that E2 activates intracellular tyrosine signaling induced by src by interacting with putative receptors located at the cellular membrane, and this specific interaction is most probably responsible for the regulation of VDR expression. Flores-Delgado *et al.* (2001) reported that the nongenomic action of estrogen regulates tyrosine phosphorylation of cytoplasmic proteins in lung myofibroblasts, a finding similar to our observations. Migliaccio *et al.* (2003) also reported that sex steroids, e.g. estradiol, could act as a growth factor and upregulate signal-transduction pathways.

When HT29 cells were treated with an E2-BSA conjugate, a compound unable to traverse the plasma membrane, the conjugate was able to upregulate both enhanced VDR expression and ERK phosphorylation, in a fashion mimicking almost exactly the effect of the free nonconjugated E2. This finding strongly suggests that the initial signal in ERK 1/2 phosphorylation induced by E2 is generated at the plasma membrane. The effect of the E2-BSA conjugate, together with the suppression of ERK 1/2 activation by suramin and PP2 in HT29 cells and the concomitant inhibition of VDR expression, further suggests that the E2-mediated signal-transduction pathway in colon and breast cancer cells is linked to the activation of putative membrane-receptor-associated tyrosine kinase activity.

Additional upstream events to MAPK phosphorylation reactions (Raf-1 phosphorylation) and downstream to MAPK phosphorylation (c-Jun phosphorylation) demonstrate that E2 induces rapid signaling effects that culminate in phosphorylation of nuclear transcription factors able to induce transcriptional activation of AP-1 sites (Monje *et al.* 2003). AP-1 sites are present within the VDR receptor and are involved in regulation of VDR transcription (Qi *et al.* 2002). The specific c-Jun phosphorylation inhibitor SP600125 dose dependently inhibited c-Jun phosphorylation and VDR expression. The MAPK/ERK kinase inhibitor PD 98059 downregulated both c-Jun phosphorylation and VDR expression. These experiments allow us to conclude that upstream and downstream events in the signaling cascade are all interrelated and all participate in the control of VDR expression.

To demonstrate that E2-ER interactions leading to enhanced VDR expression take place at the cell surface we conducted various cellular fractionation analyses to demonstrate the exact localization of ER. ERα and β were

