[CANCER RESEARCH 61, 6360–6366, September 1, 2001]

Advances in Brief

Estrogens Do Not Modify MAP Kinase-dependent Nuclear Signaling during Stimulation of Early G_1 Progression in Human Breast Cancer Cells[1]

Silvana Caristi,[2] Juan Leyva Galera, Filomena Matarese, Manami Imai,[3] Simona Caporali,[4] Massimo Cancemi, Lucia Altucci, Luigi Cicatiello, Diana Teti, Francesco Bresciani, and Alessandro Weisz[5]

Dipartimento di Patologia generale, Seconda Università degli Studi di Napoli, 80138 Napoli [S. Car., J. L. G., F. M., M. I., S. Cap., M. C., L. A., L. C., F. B., A. W.], and Dipartimento di Patologia e Microbiologia sperimentale, Università degli Studi di Messina, 98125 Messina [D. T.], Italy

Abstract

Estrogens are direct mitogens for hormone-responsive human breast cancer cells, where they promote cell cycle progression and induce transcriptional activation of "immediate early" and cyclin genes. Nongenomic signaling by estrogens, including rapid changes of mitogen-activated protein (MAP) kinase and other signal-transduction-cascades activity, has been proposed to be essential for the mitogenic actions of these hormones and their nuclear receptors. Because regulation of gene transcription is considered a key step in cell cycle control by mitogenic protein kinase cascades, here we investigated the possibility that estrogen might induce the activation of extracellular signal-regulated kinase (Erk) 1/2-, c-Jun NH_2-terminal kinase-, p38- or protein kinase A-responsive transcription factors in the cell nucleus during stimulation of early G_1 progression, a timing coincident with the maximum effects of these hormones on such enzyme activity. No significant changes in protein kinase-mediated transcription factor activity could be detected here after estrogen stimulation of either MCF-7 or ZR-75.1 cells. Furthermore, these steroids were able to induce activation of the human *CCND1* gene promoter, accumulation of cyclin D1 and pRb phosphorylation, all key events in cell cycle stimulation by mitogens, even in the presence of Erk1/2 activation blockade by a MAP kinase-activating kinase (Mek)1/2 inhibitor. Thus, estrogens do not appear to convey significant protein kinase-dependent signaling to the cell nucleus during the early phases of human breast cancer cell stimulation. Furthermore, hormonal regulation of G_1 gene transcription can occur even without additional activation of the Mek-Erk1/2 pathway by estrogen receptors.

Introduction

Control of cell cycle progression by estrogen hormones, a crucial event in breast and endometrial carcinogenesis and tumor progression, involves hormonal regulation of cell cycle gene expression. Primary events in mitogenic stimulation of target cells by estrogen include transcriptional regulation of growth-controlling protooncogene and cyclin genes by ERs[6] (1–5). Although it is widely accepted that estrogen-mediated gene regulation is responsible for hormone-dependent cell proliferation, the mechanisms that underlie cell cycle control by these steroids are still not fully defined. Hormone-responsive cells are endowed with ERs, members of the nuclear receptors superfamily of transcription factors which modulate the activity of target gene promoters upon activation by their cognate ligands (6). In addition to this "genomic" pathway of estrogen action, a mounting body of evidence suggests that these hormones also can act via additional signaling pathways involving plasma membrane ERs, both identical and distinct from the nuclear subtypes described above (7). These, so called, "nongenomic" actions of estrogen in HBC cells include immediate and transient (lasting $<$10 min) activation of Erk1/2 MAP kinases, first reported in HBC cells (8) and then shown to occur with similar kinetics also in other hormone-responsive cell types, even independently of their growth response to the hormone. Furthermore, in HBC and other cell types, estrogens and ERs have been shown to stimulate adenylate cyclase and cAMP-dependent signaling (9), to induce activation of p38 (10), and to prevent activation of Jnk (11) MAP kinases. It is not clear at present how the above-mentioned genomic and nongenomic pathways of estrogen action integrate each other to achieve the full cellular response to the hormone and how these kinase cascades contribute to activation of cell cycle gene networks by estrogen in stimulated cells. In the case of the Mek-Erk1/2 cascade, it has been shown that inhibition of this pathway can prevent hormone-mediated HBC cell growth (12, 13), although cell cycle progression (14, 15) and protooncogene or *cyclin D1* gene regulation during G_1 (3, 13, 14) can occur in estrogen-stimulated cells even independently of Erk activation. Jones and Kazlauskas (16) have recently demonstrated that only growth factors capable of inducing prolonged Erk1 and -2 activation ($>$90 min) can promote S-phase entry in target cells, whereas those that induce short-lasting responses by these enzymes ($<$30 min) fail to act as full mitogens, suggesting that Erk or other signaling-enzyme activation can be dissociated from the promotion of G_1-phase completion. This is explained by the fact that only their strong and prolonged activation allows translocation of Erks and other MAP kinases to the cell nucleus, where they can control gene transcription by phosphorylating, and thereby activating, DNA-bound transcription factors (17). This last, in fact, is an essential step in mitogen-induced gene expression and cell cycle progression (18). This study was thus designed to investigate in ZR-75.1 and MCF-7 cells, two ER-positive HBC cell lines responsive to direct mitogenic stimulation by estrogen, whether hormone-induced changes in MAP kinase and PKA activity result in consequent signaling to the cell nucleus. In addition, we exploited *cyclin D1* gene activation by estrogen as a model to assess the role of Mek-Erk1/2 cascade activa-

Received 6/18/01; accepted 7/18/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the European Community (Contract BMH4-CT98-3433), the Associazione Italiana Ricerca sul Cancro (Grant 98-00), the Ministero dell'Università e Ricerca Scientifica e Technologica (COFIN 99-00), and Seconda Università di Napoli (Fondi Ricerca di Ateneo).

[2] Permanent address: Dipartimento di Patologia e Microbiologia sperimentale, Università degli Studi di Messina, Via Consolare Valeria, 98125, Messina, Italy.

[3] Permanent address: Department of Obstetrics and Gynecology, Kitasato University, Kitasato 1-15-1 Sagamihara, Kanagawa, Japan.

[4] Present address: Istituto Dermopatico dell'Immacolata, Via Monti di Creta 104, 00167 Rome, Italy.

[5] To whom requests for reprints should be addressed, at Dipartimento di Patologia generale, Seconda Università degli Studi di Napoli, L.tto S. Aniello a Caponapoli, 2, 80138 Napoli, Italy. Phone/Fax: (0039) 081-566-5702; E-mail: alessandro.weisz@unina2.it or weisz@na.cybernet.it.

