# Estrogen-Induced Activation of Erk-1 and Erk-2 Requires the G Protein-Coupled Receptor Homolog, GPR30, and Occurs via *Trans*-Activation of the Epidermal Growth Factor Receptor through Release of HB-EGF

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**Estrogen rapidly activates the mitogen-activated protein kinases, Erk-1 and Erk-2, via an as yet unknown mechanism. Here, evidence is provided that estrogen-induced Erk-1/-2 activation occurs independently of known estrogen receptors, but requires the expression of the G protein-coupled receptor homolog, GPR30. We show that 17$\beta$-estradiol activates Erk-1/-2 not only in MCF-7 cells, which express both estrogen receptor $\alpha$ (ER$\alpha$) and ER$\beta$, but also in SKBR3 breast cancer cells, which fail to express either receptor. Immunoblot analysis using GPR30 peptide antibodies showed that this estrogen response was associated with the presence of GPR30 protein in these cells. MDA-MB-231 breast cancer cells (ER$\alpha^-$, ER$\beta^+$) are GPR30 deficient and insensitive to Erk-1/-2 activation by 17$\beta$-estradiol. Transfection of MDA-MB-231 cells with a GPR30 complementary DNA resulted in overexpression of GPR30 protein and conversion to an estrogen-responsive phenotype. In addition, GPR30-dependent Erk-1/-2 activation was triggered by ER antagonists, including ICI 182,780, yet not by 17$\alpha$-estradiol or progesterone. Consistent with acting through a G protein-coupled receptor, estradiol signaling to Erk-1/-2 occurred via a G$\beta\gamma$-dependent, pertussis toxin-sensitive pathway that required Src-related tyrosine kinase activity and tyrosine phosphorylation of tyrosine 317 of the Shc adapter protein. Reinforcing this idea, estradiol signaling to Erk-1/-2 was dependent upon *trans*-activation of the epidermal growth factor (EGF) receptor via release of heparan-bound EGF (HB-EGF). Estradiol signaling to Erk-1/-2 could be blocked by: 1) inhibiting EGF-receptor tyrosine kinase activity, 2) neutralizing HB-EGF with antibodies, or 3) down-modulating HB-EGF from the cell surface with the diphtheria toxin mutant, CRM-197. Our data imply that ER-negative breast tumors that continue to express GPR30 may use estrogen to drive growth factor-dependent cellular responses. (Molecular Endocrinology 14: 1649–1660, 2000)**

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## INTRODUCTION

Estrogen exerts multiple biological effects on a diverse array of target tissues. Its actions are required for the development and maintenance of reproductive tissues, and in some instances are essential for the growth and survival of tumors that arise from these tissues. In addition to its impact on the reproductive system, estrogen regulates bone structure (1), cardiovascular function (2), and the central nervous system (3). At present, it is unclear whether these diverse estrogen-mediated biological effects are entirely manifested via the known estrogen receptors, ER$\alpha$ and ER$\beta$. These ERs belong to the steroid hormone receptor superfamily (reviewed in Ref. 4) and function as

ligand-activated transcription factors. Upon interaction with estrogen, they undergo conformational changes that result in their ability to bind DNA and promote gene transcription. In this sense, estrogen appears to bypass second messenger signaling and directly promote the transcription of genes required for estrogen-dependent proliferation. However, in addition to its ability to promote ER-dependent gene transcription, estrogen rapidly triggers a variety of second messenger signaling events, including mobilization of intracellular calcium (5), production of cAMP (6), generation of inositol phosphate (7), and activation of the mitogen-activated protein (MAP) kinases, Erk-1 and Erk-2 (8–10). Although the mechanism by which these rapid signaling events occur is unknown, due to the rapidity (within 5 min) by which they are activated it is presumed that they are initiated at the plasma membrane and do not involve ER-mediated gene transcription.

Several studies have suggested that ER$\alpha$ and ER$\beta$ facilitate this rapid estrogen-stimulated signaling and activation of Erk-1/-2 (5, 11, 12). However, ER$\alpha$ and ER$\beta$ proteins lack known functional motifs that would allow for nongenomic mechanisms of estrogen action (13). Further questions regarding the roles of ER$\alpha$ and ER$\beta$ in rapid estrogen signaling are suggested by the effect of pure ER antagonists, such as ICI 182,780 and ICI 164,384, on these second messenger signaling pathways. It has been reported that ICI 182,780 prevents estrogen-induced activation of Erk-1 and Erk-2 (5, 8). In contrast, it has also been noted that this antiestrogen activates MAP kinases (MAPKs) and releases intracellular calcium stores (5). Similarly, ICI 164,384 has been shown to potentiate activation of adenylyl cyclase (6). These observations parallel other studies that have shown that several steroid hormones and their antihormones may act through membrane receptors to facilitate rapid nongenomic signaling (14–17). Because rapid activation of diverse second messenger signaling pathways by a single ligand is often mediated by G protein-coupled receptors (GPCRs), many have speculated that rapid steroid hormone signaling events may use GPCR signaling mechanisms. This idea is consistent with data that have implicated G proteins in second messenger signaling by androgens (18) or progesterone (19).

