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Hyperactivation of MAPK Induces Loss of ERα Expression in Breast Cancer Cells

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ERα-negative breast tumors tend to overexpress growth factor receptors such as epidermal growth factor receptor or c-erbB-2. Raf-1 is a key intermediate in the signal transduction pathways of these receptors. High levels of constitutive Raf kinase (Δraf) activity imparts ERα- positive MCF-7 breast cancer cells with the ability to grow in the absence of estrogen. Δraf transfectants maintained in estrogen-depleted media showed greatly diminished responses to 17β-estradiol or the pure antiestrogen ICI 182,780. Western blotting, ligand binding, and immunohistochemistry assays revealed a loss of ERα protein expression, and ribonuclease protection assays indicated that this correlated with loss of ERα message. In examining the basal expression of estrogen-induced genes in the stable transfectants or in transient cotransfection assays with an estrogen-response element-reporter construct and Δraf or constitutively active MAPK kinase (ΔMEK), no ligand- independent activation of ERα was observed. Transient expression of Δraf and double-label immunostaining showed ERα was lost in those cells that transiently expressed Δraf. Abrogation of Raf signaling via treatment with the MEK inhibitors PD 098059 or U0126 resulted in reexpression of ERα. Similar studies performed with MCF-7 cells overexpressing epidermal growth factor receptor or c-erbB-2 confirmed that hyperactivation of MAPK resulted in down-regulation of ERα that was reversible by MEK inhibition or transfection with dominant negative ERK1 and ERK2 constructs. These data suggest that the hyperactivation of MAPK in epidermal growth factor receptor- or c-erbB-2-overexpressing breast cancer cells is directly responsible for generation of an ERα-negative phenotype and, more importantly, that this process may be abrogated by inhibiting these pathways, thus restoring ERα expression. (*Molecular Endocrinology* 15: 1344–1359, 2001)

CLINICALLY, BREAST CANCER presents as either ERα positive or as ERα negative. The presence of ERα is correlated with a better prognosis both in terms of increased disease-free survival and overall survival and predicts for response to hormonal therapies such as tamoxifen (1–4). Tamoxifen as an adjuvant therapy is effective in both pre- and postmenopausal patients with ERα+ tumors (5); however, 25–35% of all ERα+ tumors do not respond to tamoxifen (*de novo* resistance), and even those that do initially respond ultimately develop resistance (acquired resistance) (6). Fifty percent of patients with ERα+ primary tumors that relapse after adjuvant tamoxifen therapy have recurrent tumors in which ERα expression is lost (7, 8). In the metastatic setting, tamoxifen has also proven effective, with about 30% of ERα+ patients demonstrating response. In this case, 30% of the initially tamoxifen-responsive metastatic tumors that subsequently develop resistance to tamoxifen have lost ERα expression. It may also be possible that ERα-negative tumors arise *de novo*. Regardless of whether the ERα-negative phenotype is acquired or *de novo*, the lack of ERα expression precludes the use of tamoxifen, as tamoxifen has not been demonstrated to have a therapeutic benefit in ERα-negative patients (9, 10). The reexpression of ERα, therefore, in tumors in which ERα expression has been lost or is not expressed could allow for restoration of tamoxifen sensitivity, and maintenance of ERα expression may provide a means of prolonging response to this well tolerated drug.

ERα-negative tumors frequently overexpress growth factor receptors, such as the epidermal growth factor receptor [EGFR (11)] or c-erbB-2, as do many ERα-negative breast cancer cell lines, suggesting that upregulated growth factor signaling may provide an alternative growth stimulus. The presence of high levels of these receptors is also an important prognostic indicator. For example, in breast tumors, the overexpression of EGFR is inversely correlated with ERα: in the majority of patients breast tumors are either ERα+/EGFR− or ERα−/EGFR+ (11), and EGFR+ tumors have a poor prognosis independent of ERα status (12–16). Similarly, tumors that overexpress c-erbB-2 have a poorer prog-

Abbreviations: BBS, *N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid-buffered saline; CCS, charcoal-stripped serum; DMSO, dimethylsulfoxide; dn, dominant negative; EGF, epidermal growth factor; EGFR, EGF receptor; ERE, estrogen response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRG, heregulin; IMEM, improved MEM; MAb, monoclonal antibody; ΔMEK, constitutively active MAPK kinase; MMTV, mouse mammary tumor virus; PRF-IMEM, phenol red-free IMEM; Δraf, constitutive Raf kinase; TBST, 10 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween-20.

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nosis and tend to be ERα− (17–19). The protooncogene Ras, the downstream mediator of growth factor receptor activation, is overexpressed in approximately 70% of breast cancer (20, 21), further implicating growth factor signaling mechanisms in breast cancer. Double-label immunohistochemical detection of ERα and EGFR in breast tumor specimens and breast cancer cell lines confirms the inverse correlation of expression (22–24). Furthermore, in ERα+/EGFR+ tumors, individual tumor cells express high levels of only ERα or EGFR, but never both (22, 23). The EGFR+ cells in these tumors are also associated with a higher growth rate than the ERα+/low EGFR cells (25, 26). Interestingly, ERα and EGFR expression in the same cell is observed in normal and benign breast specimens (23), suggesting that the interaction between these two signaling pathways is altered in breast cancer cells.

Experimentally, the induction of estrogen-independent growth in the ERα+, estrogen-dependent MCF-7 human breast cancer cell line via stable transfection of a variety of growth signaling factors also frequently results in tamoxifen resistance and decreased levels of ERα (27–32). In addition, MCF-7 cells selected for their ability to grow in the absence of estrogen (33–35), as well as selected for resistance to adriamycin (36), frequently up-regulate signaling molecules such as EGFR, Raf, and TGFα (36, 37). Up-regulation of these pathways may therefore be an early event in progression to ERα negativity, resulting in an intermediate ERα+/estrogen-independent phenotype. Understanding the mechanisms underlying the role of up-regulated growth factor signaling in estrogen-independent growth might lead to methods of reversing this in the earlier stages when ERα is still expressed and might provide clues on the underlying mechanism of loss of ERα expression.

We previously established a model of up-regulated growth factor signaling in the ERα-positive, estrogen-dependent MCF-7 human breast cancer cell line by stably expressing a constitutively active Raf kinase (designated Δraf), an important downstream effector of tyrosine kinase receptor signaling. Δraf-expressing MCF-7 cells exhibited increased anchorage-dependent and anchorage-independent growth in the absence of estrogen (38). In the current study, we demonstrate that estrogen-independent growth of Δraf clones results in the loss of ERα expression. This loss in expression occurs at both the protein and message levels. Transient expression of Δraf or constitutively active MAPK kinase (ΔMEK) in MCF-7 cells also leads to down-regulation of ERα expression. It is MAPK (or ERK) activity that is responsible for this down-regulation since abrogation of MAPK activity via direct inhibition of MEK or dominant negative ERKs results in rapid reexpression of ERα. Constitutive activation of stably transfected c-erbB-2 or ligand-induced activation of stably transfected EGFR also leads to a MAPK-induced down-regulation of ERα that is reversible via abrogation of MAPK activity. These data suggest that up-regulated growth factor signaling via MAPK is directly linked to loss of ERα expression and generation of the ERα-negative phenotype and that at some stage in the progression pathway, the ERα-negative phenotype may not be permanent.

RESULTS

Δraf clones maintained in charcoal-stripped serum (CCS) have significantly diminished ERα expression. We have previously shown that the initial clonal isolates of Δraf transfected MCF-7 cells had decreased doubling times in estrogen-depleted media compared with a pooled population of parental vector-control transfected cells, the HCopool cell line (38). But these Δraf transfectants still responded to estrogen by further increasing their growth rate. Each of these clones has been maintained in the absence of estrogen [phenol red-free IMEM (PRF-IMEM) supplemented with 10% CCS]. The HCopool cell line was adapted long term for growth in the absence of estrogen to serve as a CCS control (HCopool*c*). When these CCS Δraf transfected clones (or Δraf*c* clones) were analyzed for their growth properties in the presence of estrogen or antiestrogens, they exhibited significantly blunted or no responses to estrogen and antiestrogens (data not shown). This nonresponsiveness of the Δraf*c* clones suggested that the cells might no longer be expressing functional ERα. To determine whether this was indeed the case, Δraf*c* clones were assessed for ERα levels, both at the protein and mRNA level. ERα protein was analyzed both by steroid-binding assay, by Western blotting, and by immunohistochemistry. Results from these assays are shown in Fig. 1A and B. MCNC4 cells are a clonal population of parental vector-control transfected cells adapted long-term for growth in CCS and thus grow continuously in CCS like the Δraf*c* clones. While the MCNC4 line exhibits approximately 206 fmol ER/mg protein, each of the Δraf*c* clones has significantly reduced levels of ERα expression, with Raf 14*c*, the clone exhibiting the highest Δraf expression, having only approximately 10 fmol/mg protein. Thus, all of the Δraf*c* clones had significantly reduced ERα protein expression. Immunohistochemistry with the H222 anti-ER monoclonal antibody (MAb) also demonstrates loss of ERα expression in Δraf-expressing cells. Nuclear immunoreactivity for ERα is clearly observed in the HCopool cell line, increased immunoreactivity is seen in HCopool*c* cells, and no nuclear immunoreactivity is observed in Raf 14*c* cells.

