

# Prolonged Extracellular Signal-Regulated Kinase 1/2 Activation during Fibroblast Growth Factor 1- or Heregulin $\beta$ 1-Induced Antiestrogen-Resistant Growth of Breast Cancer Cells Is Resistant to Mitogen-Activated Protein/Extracellular Regulated Kinase Kinase Inhibitors

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## ABSTRACT

Increased growth factor receptor signaling is implicated in antiestrogen-resistant breast tumors suggesting that abrogation of such signaling could restore or prolong sensitivity to antihormonal agents. Activation of the mitogen-activated protein/extracellular regulated kinase kinase (MEK)-extracellular regulated kinase (ERK)1/2 cascade is a common component of such pathways. We investigated the ability of the MEK activation inhibitor U0126 to block the increased growth of estrogen receptor-positive MCF-7 breast cancer cells caused by fibroblast growth factor 1 (FGF-1), heregulin  $\beta$ 1 (HRG $\beta$ 1), and epidermal growth factor (EGF) in the presence of the pure antiestrogen ICI 182780 (Faslodex; fulvestrant). We found that either FGF-1 or HRG $\beta$ 1 but not EGF substantially reduced the inhibitory effects of U0126 on growth and ERK1/2 activation, including the combined inhibitory effects of U0126 and ICI 182780. FGF-1 and HRG $\beta$ 1 also reduced the inhibition of ERK1/2 phosphorylation by the MEK inhibitors PD98059 and PD184161. Interestingly, a transiently transfected dominant-negative MEK1 completely abrogated activation of a coexpressed green fluorescent protein-ERK2 reporter by all three of the factors. Despite a short-lived activation of Ras and Raf-1 by all three of the growth factors, both FGF-1 and HRG $\beta$ 1, unlike EGF, induced a prolonged activation of MEK and ERK1/2 in these cells. Thus, activation of FGF-1- and HRG $\beta$ 1-specific signaling causes MEK-dependent prolonged activation of ERK1/2, which is incompletely susceptible to known MEK inhibitors. We also demonstrate that the cytosolic phospholipase A2 inhibitor arachidonyl trifluoro methyl ketone and the pan PKC inhibitor bisindolymaleimide abrogated U0126-resistant phosphorylation of ERK1/2 induced by HRG $\beta$ 1 but not by FGF-1. Phosphorylation of ERK5 by all three of the factors was also resistant to U0126 suggesting that its activation is not sufficient to overturn growth inhibition due to diminished ERK1/2 activation. Therefore, therapy combining antiestrogens and MEK inhibitors may be ineffective in some antiestrogen-resistant estrogen receptor-positive breast cancers.

## INTRODUCTION

Estrogen receptor (ER)-positive breast cancers that initially respond to antiestrogens like tamoxifen frequently become resistant to it and to subsequent hormonal therapies. Understanding the mechanisms of development of antiestrogen resistance could lead to therapeutic strategies that might overcome such resistance in patients. Animal models of acquired tamoxifen resistance have shown that development of resistance is associated with an increase in the expression of erbB2

tyrosine kinase receptor (also called HER2/*neu*; Ref. 1). Whereas it was shown recently that long-term estrogen deprivation of ER-positive MCF-7 breast carcinoma cells can result in enhanced erbB2 expression (2), others have found that long-term treatment of these cells with antiestrogen did not result in altered levels of any of the erbB family members (3). In addition, studies with patient samples suggest that tumors that are initially negative for expression of HER2 do not acquire expression during tamoxifen treatment and subsequent relapse, suggesting that additional mechanisms might also account for such resistance (4).

Heregulin  $\beta$ 1 (HRG $\beta$ 1), which binds to HER2/HER3 and HER2/HER4 heterodimers (5), has also been shown to induce antiestrogen-resistant growth in MCF-7 cells (3, 6). In addition, epidermal growth factor (EGF) and transforming growth factor  $\alpha$ , known ligands of EGF receptor/HER1 have also been shown to induce either estrogen-independent or antiestrogen-resistant growth in MCF-7 cells (6, 7). Thus, these studies suggest that overexpression of the ligands themselves or aberrant expression or activity of other erbB receptor family members could also participate in the development of antiestrogen resistance.

A number of investigators have shown that different growth factors induce antiestrogen-resistant growth to varying degrees (8–13). Our laboratory has shown that signaling mediated by fibroblast growth factor (FGF)-4 and FGF-1 can also provide an alternative growth stimulus to that provided by ER activation in MCF-7 breast adenocarcinoma cells, which results in antiestrogen resistant growth (8, 14–16). Cross-talk between growth factor signaling and ER can also involve ER activation by phosphorylation independent of estrogen or phosphorylation of coactivators (17–19).

Distinct growth factors including FGFs, EGF, and HRG $\beta$ 1 are thought to regulate proliferation by activating tyrosine kinase receptors that result in the activation of a common signaling pathway involving mitogen-activated protein kinases (MAPK) extracellular regulated kinase (ERK)1 and ERK2 (20–24). Moreover, constitutively active Raf-1 and constitutively active MEK1 have also been shown to mediate antiestrogen-resistant growth in MCF-7 cells (25, 26). Therefore, it was reasonable to speculate that the MEK-ERK1/2 cascade represented a point of convergence of many signaling pathways that when blocked could abrogate multiple antiestrogen resistance mechanisms. U0126, a small-molecule inhibitor of the MAPK signal transduction pathway, used in nanomolar to low micromolar doses, has been shown to abrogate growth stimulation in response to a number of growth factors including factors that activate receptor tyrosine kinases (27, 28). U0126 has been shown to specifically inhibit the MAPK kinases mitogen-activated protein/ERK kinase (MEK)1 and MEK2 both *in vitro* and *in vivo* (29). Blockade of the MAPK pathway using the MEK inhibitor PD 184352 (a structural analog of PD 184161), has also been shown to suppress growth of colon tumors and leukemic cell lines, and tumor growth reduction has been correlated with a dose-dependent reduction in levels of activated

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MAPKs (30, 31). Therefore, combining MEK blockers with drugs like tamoxifen (or the pure ER antagonist ICI 182780) might improve therapy by blocking nonestradiol (E2)-dependent MAPK signaling that could potentially cause antiestrogen resistance in ER-positive breast cancers. Indeed, in two separate studies U0126 has been demonstrated to enhance the effects of tamoxifen and ICI 182780, respectively, against HER2-overexpressing ER-positive breast cancer cells *in vitro* (26, 32).

To assess whether MEK inhibitors might be useful in combination with antiestrogens we investigated the role of the MEK-ERK1/2 cascade in antiestrogen-resistant growth of MCF-7 cells in response to EGF, FGF-1, and HRG $\beta$ 1. Transient cotransfection of a green fluorescent protein (GFP)-ERK2 fusion construct with a dominant-negative MEK1 indicated that the ERK1/2 activation induced by all of these factors was MEK1/2-dependent. However, contrary to our expectation, we found that the MEK inhibitor U0126 was not able to completely block ERK1/2 activation and antiestrogen-resistant growth induced by either FGF-1 or HRG $\beta$ 1. We also found that the MEK inhibitors PD98059 and PD184161 were not able to completely block ERK1/2 activation induced by these factors. This appeared to be related to the prolonged activation of ERK1/2 that occurs after addition of either factor. However, the underlying mechanism for the prolonged activation of the MEK-ERK1/2 cascade by these factors appears to be different. ERK1/2 phosphorylation induced by HRG $\beta$ 1 but not by FGF-1 was abrogated by cotreatment with U0126 plus either the cytosolic phospholipase A2 (cPLA2) inhibitor arachidonyl trifluoro methyl ketone (AACOCF3) or the pan-conventional protein kinase C (PKC) inhibitor bisindolylmaleimide. Thus, FGF-1 and HRG $\beta$ 1-induced ERK 1/2 activation is prolonged and U0126-resistant but not MEK-independent. Instead, we suggest that factor-specific MEK1/2 activation mechanisms, which, in the case of HRG $\beta$ 1, is PKC- and PLA2-dependent, leads to incomplete susceptibility to U0126-mediated inhibition. Therefore, MEK inhibitors may not be very effective in reducing antiestrogen-resistant growth phenotypes induced by FGF-1- and HRG $\beta$ 1-mediated pathways in ER-positive breast cancer.

## MATERIALS AND METHODS

**Chemicals, Growth Factors, and Antibodies.** U0126 was obtained from Promega (Madison, WI), PD98059 was from Calbiochem (La Jolla, CA), and PD184161 was a gift from Pfizer Inc. (Groton, CT). Recombinant human FGF-1 and EGF were obtained from Promega, and recombinant human HRG $\beta$ 1 (residues 176–246 corresponding to the EGF-like domain) was from R&D Systems (Minneapolis, MN). ICI 182780 (Faslodex) was a gift from AstraZeneca (Macclesfield, Cheshire, United Kingdom) and was dissolved in ethanol before use. Bisindolylmaleimide and AACOCF3 were obtained from Calbiochem. Phenol red-free and phenol red containing Improved Modified Eagle's Medium (IMEM) were obtained from Invitrogen (Carlsbad, CA). In some experiments charcoal-stripped calf serum was used to strip cells of endogenous estrogens. Calf serum was treated with dextran-coated activated charcoal as described before (8, 33) to obtain charcoal-stripped calf serum. Anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-MEK, anti-MEK, anti-phospho p90 Rsk-1, and anti-p90 Rsk-1 were obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-ERK5 and anti-ERK5 were obtained from Biosource International (Camarillo, CA).