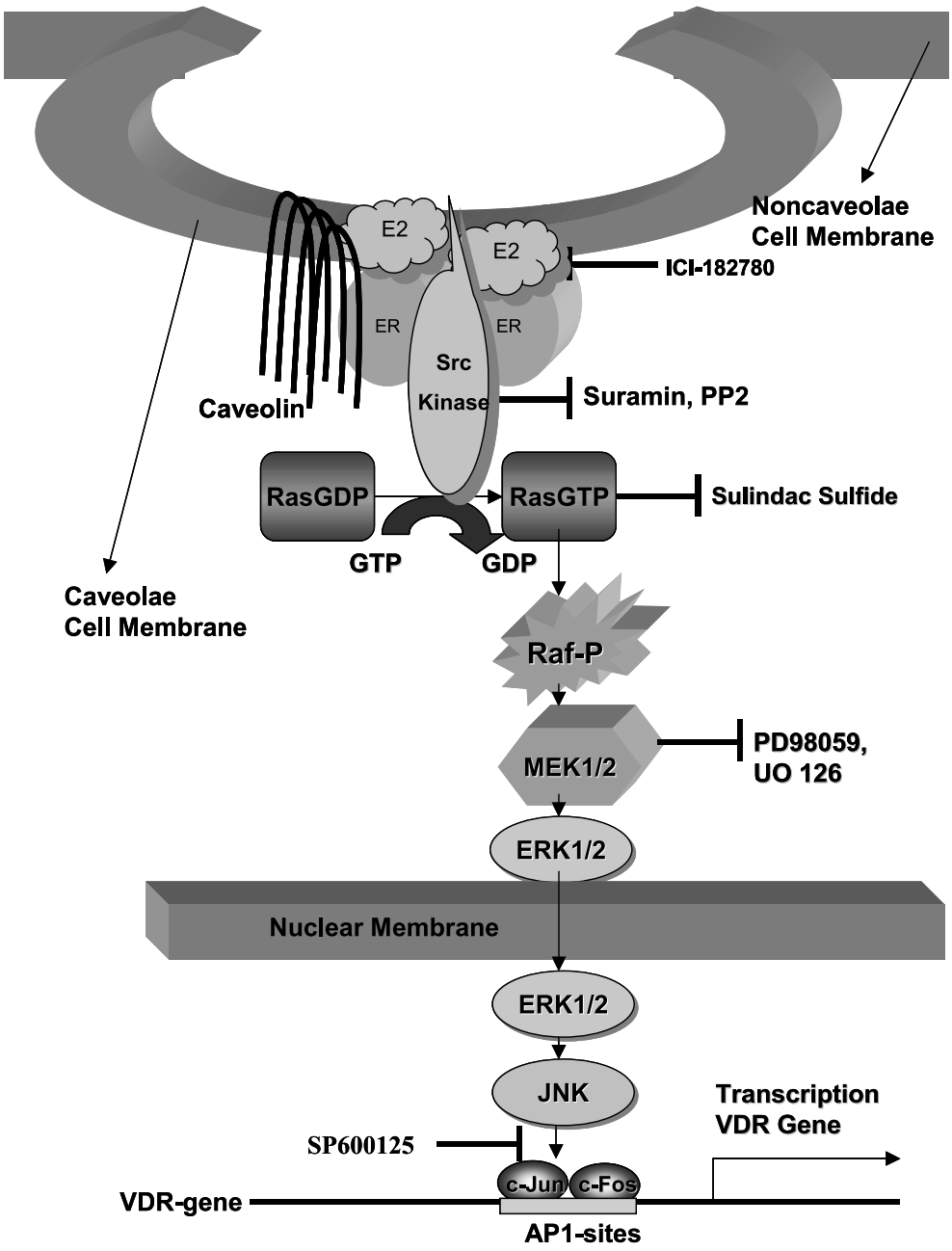

Figure 17 Proposed hypothesis to account for the effect of E2 on ERK 1/2 phosphorylation and concomitant enhanced VDR expression. E2 following binding to caveolae membranal estrogen receptors ERα and ERβ (in the form of homo- or heterodimers of ER's) induces activation of Src-associated tyrosine phosphorylation, a process that can be inhibited by Src inhibitors such as suramin and PP2. This tyrosine phosphorylation activity triggers the Ras–Raf activation, which can be obliterated by sulindac sulfide. Thereafter MAPK–ERK pathway is activated and as a result c-Jun is phosphorylated inducing it's binding to DNA binding domains such as AP-1 present within the VDR promoter. This interaction finally results in upregulation of transcription and expression of the VDR gene. GDP, guanosine diphosphate, GTP, guanosine triphosphate.

detected in cellular plasma membrane, cytosol and nucleus of MCF-7 and HT29 cells respectively, revealing identical ERα and ERβ protein species in all cellular compartments. Since signaling molecules are preferentially located in caveolae we also tested whether ERα and β are expressed in caveolae of HT29 and MCF-7 cells. During recent years, more and more reports confirmed that many signaling molecules concentrate in lipid rafts/caveolae, which serve as platforms and play an important role in regulating signal cascades. Signal molecules, such as heterotrimeric G-proteins (Li *et al.* 1995), protein kinase C (Smart *et al.* 1995), Raf1 (Mineo *et al.* 1996), and Src family tyrosine kinases (Robbins *et al.* 1995), are recruited into caveolae by caveolins, which, through the scaffolding domain, interact with the caveolin-binding motifs in these signal molecules (Couet *et al.* 1997). These clusters of signal molecules can form 'preassembled signaling complexes' on the plasma membrane. Thus, the enrichment of receptors and signal molecules in lipid rafts/caveolae enables them to be in close contact with each other and makes lipid rafts/caveolae the gateways for signals entering into the cells. We clearly demonstrate that in HT29 and MCF-7 cells treated with E2 a significant fraction of ERα and ERβ is expressed in caveolae membranes, while in untreated cells the expression is barely discernible. We assume that caveolin may anchor ER proteins to the membrane similarly to that suggested for G-protein α subunit (Couet *et al.* 1997).

We do not provide information regarding the precise side of the caveolae membrane the estrogen nuclear receptors are localized; knowing that there are at least three possibilities according to Norman *et al.* (2004). One possibility is that the receptor is located in the outside periphery of the cell but inside the curvature of the caveola interacting with the outer plasma membrane. A second model assumes the existence of an adaptor/scaffold protein that might form a heterodimer with a nuclear receptor to tie it to or near the caveolae. It is known that in Src tyrosine kinase, Gα subunits and Ras each engage in protein–protein interactions with caveolin, the integral membrane protein of caveolae. An additional model proposes that palmitoylation could bind the receptor to the inner surface of the caveola.