RNase protection assays were performed to determine ERα mRNA levels in the clones. Compared with either the HCopool (grown in FBS and stripped of estrogens for 5 d) or the MCNC4 (grown continuously in CCS) control transfected cells or to the initial Δraf clonal isolates, the Δraf*c* clones exhibited drastically reduced ERα mRNA levels (Fig. 1C). Again, the effect was most readily detectable in Raf 14*c* where very little ERα mRNA was detected. Furthermore, the significant decrease in ERα

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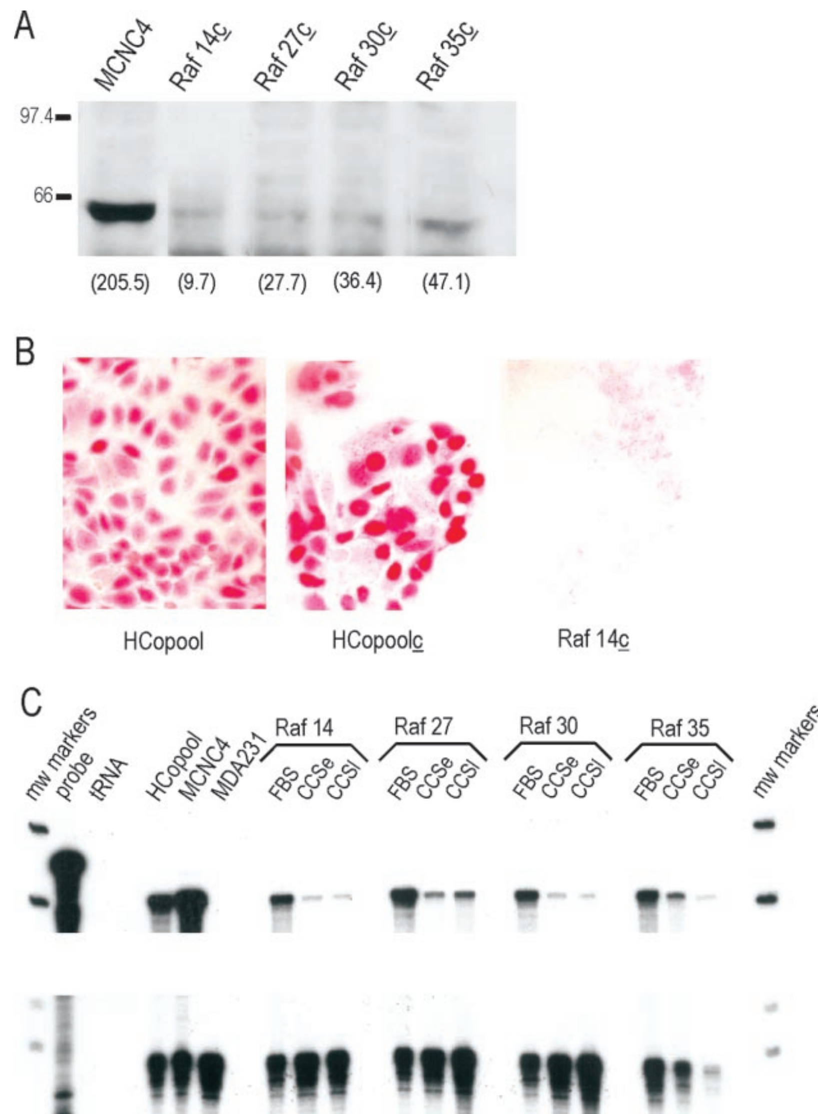

Fig. 1. Δ-rafc Clones Exhibit Significantly Reduced ERα Protein and mRNA Levels

A, Whole-cell lysates (150 μg) were electrophoresed through 10% polyacrylamide gels, transferred, and probed with an anti-ERα MAb (H151 from Dean Edwards). *Numbers in parentheses below* represent femtomoles/mg protein of ERα determined from a ligand binding assay. B, Cells were plated in Falcon chamber slides and stained for ERα expression using an anti-ERα MAb (H222 from Geoffry Greene). C, RNA was prepared from the initial clonal isolates growing in FBS and from early (CCS*e*, about 4 passages) and late passage (CCS*l*, about 16 passages) of clones growing in CCS and analyzed for ERα expression via RNase Protection Assay. The ERα-negative MDA-MB-231 cell line was used as a hybridization control. GAPDH (*lower panel*) was used as a loading control.

message occurred relatively early upon switching to growth in CCS (CCS *e*, four passages in CCS) since further significant reduction was not seen after prolonged growth in CCS (CCS *l*, 16 passages in CCS).

Δraf-induced down-regulation of ERα Does Not Involve Ligand-Independent Activation of ERα Transcriptional Activity

Because estrogen-induced activation of ERα results in subsequent down-regulation of ERα (39–45), and because it has been demonstrated in other systems that growth factor signaling via MAPK can induce ligand-independent activation of ERα (46), we assessed whether Δraf signaling could activate ERα in the absence of estrogen. We examined the basal expression of three estrogen-regulated genes, pS2, cathepsin D, and PR, in our Δrafc clones. If ligand-independent activation were occurring, one would expect higher basal levels of estrogen-induced genes in the absence of estrogen than that observed in control transfected cells in the absence of estrogen. None of the Δrafc clones displayed significantly increased basal levels of pS2 or cathepsin D mRNA compared with the control

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vector-transfected MCNC4 cell line which, like the Δraf*c* transfectants, grows continuously in the absence of estrogen (Fig. 2A). Like other MCF-7 cell lines adapted for growth in estrogen-depleted conditions, MCNC4 cells exhibit increased basal expression over control MCF-7 cells as a result of the adaptation (data not shown). Except for Raf 14*c* cells, which express less than 10 fmol/mg protein ERα and behave more like ERα-negative cells in that they express very low levels of pS2 mRNA, all of the clones expressed similar levels of pS2 and cathepsin D mRNA as MCNC4 cells. Analysis of PR mRNA expression in the Δraf*c* clones revealed that like the control MCNC4 cells, none of the raf clones expressed PR in the absence of estrogen. Furthermore, while MCNC4 cells were induced by estrogen to express PR, none of the clones expressed PR in response to estrogen (Fig. 2B). Collectively, these data suggest that constitutive activation of the Raf/MEK/MAPK pathway in MCF-7 cells does not result in ligand-independent expression of estrogen-target genes.

Because the estrogen target genes we examined contain complex promoters with multiple regulatory factors, it is possible that an effect on ERα activity could be masked by effects on other factors as well, or that small variations in expression levels were due to other growth factor-stimulated effectors such as activator protein 1. Therefore, we next examined whether Δraf or a constitutively active MEK-1 construct (ΔMEK) (47) was capable of inducing ligand-independent activation of ERα through an estrogen response element (ERE)-luciferase construct. The ERE-luciferase construct is an mouse mammary tumor virus (MMTV)-driven plasmid in which the endogenous glucocorticoid response elements (GREs) have been deleted and a double ERE consensus sequence has been inserted in their place (48, 49). A control luciferase construct was also used where the double ERE consensus sequence has been scrambled to a nonsense sequence (NON-luciferase). The experiment shown in Fig. 3A depicts a transient cotransfection assay of MCF-7 cells with either Δraf, ΔMEK, or the parental control vector pCHC6 and an ERE-luciferase vector or the control NON-luciferase vector. As can be seen, cotransfection of pCHC6 with ERE-luc results in very little luciferase activity in the absence of estrogen (Co) while estrogen is able to induce significant luciferase activity. The antiestrogens 4-hydroxytamoxifen and ICI 182,780 do not have a significant effect on activity. When Δraf is cotransfected with ERE-luc, there is no more luciferase activity in untreated cells (Co) than when the control pCHC6 plasmid was used. These data indicate that Δraf is not inducing ligand-independent activation of ERα. Of interest, however, Δraf in conjunction with estrogen treatment represses the estrogen induction compared with that with pCHC6. While the absolute values vary from experiment to experiment, the Δraf-induced repression in the experiment depicted in Fig. 3 is approximately 47%, and this is statistically significant ($P < 0.005$). When a constitutively active MEK is used in the same assay, we again observe no ligand-independent activation of ERα (Co with ΔMEK *vs.* with pCHC6), and ΔMEK almost completely represses the estrogen induction—the repression in this case is approximately 97% ($P < 0.001$). These experiments have also been performed in T47D cells with similar results: neither Δraf nor ΔMEK induced ligand-independent activation, and both inhibited estrogen-induced activity (data not shown). These data further support the finding that

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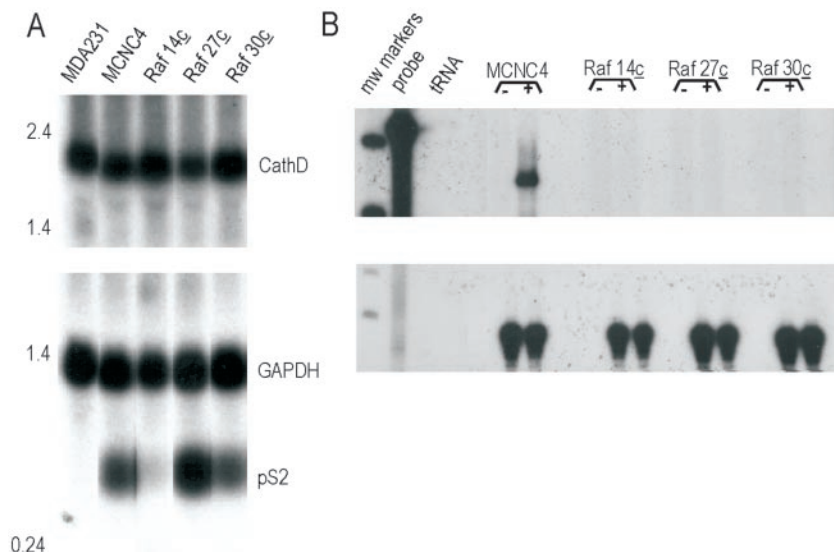

Fig. 2. Δraf*c* Clones Do Not Exhibit Ligand-Independent Induction of Estrogen-Induced Genes pS2, Cathepsin D, and PR

A, RNA was prepared from clones and analyzed for expression of pS2 and cathepsin D by Northern blotting. GAPDH was used as a loading control. B, RNA was prepared from clones treated (+) or not (−, vehicle) with 10^{-8} M estradiol for 72 h and analyzed for PR expression by RNase Protection Assay. GAPDH was used as a loading control.

