The mechanisms involved in resistance to estrogen deprivation are of major importance for optimal patient therapy and the development of new drugs. Long term culture of MCF-7 cells in estrogen (E2)-depleted medium (long term estrogen deprivation; LTED) results in hypersensitivity to E2 coinciding with elevated levels of estrogen receptor (ER) α phosphorylated on Ser118 and MAPK, together with several of its downstream targets associated previously with ERα phosphorylation. Our data suggest elevated MAPK activity results from enhanced ERBB2 expression in the LTED cells versus the wild-type (wt), and treatment with the tyrosine kinase inhibitor ZD1839 revealed increased sensitivity in both transcription and proliferation assays. Similarly the MEK inhibitor U0126 decreased transcription and proliferation in the LTED cells and reduced their sensitivity to the proliferative effects of E2, while having no effect on the wt. However, the complete suppression of MAPK activity in the LTED cells did not inhibit ERα Ser118 phosphorylation suggesting that ER activity remained ligand-dependant. The LTED cells also expressed elevated levels of insulin-like growth factor-1R, and inhibition of phosphatidylinositol 3-kinase activity with LY294002 reduced basal ERα transactivation by 70% in the LTED cells compared with the wt. However, LY294002 had no effect on ERα Ser118 phosphorylation. These data suggest that although elevated levels of MAPK occur during LTED and influence the phenotype, this is unlikely to be the sole pathway operating to achieve adaptation.

The knowledge that steroids play a pivotal role in the development of breast cancer has been exploited clinically by the development of endocrine treatments (1). These have sought to perturb the steroid hormone environment of the tumor cells, predominately by withdrawal or antagonism of estrogen. Unfortunately, the beneficial actions of existing endocrine treatments are attenuated by the ability of tumors to circumvent the need for steroid hormones, while in most cases retaining the nuclear steroid receptors (2). The identification of the factors and pathways responsible for the development of these resistant conditions is therefore paramount for the design of new diagnostics and therapeutic regimes (reviewed by Ali and Coombes (3)).

In an attempt to elucidate these mechanisms, our laboratory and others (4–6) have developed in vitro models to study the molecular changes associated with long term estrogen deprivation (LTED).1 Our previously published studies demonstrated that MCF-7 cells deprived of E2 for over 80 weeks passed through three distinct phases: quiescent (LTED-Q) followed by a hypersensitive phase (LTED-H), where basal cell growth was stimulated by the addition of E2 at concentrations below $10^{-13}$ M, and finally an apparent independent phase (LTED-I) in which exogenous E2 no longer affected their growth. Our studies also revealed that the LTED MCF-7 cells expressed elevated levels of ERα that was phosphorylated on Ser118 in the absence of exogenous E2 suggesting this was a contributing factor to the acquired hypersensitivity of these cells (6).

It has been postulated that in the majority of endocrine-resistant tumors, control over growth is assumed by locally acting autocrine or paracrine peptide growth factors. These in turn activate the cell signal transduction pathways by binding to receptors on the cell surface. Several studies have suggested a potential role for the p42/p44 MAPK signaling pathway in the initiation and pathogenesis of breast cancer. For instance p42/p44 MAPK activity appears to be elevated in primary breast cancer compared with benign breast tissue (7). Activation of the p42/p44 MAPK cascades modulates the phosphorylation and hence activity of several nuclear transcription factors that in turn regulate a series of genes. The various MAPK family members play a complex role in the determination of cell growth, differentiation, and programmed cell death, and this is thought to involve a balance between competing MAPK pathways (8).

ERα is functionally regulated via phosphorylation by several protein kinases (9–17). These phosphorylation events are believed to play a pivotal role in regulating many aspects of steroid hormone receptor function including DNA binding and transcriptional activation. Ser118 within the AF-1 domain of the ER has generated much interest. Phosphorylation of Ser118