[6] The abbreviations used are: ER, estrogen receptor; MAP kinase, mitogen-activated protein kinase; HBC, human breast cancer; Erk, extracellular signal-regulated kinase; Mek, MAP kinase-activating kinase; Mekk, Mek kinase; E_2, 17β-estradiol; PKA, protein kinase A; IGF-I, insulin-like growth factor I; DCC FCS, dextran-coated charcoal-treated fetal calf serum; pRb, retinoblastoma protein; Cdk, cyclin-dependent kinase; Jnk, c-Jun NH_2-terminal kinase; SAPK, stress-activated protein kinase; CREB, cAMP response element binding protein; EtOH, ethanol; CHOP, C/EBP homologous protein.

tion by these hormones in the control of G_1-regulatory gene transcription and cell cycle control pathways in growth-responsive HBC cells.

Materials and Methods

Reagents. Simvastatin lactone (Merck, Sharp, & Dome Italia) was activated before use, as described (16). Stock solutions (1000×) of E_2 (Sigma-Aldrich Italia; 10^{-5} M in 100% absolute EtOH) and human IGF-I (Ref. 16; 20 ng/ml in PBS) were stored frozen until use. All other reagents were of analytical grade and provided by major suppliers.

Cells and Culture Conditions. MCF-7 and ZR-75.1 cells were propagated as monolayer cultures in complete DMEM + 5% FCS medium. Cells were routinely tested for mycoplasma infection, and cultures were renewed, from frozen stocks, every 2–3 months. For use, except where otherwise indicated, cells were maintained for 4–5 days in estrogen- and phenol red-free medium as described (14). Serum-free medium included 0.25% cell-culture grade BSA (Sigma-Aldrich Italia). Simvastatin-mediated growth arrest and release from this cell cycle block by either estrogen or mevalonate were carried out as described earlier (1, 14).

Transient and Stable Transfections. Liposome-mediated transient gene transfer was carried out with DOTAP (Roche Italia) for ZR-75.1 cells and FuGENE6 (Roche Italia) for MCF-7 cells, as described by the manufacturer. First, preliminary tests were carried out to define the optimal transfection conditions. Transfected DNAs included *pFR-luc* (0.5 μg), a luciferase reporter gene including multimerized GAL4 UASs upstream of a minimal promoter, the indicated GAL *trans*-activator (50 ng), pSV-nlsLacZ DNA, a β-galactosidase expression vector (0.5 μg) and "empty" plasmid DNA (pBSM), to a final concentration of 3.5 μg/3.5-cm culture plates. Fifty ng of cytomegalovirus based expression vectors encoding Mek1, Mekk1, Mek3, or PKA were also transfected where indicated. All of the above vectors were part of the PathDetect *trans*-Reporting System (Stratagene, La Jolla, CA); before use, they were amplified, controlled by restriction mapping, and test-transfected. Six h after addition of the liposome-DNA mixture, cells were washed twice with PBS and stabilized for 12 h in the indicated culture medium before stimulation with E_2 (10^{-8} M in 0.1–0.01% ethanol), IGF-I (2 pg/ml), ethanol (0.1–0.01%), or DCC FCS (10%), as indicated. Cells were harvested either 24 h after transfection or after incubation with each inducer, as indicated, by washing and scraping in lysis buffer (Promega Italia) and then three cycles of rapid freeze-thawing. After clearing the cell lysates by a brief centrifugation at 4°C, the protein concentration was determined in the crude extracts with a colorimetric assay (BioRad Italia), luciferase and β-galactosidase activities were assayed in 100 μg protein extract as described earlier (1).

MCF-7 cell clones stably transfected with reporters pD1Δ-944 or pD1Δ-18 (including only the minimal human *cyclin D1* gene promoter, to position −18) were prepared and tested as described previously (1). Where indicated, 100 μM PD98059 was added to the culture medium 1 h before the beginning of additional stimulation or incubation.

Preparation of Whole Cell Extracts for Immunoblotting, Immunoprecipitation, and Kinase Assays. Cell extracts were prepared and analyzed by WB as described (1). Primary antibodies for immunodetection or immunoprecipitation were as follows: cyclin D1 (sc-92), pRb (sc-50), Cdk2 (sc163), Cdk4 (sc601), Erks (sc-94), pp-Jnks (sc-6254), and p-p38 (sc7973) from Santa Cruz Europe; p-Jun (06-828) from Upstate Biotech, Lake Placid, NY; phopho-Erk1/2 pathway (9911) from Cell Signaling, Beverly, MA; phospho-SAPK-Jnk Pathway (9912) from New England Biolabs, Beverly, MA; Cdc2 (Ab-4) from NeoMarkers, Union City, CA; and ppErks (V6671) from Promega Italia. Anti-p38, anti-SAPK-Jnks, and anti-pp70^{S6K} and pp90^{S6K} antibodies were a kind gift of Jiahuai Han (Scripps Research Institute, La Jolla, CA), John M. Kyriakis (Harvard Medical School, Charlestown, MA) and John Blenis (Harvard Medical School, Boston, MA), respectively. Peroxidase-labeled antirabbit or antimouse immunoglobulin antisera were used according to the manufacturer's (Amersham Italia) instructions. Densitometric analysis was performed with an Arcus-L scanner (Agfa, Germany), and subsequent data analysis was performed with a Gel-PRO Analyzer program (Media Cybernetics).

Cdk immunoprecipitation was carried out essentially as described (1–14); Cdk enzyme activity was assayed *in vitro* with SignaTECT (Promega Italia). Erk kinase activity was quantitated *in vitro* with the Biotrak p42/p44 Assay System (Amersham Italia).