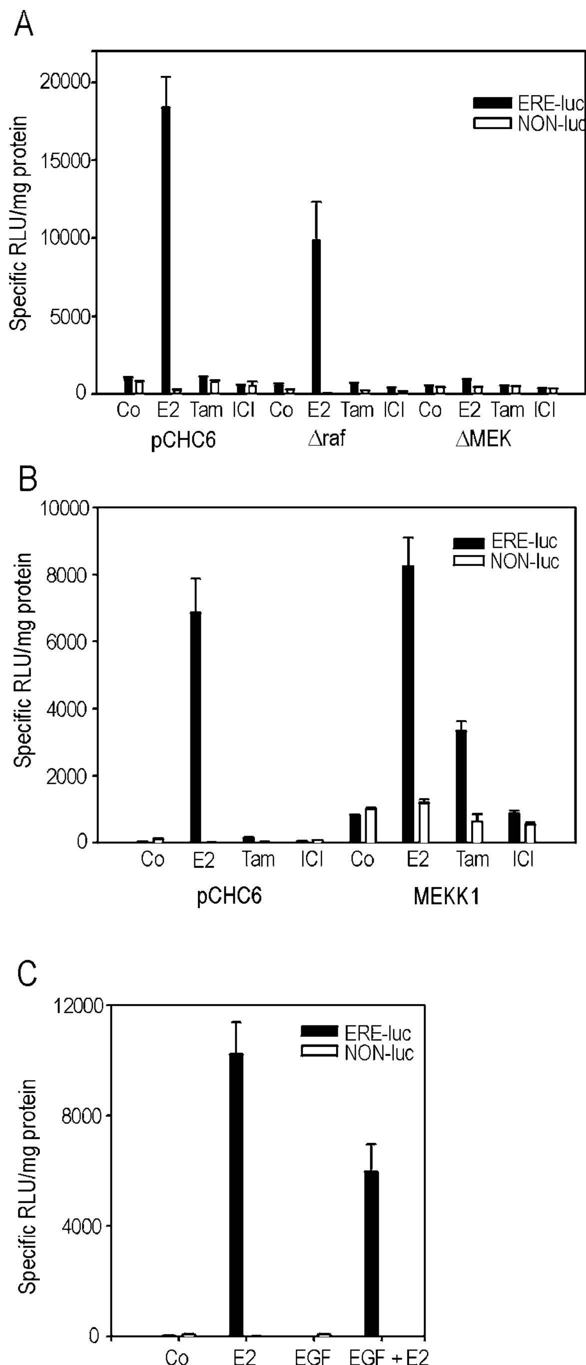

Fig. 3. Δraf and ΔMEK Do Not Induce Ligand-Independent Activation of an ERE-Reporter Construct

A, MCF-7 cells were quick-stripped of estrogens as described in *Materials and Methods* and transiently cotransfected with 2.5 μg of either parental vector pCHC6, Δraf, or ΔMEK and 2.5 μg of either ERE-luciferase or NON-luciferase. Post-transfection treatments were vehicle (Co), 10^{-8} M estradiol (E2), 10^{-7} M 4-OH-tamoxifen (Tam), or 10^{-7} M ICI 182,780 (ICI). B, MCF-7 cells cotransfected with pCHC6 or ΔMEKK1. C, MCF-7 cells transfected with ERE-luc or NON-luc were treated with vehicle (Co), 10^{-8} M estradiol (E2), 12 ng/ml EGF (EGF), or 10^{-8} M estradiol plus 12 ng/ml EGF (E2+EGF).

strong signaling via the Raf/MEK/MAPK pathway does not induce ligand-independent activation of ERα in breast cancer cells. Instead, signaling through this pathway appears to decrease ERα activity.

Constitutive MEKK1 Activity Does Not Repress ERα Activity

Since growth factor activation of Ras can activate other signaling pathways in addition to Raf, one question arising from these data is the role of other growth factor signaling pathways initiated by growth factor binding or activation of Ras in inducing ligand-independent activation or down-regulation of ERα. Transient cotransfection of a constitutively active MEKK1 with either ERE-luciferase or NON-luciferase indicates that MEKK1 signaling does not repress ERα activity (Fig. 3B). The activity of both the ERE-luciferase and the NON-luciferase constructs increased when cotransfected with MEKK1, most likely a reflection of the two imperfect activator protein 1 or serum response element sites that lie in the MMTV promoter driving these two reporter plasmids. These data suggest that the effect of Δraf and ΔMEK on ERα activity and expression is specific to signaling via MAPK.

We then moved upstream of Raf or MEKK1. MCF-7 cells transiently transfected with either the ERE- or NON-luciferase vector were treated with EGF, a ligand whose tyrosine kinase receptor signals through Raf-1 but also activates other pathways such as MEKK1. Again, no ligand-independent activation of ERα was observed (Fig. 3C). Instead, EGF treatment with estrogen resulted in a repression of estrogen's ability to activate ERα. These data indicate that EGF signaling in MCF-7 cells does not result in ligand-independent activation of ERα.

Transient Expression of Δraf Results in Down-Regulation of ERα Expression

To examine more closely the temporal component of Δraf-mediated loss of ERα expression, Δraf was transiently transfected into MCF-7 cells or HCopoolc cells and followed by double-label immunohistochemistry for both ERα with the 1D5 anti-ER MAb and the transfected Δraf. Those cells that stain for the transfected Δraf no longer display nuclear immunoreactivity for ERα (Fig. 4). We have repeated this using ΔMEK and obtain the same results. Interestingly, in all cases, we always observe that some cells immediately adjacent to the transfected cells also exhibit decreased nuclear staining, suggesting that perhaps over the 24–48 h post transfection, cell-cell signaling is occurring. To quantitate the effect of this transient expression of Δraf on ERα expression, 11 low-power fields with a total of 4,459 cells were assessed. Δraf expression was observed in 369 of these cells. Of these Δraf-expressing cells, 94.1% displayed no nuclear immunoreactivity for ERα. Only 3% of the non-Δraf-expressing cells

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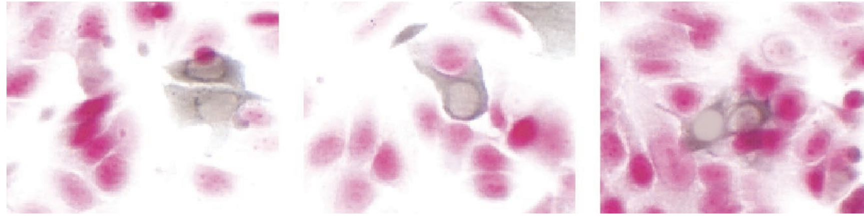

Fig. 4. Transient Expression of Δraf Results in Down-Regulation of ERα Expression

MCF-7 cells plated in Falcon chamber slides were transiently transfected with Δraf. Transfected cells were then double stained for Δ-raf expression (*gray staining*) and ERα expression (*red staining*). Three different fields are shown.

displayed this lack of ERα immunoreactivity, indicating a specific effect of Δraf expression on ERα expression.

Abrogation of Raf Signaling by a MEK Inhibitor Results in Reexpression of ERα

To assess the effect of transient shut down of Δraf signaling on ERα expression in Raf 14*c* cells, we used the MEK-specific inhibitor, PD 098059, to suppress the activity of this Raf effector as monitored by expression of activated, phosphorylated MAPK (Fig. 5A). PD 098059-treated and untreated Raf 14*c* cells were then analyzed for ERα expression by immunohistochemistry with the H222 anti-ER MAb. Untreated cells are ERα negative. However, ERα is reexpressed in approximately 60% of the cells after 12 h of continuous MEK inhibition (Fig. 5B). The staining intensity indicates that ERα is expressed at a relatively high level in some cells, but not every cell returns to ERα positivity during this time frame, suggesting a cell cycle component to the ERα reexpression mediated by MEK inhibition in this cell line, which has a doubling time of approximately 22 h. We have also assessed the time course in which ERα is reexpressed in Raf 14*c* cells in response to MEK inhibition, this time using the newer, more potent MEK inhibitor U0126. Raf 14*c* cells were treated with U0126 for 1 h, and treated cells were analyzed for ERα expression at various times post U0126 treatment. A modest increase in ERα levels is observed at 1 or 3 h post U0126 treatment, but a significant increase in ERα expression to levels comparable to that of MCF-7 cells is observed at 7 h post U0126 treatment. Thus, the abrogation of Raf signaling via MEK and MAPK is sufficient to allow for reexpression of ERα.