Recently, Weigel and colleagues isolated a complementary DNA (cDNA) encoding an orphan member of the G protein-coupled receptor superfamily, termed GPR30, whose expression is elevated in some ER-positive *vs.* ER-negative human breast tumors and cell lines (20). Here, we test the hypothesis that GPR30 may promote rapid estrogen-induced activation of Erk-1 and Erk-2. We provide several lines of evidence that, independent of ER$\alpha$ or ER$\beta$, estrogen activates the MAPK pathway via rapid, GPR30-dependent activation of an HB-EGF autocrine loop.

## RESULTS

### Estrogen-Induced Erk-1/-2 Activation Is ER Independent and Requires Expression of the GPCR Homolog, GPR30

Estrogen promotes rapid activation of the MAPKs, Erk-1 and -2 (5, 8–10). At present, it is unclear whether ER$\alpha$ or ER$\beta$ is required for this mechanism of estrogen action. To determine whether estrogen-induced Erk activation is associated with expression of ER$\alpha$ or ER$\beta$, human breast cancer cell lines exhibiting different ER expression profiles were tested for their ability to activate Erk-1/-2 after exposure to estrogen. Detergent lysates were prepared from quiescent cells that were either untreated or exposed to estrogens or EGF for various lengths of time. Erk activity and expression in these cellular lysates were measured by immunoblotting using phosphorylation state-dependent and -independent antibodies. In agreement with observations by others (5, 8), 1 nM 17$\beta$-estradiol induced a rapid, 5- to 10-fold increase in the phosphorylation state of Erk-1 and Erk-2 in MCF-7 cells, which express both ER$\alpha$ and ER$\beta$ protein (21) (Fig. 1). Surprisingly, however, 17$\beta$-estradiol induced a similar rapid increase in Erk-1/-2 in SKBR3 cells (Fig. 1) that express neither ER$\alpha$ nor ER$\beta$ messenger RNA (22). Erk-1 and -2 activation in these cell types could also be achieved using 1 $\mu$M of the pure anti-estrogen ICI 182,780, a concentration that blunts both ER$\alpha$ and ER$\beta$ (23). Although, in general, the activation kinetics for Erk-1/-2 phosphorylation by estradiol were similar in each of these cell lines, minor differences in the onset of Erk phosphorylation were observed. These differences appeared to be associated with the level of baseline phosphorylated Erk-1/-2 expressed before estrogen stimulation. In contrast, 17$\beta$-estradiol and ICI 182,780 each failed to activate Erk-1/-2 in MDA-MB-231 cells, which express only ER$\beta$ (21). Yet, suggesting no global defect in signaling to MAPKs in these cells, EGF strongly activated Erk-1/-2 (Fig. 1). Collectively, these results support the hypothesis that estrogen-induced Erk-1/-2 activation occurs via a non-ER-dependent mechanism.

Because a diverse array of extracellular ligands (reviewed in Refs. 24 and 25) signal through GPCRs to activate the MAPKs, Erk-1 and Erk-2, we questioned whether activation of these MAPKs by estrogen may also occur through a GPCR mechanism. Although there are many orphan receptors that could be considered candidates for promoting estrogen-induced activation of Erk-1/-2, we speculated that GPR30, an orphan GPCR whose expression is elevated in MCF-7 cells relative to MDA-MB-231 cells (20), may promote estrogen-induced MAPK activation. Therefore, to test this possibility we asked whether MDA-MB-231 cells, when forced to overexpress GPR30 protein, would acquire the capacity to activate Erk-1/-2 in response to estrogen stimulation.