**Plasmids, Cell Lines, and Cell Culture.** ML20 is a clonally isolated estrogen-responsive line derived from MCF-7 breast adenocarcinoma cells that has a stably transfected *LacZ* gene and has been described previously (14, 15, 33). ML20 cells are sensitive to both tamoxifen and the pure antiestrogen ICI 182780 both *in vitro* and *in vivo* (14, 15, 33). ML20 cells were maintained in IMEM containing 5–10% fetal bovine serum (FBS). GFP-ERK2 plasmid (34) was obtained from Dr. Eisuke Nishida (Kyoto University, Kyoto, Japan). The pCMV-dnMEK plasmid with S218A and S222A substitutions was obtained

from Dr. Kun Liang Guan (University of Michigan Medical School, Ann Arbor, MI).

**U0126 Treatment, 3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxy-Phenyl)-2-(4-Sulfonyl)-2H-Tetrazolium (MTS) Assays, and Growth Curves.** Subconfluent ML20 cells were stripped of estrogens by four changes with 5% charcoal-stripped calf serum over a period of 3 days. Cells were plated in this medium in 96-well plates. Cells (5000) were plated in each well and were allowed to attach overnight. The next day (day 0) the medium was aspirated, and 200  $\mu$ l of fresh medium with various conditions (listed in Table 1) was added to each well. On day 5, 40  $\mu$ l of MTS assay reagent (CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit; Promega) was added to each well, and the plates were incubated at 37°C for 2 h. Absorbance at 490 nm was read with a 96-well plate reader.

**Transfections.** Transient transfection assays were used to test whether a dominant-negative MEK1 can block the phosphorylation of a GFP-ERK2 fusion protein. ML20 cells maintained in 5% FBS containing IMEM medium were used in these assays. ML20 cells ( $1.2 \times 10^6$ ) were plated in 10-cm tissue culture dishes. They were transfected the next day using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's instructions in serum-free IMEM. Cells were transfected with 2  $\mu$ g of GFP-ERK2 plasmid and 8  $\mu$ g of either pCMV-dnMEK or a control pCMV plasmid. Transfected cells were incubated for 48 h in 5% FBS containing IMEM to allow for expression. The transfected cells were then stripped of estrogens with four changes of 5% charcoal-stripped calf serum containing medium and then left overnight in serum-free IMEM. The following day, the cells were treated with EGF, FGF-1, or HRG $\beta$ 1 in 5% FBS containing medium. The cells were incubated in these various conditions for 30 min or 2 h. Subsequently, cells were lysed using  $1 \times$  Cell Lysis buffer (Cell Signaling Technology) supplemented with 1 mM phenylmethylsulfonyl fluoride. Lysates (40  $\mu$ g) were then analyzed by Western Immunoblotting as described below.

**Immunoblotting Analyses.** For the analysis of phospho-ERK1/2, phospho-MEK1/2, phospho-p90 Rsk, and phospho-ERK5, Western blotting was performed on lysates prepared from ML20 cells treated with the appropriate conditions. Forty  $\mu$ g of cell lysate was run on Criterion precast 10% SDS-PAGE gels (Bio-Rad, Hercules, CA). Proteins were transferred onto Protran filters (Bio-Rad) by electroblotting. The blots were then probed with phosphospecific antibodies against the following: phospho-MAPK p44/42, phospho-MEK, phospho-p90 Rsk-1 (Cell Signaling Technology), or phospho-ERK5 antibodies (Bio-Source International). After probing with phospho-MAPK p44/42 antibodies, blots were stripped with Restore stripping solution (Pierce, Rockford, IL) and reprobed with antibodies against total ERK1/2. Blots that were first probed for phospho-MEK, phospho-p90 Rsk-1, or phospho-ERK5 were subsequently reprobed for total MEK, total p90 Rsk-1, and total ERK5, respectively, after stripping. Enhanced chemiluminescence with the Supersignal kit (Pierce) was used to detect bands. Densitometric analysis of the Western blots that examined the time course of activation of ERK1/2 and MEK was performed by using the NIH Image J Program. The sum of the intensities of the phospho-ERK1/2 bands were normalized to that of the total ERK1/2 bands for every time point. An average of all of the normalized values for the zero time point was then obtained. The normalized values for each time point in each of the treated conditions was then expressed as fold change over the averaged normalized intensity for the zero time point. Means  $\pm$  SE of the fold changes were subjected to the Student's *t* test and were represented graphically (SigmaPlot v8.0, SPSS Inc.) as a function of time post-treatment.

Table 1 Description of estradiol-, ICI 182780-, and growth factor-supplemented medium conditions used

Nomenclature	Components in the medium
F	5% FBS <sup>a</sup>
FI	5% FBS, ICI 182780 ( $10^{-7}$ M)
FIE	5% FBS, ICI 182780 ( $10^{-7}$ M) and EGF 10 ng/ml
FIF	5% FBS, ICI 182780 ( $10^{-7}$ M) and EGF-1 20 ng/ml
FIH	5% FBS, ICI 182780 ( $10^{-7}$ M) and HRG $\beta$ 1 25 ng/ml
FIE2	5% FBS, ICI 182780 ( $10^{-7}$ M) and 17 $\beta$ -Estradiol ( $10^{-8}$ M)
FE	5% FBS and EGF 10 ng/ml
FF	5% FBS and FGF-1 20 ng/ml
FH	5% FBS and HRG $\beta$ 1 25 ng/ml

<sup>a</sup> FBS, fetal bovine serum; EGF, epidermal growth factor; HRG $\beta$ 1, heregulin  $\beta$ 1; FGF-1, fibroblast growth factor 1.

The Western blots examining MEK activation were subjected similarly to densitometric analysis.

**Ras Activation Assays.** Ras activation was assessed by using a Raf-1 ras binding domain affinity binding assay. Cells were lysed in 1× lysis buffer (Cell Signaling Technology) supplemented with 1 mM of phenylmethylsulfonyl fluoride, and 500 μg of cellular lysate was incubated with 10 μl Raf-1 ras binding domain agarose from the Ras activation kit (Upstate Biotechnology, Lake Placid, NY) at 4°C for 30 min. The agarose pull-downs were washed three times with lysis buffer and boiled with 2× Laemmli sample buffer [10% glycerol, 150 mM β-mercaptoethanol, 3% SDS, and 150 mM Tris-HCl (pH 6.8)]. Proteins were fractionated on 12% SDS-PAGE gels and transferred to Protran nitrocellulose membranes (Bio-Rad), followed by immunoblotting with anti-Ras antibodies (Upstate Biotechnology).

**Raf-1 Activation Assays.** Raf-1 was immunoprecipitated using an antibody directed against the COOH terminus of Raf-1 (sc-133; Santa Cruz Biotechnology, Santa Cruz, CA) coupled to protein G-Sepharose. Immunoprecipitates were washed twice with lysis buffer and then incubated with 30 μl kinase assay buffer {25 mM HEPES (pH 7.4), 10 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 1 mM DTT, 1 μM ATP, and 30 μCi [<sup>γ</sup>-<sup>32</sup>P]ATP} containing inactive MEK1 expressed in *Escherichia coli* as a fusion with glutathione S-transferase (US Biologicals, Swampscott, MA) as substrate. After incubating for 30 min at 30°C, reactions were spotted onto P81 phosphocellulose squares, which were subsequently washed 10 times with 150 mM phosphoric acid. Radioactivity on the P81 squares was counted using a Bioscan QC 4000 Benchtop Radioisotope Counter (Washington, DC). A protein-G Sepharose precipitate in the absence of Raf-1 antibody was used to obtain background cpm values and was subtracted from each experimental value.

## RESULTS

**FGF-1 and HRG β1 Induce Both Antiestrogen-Resistant and U0126-Resistant Growth in MCF-7 Cells.** To assess whether MEK blockade would restore or prolong sensitivity of ER-positive breast cancer cells to antiestrogens, we tested the ability of U0126 to block FGF-1-induced growth of ML20 cells in the presence and absence of ICI 182780 in an exponential growth assay. Estrogen-deprived ML20 cells were plated in a 24-well format and were then fed with 5% FBS-containing medium, either supplemented or not with FGF-1, in the presence or absence of ICI 182780. These conditions are listed in Table 1. Parallel sets of wells were treated with increasing doses of U0126 for the entire duration of the experiment (5 days) with a pretreatment 30 min before addition of conditions. As shown in Fig. 1A, ICI 182780 ( $1 \times 10^{-7}$  M) inhibited growth of ML20 cells by ~50% by day 5 in this assay consistent with the fact that serum estrogens mediate a majority of the growth signaling in this line (Fig. 1A, compare FI condition with F). Exogenous supplementation with FGF-1 could overcome the effects of ICI 182780, bringing the growth rate approximately to levels observed with 5% FBS (Fig. 1A, compare FIF with FI and F).