Results and Discussion

Analysis of Estrogen Effects on Erk1/2 Activity and on Mek/Erk1/2, SAPK-Jnk, p38, and PKA Nuclear Signaling in Hormone-Responsive HBC Cells. ZR-75.1 and MCF-7 are ERα-expressing HBC cell lines that, upon estrogen deprivation, cease to proliferate and accumulate at the G_0–G_1 border, from where they can readily resume cell cycle progression by stimulation with a mitogenic dose (10^{-8} M) of E_2 (19). Estrogen stimulation of G_1 progression in these cells is mediated by direct activation of "immediate-early" and D-type cyclin gene transcription (1, 6, 16) and of the cyclin/Cdk/pRb cell cycle control cascade (1, 20). G_1 phase completion occurs, under these experimental conditions, by 12–15 h of hormonal stimulation, as demonstrated by sequential activation of cyclin D-Cdk4 and cyclin-E Cdk2 activity (Refs. 1 and 20 and data not shown). It has been reported that exposure of estrogen-deprived MCF-7 cells to E_2 is immediately followed by transient activation of Erk1/2 activity, maximal after 2–5 min of stimulation and lasting up to 10–30 min (7, 8). As shown in Fig. 1*a*, a 1.8–2.4-fold increase in Erk1/2 activity also can be detected in ZR-75.1 cells 2 min after the addition of 10^{-8} M E_2 to the cultures. Enzyme activity falls rapidly to uninduced levels, within 5–30 min. During these tests, we observed that Erk1/2 activation by E_2 was poorly reproducible, in as much as in several experiments it was undetectable, contrary to increases in Cdks and *cyclin D1* gene activity, or promotion of cell growth by E_2, which were instead consistently observed in all experiments (data not shown). The response of these MAP kinases to estrogen seemed distinguishable from the same response observed in hormone-responsive HBC cells after stimulation with "canonical" activators of this cascade (21), which induced very strong and longer-lasting Erks activation. Indeed, stimulation of ZR-75.1 or MCF-7 cells with IGF-I (20 pg/ml) for 5 min resulted in longer-lasting, reproducible enzyme activation (Fig. 1, *a–c* and data not shown). Furthermore, "mock" stimulation of ZR-75.1 cells with 0.01–0.1% (EtOH), the same concentrations of solvent used to complement the medium with the hormone, also resulted at times in slight (1.2–1.6-fold) and short-lasting (<5 min) activation of Erk enzymes (Fig. 1*a* and data not shown). Phosphorylated Erk1 and -2 were not detectable by Western blotting analysis in estrogen-stimulated cells (Fig. 1, *b* and *c*), indicating that, if this posttranslational modification of the Erk proteins by upstream regulators such as Mek1 and -2 indeed occurs, it concerns only a very small fraction of the Erk molecules present in the cell or, alternatively, that it might result from protein modifications not detectable with this assay. On the contrary, changes in Erk1 and -2 phosphorylation were easily detectable in parallel cultures stimulated with IGF-I (Fig. 1, *b* and *c*), insulin, or EGF (data not shown) and FCS (Fig. 3*a*).

To test whether estrogen-mediated Erk1/2 signaling can reach the transcriptional machinery in the cell nucleus, we exploited the ability of the activated forms of these enzymes to phosphorylate the *trans*-activation domain of the Elk1 transcription factor and thereby enhance its activity on gene transcription (17). To this aim, we expressed in ZR-75.1 cells the Erk1/2-responsive GAL-Elk chimeric transcription factor, comprising the DNA-binding domain of yeast GAL4 fused to the Erk-responsive *trans*-activation domain of human *Elk1*, and measured its activity *in vivo* on the expression of a GAL4-binding reporter gene during early E_2-induced G_1-phase progression (up to 6 h into stimulation), a time interval when both maximal activation of Erks and cell cycle gene transcription in response to estrogen occur (Refs. 1, 4, and 8 and Fig. 1*a*). The tests were carried out in the presence and in the absence of 5% serum in the cell-culture medium to control for possible interference of serum factors on estrogen-mediated Erk path-

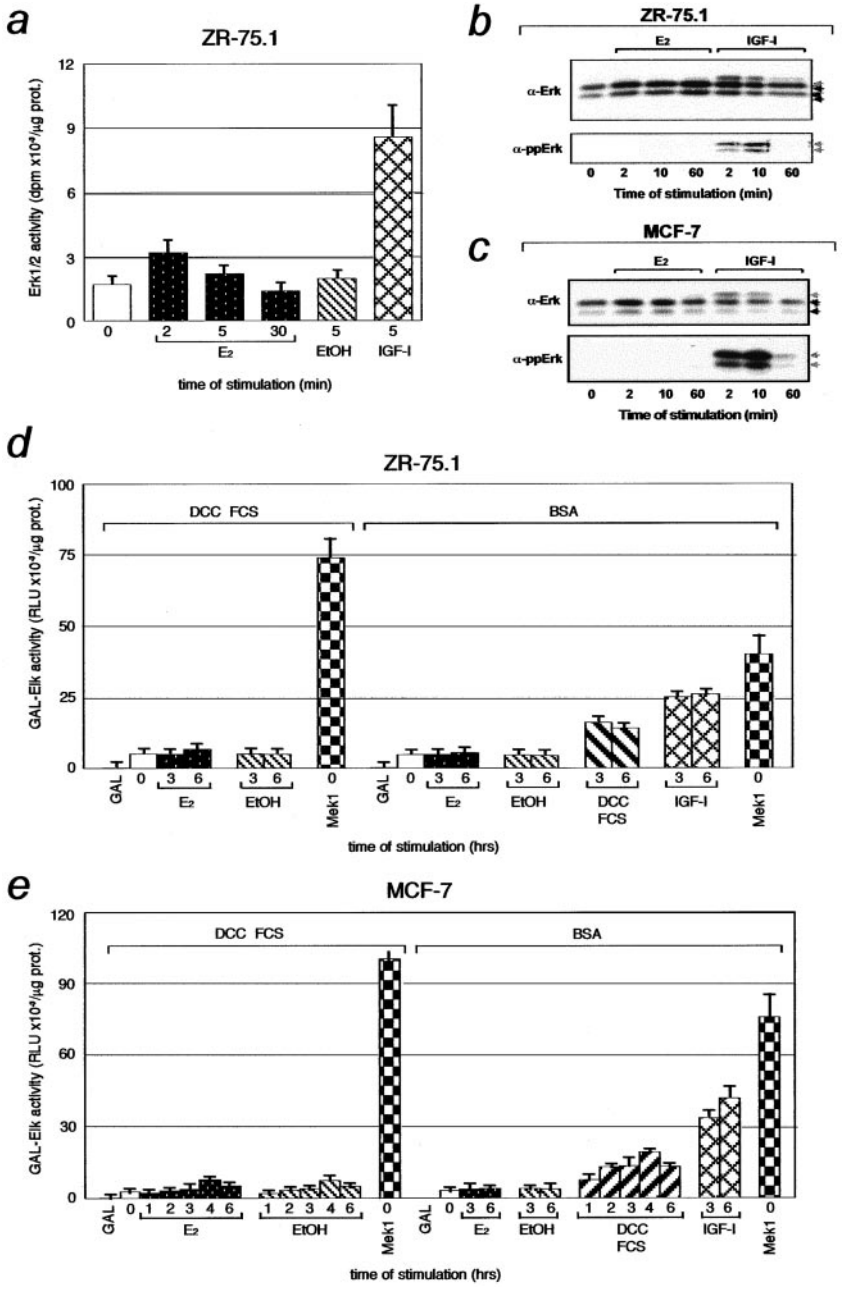

Fig. 1. Estrogen effects on Erk1 and 2 activity, phosphorylation status, and nuclear signaling during promotion of early G_1 phase progression in HBC cells. *a*, test of Erk1/2 enzyme activity in whole-cell extracts from control- or mitogen-stimulated HBC cells. *b* and *c*, high-resolution Western blot analysis of Erk1/2 phosphorylation status after estrogen or IGF-I stimulation of quiescent cells. *d* and *e*, each cell line was transfected with a GAL-TATA-luciferase reporter gene, an expression vector encoding GAL-Elk, or, where indicated (*GAL*), the GAL4 DNA binding domain, without or with (*Mek1*) an additional vector encoding human Mek1, and a bacterial β-galactosidase expression vector. After transfection, the cell cultures were stabilized either in estrogen-free full medium (*DCC FCS*) or in estrogen- and serum-free medium (*BSA*) for 12 h, before stimulation as indicated. Data (± SE) are expressed as luciferase units (*RLU*)/protein concentration unit and are representative of two to three experiments performed in duplicate. β-galactosidase levels were used as an internal control for transfection efficiency between different tests and within duplicates but were greatly affected by Mek1, DCC FCS, or IGF-I and, for this reason, could not be used to normalize data across the whole experiment.