The absolute requirement of caveolae membrane localization of ERα and ERβ in HT29 and MCF-7 cells treated with E2 in the context of signal transduction and VDR expression was further clarified using the cholesterol binding reagent β-methylcyclodextrin, experiments that demonstrated that inhibition of caveolae membrane localization of ERα and ERβ directly impinges on VDR expression and MAPK phosphorylation.

Cumulatively our data led us to suggest the hypothesis presented in Fig. 17, namely, that E2 following binding to caveolae membranal estrogen receptors (in the form of homo- or heterodimers of ER's) induces activation of Src-associated tyrosine phosphorylation, a process that can be inhibited by Src inhibitors such as suramin and PP2. Tyrosine phosphorylation activity triggers the Ras-ERK pathway, which ultimately can activate transcription factors such as c-Jun or c-Fos to bind specific sequences within the VDR gene (such as the AP-1 binding site) and finally induce upregulation of transcription and expression of the VDR gene. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

Arai H, Miyamoto K & Taketani Y 1997 A vitamin D receptor gene polymorphism in the translational initiation codon: effect on protein activity and relation to bone mineral density in Japanese women. *Journal of Bone and Mineral Research* **12** 915–921.

Arai N, Strom A, Rafter JJ & Gustafsson JA 2000 Estrogen receptor β mRNA in colon cancer cells: growth effects of estrogen and genistein. *Biochemical and Biophysical Research Communications* **270** 425–431.

Aronica SM, Kraus WL & Katznellenbogen BS 1994 Estrogen action via cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *PNAS* **91** 8517–8521.

Avioli LV, McDonald JE & Lee SIN 1965 The influence of age on the intestinal absorption of ^{47}Ca in women and its relation to ^{47}Ca absorption in postmenopausal osteoporosis. *Journal of Clinical Investigation* **44** 1960–1967.

Berthois Y, Pourreau-Schneider N, Gaudilhon P, Mittre H, Tubiana N & Martin PM 1986 Estradiol membrane binding sites on human breast cancer cell lines. Use of fluorescent estradiol conjugate to demonstrate plasma membrane binding systems. *Journal of Steroid Biochemistry* **25** 963–972.

Blackmore PF, Neulen J, Lattanzio F & Beebe SJ 1991 Cell surface-binding sites for progesterone mediate calcium uptake in human sperm. *Journal of Biological Chemistry* **266** 18655–18659.

Bullamore JR, Gallagher JC, Wilkinson R, Nordin BEC & Marshall DH 1970 Effect of age on calcium absorption. *Lancet* **2** 535–537.

Byrne MI, Flanagan L, Tenniswood MPR & Welsh J 2000 Identification of a hormone- responsive promoter immediately upstream of exon 1c in the human vitamin D receptor gene. *Endocrinology* **141** 2829–2836.

Campbell-Thompson M, Lynch IJ & Bhardwa B 2001 Expression of estrogen receptor (ER) subtypes and ERβ isoforms in colon cancer. *Cancer Research* **61** 632–640.

Chaban VV, Lakhter JA & Micevych P 2004 A membrane estrogen receptor mediates intracellular calcium in astrocytes. *Endocrinology* **149** 1–23.

Chang WJ, Ying Y, Rothberg KG, Hooper NM, Turner AJ, Gambliel HA, De Gunzburg J, Mumby SM, Gilman AG & Anderson RG 1994 Purification and characterization of smooth muscle cell caveolae. *Journal of Cell Biology* **126** 127–138.

Civitelli R, Agnusdei D, Nardi P, Zacchei F, Avioli LV & Gennari C 1988 Effects of one-year treatment with estrogens on bone mass, intestinal calcium absorption, and 25-hydroxyvitamin D-1 alpha-hydroxylase reserve in postmenopausal osteoporosis. *Calcified Tissue International* **42** 77–86.

Couet J, Li S, Okamoto T, Ikezu T & Lisanti MP 1997 Identification of peptide and protein ligands for the caveolin-scaffolding domain. Implications for the interaction of caveolin with caveolae-associated proteins. *Journal of Biological Chemistry* **272** 6525–6533.

Csikos T, Chung O & Unger T 1998 Receptors and their classification: focus on angiotensin II and the AT2 receptor. *Journal of Human Hypertension* **12** 311–318.

Cullen R, McGuire T, Diggin P, Hill A, McDermott E, O'Higgins N & Duffy MJ 2000 Detection of estrogen receptor beta mRNA in breast cancer using RT-PCR. *International Journal of Biological Markers* **15** 114–115.