When increasing doses of U0126 were added, growth in 5% FBS medium (either containing ICI 182780 or not) was drastically inhibited (growth in F condition was 55%, 40%, and 20% of control when cells were treated with 3, 10, and 30 μM U0126, respectively; Fig. 1A). Surprisingly, despite the established role of ERK1/2 activation in FGF signaling in other systems, U0126 only modestly inhibited growth in FGF-1 supplemented conditions, both in the presence and absence of ICI 182780 (Fig. 1A, compare FF with F and FIF with FI in U0126-treated cells). Importantly, we demonstrate that even a 3 μM U0126 dose drastically inhibited growth of ML20 cells growing in FBS, and also when E2 ( $1 \times 10^{-8}$  M) was added to negate the effects of the antiestrogen (Fig. 1A, FIE2 condition in U0126 treated *versus* untreated). FGF-1 supplemented cells on the other hand exhibited increased growth even in the presence of U0126 doses as high as 30 μM when compared with cells that did not receive the factor or received E2 instead (Fig. 1A).

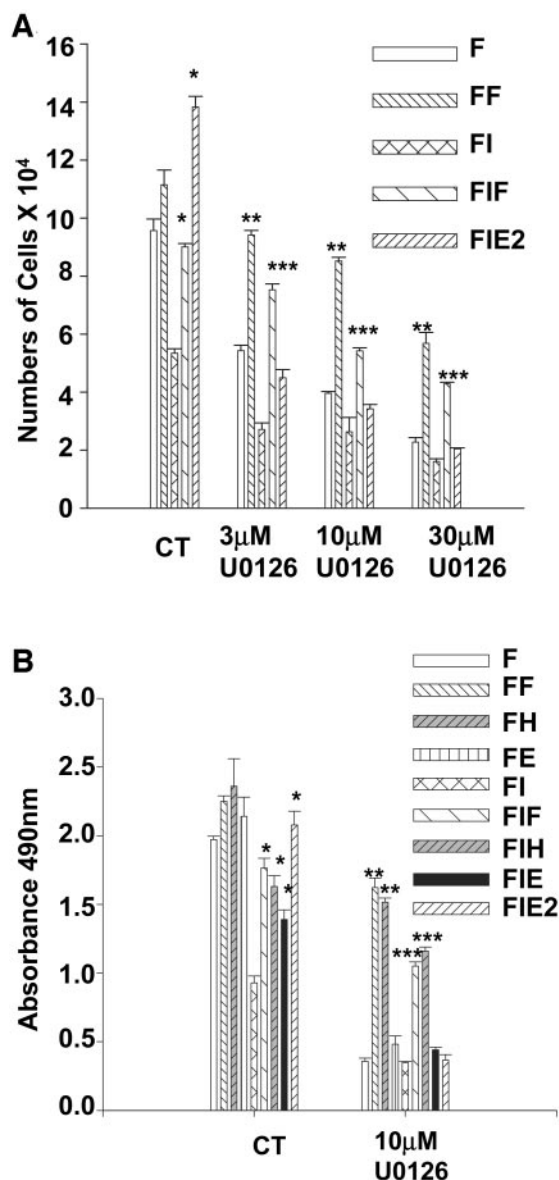
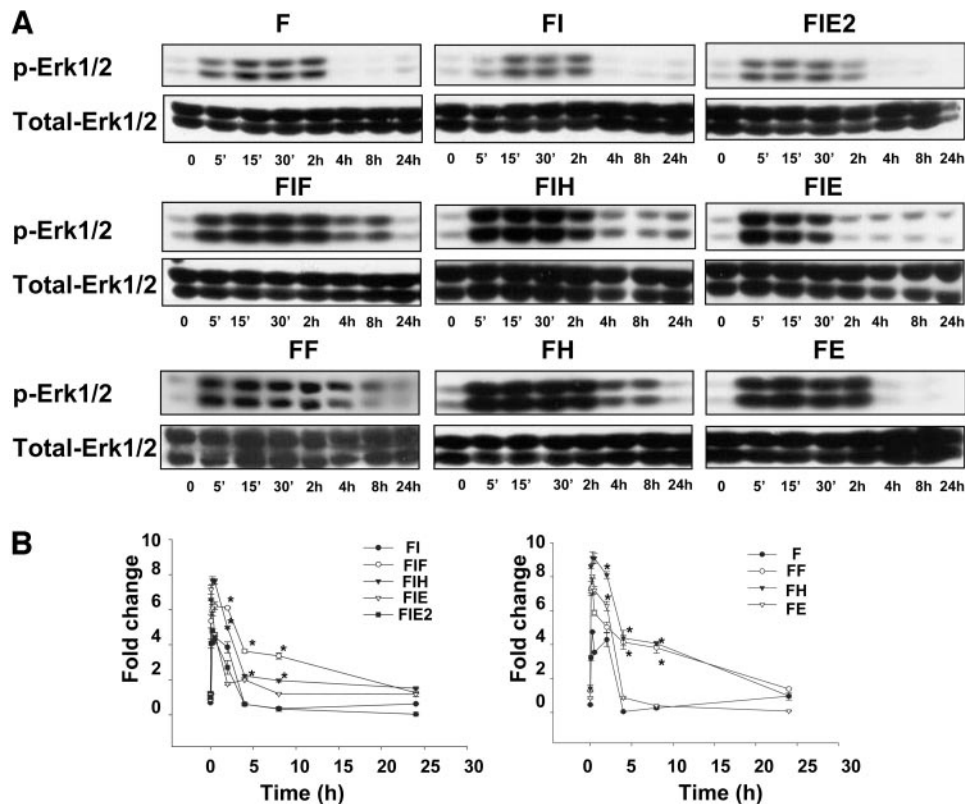


Fig. 1. Fibroblast growth factor 1 and heregulin β1 selectively induce 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenyl]thiobutadiene (U0126) and ICI 182780-resistant growth in estrogen-responsive breast cancer cells. A, ML20 cells (MCF-7 transfected with LacZ) were stripped of estrogens with four changes of 5% charcoal-treated calf serum-containing medium and incubated in the same medium for 3 days to reduce basal levels of growth and then plated in 24-well plates. Cells were then treated with the indicated conditions (see Table 1 for description of abbreviations) in the presence of increasing concentrations of U0126 for 5 days, or in its absence (CT). On day 5 cells were counted by a Coulter counter. B, ML20 cells were estrogen-stripped as described above in A and plated in 96-well plates and treated with the indicated conditions in the presence of U0126 (10 μM) or in its absence (CT). On day 5, 40 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium assay reagent was added, and absorbance at 490 nm was recorded. Results in both panels depict means from three independent replicates; bars, ±SE (\*, significantly different from FI in CT group,  $P < 0.05$ ; \*\*, significantly different from F within each U0126 dose,  $P < 0.05$ ; and \*\*\*, significantly different from F and FI within each U0126 dose,  $P < 0.05$ ).

Unpublished data from our laboratory and reports from other investigators have shown that EGF and HRGβ1 can also induce antiestrogen-resistant growth in these cells (3, 6, 7). To test whether these factors would also induce U0126-insensitive growth, we performed a MTS growth assay with estrogen-deprived ML20 cells treated with all three of the growth factors in the presence and absence of ICI 182780 in 5% FBS containing medium. We found that similar to its modest effects in FGF-1 supplemented medium, U0126 only moderately

Fig. 2. Fibroblast growth factor 1 and heregulin  $\beta 1$  induce prolonged extracellular regulated kinase (ERK)1/2 activation in the presence and absence of ICI 182780. A, ML20 cells were stripped of estrogens with four changes of 5% charcoal-treated calf serum-containing medium and then incubated overnight in serum-free medium. They were then treated with F, FI, FIF, FIH, FIE, FIE2, FF, FH, or FE medium (see Table 1 for a description of these media) for the indicated times. The cells were then lysed, and lysate aliquots (40  $\mu$ g) were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) and total ERK1/2. Blots shown are representative of at least 3 separate experiments. B, densitometric analysis of Western blots from three separate experiments. \*, significantly different from the normalized phospho-ERK1/2 levels in FI condition (left) or F condition (right) at that time point;  $P < 0.05$ ; bars,  $\pm$ SD.