way regulation. Preliminary to this investigation, we tested the possibility that transfection might influence cell cycle kinetics or the growth response to estrogen of HBC cells. For this, we fluorescently labeled both ZR-75.1 and MCF-7 cells by transient transfection with an expression vector encoding a green fluorescent protein, and then we analyzed cell cycle parameters before and after E_2 stimulation of both green fluorescent protein-positive and -negative liposome-treated cells by cytofluorometry. Results showed that the transfection procedure did not affect cell cycle kinetics and hormonal regulation of the cells, which behaved similarly to nonmanipulated controls.[7] As shown in Fig. 1, *d* and *e*, basal activity of the exogenous GAL-Elk fusion *trans*-activator on reporter gene transcription could be detected in both cell lines, consistent with reports of constitutive Erk1/2 activity in hormone-responsive cells (21). Estrogen stimulation of transfected cells induces a very small increase in Elk-mediated *trans*-activation of the reporter, whereas forced expression of Erk1/2-activating Mek1 enzyme, as well as serum and IGF-I stimulation of the cells, did produce quantifiable gene responses. It should be noted that the slight increase of GAL-Elk activity subsequent to E_2 treatment, more evident here for MCF-7 cells, could be artefactual, because it could be observed also upon mock stimulation of the cells with the same, very low, amount of EtOH required to complement the medium with the steroid (EtOH, Fig. 1*e*) and by simple manipulations of the culture plates, such as extraction from the CO_2 incubator for 1 min or for a few minutes or medium changes (data not shown). Contrary to what was observed for GAL-Elk, the E_2-responsive GAL-ERα(EF) fusion protein, comprising the hormone-responsive AF-2 of human ERα, was strongly activated by the hormone, both in the presence and in the absence of serum (>150-fold) in ZR-75.1 (Fig. 2*a*) or MCF-7 cells (data not shown). Forced expression of Mek1, Mekk1, and PKA, but not Mek3, or cell stimulation with IGF-I-induced, hormone-independent activation of the fusion protein (5–12-fold), consistent with the

[7] M. Cancemi *et al.*, manuscript in preparation.

known modulation of ER AF-2 activity by phosphorylation (19). When combined, these results indicated that activation of Erk1/2 by polypeptide growth factors and ER AF-2 by estrogen did occur in the cell nucleus and could be detected by this assay, whereas it was not possible to detect any direct effect of the hormone on Erk1/2-mediated nuclear signaling.

Estrogen and ERs have been reported to influence the activity of key components of other cytoplasmic signaling cascades in HBC or other cell types, including, in particular, stimulation of adenylate cyclase- and cAMP-dependent signaling (10), induction of p38 MAP kinase activation (10), and prevention of Jnk activation by noxious stimuli (11). By exploiting the transcription assay described above, we also set forth to measure possible changes in signaling to the cell nucleus by these transduction pathways during estrogen-induced early G_1 progression in ZR-75.1 and MCF-7 cells. For these tests, the *trans*-reporting fusion proteins GAL-Jun (responsive to the Mekk1/SAPK-Jnk pathway), GAL-CREB (responsive to cAMP/PKA-dependent signaling) and GAL-CHOP (responsive to p38 kinases via Mek3, S6k, and Erks) were used, and the experiments were carried out essentially as describe above for GAL-Elk. In Fig. 2, *b–d*, are reported the results obtained in ZR-75.1 cells, which were similar to what was observed in MCF-7 cells and which, for this reason, will only be mentioned here. These can be summarized as follows: (*a*) a measurable basal activity of GAL-Jun and GAL-CREB could be detected in the absence of hormone (compare in Figs. 2*b* and 2*c*, luciferase reporter gene expression in the presence of GAL-dbd alone—GAL- with that in the presence of GAL-Jun or GAL-CREB fusion proteins), which were significantly higher in the presence than in the absence of serum; (*b*) estrogen treatment induced a very slight decrease (8–15%) in GAL-Jun activity and no changes in GAL-CREB-mediated *trans*-activation, whereas forced expression of their activating protein kinases, Mekk1 and PKA, respectively, resulted in marked transcriptional enhancements; (*c*) basal GAL-CHOP activity was very high in these cells, in particular in the absence of serum, and was unresponsive to additional stimulation by either growth factors, forced expression of Mek3, or estrogen. When the phosphorylation status of Jnk1, Jnk2, and c-Jun was determined by Western blotting, no changes could be detected within the first 0.5–8 h of hormonal stimulation in both cell lines (data not shown), confirming the results obtained with the transcriptional assay. As mentioned above, CHOP is a target of phosphorylation by p38 MAP kinases, which in turn can be activated not only by Erks and Mek3, but also by ribosomal protein S6 kinases. The gene encoding the S6 kinase isoforms p70 and p85 is amplified and overexpressed in ZR-75.1 and MCF-7 cells (22). Indeed, when the cellular concentration and phosphorylation status of p38, p70, and p85 were measured in ZR-75.1 and MCF-7 cells by Western blotting, phosphorylated p38 isoforms were easily detectable in these, and the same was true for $p70^{S6K}$ and $p85^{S6K}$, which, in addition, were highly expressed when compared with their level in human fibroblasts or mammary epithelial MCF-10A cells (data not shown). p70 and p85 phosphorylation status was modulated by serum and growth factors in MCF-10A cells but not in ZR-75.1 or MCF-7 cells, where it was found to be very high and responsive (with a >90% reduction) to treatment of the cultures with rapamycin, an upstream inhibitor of the S6 kinase activation pathway (data not shown). This last result indicates that, in addition to *S6* gene amplification and overexpression, the enzymes this gene encodes are constitutively phosphorylated in ZR-75.1 and MCF-7 cells via rapamycin-sensitive pathways, providing a likely explanation for the high GAL-CHOP activity detected here.