Overexpression and Hyperactivation of Growth Factor Signaling Does Not Result in Ligand-Independent Activation of ERα, but Rather MAPK-Induced Loss of ERα Expression

MCF-7 cells express relatively low levels of growth factor receptors such as EGFR or c-erbB-2, and the above studies were performed where only one arm of signaling was hyperactivated (*i.e.* Δraf or ΔMEKK1). Therefore, we were interested in determining whether overexpression of these receptors and the subsequent concomitant hyperactivation of growth factor signaling pathways that is found in some breast tumors would induce either ligand-independent activation of ERα or, alternatively, down-regulation of ERα protein and activity. To do this, we have used two other MCF-7 stably transfected cell lines, MCE5 cells (stably overexpressing EGFR) and MB3 cells (a clone stably overexpressing c-erbB-2 with high levels of constitutive autophosphorylation) (29, 30). Like our Δraf*c* clones, overexpression of EGFR and c-erbB-2 facilitates the continuous growth of both cell lines in the absence of estrogen. The original ER assays performed with the initial isolates of these cell lines indicated that the EGFR overexpressing cell line (MCE5) displayed a modest reduction in ERα levels compared with control transfected cells adapted for growth in estrogen-free conditions (29), while the c-erbB-2 overexpressing cell line with high levels of constitutive autophosphorylation (MB3) displayed a significant reduction in ERα levels compared both to control transfected cells and to two c-erbB-2 overexpressing clones not exhibiting high constitutive activity (30). MCE5s and MB3s were transiently transfected with the ERE- or NON-luciferase vectors and analyzed for luciferase activity in the absence or presence of estrogen. Neither of these cell lines exhibits significant activation of ERE-luciferase in the absence of estrogen (Fig. 6A) indicating there is no ligand-independent activation. The estrogen-induced activation in MB3 s was blunted, corresponding to the significantly lower levels of ERα expressed by these cells. Since there is little EGF in the media for these cells, it was possible that the majority of the overexpressed EGFR in MCE5 cells was not activated. To address this, the same transient transfections were repeated with MCE5 cells in the absence and presence of EGF. Again, no ligand-independent activation is seen with EGF treatment (Fig. 6B), and furthermore, the addition of EGF actually repressed the estrogen-induced activation as we had seen with Δraf and ΔMEK.

We were next interested in determining whether there was a correlation between ERα status and MAPK activity in these cells. We have repeated the ERα analysis and assessed the phospho-MAPK expression of these cell lines alongside our Raf 14*c* cells. Ligand-binding assay results confirmed the earlier ERα analysis of these cell lines. Raf 14*c* cells were

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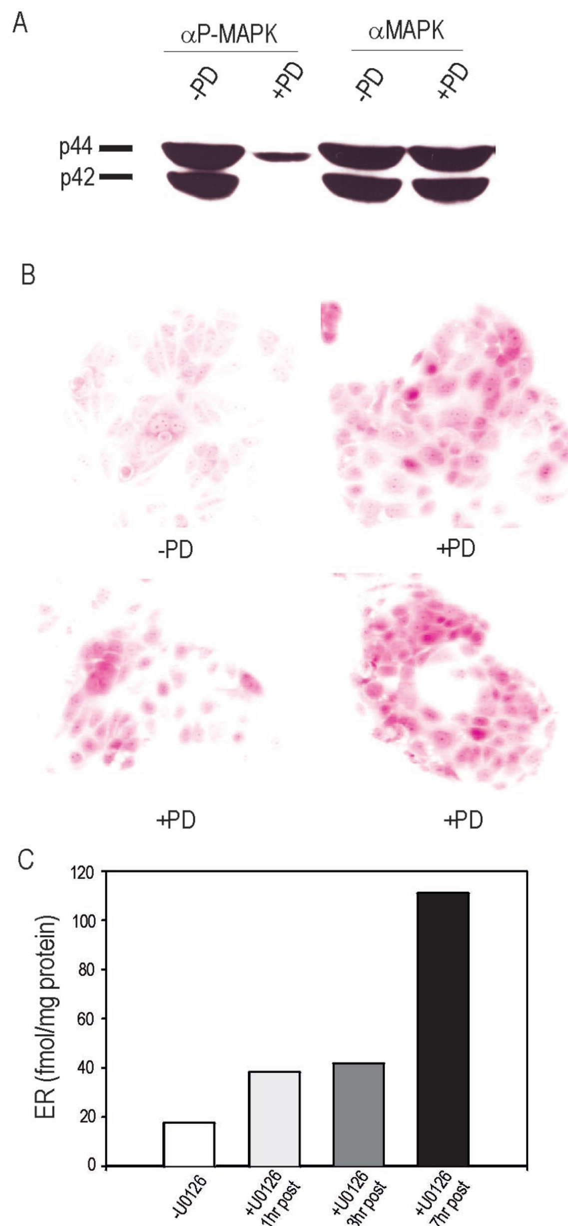

Fig. 5. Transient Abrogation of Δ-raf Signaling via the MAPK Pathway in Raf 14*c* Cells Results in Reexpression of ERα

Raf 14*c* cells were treated with 200 μM PD 098059 (+PD) or vehicle (−PD) every 3 h for a total of 12 h. A, Whole-cell lysates were prepared and 50 μg were electrophoresed through 10% polyacrylamide gels, transferred and probed. The *left-hand* gel was probed with an anti-phospho MAPK polyclonal antibody (αP-MAPK) and the *right-hand* gel was probed with an anti-MAPK polyclonal antibody (αMAPK) to demonstrate that the levels of MAPK protein remained unchanged. B, Cells were immunostained for ERα expression using the H222 anti-ERα MAb. Magnification is 100×. C, Raf 14c cells were treated for 1 h with U0126 (+U0126) or vehicle (−U0126). U0126-treated cells were then analyzed 1 (1 h post), 3 (3 h post), or 7 (7 h post) hours post U016 treatment for ERα expression by ligand binding assay. ERα levels are expressed as femtomoles/mg protein.

close to ERα negative, the MB3 cells that contain the constitutively active c-erbB-2 exhibit significantly reduced ERα levels, and the EGFR-overexpressing MCE5s display moderately reduced levels compared with their control-transfected cells (Fig. 6C). Analysis of the MAPK activity levels in MB3s and MCE5s revealed that MB3s had significantly more activity, similar to that observed with Raf 14*c* and other ERα-negative breast cancer cells, than ERα+ control cells or MCE5s (Fig. 6D). These data suggested that a threshold level of hyperactivation of downstream signaling pathways, not mere overexpression, was involved in down-regulating ERα levels. In support of this, EGF treatment of MCE5s to activate the EGFR resulted in a significant increase in MAPK activity, which corresponded to a significant reduction in ERα levels at 8 h post EGF treatment, from 223 fmol/mg protein to 5 fmol/mg protein (Fig. 6E).

Abrogation of MAPK Activity in MCE5 and MB3 Cells Reverses the ERα Down-Regulation

To confirm that it was high MAPK activity that was responsible for the negative effects on ERα expression and activity in the EGFR and constitutively active c-erbB-2-overexpressing MCF-7s, MAPK activity was abrogated using dominant negative ERK1 and ERK 2 constructs (50). MCE5s were again transiently cotransfected with ERE-luciferase or NON-luciferase and with dominant negative (dn) ERK1 and ERK2 before hormonal treatments. Cotransfection of the dnERKs had no effect on ERE-luciferase activity in the absence of estrogen, but did result in significant enhancement of estrogen-induced activity, suggesting that even the modest reduction in ERα expression in MCE5s is a result of increased MAPK activity (Fig. 7A). We did not obtain a significant EGF repression of estrogen-induced activity, probably due to the fact that with continued culture, these cells produce TGFα (D. Miller, unpublished data). In support of this, the MCE5 cells used in this experiment exhibited significantly higher levels of MAPK activity in the absence of EGF, and addition of EGF did not result in a further significant induction of this MAPK activity (data not shown). Use of the MEK inhibitor U0126 in these experiments gave similar results (data not shown). To assess the effects of MAPK abrogation on ERα levels directly, ligand-binding assays were performed with EGF-treated and untreated MCE5 cells transiently transfected with control vector or dnERK1 and dnERK2 constructs. Again, abrogation of MAPK activity resulted in increased ERα expression in the presence or absence of EGF (Fig. 7B). Most impressive was the increase in ERα levels in cells transiently transfected with the dnERKs, considering that only a fraction of the cells are taking up the vectors. Similar data was also obtained with the constitutively active c-erbB2-overexpressing MB3 cells (Fig. 7, C and D). These results indicate that even with activation of several signal transduction cascades, the