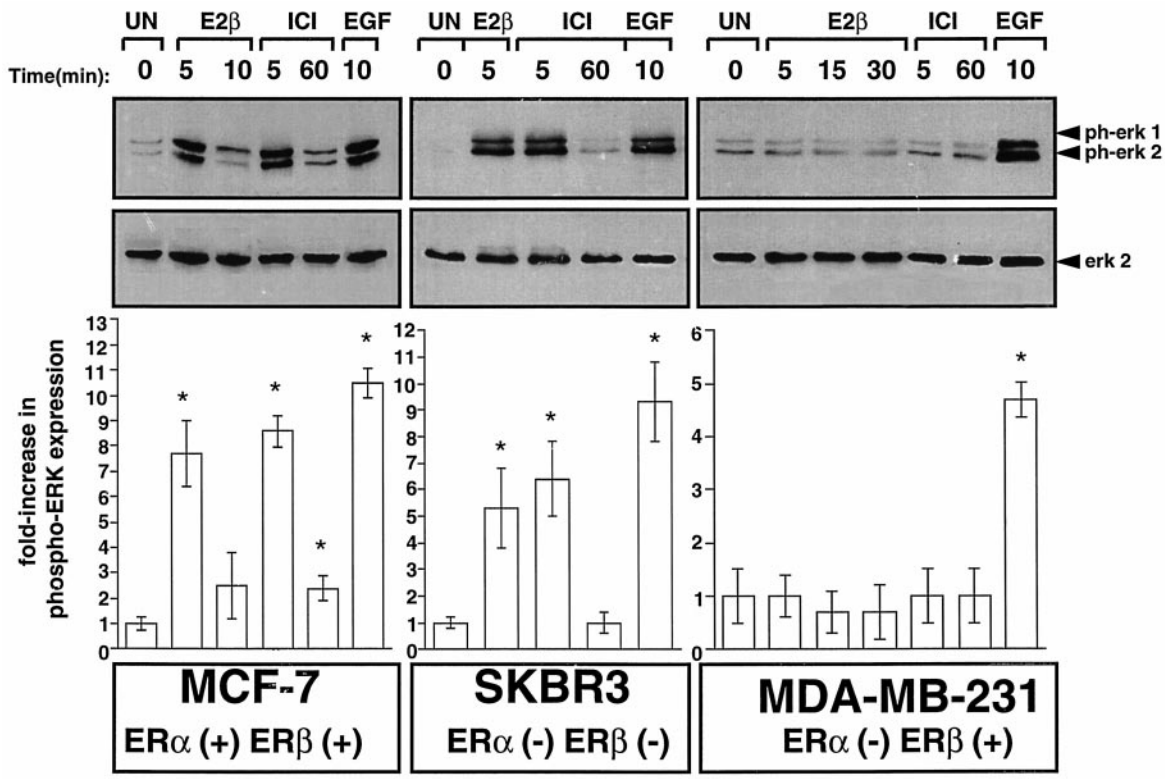


**Fig. 1.** Activation of Erk-1/-2 by Estrogens or Antiestrogens Does Not Correlate with ER Expression

Human MCF-7, SKBR3, or MDA-MB-231 breast carcinoma cells, untreated or exposed to 1 nM 17$\beta$-estradiol, 1 $\mu$M ICI 182,780, or 1 ng/ml EGF for the lengths of time indicated (minutes), were lysed in ice-cold RIPA detergent. Cellular proteins (50 $\mu$g) were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies specific for phosphorylated Erk-1 and -2. The nitrocellulose membrane was then stripped and reprobed with antibodies that recognize total (phosphorylation state-independent) Erk-2 protein. The relative positions of phosphorylated Erk-1 and -2 and total Erk-2 proteins are designated at the *right*. The ER expression profile of each cell line is indicated. The data shown *above* are representative of at least three independent experiments. *Below*, Band intensities from these individual experiments were quantified using NIH Image software. Results were normalized to total Erk-2 expression in each sample and are plotted with SEM. *, Erk-1/-2 activation significantly ($P < 0.05$, by Student's *t* test) greater than that in unstimulated cells.

To facilitate the study of GPR30 expression, antibodies were raised in rabbits to a C-terminal peptide derived from the deduced amino acid sequence of GPR30. These peptide antibodies identified a single 38-kDa band that was abundant in MCF-7 and SKBR3 cells and in MDA-MB-231 cells that had been transfected with a GPR30 expression vector, but was barely detected in vector-transfected MDA-MB-231 cells (Fig. 2A). The apparent molecular mass of the 38-kDa band closely approximates the predicted molecular mass (39,815 Da) of the mature 351-amino acid GPR30 polypeptide. Although ER$\alpha$ was readily detectable in MCF-7 cells, it was not detected in MDA-MB-231 or SKBR3 cells and was not reacquired in MDA-MB-231 cells upon transfection with GPR30 cDNA (Fig. 2A). A small amount of ER$\beta$ protein was detected in lysates from MCF-7 and MDA-MB-231 cells; however, no detectable ER$\beta$ protein was present in lysates from SKBR3 cells, consistent with a recent report (22).