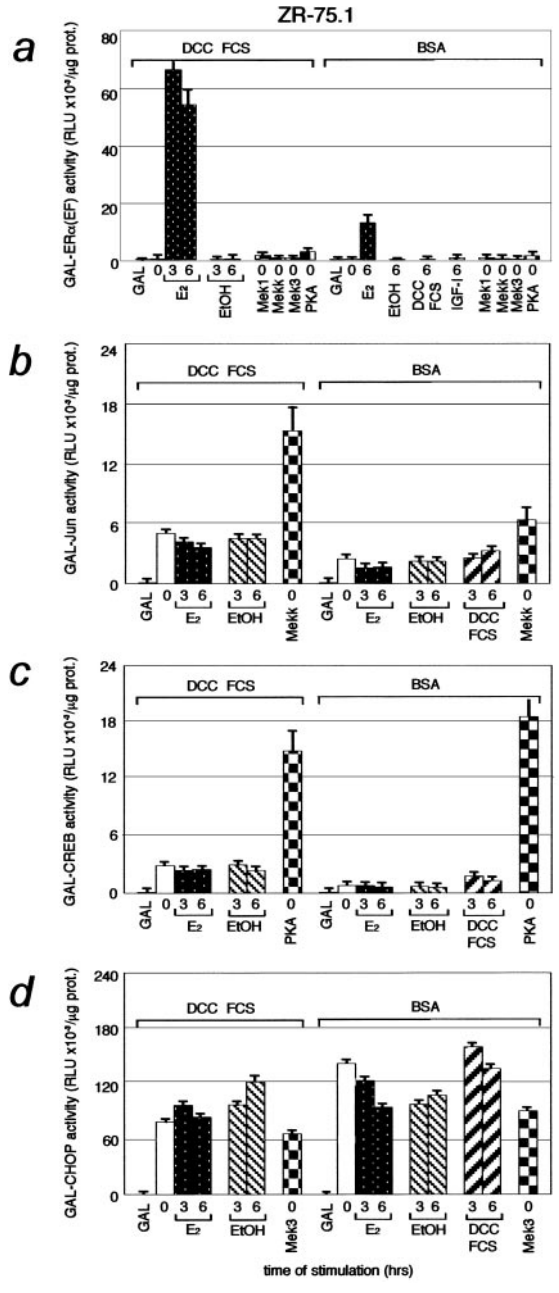

Fig. 2. Effects of estrogen on ERα AF2 activity and on nuclear signaling by the Mekk1/SAPK-Jnk-, cAMP-PKA-, and p38-dependent pathways in HBC cells. ZR-75.1 cells were transfected with a GAL-TATA-luciferase reporter gene and expression vectors encoding GAL-ERα(EF) (*a*), GAL-Jun (*b*), GAL-CREB (*c*), GAL-CHOP (*d*) or, where indicated (*GAL*), the GAL4 DNA binding domain, without or with vectors encoding the indicated protein kinases, and a bacterial β-galactosidase expression plasmid. After transfection, the cell cultures were stabilized either in estrogen-free full medium (*DCC FCS*) or in estrogen- and serum-free medium (*BSA*) for 12 h before stimulation as indicated. Data ($\pm$ SE) are expressed as luciferase units (*RLU*)/protein concentration unit and are representative of three experiments performed in duplicate. β-galactosidase levels were used only as an additional internal control for transfection efficiency and to compare different tests and duplicates.

Estrogen Can Induce Cyclin D1 Gene Activation and pRB Phosphorylation during Early G_1 Progression Even in the Presence of Mek-1 and -2 Inhibition. Given the known role of Erk1/2 MAP kinases in mediating mitogenic responses to extracellular stimuli, it has been proposed that activation of these enzymes might be part of the growth-promoting action of estrogen. One of the best-characterized mitogenic effects of these steroids is represented by recruitment of quiescent cells in the cycle and promotion of early the G_1 progression of cycling cells (5). We thus assumed that if Mek-induced Erks activation is indeed essential for growth stimulation by estrogen, blockade of this pathway should interfere with estrogen stimulation of early G_1-phase progression. We tested this hypothesis