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1 The abbreviations used are: LTED, long term estrogen deprivation; ER, estrogen receptor; E2, estradiol; MAPK, mitogen-activated protein kinase; CBP, cAMP-response element-binding protein-binding protein; PI3 kinase, phosphatidylinositol 3-kinase; p90RSK, 90-kDa ribosomal S6 kinase RSK; wt, wild-type; EGFR, epidermal growth factor receptor; IGF, insulin-like growth factor; MEK, MAPK/extracellular signal-regulated kinase kinase; FBS, fetal bovine serum; ERE, estrogen response element.
is mediated by cyclin-dependent kinase-7 in response to E2 and is also phosphorylated by p42/p44 MAPK in a ligand-independent manner (9, 18). Interestingly Ser<sup>167</sup> is phosphorylated by p90<sub>RSK</sub>, which itself is activated by p42/p44 MAPK; hence increased MAPK activity could result in endocrine resistance. Another pathway thought to play an important role in ER activity and endocrine resistance is the PI3 kinase signaling pathway, which, together with its downstream target AKT (19), promotes cellular proliferation and anti-apoptotic responses. Recent studies have demonstrated phosphorylation of ER by AKT on Ser<sup>167</sup> also results in ligand-independent activation (17, 20). Although ER activity has traditionally been associated with transcriptional regulation, several studies have implicated ER in non-genomic effects and demonstrated the ability of estrogens mediated by ER to activate the MAPK and PI3 kinase signaling pathways (21–24).

The exact mechanism responsible for the development of the LTED phenotype and the presence of phosphorylated ERα is poorly understood. We postulate that the mechanism involves a dynamic interplay between several signal transduction pathways participating in ERα phosphorylation and cell growth. To address this, we characterized and compared the p42/p44 MAPK and PI3 kinase signal transduction pathways, in LTED-I and wild-type MCF-7 cell lines, to determine whether changes in activity of these phospho-proteins contributed to the development of the apparent estrogen independent phenotype noted in the LTED-I cell line (6).

EXPERIMENTAL PROCEDURES

Materials—Antibodies for phosphorylated and total EGFR and ERBB2 were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-IGF-1R was supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-specific p216/p41 MAPK and anti-β actin antisera was purchased from Sigma, and anti-extracellular signal-regulated kinase (ERK) 1 was obtained from (Zymed Laboratories Inc.). Phospho-specific MEKI, c-Myc, p90<sub>RSS</sub>, AKT, and total AKT polyclonal antisera were purchased from New England Biolabs (Beverly, MA). MEKI inhibitor U0126 was supplied by Promega, and PI3 kinase LY294002 was purchased from New England Biosystems. 17-β-Estradiol was obtained from Sigma. Dr. A. Wakeling (AstraZeneca Pharmaceuticals, Alderley Edge, United Kingdom) kindly provided ICI 182780 and ZD1839 (EGFR tyrosine kinase inhibitor).

Tissue Culture—Wild-type (wt) MCF-7 human breast cancer cells were maintained in phenol red-free RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10 μg/ml insulin, 2 mM glutamine, 100 units of penicillin-streptomycin, and 10<sup>–9</sup> M estradiol. Long term estrogen-deprived (LTE-D-I) MCF-7 cells were maintained in medium depleted of steroids and referred to as DCC medium. This comprised RPMI 1640 deprived (LTED-I) MCF-7 cells were maintained in medium depleted of penicillin-streptomycin, and 10<sup>–9</sup> M insulin for 3 days prior to seeding 12-well plates. The cells were allowed to grow, whereas concentrations in excess of 10<sup>–8</sup> M suppressed the cell growth stimulated by 10<sup>–7</sup> M E2 (Fig. 2A). In contrast treatment of the LTED-I cells with ICI 182780 alone markedly

Preparation of Whole Cell Extracts for Immunoblotting—Cell monolayers were washed with ice-cold phosphate-buffered saline and then lysed in extraction buffer (1% (v/v) Triton X-100, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM sodium fluoride, 2 mM Na<sub>3</sub>VO<sub>4</sub>, and one tablet of Complete<sup>TM</sup> inhibitor mix (Roche Applied Science) per 10 ml of buffer) and homogenized by passage through a 26-gauge needle six times. The lysate was incubated on ice for 10 min and then clarified by centrifugation (14,000 rpm for 10 min at 4°C). The protein concentration was then quantified using bovine serum albumin reagent (Bio-Rad). Equal amounts of protein (50 μg unless stated otherwise) were resolved by SDS-PAGE and then subjected to immunoblot analysis. Antibody reactivity was detected with Super-signal reagent (Pierce). Chemiluminescence was quantified using Fluor-S and analyzed using Quantity One software (Bio-Rad).