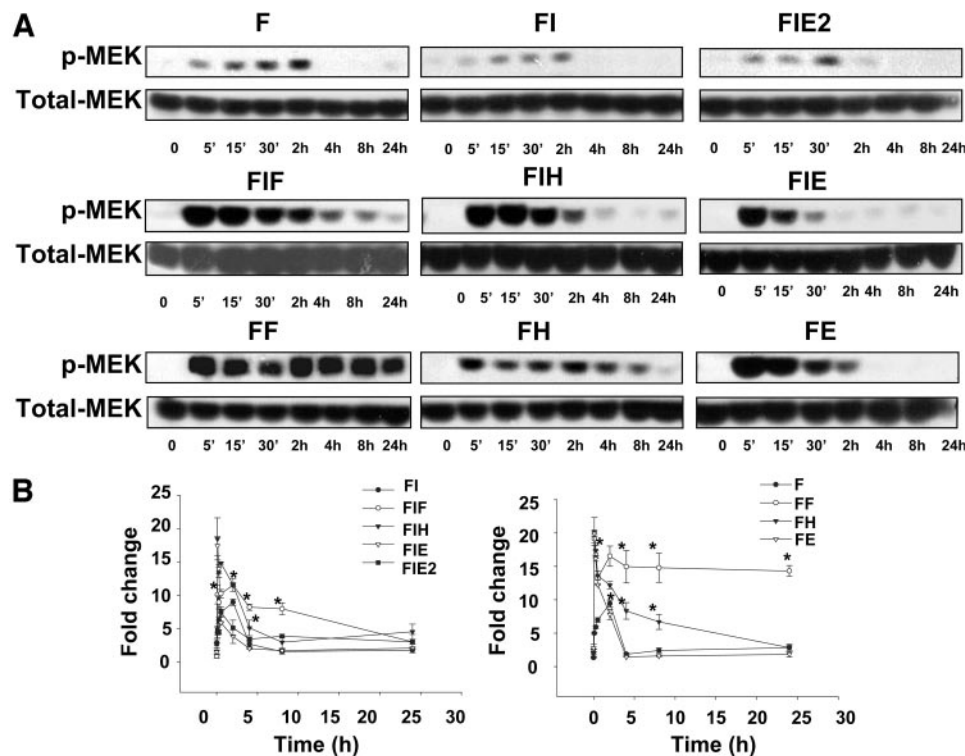


inhibited growth in HRG $\beta 1$ -containing conditions (Fig. 1B, compare FH with F and FIH with FI in U0126-treated cells). However, U0126 inhibited growth in EGF supplemented medium, both in the presence and in the absence of ICI 182780 to the same extent as the inhibition in 5% FBS medium (Fig. 1B, FIE and FE conditions). Thus, like FGF-1 and HRG $\beta 1$ , EGF was equally capable of inducing antiestro-

gen-resistant growth. However, unlike FGF-1 or HRG $\beta 1$ , and like E2, the growth stimulatory effect of EGF was very sensitive to the effects of U0126.

**FGF-1 and HRG $\beta 1$  Induces Prolonged ERK1/2 and MEK1/2 Activation Even in the Presence of ICI 182780.** S-phase transit in some cells requires ERK1/2 activation for several hours subsequent to

Fig. 3. Fibroblast growth factor 1 and heregulin  $\beta 1$  induce prolonged mitogen-activated protein/extracellular regulated kinase kinase (MEK) activation in the presence and absence of ICI182780. A, ML20 cells (MCF-7 transfected with LacZ) were stripped of estrogens with four changes of 5% charcoal-stripped calf serum-containing medium and then incubated overnight in serum-free medium. They were then treated with F, FI, FIF, FIH, FIE, FIE2, FF, FH, or FE medium (see Table 1 for a description of these media) for the indicated times. The cells were then lysed, and lysate aliquots (40  $\mu$ g) were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) and total MEK. Blots shown are representative of at least 3 separate experiments. B, densitometric analysis of Western blots from 3 separate experiments. \*, significantly different from the normalized phospho-MEK levels in FI condition (left) or F condition (right) at that time point;  $P < 0.05$ ; bars,  $\pm$ SD.



growth factor application (35–38). Therefore, we first established the magnitude and longevity of phosphorylation of ERK1/2 and its upstream activators MEK1/2 subsequent to growth factor or E2 exposure in the presence or absence of ICI 182780 in ML20 cells. Estrogen-deprived ML20 cells that had been serum-starved for 24 h were subjected to the various conditions listed in Table 1. Treatment with 5% FBS causes a rapid but modest activation of ERK1/2 that is sustained for at least 2 h (Fig. 2, A and B, F condition). ICI 182780 ( $1 \times 10^{-7}$  M) does not affect this activation (Fig. 2, A and B, FI condition) nor does E2 alter it appreciably (Fig. 2, A and B, FIE2 condition). FGF-1 supplementation in the presence or absence of ICI 182780 causes a more robust and prolonged activation of ERK1/2 for at least 8 h (Fig. 2, A and B, FIF and FF conditions) as compared with 5% FBS alone. HRG $\beta$ 1 supplementation in these conditions also caused a very robust and prolonged activation of ERK1/2 (Fig. 2, A and B, FIH and FH conditions). In contrast, EGF supplementation in 5% FBS-containing medium resulted in a relatively short-lived, albeit robust activation of ERK1/2, which dropped down to control levels within 30 min in the presence of ICI 182780 and lasted only 2 h in its absence (Fig. 2, A and B, FIE and FE conditions). In some experiments, a second bolus of EGF was added 5 min before harvesting to the 2-h time point and all of the subsequent samples. Results from these indicated no differences when compared with experiments where only a single bolus was administered. Therefore, the data suggest that the relatively rapid drop in phospho-ERK1/2 levels in EGF-supplemented conditions could not be attributed to its half-life.

We also assessed the time course of activation of MEK1/2, which are the direct upstream activators of ERK1/2. As shown in Fig. 3, A and B, treatment of serum- and estrogen-deprived ML20 cells with 5% FBS, either in the absence or presence of ICI 182780, led to modest increases in phospho-MEK1/2 levels that lasted 2 h. Similar results were obtained in the FIE2 condition (Fig. 3, A and B). However, in the presence of FGF-1 supplemented medium, phospho-MEK1/2 levels remained very high for at least 8 h in the presence of ICI 182780 and 24 h in its absence. HRG $\beta$ 1 also maintained sustained activation of MEK1/2, which remained high for at least 4 h in the presence of ICI 182780 and at least 8 h in its absence. EGF supplementation in serum-containing medium did not cause such a prolonged activation of MEK1/2 (even when an additional bolus of EGF was added 5 min before harvesting the 2 h and all of the subsequent samples; data not shown), which correlated with the time course of its activation of ERK1/2. EGF-induced increases in phospho-MEK levels were, however, of similar magnitude with respect to FGF-1 and HRG $\beta$ 1-induced increases at earlier time points.

**FGF-1- and HRG $\beta$ 1-Induced Ras and c-Raf-1 Activation Is Transient Unlike ERK1/2 Activation.** To determine whether the differential duration of MEK1/2 and ERK1/2 activation by FGF-1 or HRG $\beta$ 1 versus EGF correlated with the activation of the upstream components of this cascade, we investigated the time course of activation of Ras and Raf-1 in response to these factors in the presence of 5% FBS. The ras binding domain of Raf-1 was used to selectively capture active Ras, and anti-ras antibodies were used to detect it (Fig. 4A). The activation of Ras by 5% FBS was very minimal (Fig. 4A). On the other hand, the activation of Ras by all three of the growth factors was rapid, starting as early as 2 min in the case of HRG $\beta$ 1 and EGF and peaking at 5 min. In the case of FGF-1, active Ras levels were modestly induced and peaked at 5 min (Fig. 4A). However, Ras activation in all three of the cases returned to basal levels rapidly, with FGF-1 and HRG $\beta$ 1-induced Ras levels dropping to near basal levels by 30 min and within 2 h in the EGF-treated condition (Fig. 4A).

Active Raf-1 was determined by an *in vitro* kinase assay by using an inactive recombinant MEK1 fused to glutathione *S*-transferase (glutathione *S*-transferase-MEK), as a substrate. As shown in Fig. 4B,

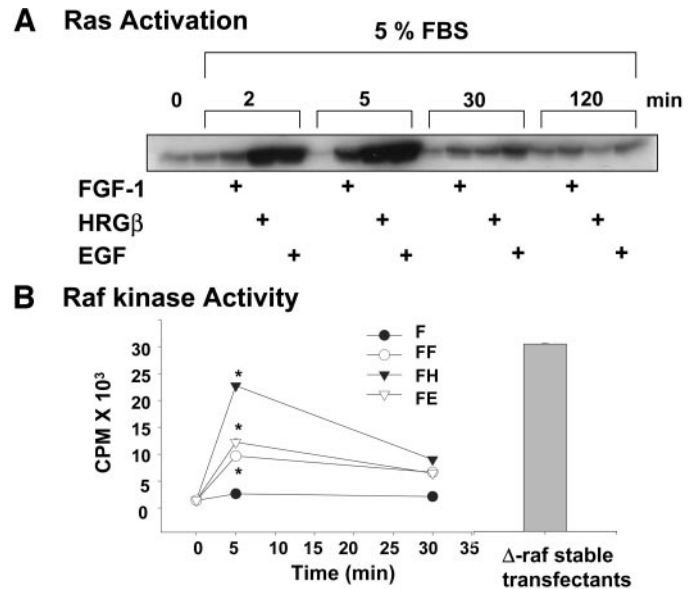


Fig. 4. Ras and c-Raf-1 activation induced by fibroblast growth factor 1 (FGF-1) and heregulin  $\beta$ 1 (HRG $\beta$ 1) is transient. A, ML20 cells were stripped of estrogens with four changes of 5% charcoal-stripped calf serum-containing medium and then incubated overnight in serum-free medium. Cells were then treated with the indicated conditions for the indicated time periods. Cells were lysed, and 500  $\mu$ g of cellular lysate was incubated with 10  $\mu$ l Raf-1 ras binding domain agarose from the Ras activation kit (Upstate Biotechnology) at 4°C for 30 min. The agarose pull-downs were washed three times with lysis buffer, boiled with 2 $\times$  Laemmli sample buffer and fractionated on SDS-PAGE gels, followed by Western immunoblotting with anti-Ras antibodies. B, Raf-1 was immunoprecipitated from cells lysed as described in A using an antibody directed against the COOH-terminus of Raf-1 coupled to protein G-Sepharose. As a positive control for Raf-1 kinase activity lysates from the Raf14c cells (25), a MCF-7 line stably transfected with a constitutively active Raf-1 deletion mutant was also immunoprecipitated. Immunoprecipitates were washed twice with lysis buffer and then incubated with 30  $\mu$ l of kinase assay buffer containing glutathione *S*-transferase-mitogen-activated protein/extracellular regulated kinase 1 as substrate. After incubating for 30 min at 30°C, reactions were spotted onto P81 phosphocellulose squares, which were subsequently washed 10 times with 150 mM phosphoric acid. Radioactivity on the P81 squares was counted. A protein G-Sepharose precipitate in the absence of Raf-1 antibody was used to obtain background cpm values and was subtracted from each experimental value. Values represent mean of cpm obtained from three separate immunoprecipitates from separate dishes; bars,  $\pm$ SE. \*, significantly different from Raf-1 activity in F condition;  $P < 0.05$ .