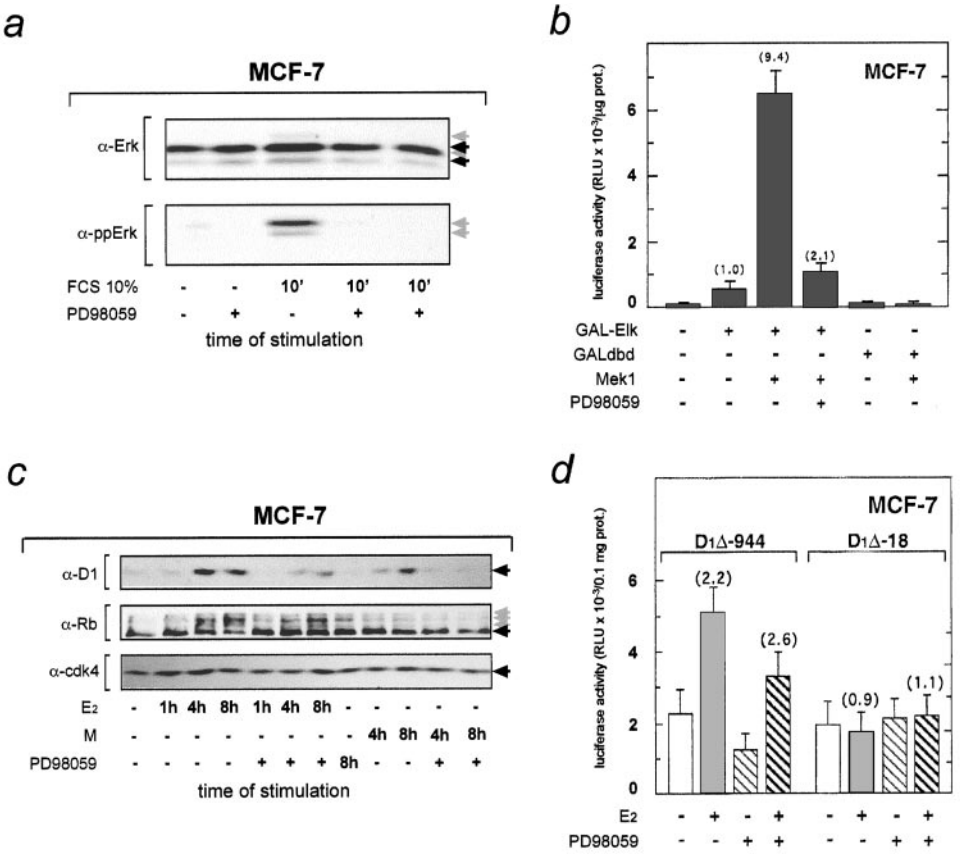

Fig. 3. Inhibition of the Erk1/2 MAP kinase pathway does not prevent cyclin D1 gene activation by estrogen in HBC cells during G_1. *a*, MCF-7 cells were maintained in serum- and phenol red-free medium for 24 h before addition of the same without (−) or with 10% DCC FCS. After the indicated time, cells were lysed and Erk-1 and -2 phosphorylation status was analyzed by high resolution Western blotting with antibody probes, detecting either total Erks (α-Erk) or phosphorylated, active Erk molecules (α-ppErk). *Gray arrows* on the *left* indicate the positions of phosphorylated Erk-1 and -2 proteins, respectively. Where indicated (+*PD*) 50 (*left*) or 100 μM (*right*) PD98059 was also added to the culture medium 1 h before additional treatments. *b*, cells were transiently transfected with a GAL4-binding luciferase reporter gene DNA and, where indicated (+), with 50 ng expression vector DNA encoding the indicated proteins, as described in the legend to Fig. 1. After 24 h, cells were collected, and luciferase and β-galactosidase (internal control) activities were assayed in the crude lysates. Data (± SE) are reported as luciferase units (*RLU*s)/protein concentration unit and refer to two experiments performed in duplicate. Where indicated (+*PD*), 100 μM PD98059 was added to the culture medium immediately after transfection. Numbers between parentheses indicate reporter-gene activity relative to cells transfected with the GAL-Elk expression vector. *c*, cells were maintained in estrogen-free medium before G_1 arrest with Simvastatin and then stimulation with 17β-estradiol (+*E2*) or release from the cell cycle block by the addition of 2 mM mevalonate to the medium (+*M*). At the indicated times, cells were lysed and 20 μg of total cellular proteins were analyzed by Western blotting as described in "Materials and Methods" (the antibodies used for immunodetection are indicated on the *left* side of each autoradiograph, and the position of the bands corresponding to specific immunocomplexes is indicated by *arrows* on the *right* side). Where indicated (+*PD*) 100 μM PD98059 was also added to the culture medium 1 h before induction. *d*, stable cell clones carrying the indicated reporter genes were maintained in estrogen-free medium for 4–5 days before complete cell cycle arrest with Simvastatin, stimulation with E_2 for 12 h in the presence or absence of 100 μM PD98059 (added to the culture media 1 h before the hormone), and luciferase analysis in whole-cell extracts. Data (± SE) are expressed as luciferase units (*RLU*)/protein concentration unit and are representative of two independent experiments, each performed in quadruplicate. Numbers in *parentheses* indicate the quantitative effect of estrogen on transfected reporter gene activity (ratio between luciferase activity in estrogen-treated *versus* control cells).

by comparing the activity of the *CCND1* gene, accumulation of the cyclin it encodes (D1) and pRb phosphorylation during the first 8 h of E_2-induced G_1 progression in synchronized MCF-7 cell cultures in the absence and in the presence of Mek1 and -2 blockade by PD98059. This compound, shown to be nontoxic and effective in HBC cells (21), binds to Mek1 and, with less affinity, Mek2 and thereby prevents activation of Erk1 and -2 by these enzymes. We first controlled PD98059 efficacy by two independent assays, whereby this compound was found to prevent serum-mediated Erk1 and -2 phosphorylation (Fig. 3*a*) as well as GAL-Elk activation by Mek1 in this cell line. To test the effects of Mek/Erk1/2 blockade on estrogen-mediated regulation of *CCND1* gene activity and expression, MCF-7 cell cultures were growth-arrested at the G_0-G_1 border by exposure to the cell cycle inhibitor Simvastatin, as described earlier (14), before stimulation with either 10^{-8} M E_2 or serum mitogens (by reversal of the Simvastatin-induced cell cycle block with mevalonate, as described in Ref. 14 and references therein). We have shown earlier that these experimental conditions are fully permissive for estrogen- or serum-mediated mitogenesis, allowing clear and reproducible detection of mitogen-mediated cell cycle effects (1, 14). *Cyclin D1* gene activation and expression, as well as pRb phosphorylation and cell cycle completion, are more easily detectable, under these conditions, in response to estrogen than to serum mitogens that are, however, very efficient inducers of cell cycle progression (Ref. 14 and references therein). This is attributable to the fact that synchronization of the cells at the G_0-G_1 border is a permissive condition for estrogen-signaling in these cells and, for this reason, allows easier detection of hormone-induced cell cycle changes (1, 14). The tests were carried out in the presence and in the absence of 100 μM PD98059, which was added to the culture medium 1 h before mitogenic stimulation. E_2 was able to enhance cyclin D1 accumulation in MCF-7 cells the presence of PD98059 (1.5–2-fold above control after 4 h and 3–4-fold increase by 8 h) with kinetics comparable with those detected in control cells (Fig. 3*c*). The Mek inhibitor, however, reduced basal cyclin D1 levels in quiescent cells by ~50% and, as a consequence, maximal accumulation on this protein after estrogen stimulation. Estrogen-induced pRb phosphorylation, coincident with cyclin D1 accumulation, was also detectable in the presence of 100 μM PD98059 (Fig. 3*c*), suggesting that G_1-specific Cdks were also activated. Contrary to what was observed for estrogen, cyclin D1 accumulation in response to serum

mitogens, detectable under these conditions upon addition of mevalonate to the cell cultures and consequent to reversal of serum mitogen inhibition (14), was completely abolished by preincubation of the cells with 100 μM PD98059 (Fig. 3*c*). We have previously shown that, under these conditions, pRb phosphorylation is less readily affected by serum mitogens during the first 12 hours of mevalonate treatment (1), and for this reason did not reach detectable levels here. Comparable results were obtained also in ZR-75.1 cells, where estrogens do activate this cyclin/Cdk/pRb cascade also in the absence of Simvastatin treatment (20); and this was, once again, not inhibited by 100 μM PD98059 (data not shown). When combined, the results described above suggest that serum, but not estrogen, requires Erk1/2 activation by Mek1/2 to promote cyclin D1 accumulation in HBC cells. To test whether the effects of estrogen and PD98059 on cyclin D1 levels were also detectable on the *CCND1* gene promoter, we measured the response to E_2 of *pD1*Δ*-944*, an estrogen-responsive human D1 promoter-luciferase reporter gene (1), after its stable insertion into the MCF-7 cells genome. As shown in Fig. 3*d*, *pD1*Δ*-944* activity is enhanced by estrogen both in the presence and in the absence of the Mek inhibitor, which, however, diminishes basal promoter activity. The mutant reporter *pD1*Δ*-18*, lacking both E-responsive and other *cis*-acting elements of the promoter, showed lower basal activity, which was unaffected by either E_2 or PD98059. The significance of this finding is discussed below.