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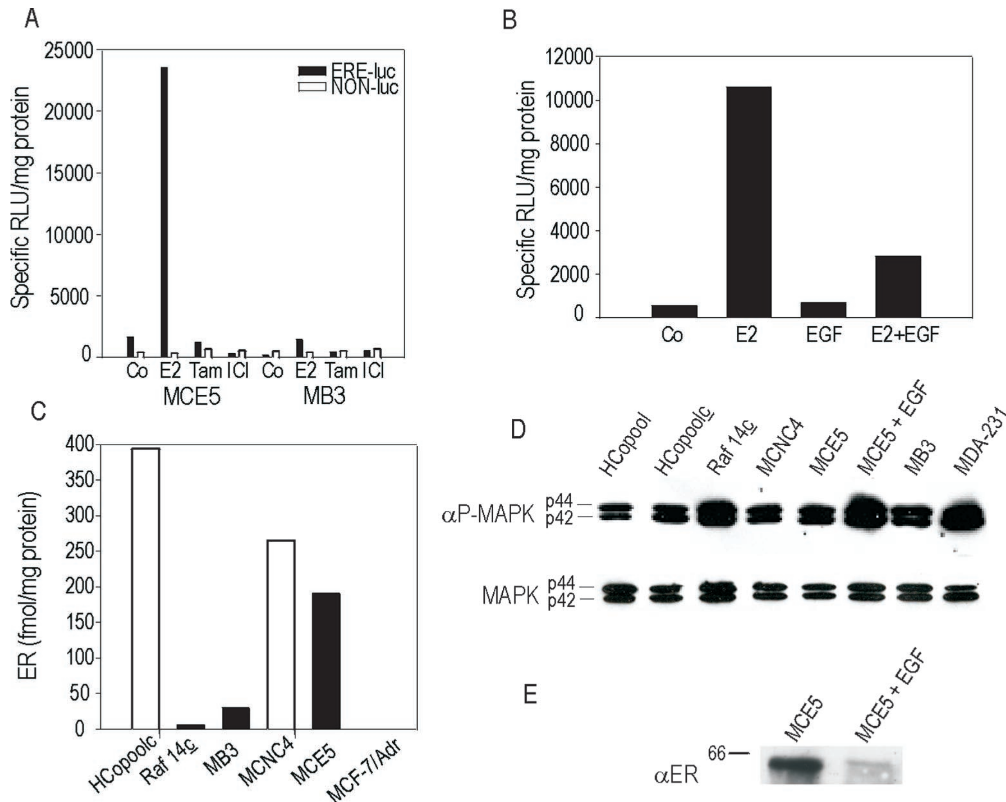

Fig. 6. Growth Factor Signaling Represses ERα

A, MCE5 and MB3 cells were transfected with 2.5 μg of either ERE-luc or NON-luc. Post-transfection treatments were for 48 h in media plus vehicle (Co), 10^{-8} M estradiol (E2), 10^{-7} M 4-OH tamoxifen (Tam), or 10^{-7} M ICI 182,780 (ICI). B, MCE5 cells were transfected with ERE-luc and treated with vehicle (Co), 10^{-8} M estradiol (E2), 12 ng/ml EGF (EGF), or 10^{-8} M estradiol plus 12 ng/ml EGF (E2+EGF). C, ERα levels were determined for cell lines by ligand-binding assay and are expressed as femtomoles/mg protein. D, Whole-cell lysates (2.5 μg) were electrophoresed in 10% polyacrylamide gels, transferred, and probed with an antiphospho-MAPK antibody. MCE5 cells were treated or not with 12 ng/ml EGF for 10 min. E, Whole-cell lysates (150 μg) from MCE5 cells treated or not with 12 ng/ml EGF for 8 h were electrophoresed in 10% polyacrylamide gels, transferred, and probed with H151 anti-ERα MAb.

resultant high MAPK activity in these cells results in loss of ERα expression and suggests that ERα-negative, EGFR, or c-erbB-2-overexpressing breast cancer cells are ERα negative due to the direct down-regulation of ERα induced by the high MAPK activity.

Abrogation of MAPK Activity in Conjunction with Demethylation of the ERα Promoter Results in Reexpression of ERα in MDA-231 Cells

To extend further our hypothesis of hyperactivation of MAPK being responsible for down-regulation of ERα expression, we were interested in whether the ERα-negative phenotype of established ERα-negative breast cancer cells lines could be reversed by abrogation of MAPK activity. However, most ERα-negative breast cancer cell lines, such as MDA-MB-231 cells, MCF-7/Adr cells, MDA-MB-468, and SkBr3 cells, exhibit site- specific methylation of CpG islands in the ERα promoter, thus requiring treatment with a demethylating agent such as 5-aza-cytidine to obtain reexpression of ERα in these cells (51, 52). This reexpressed ERα should then be down-regulated again due to the high levels of MAPK activity present in these cells while inhibition of this high MAPK activity would allow for the continued reexpression of ERα. MDA-231 cells treated for 3 d with 5-aza-cytidine to allow for the reexpression of ER were then treated with the MEK inhibitor U0126 for varying times. As demonstrated in Fig. 8, a 3-d treatment with 5-aza-cytidine results in ERα being expressed. Further treatment of these cells with U0126 results in an increased expression of ERα at 8 h (a 1.5-fold increase), with expression then decreasing over the next 4–16 h back to pre-U0126 treatment levels. This 8-h time frame for increased ERα expression is highly consistent with the U0126 and/or dnERK data in our Raf, EGFR, and c-erbB-2 MCF-7 cell models. Our models, however, would have predicted that because of the high MAPK activity in MDA-231 cells, demethylation of the ERα promoter alone should not have resulted in detectable ERα expression at the 3-d time point of 5-aza-cytidine treatment; *i.e.* concomitant abrogation of MAPK activity

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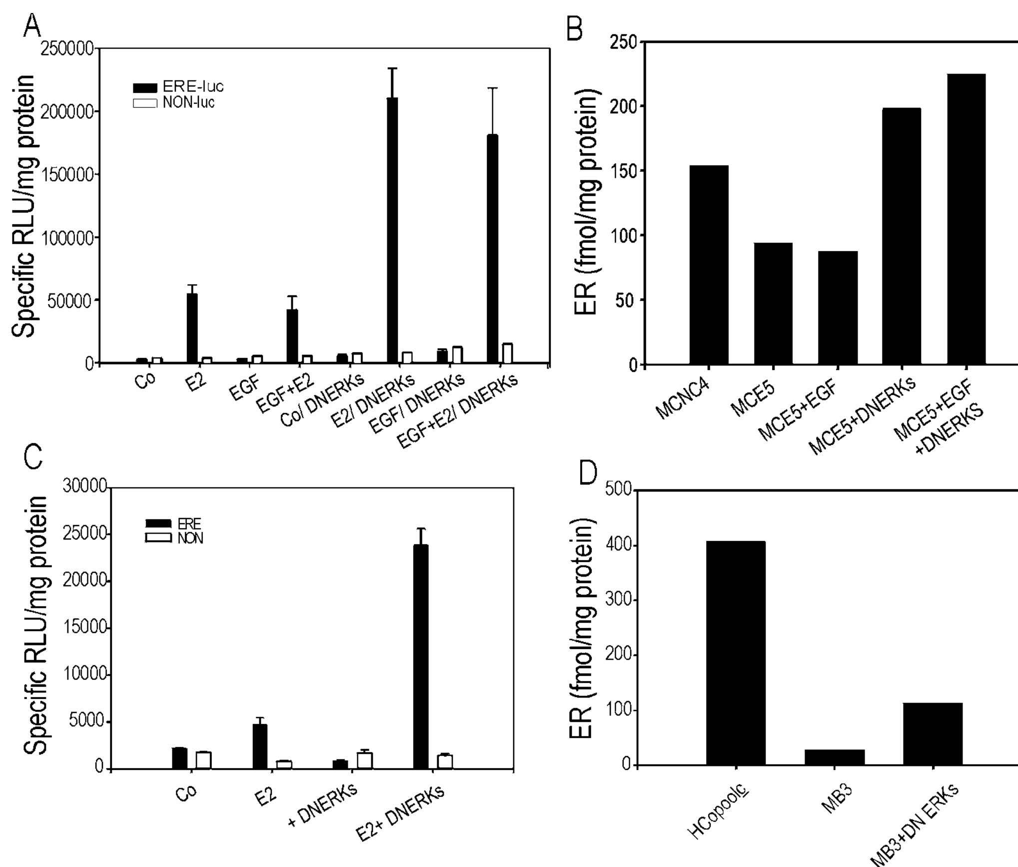

Fig. 7. Abrogation of EGFR- or c-erbB-2-Induced MAPK Activity Restores ERα Expression and Activity

MCE5 (panel A) and MB3 (panel C) cells were cotransfected with 2.5 μg ERE-luc or NON-luc and 2.5 μg of control vector pCHC6 or dnERKs (1.25 μg of both dnERK1 and dnERK2 were used) as indicated. Post-transfection treatments were as indicated. ERα levels in treated, untreated and transfected MCE5 (panel B) or MB3 (panel D) cells were determined by ligand binding assay.

would be necessary to detect ERα expression. Interestingly, analyses of the MAPK activity from these MDA-231 cells demonstrated that there is a significant decrease in the MAPK activity of MDA-231 treated with 5-aza-cytidine alone, a 9.4-fold decrease compared with untreated cells. Treatment with U0126 results in further reduction in MAPK activity of 4.4-fold at 4 h with activity returning to pre-U0126 levels at the longer time points. The inhibition of MAPK activity over the first 4 h of U0126 treatment is sufficient to detect an increase in ERα expression at 8 h. The subsequent return of the MAPK activity to pre-U0126-treated levels then results in ERα expression reduced to that of pre-U0126-treated levels at later time points. Both the U0126-induced increase in ERα expression and decrease in MAPK activity were specific since neither ERα expression nor MAPK activity varied over the time course in the 5-aza-cytidine-treated cells incubated with DMSO as the vehicle control (data not shown). These data indicate that abrogation of MAPK in an established ERα-negative breast cancer cell line with methylation of the ERα promoter correlates with reexpression of ERα.