GPR30-transfected MDA-MB-231 cells supported rapid activation of Erk-1/-2 in response to either 17$\beta$-estradiol or ICI 182,780 (Fig. 2B). These hormones did not promote Erk-1/-2 activation in MDA-MB-231 cells transfected with the empty vector (data not shown). Activation of Erk-1/-2 in MDA-MB-231 (GPR30) cells was also induced by 1 $\mu$M tamoxifen (partial agonist), but not by the inactive estrogen isomer, 17$\alpha$-estradiol, or other sex steroid hormones, such as progesterone (Fig. 2C). No differences were observed in total Erk-2 protein expression under any of these conditions (Fig. 2, B and C). Thus, these data suggest that when forced to express levels of GPR30 protein comparable to those expressed in MCF-7 or SKBR3 cells, MDA-MB-231 cells become estrogen responsive with respect to their ability to activate Erk-1/-2.

### Estrogen-Mediated GPR30-Dependent Erk-1/-2 Activation Occurs via a G$\beta\gamma$, Pertussis Toxin-Sensitive Pathway That Requires Src-Related Tyrosine Kinase Activity and Shc

Activation of Erk-1/-2 by GPCRs is mediated through the action of receptor-associated heterotrimeric G proteins (reviewed in Ref. 24). After ligand-receptor