Dokoh S, Donaldson CA & Haussler MR 1984 Influence of 1,25-dihydroxyvitamin D3 on cultured osteogenic sarcoma cells: correlation with the 1,25-dihydroxyvitamin D3 receptors. *Cancer Research* **44** 2103–2109.

Duncan WE, Glass AR & Wray HL 1991 Estrogen regulation of the nuclear 1,25-dihydroxyvitamin D3 receptor in rat liver and kidney. *Endocrinology* **129** 2318–2324.

Endoh H, Sasaki H, Maruyama K, Takeyama KI, Waga I, Shimizu T, Kato S & Kawashima H 1997 Rapid activation of MAP kinase by estrogen in bone cell line. *Biochemical and Biophysical Research Communications* **235** 99–102.

Escaleira MT, Sonohara S & Brentani MM 1993 Sex steroids induced up-regulation of 1,25-(OH)2 vitamin D3 receptors in T 47D breast cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* **45** 257–263.

Fielding CJ & Fielding PE 2000 Cholesterol and caveolae: structural and functional relationships. *Biochimica et Biophysica Acta* **1529** 210–222.

Fiorelli G, Picariello L, Martineti V, Tonelli F & Brandi LM 1999 Functional estrogen receptor β in colon cancer cells. *Biochemical and Biophysical Research Communication* **261** 521–527.

Flores-Delgado G, Bringas P, Buckley S, Anderson KD & Warburton D 2001 Nongenomic estrogen action in human lung myofibroblasts. *Biochemical and Biophysical Research Communications* **283** 661–667.

Francavilla A, Di Leo A, Polimeno L, Conte D, Barone M, Fanizza G, Chiumarulo C, Rizzo G & Rubino M 1987 Nuclear and cytosolic estrogen receptors in human colon carcinoma and in surrounding noncancerous colonic tissue. *Gastroenterology* **93** 1301–1306.

Francis RM, Peacock M, Storer JH & Nordin BEC 1984 Calcium malabsorption in elderly women with vertebral fractures: evidence for resistance to the action of vitamin D on the bowel. *Clinical Science* **66** 103–107.

Fujiuchi S, Ohsaki Y & Kikuchi K 1997 Suramin inhibits the growth of non-small-cell lung cancer cells that express the epidermal growth factor receptor. *Oncology* **54** 134–140.

Gallagher JC, Riggs BL, Eisman J, Hamstra A, Amoud SB & Deluca HF 1979 Intestinal calcium absorption and serum vitamin D metabolites in normal subjects and osteoporotic patients. *Journal of Clinical Investigation* **64** 729–736.

Gennari C, Agnusdei D, Nardi P & Civitelli R 1990 Estrogen preserves a normal intestinal responsiveness to 1,25-dihydroxyvitamin D3 in overectomized women. *Journal of Clinical Endocrinology and Metabolism* **71** 1288–1293.

Glass CK, Rose DW & Rosenfeld MG 1997 Nuclear receptor coactivators. *Current Opinion in Cell Biology* **9** 222–232.

Gross C, Eccleshall TR, Malloy PJ, Villa ML, Marcus R & Feldman D 1996 The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women. *Journal of Bone and Mineral Research* **11** 1850–1855.

Halachmi S, Marden E, Martin G, MacKay H, Abbondanza C & Brown M 1994 Estrogen receptor associated proteins: possible mediators of hormone-induced transcription. *Science* **264** 1455–1458.

Harris SS, Eccleshall TR, Gross C, Dawson-Hughes B & Feldman D 1997 The vitamin D receptor start codon polymorphism (FokI) and bone mineral density in premenopausal American black and white women. *Journal of Bone and Mineral Research* **12** 1043–1048.

Heaney RP, Recker RR & Saville PD 1978 Menopausal changes in calcium balance performance. *Journal of Laboratory and Clinical Medicine* **92** 953–963.

Hendrickse CW, Jones CE, Donovan IA, Neoptolemos JP & Baker PR 1993 Oestrogen and progesterone receptors in colorectal cancer and human colonic cancer cell lines. *British Journal of Surgery* **80** 636–640.

Herrmann C, Block C, Geisen C, Haas K, Weber C, Winde G, Moroy T & Muller O 1998 Sulindac inhibits Ras signaling. *Oncogene* **17** 1769–1776.