Raf-1 activation was maximal in the case of all three of the factors by 5 min and dropped rapidly by 30 min, consistent with Ras activation (Fig. 4A). The peak active Raf-1 levels induced by HRG $\beta$ 1 were the highest and were almost as high as active Raf levels measured in a MCF-7 clone stably expressing a constitutively active Raf-1 NH $_2$ -terminal deletion mutant (Fig. 4B). Peak active Raf-1 levels induced by FGF-1 were lower than that induced by EGF or HRG $\beta$ 1. Active Raf-1 induced by serum alone was minimal, again consistent with Ras activation levels.

**HRG  $\beta$ 1 and FGF-1 but not Serum or EGF Can Stimulate MAPK and p90 Rsk-1 Activation in the Presence of U0126.** To determine whether the reduced growth-inhibitory effect of U0126 in FGF-1- or HRG $\beta$ 1-containing medium correlated with an inability to effectively reduce the prolonged MAPK activity, we examined phospho-ERK1/2 levels under these conditions (Fig. 5, A and B). We used serum and estrogen-deprived ML20 cells (Fig. 5, A and B) and treated them with 5% FBS supplemented with the three factors for 2 h, a time point at which substantial phospho-ERK1/2 levels are found under all of the conditions (Fig. 5, A and B, and Fig. 2). In parallel, dishes with the same conditions were also treated with 10  $\mu$ M U0126 for 2 h (with a 30-min pretreatment before addition of conditions). To determine the concentration of U0126 to be used in these assays we used a clonally isolated MCF-7 line called Raf14c that expresses a stably transfected constitutively active c-Raf mutant (25). Serum-starved cells from this line (lane UT, Fig. 5A, bottom) exhibit a high level of

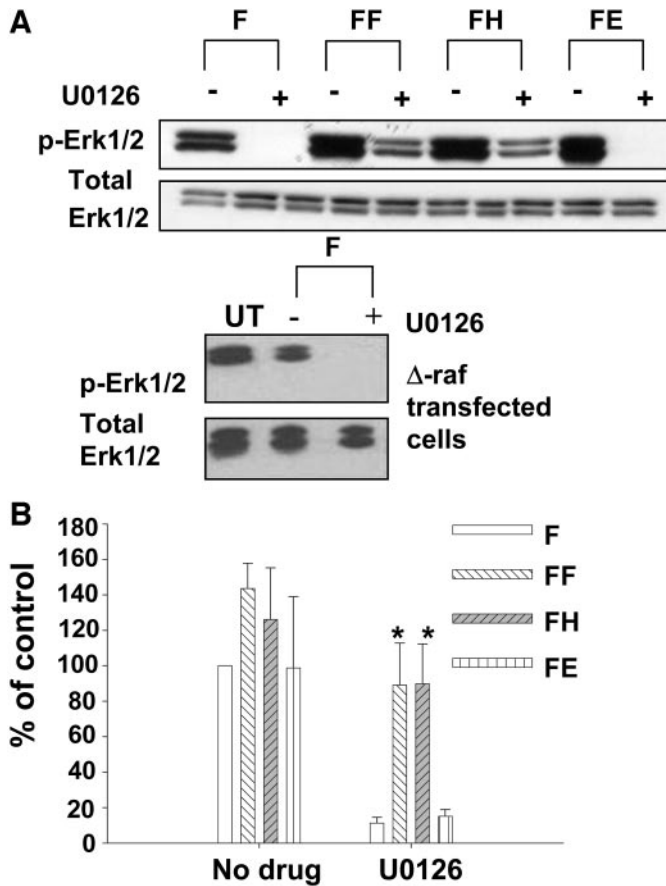


Fig. 5. 1,4-Diamino-2,3-dicyano-1,4-bis [2-aminophenyl]thiobutadiene (U0126) does not effectively block fibroblast growth factor 1- and heregulin  $\beta$ 1-stimulated extracellular regulated kinase (ERK)1/2 phosphorylation in estrogen receptor-positive breast cancer cells. **A**, ML20 cells or Raf14c, a MCF-7 line that had been stably transfected with a constitutively active c-Raf ( $\Delta$ -raf; Ref. 25), were stripped of estrogens as described in Fig. 1 legend and incubated overnight in serum-free medium. Where indicated, cells were then pretreated for 30 min with 10  $\mu$ M U0126 and then exposed to the indicated conditions (see Table 1) for 2 h in the presence of the inhibitor. The cells were then lysed, and aliquots (40  $\mu$ g) were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) and total ERK1/2. Lane marked UT represents lysates from Raf14c cells that did not receive any conditions or U0126. Blots shown are representative of at least three separate experiments. **B**, densitometric analysis of Western blots from three separate experiments. \*, significantly different from the normalized phospho-ERK1/2 levels in F and FE conditions within the U0126 treated group;  $P < 0.05$ .

phospho-ERK1/2. Ten  $\mu$ M U0126 was found to completely inhibit ERK1/2 phosphorylation in this line even in the presence of serum (Fig. 5A, bottom).

As shown in Fig. 5A, 10  $\mu$ M U0126 was sufficient to completely eliminate the phosphorylation of ERK1/2 induced by 5% FBS. At the same dose U0126 also completely inhibited ERK1/2 activation induced by EGF-supplemented medium (Fig. 5, A and B). However, U0126 only modestly inhibited ERK1/2 activation by FGF-1 and HRG $\beta$ 1 under these conditions (Fig. 5, A and B).

Data shown in Fig. 6, A and B, depict results from experiments where three structurally distinct MEK blockers (see Table 2) were used to assess whether growth factor-induced ERK1/2 activation seen in ICI 182780-treated conditions was susceptible to MEK inhibition. PD98059, the least potent MEK inhibitor (39), when used at 200  $\mu$ M, a dose shown previously to block ERK1/2 phosphorylation in Raf14c cells (40), was nearly as effective as U0126 in reducing ERK1/2 activation in the F and FI conditions (Fig. 6, A and B). In these experiments we also used a new, highly potent MEK inhibitor PD184161 at 10  $\mu$ M (41). PD184161 is a close structural analog of PD184352, which is currently in clinical trials and which directly

inhibits MEK1 with an IC<sub>50</sub> of 17 nM (30). All of the three inhibitors nearly completely abrogated phosphorylation of ERK1/2 when E2 was added to negate the effects of ICI 182780 (Fig. 6, A and B). We also found that all three of the MEK inhibitors do not effectively abrogate ERK1/2 activation induced by FGF-1- and HRG $\beta$ 1-supplemented medium in the presence of antiestrogen (Fig. 6, A and B). Even 30  $\mu$ M U0126 did not completely inhibit ERK1/2 activation by FGF-1 and HRG $\beta$ 1 in the presence or absence of ICI 182780 (data not shown).