Transcriptional Analysis—LTED-I and wt MCF-7 cells (previously stripped of steroids for 3 days) were seeded in 24-well plates at a density of 4 × 10<sup>4</sup> and 8 × 10<sup>4</sup> cells per well respectively, in DCC medium. The following day the cells were transfected with Lipofectin (Invitrogen) with 0.25 μg of EREElklu (luciferase reporter plasmid) and 0.25 μg of pCH110 (β-galactosidase for normalizing luciferase data) for 4 h. The cells were subsequently fed with DCC medium and left to recover overnight, before treatment with the appropriate concentration of E2, modulators, or vehicle. After treatment for 24 h the luciferase and β-galactosidase activity were measured using a luminometer.

RESULTS

Removal of Insulin from the Growth Medium Reduced Hypersensitivity to E2 in LTED-I Cells—Our previous study (6) of the development of the LTED phenotype showed that the cells pass from a hypersensitive phase to an apparent independent phase where the addition of E2 had no effect on cell growth. We hypothesized that rather than being ligand-independent, the LTED-I cells were in fact super-sensitized to the residual E2 in the DCC medium, to the degree that no further growth stimulation was possible with added E2.

Evidence suggests that insulin and insulin-like growth factors are potent breast cancer cell mitogens able to act synergistically with E2 (26). Because the wt MCF-7 and LTED cells were maintained in medium containing insulin, we examined whether removal of insulin would affect the basal and E2-stimulated growth rate of the two cell lines. Removal of insulin resulted in an ~50% drop in the basal growth rate of the LTED-I cells while having no effect on the wt MCF-7 (compare Fig. 1, A and B). In the absence of insulin, E2 stimulated the growth of the wt cells, and this effect was enhanced by addition of insulin (Fig. 1D). In the absence of insulin, the hypersensitivity of the LTED-I cells to the effects of E2 was restored with doses as low as 10<sup>–12</sup> providing an ~50% increase in cell growth (Fig. 1C). However, in the presence of insulin, addition of 10<sup>–12</sup> M E2 had no effect on growth compared with the untreated control whereas doses in excess of 10<sup>–10</sup> M E2 resulted in a decrease in cell growth (greater than 50%). In the absence of insulin, these elevated doses of E2 had no marked effect on cell growth compared with the control.

The removal of insulin appears to reveal a persistent hypersensitivity in the LTED-I cells, which is imperceptible in the presence of insulin, as the cells have reached their maximum growth rate. Hence cross-talk between the insulin-like growth factor and steroid signaling pathways may be involved in the adaptation of the cells to E2 deprivation.

The Pure Anti-estrogen ICI 182780 Inhibits LTED-I Cell Growth—The pure anti-estrogen ICI 182780 prevents activation of AF-1 and AF-2 and reduces the half-life of the ER. Thus if the growth of the LTED-I cells was ER-independent they would be resistant to the anti-proliferative effect of the drug. ICI 182780 alone had no major effect on the wt MCF-7 cell growth, whereas concentrations in excess of 10<sup>–9</sup> M suppressed the cell growth stimulated by 10<sup>–7</sup> M E2 (Fig. 2A). In contrast treatment of the LTED-I cells with ICI 182780 alone markedly

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FIG. 1. Removal of insulin reveals the LTED-I cells hypersensitivity to E2. Growth assays were performed as described under “Experimental Procedures.” LTED-I and wt MCF-7 cells were cultured ± insulin (ins) (10 μg/ml) and increasing doses of E2 for 6 days. The data represent the average of triplicate readings. The means ± S.E. are shown. A and B show the effect of insulin on the basal growth rate of the LTED-I and wt MCF-7. C and D demonstrate that removal of insulin reveals the hypersensitivity of LTED-I cells to E2.

**LTED-I Cells Utilize the Classical ERE Pathways in Part for Proliferation and Transcription**—Our previous studies (6) indicated that the LTED-I cells contained an elevated level of ERα, which was phosphorylated on Ser118. We envisaged this could influence the basal ER-ERE-driven transcription sensitizing the cells to the effects of residual E2 in the medium. To test this, LTED-I cells were cultured in the presence of 10−8 M ICI 182780 (demonstrated to inhibit cell growth) and increasing concentrations of E2. Doses in excess of 10−10 M E2 were able to reverse the inhibitory effect of 10−8 M ICI (Fig. 2B). From this we concluded that cell growth was mediated wholly or in part via the ER-ERE pathway.