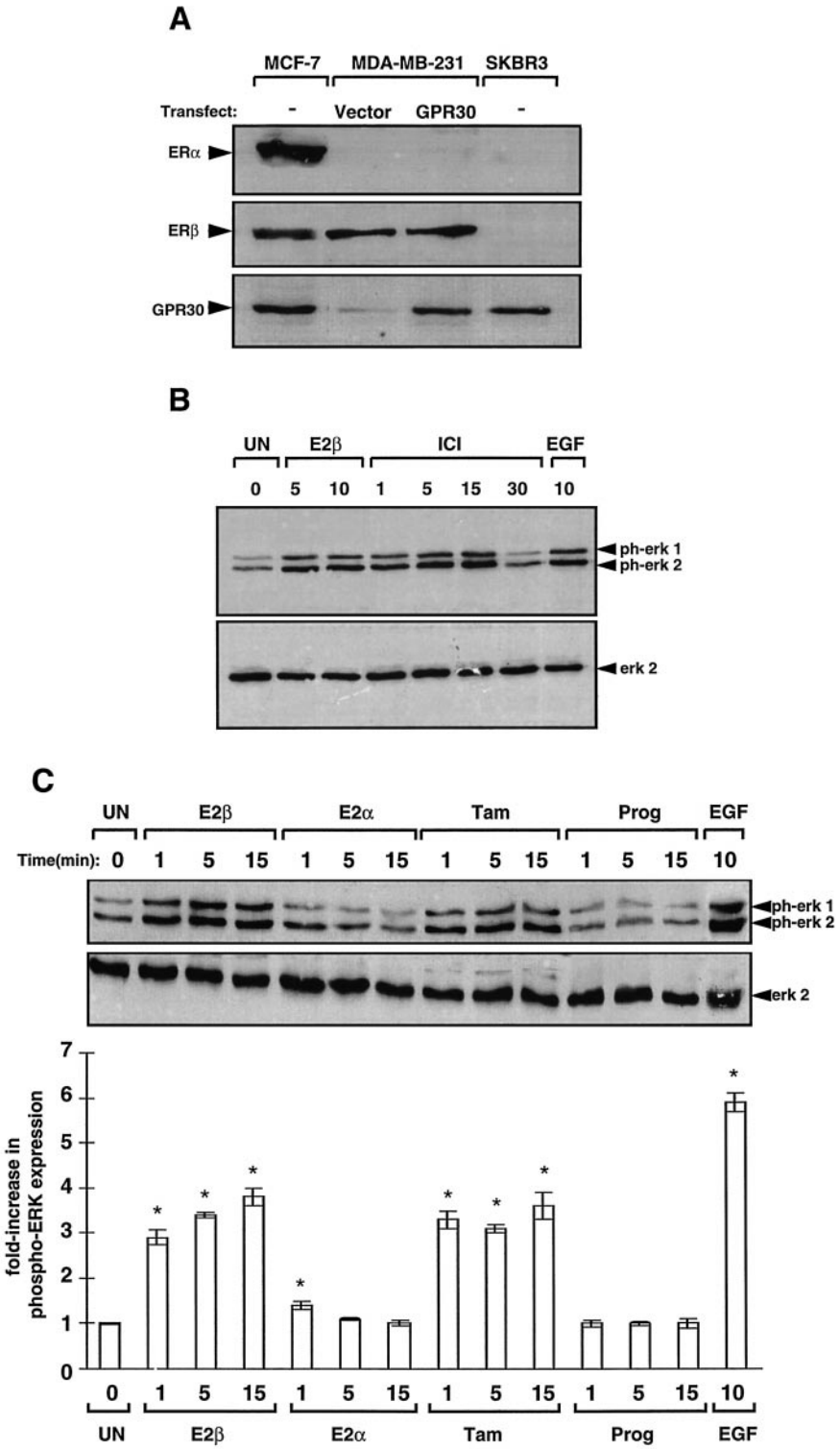


**Fig. 2.** Estrogen-Induced Activation of Erk-1/2 Requires Cellular Expression of the G Protein Receptor Homolog, GPR30

A, Expression of ERα, ERβ, and GPR30 in MCF-7, SKBR3, or MDA-MB-231 cells stably expressing GPR30 or transfected with control vector was assessed by immunoblotting with antibodies specific for ERα, ERβ, or GPR30. B and C, Using phosphorylation state-dependent or -independent antibodies, phospho-Erk or Erk expression was determined from whole cell lysates of MDA-MB-231 (GPR30) cells that were untreated, stimulated with EGF (1 ng/ml; 10 min), or exposed to 17β-estradiol (1 nM) or ICI 182,780 (1 μM), or to 17α-estradiol (1 nM), 4-hydroxytamoxifen (1 μM), or progesterone (1 nM) for the indicated times (minutes). The positions of phosphorylated Erk-1/-2 and total Erk- 2 proteins are indicated at the *right*. The data shown are representative of at least three independent experiments. As in Fig. 1, band intensities from independent experiments have been quantified by densitometry and are plotted with the SEMs. *, Erk-1/-2 activation significantly ($P < 0.05$, by Student's *t* test) greater than unstimulated cells.

interaction, the G$\alpha$-subunit protein dissociates from the heterotrimeric G$\alpha\beta\gamma$ complex. Both free G$\alpha$ and the remaining G$\beta\gamma$ complex have been shown to participate in signaling pathways that may promote Erk-1/-2 activation (reviewed in Ref. 24). These signaling mechanisms are commonly discriminated based on their sensitivity to pertussis toxin, tyrosine kinase inhibitors, and dominant negative effector proteins. Therefore, to further test the role of GPR30 in promoting estrogen-induced Erk-1/-2 activation, we examined whether estrogen-induced Erk-1/-2 activation was sensitive to inhibitors known to disrupt G protein-mediated signaling.

Erk activity and expression were assessed in GPR30-transfected MDA-MB-231 cells that were untreated or pretreated with either pertussis toxin or the Src family tyrosine kinase inhibitor, PP2 (26), before stimulation with either 17$\beta$-estradiol or EGF. As shown in Fig. 3A, pertussis toxin completely abrogated the ability of estradiol to activate Erk-1/-2, yet had no impact on EGF-mediated Erk activation. Likewise, pertussis toxin inhibited estradiol-induced Erk activation in MCF-7 cells (data not shown). Similarly, PP2 completely blocked estradiol-induced Erk activation, indicating a requirement for a Src-related tyrosine kinase (Fig. 3A). In contrast, PP2 had no discernible effect on EGF-stimulated Erk phosphorylation, consistent with a recent report that Src does not lie on the pathway from the EGF receptor to MAPKs (27). Because MAPK activation via pertussis toxin-sensitive, Src-dependent, G protein signaling commonly occurs via a G$\beta\gamma$-subunit protein-mediated pathway that uses the Shc adaptor protein (28–30), we next tested whether estrogen-induced Erk activation could be inhibited by either a G$\beta\gamma$-sequestrant peptide (31) or a dominant negative Shc protein. To accomplish this aim, MDA-MB-231 (GPR30) cells were transfected with a minigene encoding the carboxyl-terminus of the $\beta$-adrenergic receptor kinase ($\beta$ark), dominant negative Shc (shcY317F) or control vector, pcDNA3.1Zeo. Phospho-Erk and total Erk-2 protein expression were assessed in MDA-MB-231 (GPR30/$\beta$ark), MDA-MB-231 (GPR30/dnshc), or MDA-MB-231 (GPR30/Zeo) transfectants that had been stimulated with estradiol or EGF. As observed in Fig. 3B, cells expressing $\beta$ark or dominant negative Shc failed to phosphorylate Erk-1/-2 in response to estradiol stimulation, but remained fully competent to activate Erk in response to EGF stimulation. Zeo-transfected MDA-MB-231 (GPR30) cells maintained their estrogen responsiveness to Erk activation.