DISCUSSION

Raf is a downstream effector of growth factor receptors, such as EGFR or c-erbB-2, the overexpression of which is either inversely correlated with ERα expression or directly correlated with decreased sensitivity to antiestrogens (8, 12, 13, 53). We have used the expression of constitutively activated Raf as a model of up-regulated growth factor signaling to study the interaction of the growth factor and ER- mediated signaling systems in the progression of breast cancer from estrogen-dependent growth to estrogen-independent growth. We have previously found that expression of a constitutively active form of the Raf-1 kinase in MCF-7 ERα+, estrogen-dependent human breast cancer cells results in two seemingly linked phenomena. First, it induced estrogen-independent growth of these cells, both anchorage dependent and anchorage independent (38). Second, the expression of Δraf was not tolerated by the cells when grown in the presence of estrogen. In dissecting the mechanisms underlying both of these phenomena, we were

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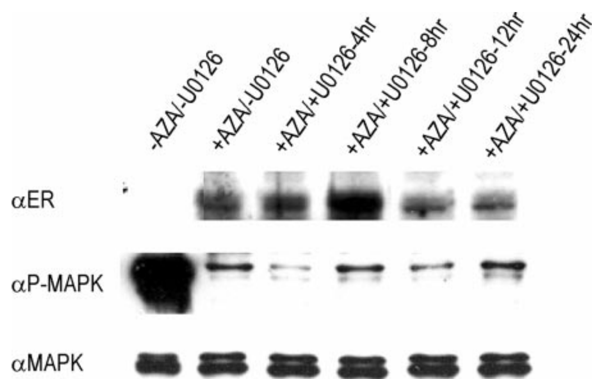

Fig. 8. Demethylation of the ERα Promoter in MDA-MB-231 Cells Alone and in Conjunction with MEK Inhibition Decreases MAPK Activity and Restores ERα Expression

MDA-MB-231 cells were treated or not with 0.5 μM 5-aza-2′-deoxycytidine for 3 d. Cells were then treated with vehicle or 10 μM UO126 and harvested at the indicated timepoints. Whole-cell extracts were prepared, and 5 μg were electrophoresed through 10% polyacrylamide gels, transferred, and probed. The gel in the *upper panel* was probed with an anti-ERα antibody, 1D5, and the gel in the *middle panel* was probed with an anti-phospho MAPK antibody. The gel in the *lower panel* was probed with an anti-MAPK antibody, demonstrating equal levels of MAPK protein among the samples.

interested in determining the direct effect of Raf signaling on ERα that imparts estrogen independence.

Δraf transfected clones growing continuously in the absence of estrogen (Δrafc) were characterized for their growth capabilities in the presence and absence of estrogen or antiestrogens. Interestingly, in addition to the fact that these cells grew in the absence of estrogen, they no longer responded positively to the addition of estrogen nor did they respond to antiestrogens as the control cells did. These data raised the possibility that the Δrafc cells no longer expressed functional ER. Analysis of the cells for ERα expression at the levels of steroid binding, overall protein by Western blotting and immunohistochemistry, and RNA all demonstrated significant reduction in ERα expression (Fig. 1). Furthermore, double-label immunohistochemistry demonstrated that high expression of both ERα and stably transfected Δraf did not occur in the same cell (data not shown). These data were quite exciting because they recapitulate results of published studies in which double-label immunohistochemical detection of ERα and EGFR in breast tumor specimens from ERα+/EGFR+ tumors revealed that individual tumor cells express high levels of only ERα or EGFR, but not both (22, 23). The EGFR+ cells in these tumors are also associated with a higher growth rate than the ERα+ cells (25, 26), suggesting that active signaling is occurring perhaps through MAPK.

Stable transfections of growth factor signaling components into ERα+ MCF-7 breast cancer cells, *i.e.* EGFR, erbB-2, heregulin (HRG), or Ras, lead to significantly decreased levels of ERα in most cases, in addition to estrogen-independent growth (27–32). For example, decreased ERα resulting from overexpression of c-erbB-2 in MCF-7 cells has been described by two different groups. In one case, an approximate 50% decrease in ERα levels was observed (31). In the second study, an approximate 4-fold reduction in ERα was found in one clone where the transfected erbB-2 exhibited a high level of constitutive autophosphorylation (30). In this latter study, clones not exhibiting constitutive activation of the transfected c-erbB-2 displayed no alteration in ERα expression, suggesting that up-regulated signaling, not merely overexpression, was linked to ERα down-regulation. In support of this, ERα levels in fibroblast growth factor-1-overexpressing MCF-7 cells were unaffected (49) and displayed a much more modest increase in MAPK activity (data not shown) while EGF treatment of EGFR-overexpressing cells resulted in both significantly increased MAPK activity and decreased ERα expression (Fig. 6, D and E). Thus, our system of chronic activation of a specific downstream growth signaling pathway resulting in both estrogen-independent growth and loss of ERα implicates the activation of MAPK as the mediator of signal transduction-induced down-regulation of ERα. This is further supported by the reversal of ERα down-regulation that occurs upon abrogation of MAPK activity either by MEK inhibition or dominant negative ERKs in our different cell line models (Figs. 5 and 7).

This loss of ERα expression could occur by one of three mechanisms. First, Δraf induction of estrogen-independent growth could be occurring through an ERα-independent mechanism, and the loss of ERα in our cells could merely be due to the fact that since the cells no longer need ERα for growth, they have down-regulated its expression. However, this would be in contrast to cells naturally selected for estrogen independence by long-term growth and selection in the absence of estrogen where these cells remain responsive to estrogen and/or antiestrogens and actually up-regulate expression of ERα (Refs.33–35, 54 and our own control cells).

Second, if Raf activation of the MAPK kinase cascade activates ERα in the absence of estrogen (ligand-independent activation), then it is likely that the constitutive activation of MAPK that occurs in our cells would result in the constitutive activation of ERα. Dopamine, which activates adenylate cyclase, has been shown to activate ERα in the absence of estrogen (55), also termed ligand-independent activation, and is able to potentiate the partial agonist activity of tamoxifen (56). In the rat uterus, where ERα and EGFR are expressed in the same cell, EGF can result in the same effects on uterine tract growth and differentiation as estrogen (57), and this effect can be decreased by pretreatment with the pure antiestrogen ICI 164,384 (58), suggesting that these effects are ERα mediated (57). The uteri of ERα knockout mice do not exhibit EGF-induced DNA synthesis or induction of PR expression (59), indicating that these EGF effects are mediated through this form of ERα. EGF or TGFα can

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also activate ERα transcriptional activity in endometrial adenocarcinoma cells and ovarian adenocarcinoma cells (60). MAPK, a downstream effector of growth factor signaling, has been shown to phosphorylate and activate ERα in *in vitro* cotransfection experiments of MAPK and ERα into HeLa or Cos cells. EGF treatment of ER-negative cells transiently transfected with ERα also activates ERα (46). Because an end result of ERα activation by estrogen is its eventual down-regulation, a constitutive and continual activation of ERα could ultimately result in the chronic and total down-regulation of ERα.

Experiments performed to examine ligand-independent activation in ERα+ breast cancer cells have given conflicting results. Agents that increase intracellular cAMP, such as IGF-I, cause increased rat uterine and MCF-7 breast cancer cell PR levels (61, 62), as well as activation of transiently transfected reporter constructs containing EREs (63, 64), in a manner similar to that of estrogen. In addition to increasing transcriptional activity of ERα, ERα phosphorylation was increased in these studies (63). This type of interaction between IGF-I and ERα signaling corresponds to the physiological relationship between these two in MCF-7 cells in which IGF-I synergizes with estrogen in inducing growth (65–69). The transfection of HRG or erbB-2 into MCF-7 cells has also been shown to result in ligand-independent activation of ERα in one study (31). However, other HRG transfection studies resulted in down-regulation of ERα expression without transcriptional activation (32, 70, 71). In addition, treatment of MCF-7 cells with EGF results in down-regulation of ERα without induction of PR (72), as does treatment with a novel 52-kDa form of HRG (73). These data suggest that signaling via these pathways in ERα+ breast cancer cells can induce the down-regulation of ERα expression without ligand-independent activation, which corresponds to the clinical observation that ERα expression is inversely correlated with EGFR or erbB-2 overexpression.

Using ERE-luciferase reporter constructs and analysis of the basal level of transcription of estrogen-induced genes, we observe no ligand-independent activation of ERα by Δraf. Similarly, when we move further downstream of Raf and analyze the effect of a constitutively active MEK or upstream and analyze the effects of EGF treatment, we also find no increase in ERα activity in ERE-luciferase reporter assays. Finally, in two cell line models exhibiting up-regulated growth factor signaling due to activation of overexpressed EGFR or to overexpression of constitutively active c-erbB-2, we do not observe any ligand-independent activation of ERα, but rather decreased ERα expression and activity. Thus, a third possibility is supported by our data, which indicate that the Ras/Raf/MEK/MAPK signaling pathway can induce the down-regulation of ERα expression without ligand-independent activation.