Inhibited cell growth. These data were consistent with the hypothesis that the LTED-I cells were super-sensitized to residual E2 in the DCC medium. We next considered the possibility that ICI 182780 may indirectly effect growth by inhibiting growth factor-mediated transcription. To test this, LTED-I cells were co-transfected with a luciferase reporter construct regulated by two EREs and test this hypothesis wt and LTED-I cells were co-transfected to inhibit residual E2 in the medium. To test this, LTED-I cells were cultured in the presence of 10−8 M ICI 182780 (demonstrated to inhibit cell growth) and increasing concentrations of E2. Doses in excess of 10−10 M E2 were able to reverse the inhibitory effect of 10−8 M ICI (Fig. 2B). From this we concluded that cell growth was mediated wholly or in part via the ER-ERE pathway.

Long Term Estrogen Deprivation Results in Alterations in the Expression of MAPK Family Members—The above data and our observation that the level of ERα phosphorylated on Ser118 was elevated 3-fold in the LTED-I compared with the wt MCF-7 cells (Fig. 4) suggested that enhanced expression of ER and its activation via specific kinases played a central role in the apparent E2-independent proliferation. Several studies have shown that p42/p44 MAPK may be involved in ligand-independent activation of ERα (9, 18). Similarly elevated levels of activated p42/p44 MAPK have been detected in cells during LTED (5, 27). We therefore examined whole cell extracts isolated from our MCF-7 cells at intervals during E2 deprivation (Fig. 4). As the expression of the phosphorylated proteins Raf, MEK1/2, MAPK, and p90Rsk appeared to fluctuate we assessed combined activation of ER and the resultant ER-driven transcription are important components of the LTED-I cell phenotype.

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LTED-I cells were cultured as described previously in the presence of increasing doses of ICI 182780 alone or in combination with E2. Basal transcription in the wt cells showed a small but statistically insignificant increase in the inhibitor whereas in the LTED-I cells ERα transcription was reduced by ~50%. Addition of high doses of E2 (10^{-9} M) readily overcame the suppressive effect of U0126 suggesting p42/p44 MAPK may be used during ER signaling in the LTED-I cells and that they are sensitized to the restorative effects of E2 consistent with the result found after treatment with ICI 182780.

Elevated p42/p44 MAPK Is Involved in the Mechanism of E2 Hypersensitivity—We postulated that if p42/p44 MAPK were directly or indirectly involved in the development of E2 hypersensitivity in response to LTED, then blocking its activity using the MEK inhibitor U0126 should shift the dose response curve to the right similar to the wt cells. To allow the continued sensitivity of the LTED-I cells to be visible, this experiment was conducted in the absence of insulin. The cells were treated over a 6-day period with increasing doses of E2 plus or minus U0126 (20 μM). The E2 dose curve (Fig. 6) in the presence of U0126 shifted to the right when compared with E2 alone, with the sensitivity altering by one log. This implies that p42/p44 MAPK is involved in sensitizing the LTED-I cells to the effects of E2.

Induction of p42/p44 MAPK Activity by Insulin Does Not Play a Role in the Phosphorylation of ERα Ser^{118}. The question remains as to what mechanism elevates MAPK activity. Studies have shown that in MCF-7 cells, insulin up-regulates the ER content and binding capacity and that this is blocked by specific tyrosine kinase inhibitors (30), and more recent studies have suggested that IGF-1R is elevated during LTED (31). As insulin has been shown to activate a spectrum of downstream signaling pathways such as MAPK, PI3 kinase, and AKT, we considered the possibility that insulin might stimulate p42/p44 MAPK activity in the LTED-I cells, leading to phosphorylation of ERα Ser^{118}. Initially we screened whole cell extracts from the LTED cell pellets for expression of IGF-1R (Fig. 7A). This indicated that there was an ~2-fold increase in IGF-1R protein content during the LTED-H and LTED-I stages compared with the parental control.

To investigate this further we next stimulated the LTED-I and wt cells with insulin over a 30-min time course (Fig. 7B). Insulin increased p42/p44 MAPK activity as expected, within 5 min. However, treatment with insulin had no effect on the phosphorylation status of ERα Ser^{118} in the wt or LTED-I cells.

Overexpression of EGFR or ERBB2 May Provide a Route to Increased p42/p44 MAPK Activity—Recent studies have suggested estrogens mediated by ER can rapidly stimulate p42/p44 MAPK activation (21, 22, 32). We postulated that this may provide a potential mechanism for elevated p42/p44 MAPK in the LTED-I cells, based on their increased sensitivity for E2 and elevated ER content. Treatment of the LTED-I and wt MCF-7 cells with E2 resulted in rapid phosphorylation of Ser^{118} but did not activate p42/p44 MAPK (data not shown). These results were similar to those reported by Joel et al. (33) and Lobenhofer and Marks (34).