Agonist stimulation of GPCRs results in rapid tyrosine phosphorylation of Shc and EGF-related receptors and the formation of Shc-EGF-related receptor complexes (32). To determine whether 17$\beta$-estradiol might similarly promote GPR30-dependent tyrosine phosphorylation of Shc and the formation of Shc-EGF receptor complexes, lysates were prepared from vector-, GPR30-, or GPR30/$\beta$ark-transfected MDA-MB-231 cells that had been either untreated or treated with 17$\beta$-estradiol or EGF, immunoprecipitated with pan-Shc antibodies, and then assayed for phosphotyrosyl proteins (Fig. 3C). Estradiol stimulated tyrosine phosphorylation of p66, p52, and p46 Shc isoforms as well as a Shc-associated 170-kDa protein present in GPR30 expressors, but not in control-transfected cells or in GPR30 cells coexpressing $\beta$ark. This same pattern of Shc-associated tyrosine-phosphorylated proteins was observed in each of these cell types when stimulated by EGF. The 170-kDa tyrosine-phosphorylated protein was identified as the EGF receptor by reblotting with EGF receptor (EGFR)-specific antibody. Reprobing this membrane with a pan-Shc antibody confirmed that there was little difference in total Shc protein recovery in these Shc immunoprecipitates. Taken together, these data suggest that GPR30 signaling occurs through a pertussis toxin-sensitive, G$\beta\gamma$-signaling mechanism that requires Src family tyrosine kinase activity and tyrosine phosphorylation of Shc on tyrosine residue 317.

### GPR30-Mediated Erk-1/-2 Activation Requires EGF Receptor Tyrosine Kinase Activity and Occurs through the Release of Cell Surface-Associated HB-EGF

To further explore the mechanism by which GPR30 promotes EGFR tyrosine phosphorylation, we examined the effect of specific tyrosine kinase inhibitors on estrogen-induced activation of Erk-1/-2 and the EGFR. MDA-MB-231 (GPR30) cells were treated with the EGF receptor kinase inhibitor, tyrphostin AG-1478; the Her-2/Neu kinase inhibitor, tyrphostin AG-879; or the Src family tyrosine kinase inhibitor, PP2, before stimulation with 17$\beta$-estradiol, the pure antiestrogen ICI 182,780, or EGF. Immunoblot analysis showed (Fig. 4A) that tyrphostin AG-1478 blocked EGF-induced as well as 17$\beta$-estradiol-induced EGFR tyrosine phosphorylation, and activation of Erk-1/-2. AG-1478 also similarly inhibited ICI 182,780-induced activation of Erk-1/-2 (Fig. 4A). In contrast, tyrphostin AG-879 did not influence either Erk-1/-2 activation or EGFR tyrosine phosphorylation by estrogen, antiestrogen, or EGF (Fig. 4A). The Src family kinase inhibitor, PP2, completely inhibited 17$\beta$-estradiol-induced EGFR tyrosine phosphorylation (Fig. 4A) and Erk-1/-2 activation (Figs. 3B and 4A). As observed previously, PP2 pretreatment did not effect EGF-induced Erk-1/-2 activation. However, PP2 did increase the mobility of the EGFR (Fig. 4A), probably due to less extensive EGFR tyrosine phosphorylation on residues 845 and 1101 (33). ICI 182,780-induced tyrosine phosphorylation of the EGF receptor does not occur in vector-transfected MDA-MB-231 cells that lack GPR30 (Fig. 4B), but this antiestrogen does promote EGFR tyrosine phosphorylation in SKBR3 cells that express elevated levels of GPR30 protein (Fig. 4B). 17$\beta$-Estradiol did not stimulate EGFR tyrosine phosphorylation in MDA-MB-231 cells (data not shown), but acted similarly to ICI 182,780 in SKBR3 cells promoting