An important aspect of this loss of ERα expression involves its reversibility. It has been demonstrated that most ERα-negative breast cancer cell lines such as MDA-MB-231 cells, MCF-7/Adr cells, and MDA-MB-468 cells exhibit site-specific methylation of CpG islands in the ERα promoter, and that reversion of this ERα negativity requires treatment with a demethylating agent such as 5-aza-cytidine (51, 52). In addition, about 25% of ERα-negative breast tumors were found to contain methylated ERα (74). More recent data using a highly sensitive methylation-specific PCR assay determined that 100% of these same ERα-negative tumors displayed some degree of methylation; however, a number of ERα-positive tumors also showed similar degrees of methylation (75). Methylation, by modifying the DNA structure assisting in the recruitment of histone deacetylases, ensures that the gene is in an inactive conformation. If methylation occurs as a step subsequent to another mechanism of ERα repression, then one would predict that some ERα-negative breast tumors at an earlier point in a progression pathway do not express ERα because one of these other mechanisms is still operative. In such tumors, it then may be possible to reverse this phenotype and restore antiestrogen sensitivity.

Therefore, we were extremely interested in determining whether the Raf-induced loss of ERα expression observed in our transfectants could be reversed. It was first determined that the cells did not contain methylated ERα (76). The resultant shut off of Δraf signaling through MEK and MAPK via treatment with PD 098059 or U0126 allowed for the reexpression of ERα, indicating that the down-regulation of ERα in our system is MEK-dependent (Fig. 5). This time frame is not inconsistent with a purely transcriptional mechanism. Alternatively, it may be that accelerated protein degradation is also involved, thus accounting for the rapid reexpression or that the return of ERα expression is cell cycle dependent.

It is important to note here that, unlike other transfections of constitutively active Raf, namely an estrogen-regulated ΔRaf:ERα ligand-binding domain version, where aberrant activation of other signaling pathways such as the c-Jun N-terminal kinase (JNK) pathway via establishment of autocrine growth factor loops has been demonstrated (77–80), our Δraf transfectants do not exhibit increased JNK, NFκB, or MEKK1 activity (our unpublished data), indicating that the effects on ERα expression in these transfectants is through Raf/MEK/MAPK. This conclusion is further supported by results of transient expression experiments with Δraf and ΔMEK vectors in which effects in the short term are similar to those seen with the stable transfectants. Finally, the experiments using dnERKs to reverse the ERα down-regulation in Raf 14c cells and in the EGFR and c-erbB-2-overexpressing cells (Fig. 7) clearly establish MAPK as the signal transduction mediator of growth factor-induced down-regulation of ERα. Furthermore, even in an established ERα-negative breast cancer cell line with methylation of the ERα promoter, the demethylation-induced increase in ERα expression correlated with significant reduction in

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MAPK activity, and further specific inhibition of MAPK resulted in further increases in ERα expression (Fig. 8).

These data raise the possibility that potentially reversible mechanisms can be responsible for the lack of ERα expression in some breast cancer cells, and thus there may exist two subpopulations of ERα-negative tumors: those in which the ERα gene is methylated, and thus permanently off, and those in which the lack of ERα expression can be reversed by blocking the mechanism responsible for its down-regulation.

MATERIALS AND METHODS

Cell Culture

HCopool cells (pooled, hygromycin-resistant, control parental vector transfectants) were maintained in IMEM (Life Technologies, Inc., Gaithersburg, MD) with phenol red supplemented with 10% FBS Intergen, Purchase, NY). For growth in the absence of estrogen, media were switched to PRF-IMEM supplemented with 10% CCS (Life Technologies, Inc.). For certain experiments, cells growing in FBS-containing media were quick-stripped of estrogen by repeated rinsing of cells in PRF-IMEM and growth in PRF-IMEM + 10% CCS (three times per day for 2 d). All other cells lines (HCopool*c*—HCopool cells long-term adapted for growth in the absence of estrogen, MCNC4—a G418-resistant clone of control vector-transfected cells long-term adapted for growth in the absence of estrogen, and Raf 14*c*, Raf 27*c*, Raf 30*c*, and Raf 35*c*—different Δraf transfected clones growing continuously in the absence of estrogen) were maintained in PRF-IMEM supplemented with 10% CCS. Hormone treatments, when performed, were with 17β-estradiol (Sigma, St. Louis, MO) at 10^{-8} M, the partial estrogen antagonist 4-hydroxy-tamoxifen at 10^{-7} M, or the pure antiestrogen, ICI 182,780 (obtained from Alan Wakeling, AstraZeneca Pharmaceuticals, Macclesfield, UK) at 10^{-7} M. Cells were plated in 75-cm^2 T-flasks (Costar Cambridge, MA) and grown in a forced-air humidified incubator at an atmosphere of 5% CO_2 and 37 C.

Gel Electrophoresis and Western Blotting

Whole cell lysates for ERα detection were prepared from cells grown to approximately 80% confluence. Cells were harvested by incubation in trypsin-EDTA (Life Technologies, Inc.), washed once in PRF-IMEM plus 10% CCS, once in PRF-IMEM, and then in TEG buffer (10 mM Tris-OH, pH 7.4, 1 mM EDTA, 10% glycerol). Cell pellets were homogenized at 0 C in TEDG (10 mM Tris-OH, pH 7.4, 1 mM EDTA, 1 mM, 10% glycerol) plus 0.5 M NaCl and a cocktail of proteolysis inhibitors (as described in Ref. 81 except that leupeptin was at 1 mg/ml). Homogenates were centrifuged at 105,000 × *g* at 4 C for 30 min in a Ti70.1 rotor (Beckman Coulter, Inc., Fullerton, CA) to yield a whole-cell extract. One hundred fifty micrograms of cellular lysate were electrophoresed through 10% SDS-polyacrylamide gels with 0.1% SDS included in the gel and running buffers. Rainbow mol wt markers were from Amersham Pharmacia Biotech (Arlington Heights, IL). Electrophoresed gels were transferred to 0.45 μm nitrocellulose (Bio-Blot NC, Costar, Cambridge, MA) for 4 h at 0.4 Amps in phosphate transfer buffer [20 mM sodium phosphate, pH 6.8, 20% methanol, 0.05% SDS (82)], and the blots were blocked in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) with 3% BSA and an additional 0.15% Tween-20 added for 60 min at room temperature. Blots were then incubated with H151, an anti-ERα MAb (made against a hinge-region peptide, kindly provided by Dean Edwards, University of Colorado Health Sciences Center, Denver, CO), diluted to 1 μg/ml in TBST plus 1% BSA overnight at 4 C. After washing the blots 3 × 5 min with TBST, they were incubated with goat antimouse antiserum linked to horseradish peroxidase (Amersham Pharmacia Biotech) diluted 1:2,000 in TBST/1% BSA for 60 min at room temperature. The blots were again washed in TBST, once for 20 min and then 3 × 5 min, and the bound secondary antibody was visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

For detection of phosphorylated MAPK, cell lysates were prepared as described previously (38). Briefly, cells were rinsed in PBS and then lysed in a modified gold lysis buffer [20 mM Tris, pH 7.9, 137 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1 mM Pefabloc A (instead of phenylmethylsulfonyl fluoride), 1 mM aprotinin, 1 mM leupeptin, 1 μM pepstatin A, 1 mM Na_3VO_4, 1 mM sodium pyrophosphate, 10 mM sodium fluoride] on ice, scraped into a microfuge tube, and centrifuged at 12,000 × *g* to pellet nuclear debris. Supernatants were analyzed for protein content using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL) and stored at −20 C. Fifty micrograms of cellular lysate were electrophoresed through 10% SDS-polyacrylamide gels as above. Electrophoresed gels were transferred for 2 h at 0.4 Amps in Towbin's buffer (20 mM Tris, 150 mM glycine, pH 8.3, 20% methanol, 0.1% SDS), and the blots were blocked as above. Blots were then incubated with an antiphospho-MAPK polyclonal antibody that specifically recognizes only the phosphorylated form of MAPK or with an anti-MAPK polyclonal antibody that recognizes all forms of MAPK (New England Biolabs, Inc., Beverly, MA) diluted 1:1,000 in TBST plus 1% BSA overnight at 4 C. Blots were processed as above except the secondary antibody was donkey antirabbit antiserum linked to horseradish peroxidase (Amersham Pharmacia Biotech) and diluted 1:4,000 in TBST/1% BSA for 60 min at room temperature.

ER Steroid-Binding Assay

Whole-cell extracts were prepared as described above for ERα Western blotting. Extracts were incubated with 10 nM [^{3}H]-17β-estradiol ± a 100-fold excess of unlabeled estradiol for 16 h at 4 C. Binding was assayed using the dextran-coated charcoal assay as described previously (83). In short, the dextran-coated charcoal was added to adsorb free hormone and was then pelleted by centrifugation. Aliquots of supernatant were removed and counted in 10 ml of liquid scintillation fluid in a Beckman Coulter, Inc. liquid scintillation counter. Values are expressed as femtomoles/mg protein. Steroid-binding assays have been performed on all cell lines a minimum of three times, with comparable results shown as a representative experiment.