It is well established that estrogen can stimulate directly normal and transformed cell proliferation, where regulation of cell cycle gene expression is a key step in the mitogenic activity of these steroids (5). Changes in the expression of estrogen-regulated genes represent the best-characterized cellular response to estrogen, sufficient to explain most long-range phenotypic cellular changes observed in response to these hormones, including cell proliferation. In addition to the well-known genomic actions of ERs nongenomic cellular responses to estrogen are being reported with increasing frequency (2, 7, 8) and have been proposed to exert a central role in target-cell responsivity to these hormones (12). Considering the role of such enzyme cascades, in particular those converging on Erk1 and -2 MAP kinases, in mitogenic signaling by mitogens acting via membrane receptors, this observation led to the proposal that direct regulation of certain MAP kinase pathways by cytoplasmic or membrane-bound ERs, or ER-like molecules, might represent a central event in cell-proliferation control by estrogen. This assumption was based primarily on the observation that pharmacological inhibition of Erk1/2 activation can prevent HBC cell proliferation in response to estrogen (12, 13). This, however, still remains questionable, because the growth of these same cells can occur even in the absence of detectable Erk1/2 activation (14, 15). Furthermore, one can assume that the MAP kinase blockade might result in the inhibition of cell cycle progression simply because it lowers the constitutive activity of these enzymes below a threshold level required to allow efficient ER-mediated canonical estrogen signaling. Indeed, it is known that ERs and key components of their genomic pathway are targets of MAP kinase-mediated phosphorylation, which either enhances or is a prerequisite for their function (3, 19, 23, 26). Furthermore, MAP kinase activity—but not activation—might be essential for the "cell cycle clock" functionality. This includes stimulus-independent, automatic effector molecules that perform the different metabolic tasks required for DNA replication and cell division. The interpretation of experimental results obtained when testing MAP kinase inhibitors effects on long-range estrogen responses, such as, in particular, cell cycle completion or cell proliferation, is made even more complex by other possible artefacts. Estrogen-stimulated HBC cells, in fact, secrete a number of growth factors of the EGF and IGF families, which can accumulate in the medium of hormone-treated cultured cells and thereby affect MAP kinase activity by autocrine mechanisms (5). Long-lasting inhibition of such signaling cascades can thus result in the inhibition of HBC-cell growth by interference with these indirect mitogenic loops, which is completely unrelated to the direct effects of estrogen on the cell cycle progression of HBC cells. An example of such mechanisms is likely to be represented by a recent report describing activation of Erk-mediated Elk1 phosphorylation and serum response factor-mediated transcription in E_2-treated HBC cells (2). In this case, the transcription assays were carried out with transfected cells maintained in the presence of hormone for nearly 2 days, a timing more consistent with indirect MAP kinase regulation via accumulation of secreted growth factors in the medium of hormone-treated cultures than with a short-lasting, direct stimulation of these pathways by hormone-activated ERs.

On the basis of the above considerations, we concluded that solid evidence supporting a primary role of direct MAP kinase activation in cell cycle control by estrogen and its nuclear receptors was still missing. For this reason, we set out to search for possible links between primary changes induced by estrogen in the MAP kinase- and cAMP-dependent signal transduction and promotion of G_1 phase progression. To this aim, we focused on the signaling pathways reported to be regulated by these hormones in HBC cells and exploited an assay which allows for efficient *in vivo* detection of nuclear signaling by key effectors of each of these pathways. As stated above, the rationale for this study resides in the fact that nuclear signaling by mitogenic signal transduction cascades is central to convert their short-lasting cytoplasmic responses to extracellular stimuli into longer-lasting changes in gene programming, which in turn is essential to allow activation of the cell cycle regulatory gene circuitries required for progression of the mitotic cycle to completion (16–18). Our results clearly show the lack of a significant correlation between estrogen-mediated early G_1 progression and increased MAP- or cAMP-dependent protein kinase signaling to the cell nucleus. Supporting this result, we found also that *cyclin D1* gene activation and pRb phosphorylation by E_2 can occur even when Erk1/2 activation by Mek1/2 is fully inhibited. As these responses of the cyclin/Cdk/pRb pathway represent molecular markers of early G_1 progression, this result indicates that Erk1/2 activation via Mek1/2 is dispensable for the estrogen-mediated promotion of cell cycle progression during this phase. When combined, these two results do not support the hypothesis that putative nongenomic actions of ERs via these pathways might exert a primary role in cell cycle gene regulation by estrogen, at least in these hormone-responsive HBC cells. In addition, as MAP and tyrosine kinase cascades are known to affect the ligand binding and *trans*-activation functions of the ER molecules (19), inhibition of estrogen-induced G_1-phase completion and G_1-S transition by blockade of key enzymes of these cascades (12, 13) are more likely to reflect an interference with the genomic pathway of estrogen action rather than attenuation of nongenomic estrogen signaling.

The data reported in Fig. 3, *c* and *d*, show that cyclin D1 induction and *CCND1* gene activation by E_2 are less pronounced in the presence of Mek1/2 inhibition. A likely explanation for this finding can be found in the following: (*a*) the promoter of the human *CCND1* gene is activated by estrogen via regulatory element(s) located between nucleotides −18 and −944 (1, 4), which, in HBC cells, involves direct interaction of ERs with this gene promoter *in vivo* (3); (*b*) this same regulatory region of the gene includes also multiple Mek-Erk1/2 kinase-responsive DNA elements (27) whose contribution to basal and ER-stimulated *CCND1* gene promoter activity is likely to be reduced by the blockade of this MAP kinase pathway; and (*c*) it was recently shown that direct activation of this gene by ERs involves AIB1 (3), a transcriptional coactivator whose functions can be controlled by these same MAP kinases (25). The lower response of the *CCND1* gene to estrogen in the presence of Erk1/2 inhibition might

thus be explained by considering that a complete blockade of constitutive Erk1/2 phosphorylation by inhibition of these enzymes is likely to interfere with the transcriptional effects of ER-AIB1 and, possibly, other ER-coactivator complexes (23, 24, 27). Efficient estrogen stimulation of cell cycle progression is thus bound to be conditioned by the status of multiple signaling pathways affecting ER function, independent of any direct effect of the hormone on the activity of these cascades.

In conclusion, the results reported here suggest a number of alternative explanations to the increasing number of reports concerning the effects of MAP kinase inhibitors on mitogenic signaling by estrogen, which might be relevant when considering the regulation of growth-independent cellular responses to these hormones and their nuclear receptors. As a consequence, we believe that some caution should be applied when considering new therapeutic approaches against hormone-responsive breast cancer based on MAP kinase pathway inhibition, as these treatments could ultimately interfere with a spectrum of beneficial effects of estrogen, dependent upon the genomic actions of ERs in many of their target tissues.

Acknowledgements

We thank Raffaele Addeo, Carmen Pacilio, and Domenico Germano for experimental assistance and stimulating discussions; C. Ambrosino (European Molecular Biology Laboratory, Heidelberg) for critical reading of the manuscript; Jiahuai Han (Scripps Research Institute, La Jolla, CA), John M. Kyriakis (Harvard Medical School, Charlestown, MA), and John Blenis (Harvard Medical School, Boston, MA) for anti-p38, anti-SAPK-Jnk, and anti-pp70^{S6K} and pp90^{S6K} antisera; and P. Chambon (Institute de Génétique et Biologie Moleculaire et Cellulaire, Strasbourg, France) for the GAL-ERα(E/F) fusion protein expression vector.