Horst RL, Goff JP & Reinhardt TA 1990 Advancing age results in reduction of intestinal and bone 1,25-dihydroxyvitamin D receptor. *Endocrinology* **126** 1053–1057.

Jehan F & DeLuca HF 1997 Cloning and characterization of the mouse vitamin D receptor promoter. *PNAS* **94** 10138–10143.

LaPushin R, Totpal K, Higuchi M & Aggarwal BB 1994 Suramin inhibits tumor cell cytotoxicity mediated through natural killer cells, lymphokine-activated killer cells, monocytes, and tumor necrosis factor. *Journal of Clinical Immunology* **14** 39–49.

Leland P, Obiri N, Aggarwal BB & Puri RK 1995 Suramin blocks binding of interleukin-4 to its receptors on human tumor cells and interleukin-4-induced mitogenic response. *Oncology Research* **7** 227–235.

Le Mellay V, Grosse B & Lieberherr M 1997 Phospholipase C beta and membrane action of calcitriol and estradiol. *Journal of Biological Chemistry* **272** 11902–11907.

Levy J, Zuili I, Yankowitz N & Shany S 1984 Induction of cytosolic receptors for 1α,25-dihydroxyvitamin D3 in the immature rat uterus by oestradiol. *Journal of Endocrinology* **100** 265–269.

Li S, Okamoto T, Chun M, Sargiacomo M, Casanova JE, Hansen SH, Nishimoto I & Lisanti MP 1995 Evidence for a Regulated Interaction between Heterotrimeric G Proteins and Caveolin. *Journal of Biological Chemistry* **270** 15693–15701.

Liel Y, Kraus S, Levy J & Shany S 1992 Evidence that estrogens modulate activity and increase the number of 1,25-dihydroxyvitamin D3 receptors in osteoblast-like cells (ROS 17/2·8). *Endocrinology* **130** 2597–2601.

Liel Y, Shany S, Smirnoff P & Schwartz B 1999 Estrogen increases 1,25-dihydroxyvitamin receptor expression and bioresponse in the rat duodenal mucosa. *Endocrinology* **140** 280–285.

Luqmani YA, Ryall G, Shousha S, Coombes RC 1992 An immunohistochemical survey of pS2 expression in human epithelial cancers. *International Journal of Cancer* **50** 302–304

Meggouh F, Lointier P & Saez S 1991 Sex steroid and 1,25-dihydroxyvitamin D3 receptors in human colorectal adenocarcinoma and normal mucosa. *Cancer Research* **51** 1227–1233.

Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E & Auricchio F 1996 Tyrosine kinase/p21^{ras} /MAPK-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO Journal* **15** 1292–1300.

Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Bottero D, Varricchio L, Nanayakkara M, Rotondi A & Auricchio F 2003 Sex steroid hormones act as growth factors. *Journal of Steroid Biochemistry and Molecular Biology* **83** 31–35

Mineo C, James GL, Smart EJ & Anderson RG 1996 Localization of epidermal growth factor-stimulated Ras/Raf-1 interaction to caveolae membrane. *Journal of Biological Chemistry* **271** 11930–11935.

Monje P, Marinissen MJ & Gutkind JS 2003 Phosphorylation of the carboxyl-terminal transactivation domain of c-Fos by extracellular signal-regulated kinase mediates the transcriptional activation of AP-1 and cellular transformation induced by platelet-derived growth factor. *Molecular and Cellular Biology* **23** 7030–7043.

Nahmias C & Strosberg AD 1995 The angiotensin AT2 receptor: searching for signal-transduction and physiological function. *Trends in Pharmacological Sciences* **16** 223–225.

Nemere I, Dormanen MC, Hammond MW, Okamua WH & Norman AW 1994 Identification of specific binding pro-branes of chick intestinal epithelium and relationship to transcaltachia. *Journal of Biological Chemistry* **269** 23750–23756.

Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M & Gustafsson JA 2001 Mechanisms of estrogen action. *Physiological Reviews* **81** 1535–1565.

Norman AW, Mizwicki MT & Norman DP 2004 Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. *Nature Reviews. Drug Discovery* **3** 27–41.

Paech K, Webb P, Kuiper GJ, Nilsson S, Gustafsson JA, Kushner PJ & Scanlan TS 1997 Differential ligand activtion of estrogen receptors ERα and ERβ at AP1 sites. *Science* **277** 1508–1510.

Pappas TC, Gametchu B & Wason CS 1995 Membrane estrogen receptors identified by multiple antibody labeling and impeded ligand binding. *FASEB Journal* **9** 404–410.