Immunohistochemistry Assay

Cells were plated in two-well chamber slides (Falcon, Becton Dickinson, Franklin Lakes, NJ), allowed to attach, and grown as a monolayer. For ERα expression, cells in FBS were quick stripped of estrogen. Cells were fixed by incubation for 10 min at room temperature with 10% formaldehyde-PBS, followed by ice-cold acetone for 15 sec. Fixed cells were then blocked by incubation for 60 min at room temperature in PBS with 1% BSA. For ERα detection, cells required permeabilization by incubation in PBS with 0.1% Triton X-100 for 5 min at room temperature. Primary antibodies were incubated overnight at room temperature in a humidified chamber and were at 2.5 μg/ml for anti-ERα (H222 kindly provided by Geoffrey Greene) or at 1:400 dilution (1D5 from Zymed Laboratories, Inc., South San Francisco, CA), and 0.5 μg/ml for anti-Raf (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted in PBS/1% BSA. Both ERα antibodies gave compa-

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rable immunostaining results. After three PBS washes, secondary antibody incubations were for 60 min at room temperature and were a 1:200 dilution of biotinylated antirat (for ERα) and a 1:300 dilution of horseradish peroxidase-linked antirabbit (for Δraf) in PBS/1% BSA. Detection of ERα required a further incubation of 30 min at room temperature with streptavidin-alkaline phosphatase (AP) and then visualization with Vector Red (to give a red color). Detection of Δraf required just visualization with Vector SG (to give a blue-gray color, Vector Laboratories, Inc.). For double-label immunohistochemistry, both primary antibodies were incubated together, followed by both secondaries together, visualization of Δraf, and then streptavidin-AP and visualization of ERα. Stained cells were then dehydrated through a graded series of ethanol, followed by xylene, and mounted in Permount. All incubations were followed by three washes of 30 sec each, and no counterstain was used.

RNA Extraction, Northern Analysis, and RNase Protection Assays

RNA was extracted using a one-step acid-guanidinium method as described in Ref. 84. For Northern analysis, 10 μg of total RNA were electrophoresed through a 1% agarose/formaldehyde gel as described previously (29). After capillary transfer to nitrocellulose, the membrane was baked at 80 C for 2 h. The blot was first probed for Cathepsin D. The Cathepsin D riboprobe was transcribed from a 319-bp *Kpn*I-*Eco*RI fragment consisting of nucleotides 90 to 409. After cloning the fragment into pBluescriptKS+ and linearizing with *Xba*I, the fragment was transcribed with T3 polymerase. After probing for Cathepsin D, the blot was stripped and reprobed for expression of pS2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as a loading control. The pS2 antisense riboprobe was transcribed from a 305-bp *Pst*I fragment, inserted into pBluescriptKS+, linearized with *Hin*dIII, and transcribed with T7 polymerase. Construction and synthesis of GAPDH antisense riboprobe has been previously described (29). Prehybridization, hybridization, and membrane stripping were carried out as previously described (29). Briefly, the blot was prehybridized in 50% formamide, 5× SSC, 5× Denhardt's, 25 mM $NaHPO_4$ for 4 h at 42 C. Hybridization was carried out in prehybridization buffer plus 10% (wt/vol) dextran sulfate overnight at 42 C. The blot was washed twice at room temperature in 0.2× SSC-0.1% SDS and twice in 0.1× SSC-0.1% SDS at 65 C for 20 min each. Blots were exposed at −70 C to x-ray film.

RNase Protection assays were performed as previously described (29). The ERα riboprobe, corresponding to Exon 5, was transcribed with T7 polymerase from pORB-300 (kindly provided by MaryBeth Martin, Georgetown University Medical Center) linearized with *Eco*RI as previously described in Ref. 85. It generates a protected fragment of 305 bases. The PR riboprobe was transcribed from an *Ava*I fragment spanning nucleotides 283–545 inserted into pGEM4. After linearization with *Eco*RI, it was transcribed using SP6 polymerase. It generates a protected fragment of 262 bases. The GAPDH riboprobe generates a protected fragment of 104 bases.

Transient Transfections and Luciferase Assays

MCF-7 cells were plated in Falcon six-well plates, allowed to attach overnight, and were then quick stripped of estrogens by repeated washing and replacing of the media with PRF-IMEM supplemented with 10% CCS three times per day for 2 d. At the end of the second day, cells were transfected by *N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid-buffered saline (BBS), calcium phosphate, 2% CO_2 protocol as described above (86) except each well was transfected with 2.5 μg of ERE or NON luciferase plasmid and 2.5 μg of Δraf, ΔMEK, or ΔMEKK1 plasmid suspended in $CaCl_2$ and mixed with BBS. The MCE5 and MB3 cells were transfected with 2.5 μg of ERE or NON luciferase. The luciferase plasmids were either pGLB-MERE or pGLB-MNON (48), obtained by inserting an altered MMTV promoter where the endogenous glucocorticoid response element is replaced with a tandem repeat of a consensus ERE (MERE) or the same sequence with the ERE palindromes scrambled (MNON) (48)] into the *Hin*dIII site of pGLB basic luciferase plasmid (Promega Corp., Madison, WI) . The Δraf plasmid is the same vector we used for generation of the stable transfectants (38). The ΔMEK construct was obtained from Natalie Ahn; it is a cytomegalovirus (CMV)-driven MEK containing two substitutions (Ser-218 and Ser-222 have been replaced with acidic residues) and a truncation from residues 32–51 (a suspected kinase regulation domain) that result in approximately 400 times the activity of wild-type MEK (47). The ΔMEKK1 plasmid is a CMV-driven constitutively active MEKK1 obtained from Stratagene (La Jolla, CA). Additional transfections included dominant negative ERK1 and ERK2 constructs (kindly provided by Peter Shaw, Max Planck Institute for Immunology). These were transfected at 1.25 μg each to give a total of 2.5 μg, and pCHC6 was again used as the vector control. The cells were incubated for 16–18 h at 2% CO_2 and 35 C, washed two times with PBS, and then incubated for 48 h in media containing vehicle (0.01% ethanol), 10^{-8} M estradiol, 10^{-7} M 4-OH-tamoxifen, 10^{-7} M ICI 182,780, 12 ng/ml EGF, or a combination of EGF and estrogen. Cells were assayed for luciferase activity (expressed as relative light units or RLU) using the Luciferase Assay System of Promega Corp. according to the manufacturer's instructions. The luciferase values were normalized for protein to obtain RLU/mg, and the RLU/mg values were adjusted to specific RLU/mg by subtracting out the value obtained with lysate prepared from mock-transfected cells. The triplicates were then averaged, and the values were plotted as specific RLU/mg protein with error bars depicting the standard error. A set of triplicate wells transfected with pCMV-luciferase was included in all transfection experiments as a measure of transfection efficiency. When transfection experiments were performed with multiple cell lines, the CMV luciferase activity was used to normalize the data for transfection efficiencies.

Transient Transfection of Δraf and Double-Label Immunohistochemistry

MCF-7 cells were plated in two-well chamber slides, allowed to attach overnight, and were then quick stripped of estrogens by repeated washing and replacing of the media with PRF-IMEM supplemented with 10% CCS 3 times per day for two days. At the end of the second day, cells were transfected by the BBS-calcium phosphate, 2% CO_2 protocol (86). Briefly, each well was transfected with 1.0 μg of Δraf suspended in $CaCl_2$ and mixed with BBS. The cells were incubated for 18 h at 2% CO_2 and 35 C, were washed two times with PBS, and then incubated for 48 h in fresh media. Cells were then processed for immunohistochemistry.

MEK Inhibition

Inhibition of MEK activity was accomplished using PD 098059 [a Parke-Davis (Morris Plains, NJ) compound obtained from New England Biolabs, Inc. (Beverly, MA)]. PD 098059 was resuspended in DMSO according to the manufacturers instructions. A 1-h treatment with 50 μM has been shown to effectively suppress the MEK induction of MAPK activity in response to various growth factors in other cell systems, but much higher doses are required to inhibit a strong or sustained activator (as described in Product Data Sheet from NEB). Since Δraf results in high and sustained MEK activity and because it was necessary to inhibit Δraf signaling for several hours to allow for ERα protein to be reexpressed, the conditions required for abrogating Δraf signaling for 12 h in Raf 14*c* cells using PD 098059 were first

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determined by analysis of the effects of varying doses and times on the phosphorylation of MAPK. It was determined that while 100 μM was sufficient to abrogate Δraf activation of MAPK for 1 h, a 200 μM dose was required to abrogate activation of MAPK in Raf 14*c* cells for 3 h. After 4 h, some return of phospho-MAPK was still observed with the 200 μM dose. Therefore, cells were treated four times consecutively with the 200 μM dose for 3 h to a total of 12 h of complete MEK inhibition. Cells were then washed in PBS and processed for immunohistochemistry. For other experiments, a newer and more effective MEK inhibitor, U0126 (Promega Corp.), was used. Experiments were performed to determine the appropriate dose and time of treatment. For all cell lines, a 30-min pretreatment with U0126 at 10 μM was sufficient to abrogate MAPK activity. In either case, control treatments were vehicle alone (DMSO).

Demethylation of the ER Promoter

MDA-MB-231 cells were plated in PRF-IMEM plus 10% CCS and grown to approximately 60% confluency. Cells were treated or not with 0.5 μM 5-aza-2′-deoxycytidine (Sigma) to return ERα expression, as described previously (52). Cells were then treated with 10 μM UO126 (Promega Corp.) and harvested at 0, 4, 8, 12, and 24 h. In parallel, cells were treated with DMSO as the vehicle control for U0126. After harvesting, whole-cell extracts were prepared and analyzed for ERα, phospho-MAPK, and MAPK expression by gel electrophoresis and Western blotting as described as above. Densitometric scanning was performed using Image Quant software.

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