We also asked whether the observed U0126-resistant phosphorylation of ERK1/2 in FGF-1- and HRG $\beta$ 1-containing medium results in activation of its downstream target p90 Rsk-1 (Fig. 7). The anti-phospho-p90 Rsk-1 antibody recognizes p90 Rsk-1 only when it is phosphorylated at Thr359/Ser363, both of which are ERK targets. As shown in Fig. 7, p90 Rsk-1 was substantially phosphorylated by FGF-1 and HRG $\beta$ 1 signaling in the presence of U0126, consistent with phospho-ERK1/2 levels. However, U0126 treatment completely

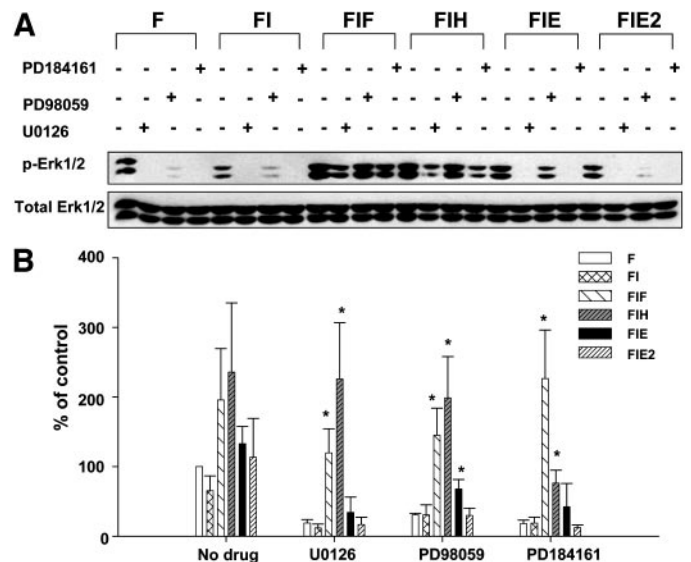


Fig. 6. The mitogen-activated protein/extracellular regulated kinase (ERK) kinase inhibitors PD98059, PD184161, and 1,4-diamino-2,3-dicyano-1,4-bis [2-aminophenyl]thiobutadiene (U0126) do not effectively block fibroblast growth factor 1- and heregulin  $\beta$ 1-stimulated ERK1/2 phosphorylation in estrogen receptor-positive breast cancer cells in the presence of the antiestrogen ICI 182780. **A**, ML20 cells were stripped of estrogens as described in Fig. 1 legend and incubated overnight in serum-free medium. Cells were then pretreated for 30 min with PD98059 (200  $\mu$ M), PD184161 (10  $\mu$ M), or U0126 (10  $\mu$ M) and were then exposed to the indicated conditions (see Table 1) for 2 h in the presence of the inhibitor. The cells were then lysed, and aliquots (40  $\mu$ g) were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) and total ERK1/2. Blots shown are representative of at least three separate experiments. **B**, densitometric analysis of Western blots from three separate experiments. \*, significantly different from the normalized phospho-ERK1/2 levels in F and FI conditions within the mitogen-activated protein/extracellular regulated kinase inhibitor-treated groups;  $P < 0.05$ .

Table 2 Inhibitors used in this study

Inhibitor	Enzyme(s) inhibited	Concentration(s) used	References
U0126	MEK <sup>a</sup> 1/2 and MEK5	10 and 30 $\mu$ M	29, 39, 48, 49, 50
PD 98059	MEK1/2 and MEK5	200 $\mu$ M	39, 49, 50
PD 184161 <sup>b</sup>	MEK1/2 and MEK5 (?)	10 $\mu$ M	41 <sup>b</sup>
AACOCF3	cPLA2	10 $\mu$ M	42
Bis-indolylmaleimide	Conventional PKCs	10 $\mu$ M	42

<sup>a</sup>MEK, mitogen-activated protein/extracellular regulated kinase kinase; AACOCF3, arachidonyl trifluoro methyl ketone; PKC, protein kinase C.

<sup>b</sup>PD184161 is a close structural analog of PD184352, which has completed Phase I trials. Whereas the ability of PD184352 to inhibit activation of MEK5-extracellular regulated kinase 5 has been established (49, 65), similar data for PD184161 have not been reported.

abrogated p90 Rsk-1 phosphorylation in F, FE, FI, FIE, and FIE2 conditions.

**MAPK Phosphorylation in HRG $\beta$ 1- and FGF-1-Stimulated MCF-7 Cells Is MEK Dependent.** The experiments with U0126 described above did not completely rule out a role for MEK1/2 in the prolonged ERK1/2 activation and growth observed in the presence of the compound in FGF-1 and HRG $\beta$ 1 containing medium, because it could be argued that factor-specific MEK activation mechanisms that are insensitive to U0126 are involved. To additionally test the MEK dependence of the prolonged ERK1/2 activation induced by FGF-1 and HRG $\beta$ 1 we did transient cotransfection assays using a GFP-ERK2 reporter and a dominant-negative MEK (S218A and S222A) construct.

The GFP-ERK2 fusion used in this assay has been shown to be a *bona fide* surrogate for the endogenous ERKs by others in similar experiments (34, 36). As shown in Fig. 8, 5% FBS either unsupplemented or with added EGF, FGF-1, or HRG $\beta$ 1 caused robust phosphorylation of GFP-ERK2 when cotransfected with the pCMV vector

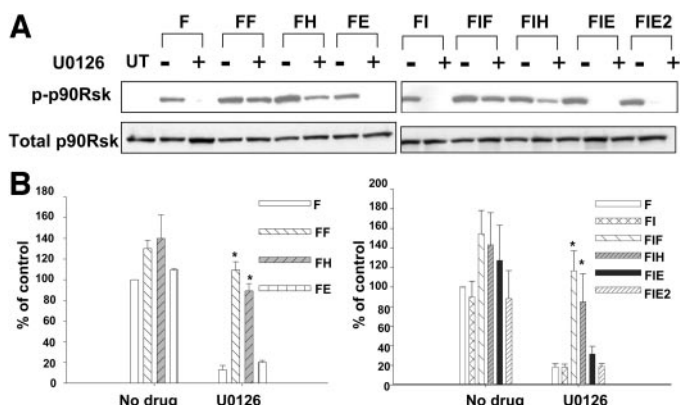


Fig. 7. 1,4-Diamino-2,3-dicyano-1,4-bis [2-aminophenyl]thiobutadiene (U0126)-resistant p90 Rsk-1 phosphorylation induced by fibroblast growth factor 1 and heregulin  $\beta$ 1. **A**, ML20 cells were stripped of estrogens as described in Fig. 1 legend and then incubated overnight in serum-free IMEM. The following day, where indicated cells were pretreated for 30 min with 10  $\mu$ M U0126 before being exposed to the indicated conditions (see Table 1) for 2 h in the presence or absence of the inhibitor. The cells were then lysed, and aliquots (40  $\mu$ g) were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) p90 Rsk-1 and total p90 Rsk-1. Lane marked UT represents lysates from cells that did not receive any conditions or U0126. Blots shown are representative of at least three separate experiments. **B**, Densitometric analysis of Western blots from 3 separate experiments. \*, significantly different from the normalized phospho-p90 Rsk-1 levels in F condition (left) and FI condition (right) within the U0126-treated groups;  $P < 0.05$ ; bars,  $\pm$ SD.

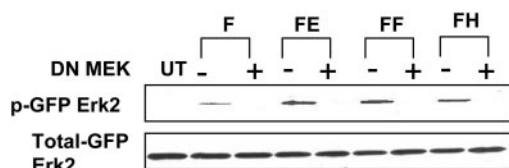


Fig. 8. Extracellular regulated kinase (ERK)1/2 phosphorylation induced by heregulin  $\beta$ 1 and fibroblast growth factor 1 is mitogen-activated protein/extracellular regulated kinase kinase (MEK)-dependent. ML20 cells ( $1.2 \times 10^6$ ) were plated in 10-cm tissue culture dishes and 16 h later transfected with 2  $\mu$ g of GFP-ERK2 plasmid and 8  $\mu$ g of either pCMV-dnMEK (+ lanes) or the control pCMV plasmid (- lanes) using LipofectAMINE Plus reagent (Invitrogen). Cells were then incubated for 48 h in 5% fetal bovine serum containing IMEM to allow for expression of transfected genes. The transfected cells were then stripped of estrogens with four changes of 5% charcoal-stripped calf serum-containing medium and then incubated overnight in serum-free IMEM to reduce basal levels of GFP-ERK2 phosphorylation. Cells were then treated with F, FH, FE, or FF medium (see Table 1 for description). The cells were incubated in these conditions for 2 h and subsequently lysed. Lysate aliquots were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) green fluorescent protein (GFP)-ERK2 and total GFP-ERK2. Lane marked UT represents lysates from cells that did not receive any conditions. Blot shown is representative of at least three separate experiments.

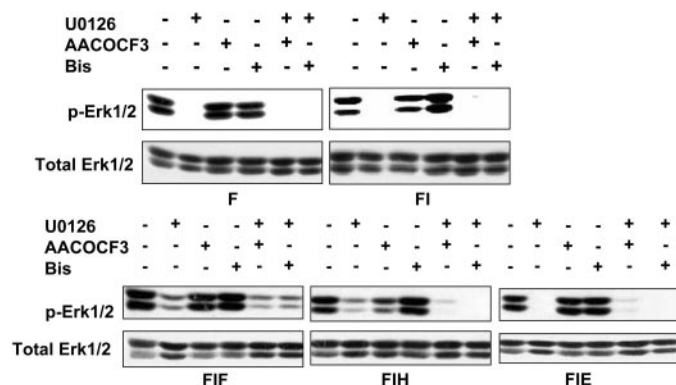


Fig. 9. 1,4-Diamino-2,3-dicyano-1,4-bis [2-aminophenyl]thiobutadiene (U0126)-resistant extracellular regulated kinase (ERK)1/2 phosphorylation induced by heregulin  $\beta$ 1 is protein kinase C and cytosolic phospholipase A2-dependent. ML20 cells (MCF-7 transfected with LacZ) were stripped of estrogens as described in Fig. 1 legend and incubated overnight in serum-free medium. Where indicated cells were then pretreated for 30 min with the indicated inhibitor combinations and were then exposed to the indicated conditions (see Table 1) for 2 h in the presence or absence of the inhibitors. U0126, bisindolylmaleimide, and arachidonyl trifluoro methyl ketone (AACOCF3) were used at 10  $\mu$ M. The cells were then lysed, and aliquots (40  $\mu$ g) were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) and total ERK1/2. Blot shown is representative of at least two separate experiments.

control. Cotransfection with the dominant-negative MEK expression vector pCMV-dnMEK completely eliminated GFP-ERK2 phosphorylation when these cells were treated with 5% FBS or EGF-supplemented 5% FBS. Whereas U0126-insensitive GFP-ERK2 phosphorylation was observed in FGF-1- and HRG $\beta$ 1-supplemented conditions (data not shown), GFP-ERK2 phosphorylation by these factors was completely abrogated by cotransfected dnMEK (Fig. 8), suggesting that these responses are MEK-dependent as well.