References

1. Altucci, L., Addeo, R., Cicatiello, L., Dauvois, S., Parker, M. G., Beato, M., Truss, M., Sica, V., Bresciani, F., and Weisz, A. 17β-estradiol induces cyclin D1 gene transcription, p36^{D1}-p34^{cdk4} complex activation and p105^{Rb} phosphorylation during mitogenic stimulation of G_1-arrested human breast cancer cells. Oncogene, *12:* 2315–2324, 1996.
2. Duan, R., Xie, W., Burghardt, R. C., and Safe, S. Estrogen receptor-mediated activation of the serum response element in MCF-7 cells through MAPK-dependent phosphorylation of Elk-1. J. Biol. Chem., *276:* 11590–11598, 2001.
3. Planas-Silva, M. D., Shang, Y., Donaher, J. L., Brown, M., and Weinberg, R. A. AIB1 enhances estrogen-dependent induction of cyclin D1 expression. Cancer Res., *61:* 3858–3862, 2001.
4. Sabbah, M., Courilleau, D., Mester, J., and Redeuilh, G. Estrogen induction of the cyclin D1 promoter: involvement of a cAMP response-like element. Proc. Natl. Acad. Sci. USA, *96:* 11217–11222, 1999.
5. Weisz, A., and Bresciani, F. Estrogen regulation of proto-oncogenes coding for nuclear proteins. Crit. Rev. Oncog., *4:* 361–388, 1993.
6. Weisz, A. Estrogen regulated genes. *In:* M. Oettel and E. Schillinger (eds.), Handbook of Experimental Pharmacology, Vol. 135/I: Estrogens and Antiestrogens, pp. 127–151. Springer Verlag, 1999.
7. Kelly, M. J., and Levin, E. R. Rapid actions of plasma membrane estrogen receptors. Trends Endocinol. Metab., *12:* 152–156, 2001.
8. Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. Tyrosine kinase/p21^{ras}/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J., *15:* 1292–1300, 1996.
9. Aronica, S. M., Kraus, W. L., and Katzenellenbogen, B. S. Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc. Natl. Acad. Sci. USA, *91:* 8517–8521, 1994.
10. Zhang, C. C., and Shapiro, D. J. Activation of the p38 mitogen-activated protein kinase pathway by estrogen or by 4-hydroxytamoxifen is coupled to estrogen receptor-induced apoptosis. J. Biol. Chem., *275:* 479–486, 2000.
11. Razandi, M., Pedram, A., and Levin, E. R. Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer. Mol. Endocrinol., *14:* 1434–1447, 2000.
12. Castoria, G., Barone, M. V., Di Domenico, M., Bilancio, A., Ametrano, D., Migliaccio, A., and Auricchio, F. Non-transcriptional action of oestradiol and progestins triggers DNA synthesis. EMBO J., *18:* 2500–2510, 1999.
13. Lobenhofer, E. K., Hupper, G., Iglehadrt, J. D., and Marks, J. R. Inhibition of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activity in MCF-7 cells prevents estrogen-induced mitogenesis. Cell Growth Differ., *11:* 99–110, 2000.
14. Bonapace, I. M., Addeo, R., Altucci, L., Cicatiello, L., Bifulco, M., Laezza, C., Salzano, S., Sica, V., Bresciani, F., and Weisz, A. 17β-estradiol overcomes a G_1 block induced by HMG-CoA reductase inhibitors and fosters cell cycle progression without inducing ERK-1 and -2 MAP kinases activation. Oncogene, *12:* 753–763, 1996.
15. Lobenhofer, E. K., and Marks, J. R. Estrogen-induced mitogenesis of MCF-7 cells does not require the induction of mitogen-activated protein kinase activity. J. Steroid. Biochem. Mol. Biol., *75:* 11–20, 2000.
16. Jones, S., and Kazlauskas, A. Growth-factor-dependent mitogenesis requires two distinct phases of signaling. Nat. Cell Biol., *3:* 165–172, 2001.
17. Karin, M., and Hunter, T. Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. Curr. Biol., *5:* 747–757, 1995.
18. Brunet, A., Roux, D., Lenormand, P., Dowd, S., Keyse, S., and Pouyssegur, J. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. EMBO J., *18:* 664–674, 1999.
19. Weigel, N. Steroid hormone receptors and their regulation by phosphorylation. Biochem. J., *319:* 657–667, 1996.
20. Cicatiello, L., Addeo, R., Altucci, L., Belsito Petrizzi, V., Boccia, V., Cancemi, M., Germano, D., Pacilio, C., Salzano, S., Bresciani, F., and Weisz, A. The antiestrogen ICI 182,780 inhibits proliferation of human breast cancer cells by interfering with multiple, sequential estrogen-regulated processes required for cell cycle completion. Mol. Cell. Endocrinol., *165:* 199–209, 2000.
21. Hermanto, U., Zong, C. S., and Wang, L. H. Inhibition of mitogen-activated protein kinase kinase selectively inhibits cell proliferation in human breast cancer cells displaying enhanced insulin-like growth factor I-mediated mitogen-activated protein kinase activation. Cell Growth Differ., *11:* 655–664, 2000.
22. Barlund, M., Forozan, F., Kononen, J., Bubendorf, L., Chen, Y., Bittner, M. L., Torhorst, J., Haas, P., Bucher, C., Sauter, G., Kallioniemi, O. P., and Kallioniemi, A. Detecting activation of ribosomal protein S6 kinase by complementary DNA and tissue microarray analysis. J. Natl. Cancer Inst., *92:* 1252–1259, 2000.
23. Ait-Si-Ali, S., Carlisi, D., Ramirez, S., Upegui-Gonzalez, L. C., Duquet, A., Robin, P., Rudkin, B., Harel-Bellan, A., and Trouche, D. Phosphorylation by p44 MAP Kinase/ERK1 stimulates CBP histone acetyl transferase activity *in vitro.* Biochem. Biophys. Res. Commun., *262:* 157–162, 1999.
24. Watters, J. J., Chun, T. Y., Kim, Y. N., Bertics, P. J., and Gorski, J. Estrogen modulation of *prolactin* gene expression requires an intact mitogen-activated protein kinase signal transduction pathway in cultured rat pituitary cells. Mol. Endocrinol., *14:* 1872–1881, 2000.
25. Font de Mora, J., and Brown, M. AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. Mol. Cell. Biol., *20:* 5041–5047, 2000.
26. Lopez, G. N., Turck, C. W., Schaufele, F., Stallcup, M. R., and Kushner, P. J. Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. J. Biol. Chem., *276:* 22177–22182, 2001.
27. Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A., and Pestell, R. G. Transforming p21^{ras} mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. J. Biol. Chem., *270:* 23589–23597, 1995.