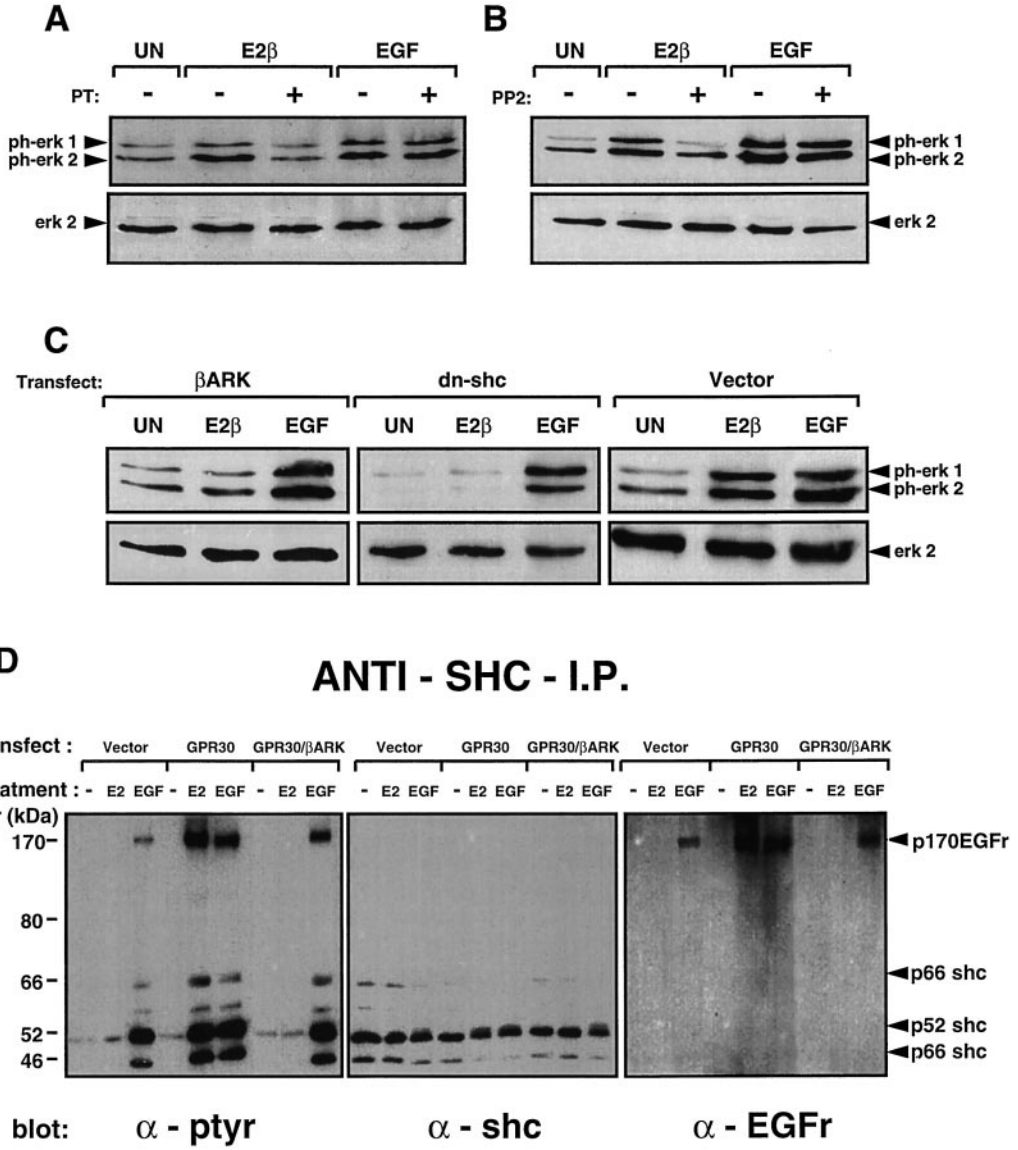


**Fig. 3.** Estrogen-Mediated, GPR30-Dependent Erk-1/-2 Activation Occurs via a G$\beta\gamma$-Subunit Protein-Src-Shc Pathway and Results in Shc-EGFR Association

A and B, MDA-MB-231 (GPR30) cells untreated or pretreated with pertussis toxin (PT; 100 ng/ml; 16 h) or the Src family tyrosine kinase inhibitor, PP2 (50 $\mu$M; 15 min), were stimulated with 17$\beta$-estradiol (1 nM; 5 min) or EGF (1 ng/ml; 15 min). Subsequently, phospho-Erk and Erk expression was determined by immunoblotting with phosphorylation state-dependent or -independent Erk-1/2 antibodies. *C*, MDA-MB-231 (GPR30) cells transfected with G$\beta\gamma$ sequestrant peptide, $\beta$ark; dominant negative Shc, GSTShc317Y/F; or control vector, pcDNA3.1Zeo were assessed for their ability to phosphorylate Erk-1 or -2 after stimulation with 17$\beta$-estradiol or EGF as described in A. D, p66Shc, p52Shc, and p46Shc proteins were immunoprecipitated using pan-Shc specific antibodies from 1 mg total cellular protein extracted in modified RIPA buffer from MDA-MB-231, MDA-MB-231 (GPR30), or MDA-MB-231 (GPR30$\beta$ark) cells, untreated or stimulated for 5 min with 17$\beta$-estradiol (1 nM) or EGF (10 ng/ml). Tyrosine-phosphorylated proteins associated with the Shc immunoprecipitates were detected by immunoblotting with the phosphotyrosine-specific monoclonal antibody, 4G10. Recovery of Shc protein in the immunoprecipitates was assessed by stripping the membrane and reprobing with antibodies to Shc. The 170-kDa tyrosine-phosphorylated protein was identified as the EGFR by reprobing the same membrane with ErbB1-specific antibodies. The positions of p66Shc, p52Shc, p46Shc, and p170EGFR are indicated at the *right*. Mol wt standards are indicated at the *left*.

EGFR activation (Fig. 4B). Considered together, these data imply that EGFR tyrosine kinase activity is required for GPR30-dependent, estrogen-induced Erk-1/-2 activation.