**U0126-Resistant MAPK Phosphorylation in HRG $\beta$ 1- but not FGF1-Stimulated Cells Is PKC and cPLA2 Dependent.** Positive feedback loops mediated by conventional PKC isoforms and by cPLA2 have been implicated in the sustained activation of ERK1/2 pathways (42–44). In addition, FGF-2 was shown recently to activate cPLA2 (45). To test whether these mechanisms could account for the U0126-resistant phosphorylation induced by HRG $\beta$ 1 and FGF-1, we subjected ML20 cells to combined treatment of U0126 plus the pan-conventional PKC inhibitor bisindolylmaleimide (10  $\mu$ M) or U0126 plus AACOCF3 (10  $\mu$ M), a cPLA2 inhibitor (Table 2), and then exposed them to the various growth factors in the presence of 5% FBS and ICI 182780. As shown in Fig. 9, cotreatment of U0126 with either bisindolylmaleimide or AACOCF3 abrogated ERK1/2 phosphorylation induced by HRG $\beta$ 1 but not by FGF-1 (compare FIF with FIH lanes). Interestingly bisindolylmaleimide alone did not substantially affect ERK1/2 phosphorylation induced in any condition, and AACOCF3 alone only partially inhibited ERK1/2 phosphorylation induced in the FIH condition (Fig. 9). This suggests that the ERK activation mechanisms induced by HRG $\beta$ 1 in MCF-7 cells are in part dependent on cPLA2 and conventional PKCs and that sustained ERK1/2 phosphorylation observed with FGF-1 treatment involves a different mechanism.

**HRG $\beta$ 1, EGF, and FGF-1 Can Overcome the Effects of Higher Doses of U0126 on ERK5 Phosphorylation.** ERK5 activation has been implicated in the proliferative effects of both EGF and HRG $\beta$ 1 (46, 47). U0126 at high doses is also an inhibitor of ERK5 activation (48–50). Therefore, it was possible that the specific effects of FGF-1 and HRG $\beta$ 1 with respect to U0126-resistant growth might be due to suboptimal inhibition of ERK5 activation at the doses used. We tested whether 30  $\mu$ M U0126 would result in different levels of phospho-ERK5 induced by these factors, as well as by FGF-1, thus perhaps explaining such factor-specific effects on growth in these cells. As

shown in Fig. 10, HRG $\beta$ 1 was the most potent in terms of induction of phospho-ERK5. However, U0126 did not decrease this (Fig. 10). Surprisingly EGF, which does not stimulate growth in U0126-treated conditions, was a potent inducer of phospho-ERK5, more so than FGF1, and even in the presence of 30  $\mu$ M U0126 (Fig. 10). U0126 did inhibit the minimal ERK5 activation observed in response to 5% FBS treatment (Fig. 10). Thus, these results indicate that ERK5 activation alone, in the absence of active ERK1/2, is not sufficient for U0126-resistant growth.

## DISCUSSION

Growth factor-induced ERK1/2 activation has been characterized as rapid and transient in some cases and prolonged in others. Prolonged ERK1/2 activation has been shown to be required for S-phase transit in some systems (35, 37, 38). We found that two factors capable of inducing antiestrogen-resistant growth, FGF-1 and HRG $\beta$ 1, are both able to induce a prolonged activation of ERK1/2 in MCF-7 cells in either the presence or absence of MEK activation blockers. The MEK-ERK1/2 pathway has also been shown to be important in the estrogen-dependent growth of the MCF-7 breast carcinoma (6, 27). In our study E2 supplementation negated the effects of ICI 182780 on growth but did not increase the amplitude nor the duration of the ERK1/2 activation response induced by 5% FBS (Fig. 2, A and B). However, U0126 completely abrogated the growth effects of E2 and the ERK1/2 activation levels in E2-supplemented conditions (Fig. 1, A and B, and Fig. 6, A and B). This suggests that the inability of E2, like EGF and unlike FGF-1 and HRG $\beta$ 1, to maintain a sustained ERK1/2 activation response in the presence of U0126 renders it unable to overcome the inhibitory effects of U0126.

Although the mechanisms regulating the differences in the magnitude and duration of MAPK signaling in MCF-7 cells subsequent to exposure with FGF-1 and HRG $\beta$ 1 *versus* EGF are still not entirely clear, a proposed scheme is depicted in Fig. 11. Neve *et al.* (51) have suggested that the EGF receptor is less able to maintain sustained effector activity, because it is rapidly internalized, ubiquitinated, and degraded upon ligand stimulation, unlike the HRG $\beta$ 1-activated ErbB2 receptor, which has a relatively slow rate of endocytosis. However, we have demonstrated that the signaling components Ras and Raf-1, which are proximal to the receptors and upstream of MEK, are activated only transiently when cells are stimulated by EGF, HRG $\beta$ 1, or FGF-1 (Figs. 4 and 11). This suggests that continually active receptor tyrosine kinase may not entirely explain the prolonged MAPK signaling. It has also been suggested that regulation and activation of another Ras-like small G-protein, Rap1, can also govern the kinetics of ERK1/2 activation (Fig. 11). Prolonged Rap1 activation is induced when nerve growth factor stimulates TrkA (unlike EGF stimulation), which then forms a complex with phosphorylated

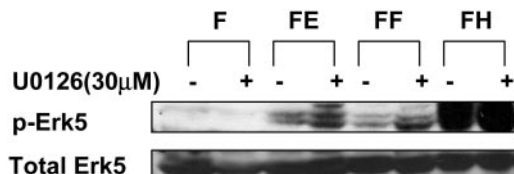


Fig. 10. Extracellular regulated kinase (ERK)5 phosphorylation induced by heregulin  $\beta$ 1 and fibroblast growth factor 1 is not reduced in the presence of 1,4-diamino-2,3-dicyano-1,4-bis [2-aminophenyl]thiobutadiene (U0126). ML20 cells were stripped of estrogens as described in Fig. 1 legend and then incubated overnight in serum-free IMEM. The following day, the cells were treated with indicated conditions for 30 min, in the presence or absence of 30  $\mu$ M U0126, following a 30-min pretreatment with the inhibitor. The cells were lysed, and lysate aliquots were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) ERK5 and total ERK5. The blot shown is representative of at least two separate experiments.

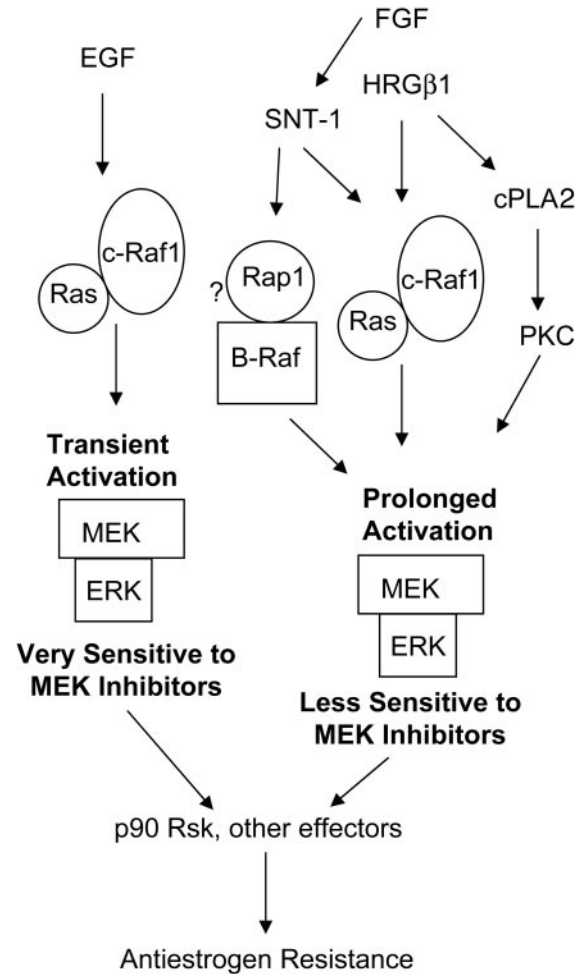


Fig. 11. Schematic representation of factor-specific mitogen-activated protein/extracellular regulated kinase (ERK) kinase (MEK)-ERK activation pathways involved in antiestrogen resistance that confer differential susceptibility to MEK inhibitors. The commonalities between the different pathways and the proposed differences that could contribute to distinct kinetics of activation and/or deactivation of MEK-ERK enzymes and their effectors are depicted. The consequence of differences in the amplitude and duration of MEK-ERK activation by these pathways is a differential susceptibility to MEK inhibitors, suggesting that to be effective, combining such drugs with antiestrogens will require a judicious strategy including examination of the causal factors for MEK activation in tumors.