Reports have suggested that cross-talk between growth factor and steroid receptors may play an important role in endocrine resistance (3). We postulated that the increase in p42/p44 MAPK activity in the LTED-I cells may be a result of increased EGFR receptor activity. Whole cell extracts from MCF-7 cells harvested at intervals during E2 withdrawal were immunoblotted with antibodies specific for phosphorylated and total EGFR. Levels of phosphorylated receptor during the LTED-Q
phase were ~50% lower compared with those in the wt cells. However, the level of phosphorylated receptor returned to wt levels by week 19 and remained so during the LTED-H and LTED-I phases (Fig. 8A). The level of total EGFR remained largely unchanged compared with the wt control.

We next investigated the level of phosphorylated and total ERBB2 in the LTED whole cell extracts versus the wt MCF-7 (Fig. 8B). During the LTED-Q phase phosphorylated ERBB2 was undetectable compared with the parental control. However, during LTED-H and LTED-I phases the level of total and phosphorylated receptor was markedly elevated compared with the wt MCF-7 cells. Fluorescent in situ hybridization analysis (using the Vysis PathVysion™ HER-2 kit) indicated that ERBB2 was not amplified in the LTED-I cells (data not shown). To investigate this further we used the tyrosine kinase inhibitor ZD1839 (Iressa). This inhibitor has been shown to be selective for EGFR (IC50 2.7 nM) (35). LTED-I and wt MCF-7 cells were treated with ZD1839 for 24 h. The data are reported as the -fold increase in activity compared with the wt MCF-7 cells. Average activity from triplicate wells ± S.E. of the means. Results were confirmed in two independent experiments.

The effect of E2 on ER-mediated transcription did not differ between wt MCF-7 and LTED-I cells. At a dose of 20 nM the basal transcription was reduced by 40% in the LTED-I cells but remained unaffected in the wt MCF-7 cells (Fig. 8D). E2 remained stimulatory in the presence of ZD1839 but was restricted by the inhibitor particularly at 10^-11 M E2. Although increases in growth factor receptor activity have been postulated previously, to our knowledge this is the first report showing elevated levels of phosphorylated ERBB2 associated with LTED phenotype, and it provides a potential mechanism for the elevated p42/44 MAPK.

Increased Activity of p42/p44 MAPK in LTED-I Cells Is Not Responsible for ERα Ser118 Phosphorylation—The above results suggested that the elevated level of p42/p44 MAPK in the LTED-I cells (possibly mediated via ERBB2) might be linked to the phosphorylation of ERα Ser118. To further test the hypothesis that elevated levels of p42/p44 MAPK were responsible for phosphorylation of Ser118 in the LTED-I cells, we compared the effects of various signal transduction pathway inhibitors on Ser118 basal phosphorylation versus E2-induced phosphorylation in the wt MCF-7 and LTED-I cell lines. Fig. 9A shows that U0126, a specific inhibitor of MEK1/2, blocked p42/p44 MAPK activity but had no effect on the basal phosphorylation status of Ser118 in the LTED-I or wt cells. More importantly even though U0126 abolished p42/p44 MAPK activity, it did not affect E2-induced Ser118 phosphorylation in the LTED-I or wt cells. To determine that the effect noted was not a time-related issue and that Ser118 remained phosphorylated for more than 30 min, negating the effect of blocking MAPK, wt and LTED-I cells were treated with U0126 over an 8-h time course (Fig. 9B). No significant reduction in Ser118 phosphorylation was noted in either cell line, whereas p42/p44 MAPK activity was abolished.