Pettersson K, Grandien K, Kuiper GJ & Gustafsson JA 1997 Mouse estrogen receptor β forms estrogen response element binding heterodimers with estrogen receptor α. *Molecular Endocrinology* **11** 1486–1496.

Pfahl M 1993 Nuclear receptor/AP1 interaction. *Endocrine Reviews* **14** 651–658.

Pietras R & Szego CM 1977 Specific binding sites for oestrogen at outer surfaces of isolated endometrial cells. *Nature* **265** 69–72.

Pietras RJ & Szego CM 1980 Partial purification and characterization of oestrogen receptors in subfractions of hepatocyte plasma membranes. *Biochemical Journal* **191** 743–760.

Qi X, Pramanik R, Wang J, Schultz RM, Maitra RK, Han J, DeLuca HF & Chen G 2002 Phosphorylation of the carboxyl-terminal transactivation domain of c-Fos by extracellular signal-regulated kinase mediates the transcriptional activation of AP-1 and cellular transformation induced by platelet-derived growth factor. *Journal of Biological Chemistry* **277** 25884–25892.

Robbins SM, Quintrell NA & Bishop MJ 1995 Myristoylation and differential palmitoylation of the HCK protein-tyrosine kinases govern their attachment to membranes and association with caveolae. *Molecular and Cellular Biology* **15** 3507–3515.

Russell JE, Morimoto S, Brige SJ, Fausto A & Avioli LV 1986 Effects of age and estrogen on calcium absorption in the rat. *Journal of Bone and Mineral Research* **1** 185–189.

Schwartz B, Smirnoff P, Shany S & Liel Y 2000 Estrogen controls expression and bioresponse of 1,25-dihydroxyvitamin D receptors in the rat colon. *Molecular and Cellular Biochemistry* **203** 87–93.

Smart EJ, Ying YS, Mineo C & Anderson RG 1995 A detergent-free method for purifying caveolae membrane from tissue culture cells *PNAS* **92** 10104–10108.

Song KS, Li S, Okamoto T, Quilliam LA, Sargiacomo M & Lisanti MP 1995 Co-purification and Direct Interaction of Ras with Caveolin, an Integral Membrane Protein of Caveolae Microdomains Detergent-free purification of caveolae membranes. *Journal of Biological Chemistry* **271** 9690–9697.

Tada H, Shiho O, Kuroshima KI, Koyama M & Tsukamoto K 1986 An improved colorimetric assay for interleukin 2. *Journal of Immunological Methods* **93** 157–167.

Takamoto S, Seino Y, Sacktor B & Liang CT 1990 Effect of age on 1,25-dihydroxyvitamin D3 receptors in Wistar rats. *Biochimica et Biophysica Acta* **1034** 22–28.

Takeda H, Yamakawa M, Takahashi T, Imai Y & Ishikawa M 1992 An immunohistochemical study with an estrogen receptor-related protein (ER-D5) in human colorectal cancer. *Cancer* **69** 907–912.

Tesarik J & Menoza C 1995 Nongenomic effects of 17 beta estradiol on maturing human oocytes: relationship to oocyte developmental potential. *Journal of Clinical Endocrinology and Metabolism* **80** 1433–1438.

Theisinger B, Guthke R, Blin N, Welter C & Seitz G 1993 Influence of steroid hormones on pS2/BCEI gene expression in xenografted colon tumors. *In Vivo* **7** 411–414.

Vincent TS, Hazen-Martin DJ & Garvin AJ 1996 Inhibition of insulin-like growth factor II autocrine growth of Wilms' tumor by suramin *in vitro* and *in vivo*. *Cancer Letters* **103** 49–56.

Walters MR 1981 An estrogen-stimulated 1,25-dihydroxyvitamin D3 receptor in rat uterus. *Biochemical and Biophysical Research Communications* **103** 721–726.

Watters JJ, Campbell JS, Cunningham MJ, Krebs GE & Dorsa DM 1997 Rapid membrane effects of steroids in neuroblastoma cells: Effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. *Endocrinology* **138** 4030–4033.

Wehling M 1995 Nongenomic aldosterone affects the cell membrane as a specific target of mineralocorticoid action. *Steroids* **60** 153–156.

Welter C, Theisinger B, Rio MC, Seitz G, Schuder G & Blin N 1994 Expression pattern of breast-cancer-associated protein pS2/BCEI in colorectal tumors. *International Journal of Cancer* **56** 52–55.

Received in final form 1 March 2005
Accepted 2 March 2005