SNT-1 and other downstream components resulting in prolonged ERK1/2 activation (52). Because SNT-1 is also phosphorylated and activated upon FGF-1 stimulation in our system<sup>3</sup> it is possible that Rap1 could also mediate FGF-1-induced prolonged ERK1/2 activation (Fig. 11). Experiments exploring the role of Rap1 in U0126-resistant induction of ERK1/2 activity by FGF-1 are in progress. In a recent report examining computer models of the Ras-MAPK pathway, Yamada *et al.* (53) have also proposed a role for SNT-1 in the prolonged activation of MAPK by FGF *versus* EGF.

Positive feedback loops generated by ERK1/2 activation of cPLA2 and PKCs have also been implicated in sustained activation of this cascade (42, 43, 54). This is a possible explanation for the sustained HRG $\beta$ 1-induced ERK1/2 activation, because treatment with bisindolymaleimide or the cPLA2 inhibitor AACOCF3 plus U0126 results in a complete inhibition of ERK1/2 phosphorylation (Fig. 9). Although FGF-2 has also been shown to activate cPLA2 (45), it does not seem to play a role in FGF1-induced ERK1/2 activation in these cells (Figs. 9 and 11). Sustained ERK1/2 activation in other systems can be

<sup>3</sup> Unpublished observations.



blocked by inhibitors of phosphatidylinositol 3'-kinase (55). However, studies in our laboratory demonstrate that neither combined treatment of U0126 plus the phosphatidylinositol 3'-kinase inhibitor LY294002 nor LY294002 alone altered phospho-ERK1/2 levels induced by FGF-1 in MCF-7 cells.<sup>3</sup>

Signal duration through the ERK1/2 cascade can also potentially reflect dephosphorylation reactions in addition to phosphorylations. Thus, it is possible that Ser-Thr phosphatases like PP2A, dual specificity phosphatases such as MAPK phosphatases, and tyrosine phosphatases such as PTP1B, can all play a role in the sustained activation of ERK1/2. Although it is widely acknowledged that activation of the MEK-ERK pathway results in increased expression, enhanced stabilization, and elevated catalytic activity of some MAPK phosphatases (56, 57), resulting in feedback inhibition of the ERKs themselves, a recent report also shows that ERK1/2 signaling can trigger MKP-1 degradation, thereby accomplishing sustained kinase activation (58). Whether the activity of these phosphatases in our system is reduced by HRG $\beta$ 1 or FGF-1 and whether it correlates with the duration of ERK1/2 activation is not known at present.

Sustained activation of MAPKs has been shown to be a requirement for S-phase entry in some systems, whereas it correlates with migration, metalloproteinase expression, invasive capacity, and various other specific biological outcomes in others (35, 59–61). In our system the sustained and stronger nature of ERK1/2 activation induced by FGF-1 and HRG $\beta$ 1 correlates with the U0126-insensitivity of this pathway. It is not clear why the amplitude and duration of MEK activation should determine the extent of inhibition by a MEK inhibitor administered for 30 min before addition of the growth factor and throughout the length of the experiment. U0126 has been used to test MEK 1/2 dependence of growth factor-elicited signaling pathways, because it can block the activity, both *in vitro* and *in vivo*, of native MEK 1/2 and also activated MEK 1/2. Interestingly, whereas only nanomolar to low micromolar concentrations of U0126 are required to inactivate MEK in stimulated cells, the IC<sub>50</sub> for inhibiting MEK catalytic activity *in vitro* is 10  $\mu$ M. In *in vitro* experiments U0126 has also been shown to block the activation of MEK by both Raf-1 and a catalytic fragment of MAPK kinase at low concentrations (39). This has led to the suggestion that in cells U0126 predominantly blocks the activation of MEK rather than blocking its enzymatic activity.

Phosphorylation of MEK1 at serines 218 and 222 and the subsequent activation is mediated directly by not only Raf (of which there are three isoforms) but also c-mos and MAPK kinase 1. It is not clearly known whether MEK blockers are equally effective in inhibiting MEK activated by all of these pathways *in vivo*, thus perhaps explaining the differential susceptibility observed in these studies. It is also not known whether in addition to these phosphorylations, interactions with other proteins in scaffolding complexes or other signaling complexes play a role in MEK activation *in vivo* (62). Both MAPK kinase 1 and Raf-1 are found in scaffolding complexes with MEK (63) along with other proteins that might serve to provide specificity in routing signals generated by different growth factors. Consequently, different conformations of MEK and protein-protein interactions in such scaffolding complexes in response to different stimuli could potentially result in differential susceptibility to MEK inhibitors. Because cells were pretreated with U0126 in these experiments for 30 min before addition of FGF-1 or HRG $\beta$ 1 containing medium, this could suggest that U0126 prebound MEK might be in rapid equilibrium with free MEK. This equilibrium is likely shifted to the right with different MEK complexes being made upon treatment with these conditions that are no longer capable of binding U0126. On the other hand it could also suggest that specific active MEK-containing complexes that are made subsequent to HRG $\beta$ 1 or FGF-1

treatment are still capable of activating ERK1/2 despite being prebound to U0126.

It is well accepted now that although U0126 inhibits Raf-1 and MAPK kinase-mediated activation of MEK, it does not block the activating phosphorylations of MEK at Ser 218 and 222 (39). It has also been suggested that sustained activation of MEK1 requires not only its phosphorylation at Ser 218 and Ser 222 but also dephosphorylation at Ser 212 (64). Thus, it is also possible that it is the increased dephosphorylation at Ser 212 that results in sustained ERK1/2 activation. It is also possible that U0126 functions by blocking this “activating” dephosphorylation at Ser 212, a residue that is normally phosphorylated in intact cells (64). Therefore, another possibility is that FGF-1- and HRG $\beta$ 1-mediated pathways can induce Ser 212 dephosphorylation of U0126-prebound MEK resulting in a partially active MEK, whereas serum, EGF, and E2 are unable to do so. Thus, mechanistic details about U0126-mediated inhibition in a cellular context need to be further elucidated.

U0126 has also been reported to inhibit the MEK5-ERK5 kinase cascade, albeit in some cells at higher doses than that required for MEK1/2 inhibition (48, 49). The MEK5-ERK5 pathway has also been implicated in the proliferative responses to EGF and HRG $\beta$ 1 in some cell types (46, 47, 65). However, it is not known whether this pathway plays a role in antiestrogen-resistant growth induced by either of these growth factors or by FGF-1 in MCF-7 cells. In our experiments we found that HRG $\beta$ 1 was the most potent inducer of ERK5 activation and that U0126 at high doses did not decrease this (Fig. 10). Surprisingly, EGF, which does not stimulate growth in U0126-treated conditions, was also a potent inducer of phospho-ERK5, even more so than FGF-1 and even in the presence of 30  $\mu$ M U0126 (Fig. 10). This lack of correlation between phospho-ERK5 levels and the growth phenotypes of these factors suggests that ERK5 activation cannot surmount the growth reduction resulting from diminished ERK1/2 activation. In fact, in certain conditions, ERK5 phosphorylation was enhanced in the presence of 10  $\mu$ M U0126 (data not shown). This is consistent with two other reports that suggest that the ERK1/2 cascade negatively regulates the ERK5 pathway (49, 66). It is possible, however, that ERK5 activation can contribute to growth in EGF, FGF-1, and HRG $\beta$ 1 conditions. However, consistent with another recent report, our data with EGF suggest that ERK5 activation is not sufficient if ERK1/2 is also not activated (66).

The implications of the studies described in this report could be important for future endocrine therapy strategies for breast cancer. Because different growth factors induce varying levels of antiestrogen resistance, it would be logical to target signaling components common to multiple pathways. Because activation of the Ras-Raf-MEK-ERK1/2 pathway represents a common paradigm in multiple proliferative pathways, inhibition of components of this cascade is now being investigated both in laboratory and clinical studies as a means of combating antiestrogen resistance (67). Increased dependence on MAPK signaling is now being recognized to play a major role in antiestrogen resistance and adaptive resistance to estrogen deprivation (2, 68). Preclinical experiments with hormone-resistant MCF-7 xenografts that use combined treatment of antiestrogens and small molecule inhibitors of components of the Ras/Raf/MEK/MAPK pathway have been performed and have demonstrated significant synergistic effects (68). Because MAPK signaling can be activated by both Ras-dependent and Ras-independent pathways, multiple approaches to blocking this cascade will need to be considered. Currently available MEK inhibitors function *in vivo* predominantly by blocking MEK activation. Our results raise the possibility that distinct factor-specific MEK activation mechanisms will likely determine the level of susceptibility to such inhibitors. Therefore, a lack of complete understanding of MEK activation mechanisms and alternate input

routes into the MAPK signaling cascade that are operative in patient tumors could possibly result in frequent failures of therapy regimens that use MEK inhibitors in combination with antihormonal agents.

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