When we investigated the effect of ZD1839 on the basal ER-dependent transcription in the LTED-I and wt MCF-7 cells. At a dose of 20 µM the basal transcription was reduced by 40% in the LTED-I cells but remained unaffected in the wt MCF-7 cells (Fig. 8D). E2 remained stimulatory in the presence of ZD1839 but was restricted by the inhibitor particularly at 10^-11 M E2. Although increases in growth factor receptor activity have been postulated previously, to our knowledge this is the first report showing elevated levels of phosphorylated ERBB2 associated with LTED phenotype, and it provides a potential mechanism for the elevated p42/p44 MAPK.
It has been suggested that the protein kinase C and PI3 kinase pathways are capable of activating p42/p44 MAPK in a MEK1/2-independent manner (36), and PI3 kinase is involved in phosphorylation of ERα/H9251 (17, 37). We therefore postulated that an alternative signaling pathway was responsible for the phosphorylation associated with Ser118. To test this hypothesis we treated the LTED-I and wt MCF-7 cells with the PI3 kinase inhibitor LY294002 (Fig. 9C). The inhibitor had no effect on basal or E2-induced Ser118 phosphorylation. From this we concluded that neither MAPK nor PI3 kinase were responsible for phosphorylation of Ser118 in this setting, confirming our view that the LTED-I cells were not ligand-independent but supersensitized to residual E2 in the DCC medium. These data also suggested that alternative kinases were responsible for the phosphorylation of Ser118.

PI3 Kinase Is Associated with ERα-directed Transcription—Our data suggested it was unlikely p42/p44 MAPK activation alone was responsible for the LTED-I phenotype. Recent data indicate that AKT is capable of phosphorylating ERα Ser167 and activating transcription (17). We wished to investigate whether signaling pathways other than Ser118 and p42/p44 MAPK were up-regulated in the LTED-I cells or whether there might be a dynamic interplay between phosphorylation events on AF-1 that could account for the LTED-I phenotype. First we assessed the whole cell extracts from time points during E2 withdrawal for expression of total and phosphorylated AKT (Fig. 10A). The overall median level of protein was unchanged when compared with the wt MCF-7 (FBS) control.

To test this hypothesis further wt and LTED-I cells were co-transfected with the ERE-driven luciferase and β-galactosidase reporter constructs as described previously. The cells were treated subsequently with the PI3 kinase inhibitor LY294002 plus or minus increasing concentrations of E2. Basal transcription in the wt cells was marginally increased by the
treated with DCC-FBS medium

transactivation was reduced by

Cells were seeded at 10^4 per well in the absence of insulin as described under “Experimental Procedures.” After 48 h cells were treated with DCC-FBS medium ± U0126 (20 μM) and increasing doses of E2. The results shown are the average of triplicate readings. Results were confirmed in two independent experiments.

Levels of IGF-1R are elevated during LTED.

Our data with U0126 suggested that Ser^{118} was not phosphorylated by p44/p42 MAPK, and indeed further studies with a number of signal transduction inhibitors suggested Ser^{118} is the target for multiple kinases, in confirmation of previous findings (33). This provided further supporting evidence that LTED-I cells are ligand-dependent rather than ligand-independent as phosphorylation of Ser^{118} in response to E2 is MAPK-independent (33). It was notable, however, that p42/p44 MAPK was involved in sensitizing the LTED-I cells to the effects of E2. This was demonstrated in the shift in dose response to E2 when p42/p44 MAPK was blocked and similarly the decrease in proliferation in response to U0126 compared with the wt cells. These data are consistent with the theory of direct interaction between ER and growth factor signaling but also with the possibility that the pathways may operate independently with
both MAPK and E2 providing mitogenic signals. However, our data showing inhibition with ICI 182780 implies that interac-
tive signaling plays a significant role.

It is clear that blocking p42/p44 MAPK may also affect other factors. For instance recent evidence suggests that the tran-
scriptional activity of AIB1, a ligand-dependent ER coactivator, is enhanced by p42/p44 MAPK phosphorylation leading to the recruitment of CBP and an associated increase in acetyl trans-
ferase activity (43). It is envisaged that the ability of growth factors to augment estrogen action may be mediated in part through p42/p44 MAPK activation of AIB1. This may also explain the selection of AIB1 amplification during progression in ER-positive breast cancers (44–46). Such a model is consist-
ent with our findings of elevated ERBB2 and MAPK activity.

Further evidence for multiple signaling pathways having a role in the LTED-I phenotype and for the theory that p42/p44 MAPK amplifies the sensitivity of the cells to E2 was provided by transcription assays. For instance LY294002 reduced basal transcription in the LTED-I cells by 70% while having a slight stimulatory effect on the wt cells (possibly because of compen-
satory alternative pathways). Furthermore a combination of U0126 and LY294002 reduced the basal transcription in the wt

Fig. 8. ERBB2 but not EGFR is elevated during LTED. A, the level of phosphorylated and total EGFR in the LTED cells. Whole cell extracts (100 μg) from representative weeks post-E2 with-
drawal were separated through 7% SDS-
PAGE gels and immunoprobed with anti-
bodies specific for phosphorylated and total EGFR. B, the level of phosphory-
lated and total ERBB2 in the LTED cells. Whole cell extracts were immuno-
probed with antibodies specific for phosphory-
lated and total ERBB2 C, the effect of ZD1839 on LTED-I, wt MCF7, and SKBR3 cell growth. Wt MCF-7, LTED-I, and SKBR3 cells were seeded at a density of 5 × 10^5 cells per well and 48 h later treated with increasing doses of ZD1839, a specific inhibitor of EGFR. Wt cells were cultured in the presence of 10^-8 M E2 whereas LTED-I cells were grown in DCC-FBS medium. SKBR3 cells were cult-
ured in RPMI 1640 containing phenol red and 10% FBS. The data represent triplicate readings, and the bars show mean ± S.E. Results were confirmed in two independent experiments. D, the effect of EGFR inhibitor ZD1839 on basal and E2-mediated ERα transactivation. Wt and LTED cells were transiently co-
transfected with ERE/IItkLuc and pCH110 in serum-free medium followed by a 24-h incubation in DCC medium con-
taining increasing doses of E2 alone or in the presence of ZD1839. Normalized lucif-
erase activity from triplicate wells was expressed relative to the vehicle-treated control (cont). Bars represent ± S.E. of the mean. Results were confirmed in two independent experiments.
and LTED-I cells by 50 and 70%, respectively. It is notable that even high doses of E2 were unable to remove the block on transcription in the wt cells but partially rescued ERα/H9251-driven transcription in the LTED-I cells. These observations and the complex pattern of changes in protein expression and phosphorylation seen during the development of the LTED phenotype suggests that a network of kinases and molecular switches operate at different stages during E2 withdrawal. It has been suggested that a complex of p42/p44 MAPK and p90RSK interact with the ER and that these kinases coordinately phosphorylate Ser118 and Ser167 (14). The presence or absence of co-ordinate phosphorylation of these residues may provide the ER with a mechanism of distinguishing signals from various second messenger pathways. To add a
further layer of complexity recent findings have shown that AKT phosphorylates Ser\textsuperscript{118} (17) and that regulation of ER\textalpha activity by AKT may be indirectly controlled by p42/p44 MAPK via its interaction with p90\textsuperscript{RSK} (47, 48). It has also been reported that p90\textsuperscript{RSK}, when phosphorylated by p42/p44 MAPK in response to growth factors, is transported into the nucleus and forms a stable complex with coactivator CBP, which then regulates transcription (49). Evidence also suggests CBP complexes with ER and that ectopic expression of CBP enhances ER\textalpha-driven transcriptional activity (50). These findings may account for the elevated basal transcription in the LTED-I cell line as these cells have elevated levels of ER, p42/p44 MAPK, and p90\textsuperscript{RSK}.

In summary we have demonstrated that the LTED-I cells are super-sensitive to the effects of E\textsubscript{2}, requiring ER for both proliferation and ER-directed transcription. This finding suggests that IC\textsubscript{182780} might be more effective in the treatment of breast cancers that acquire resistance to estrogen deprivation rather than in de novo hormone responsive disease. We have shown elevated levels of members of the MAPK kinase signaling pathway together with elevated levels of phosphorylated ER\textalpha. However, although our data show that the MAPK and PI3 kinase pathways play an integral role in the development of the LTED-I phenotype and sensitization of these cells to residual E\textsubscript{2}, neither appear responsible for the phosphorylation ER\textalpha Ser\textsuperscript{118}. We postulate an adaptive pathway similar to that shown in Fig. 11 in which the development of the LTED-I phenotype results from elevated levels of ER coupled with enhanced activation of the ER as a result of increased ER\textalpha expression and p42/p44 MAPK activity. p42/p44 MAPK may be involved in ER activation (on sites other than Ser\textsuperscript{118}) and regulation of down-stream partners such as p90\textsuperscript{RSK}, c-Myc, and AIB1 leading to increased coactivator activity and consequently providing a hypersensitive receptor to residual E\textsubscript{2}. In conclusion these data confirm the presence of cross-talk between the ER and growth factor signaling pathways during LTED and support the development of treatment strategies based on signal transduction inhibitors, which may be used to extend the duration of sensitivity to E\textsubscript{2} deprivation or to reverse resistance at its time of emergence.

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Signaling Pathways Operating during LTED

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