Recent evidence from Ullrich and colleagues suggests that GPCRs mediate EGFR *trans*-activation and downstream signaling through the release of surface-associated heparan-binding EGF (HB-EGF)

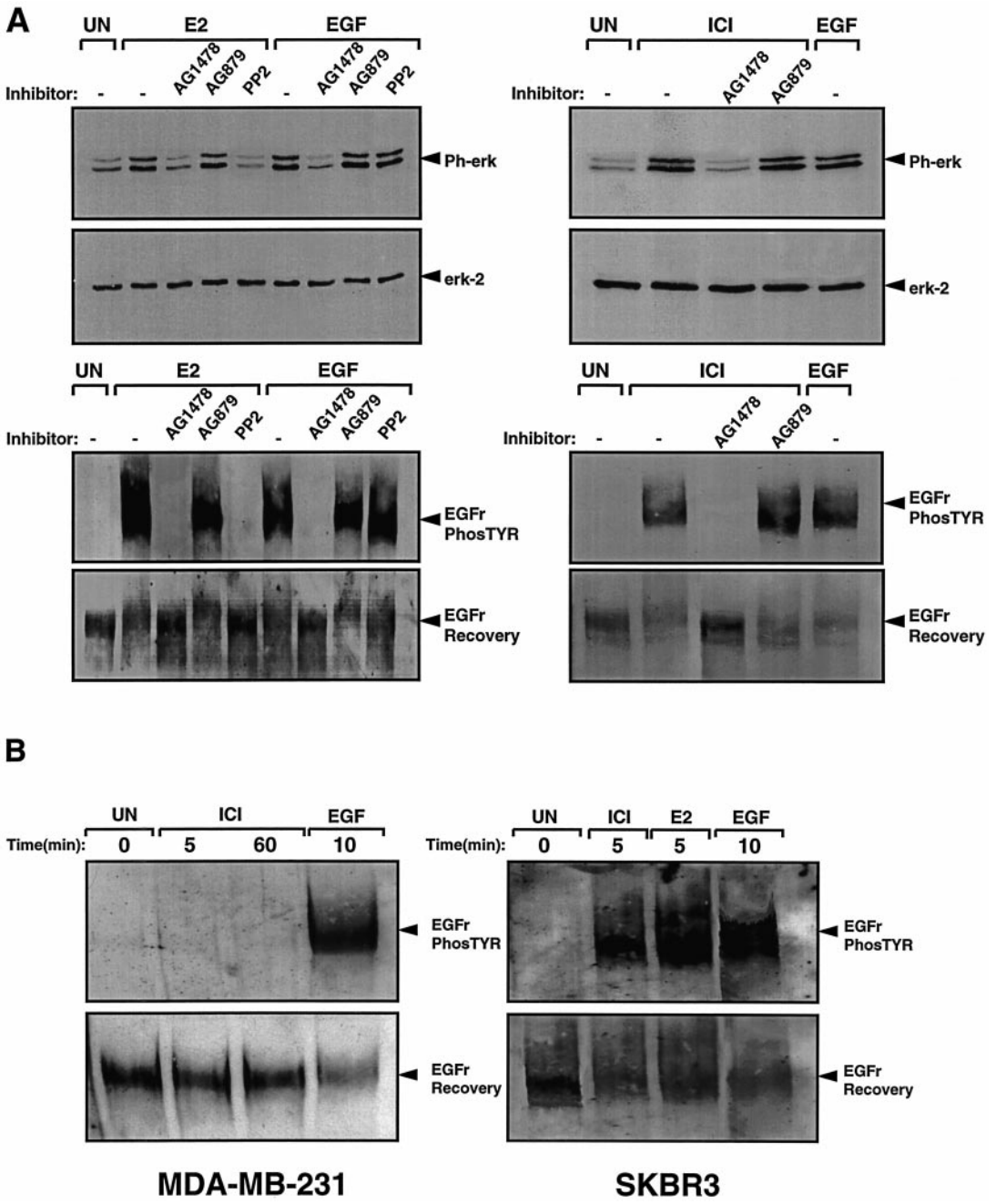


**Fig. 4.** EGF Receptor Kinase Activity Is Required for GPR30-Mediated Erk-1/-2 Activation

A, MDA-MB-231 (GPR30) cells were treated in the absence or presence (15 min) of tyrphostins AG-1478 or AG-879 (50 $\mu$M) or the Src kinase inhibitor, PP2 (50 $\mu$M), before stimulation for 5 min with 17$\beta$-estradiol (1 nM), ICI 182,780 (1 $\mu$M), or EGF (10 ng/ml). Phospho-Erk and Erk-2 expressions in these samples were determined as previously described. After immunoprecipitation with the ErbB1-specific antibody, Ab-1, tyrosine-phosphorylated EGFR was detected by immunoblotting with the phosphotyrosine-specific monoclonal antibody, PY20. EGFR recovery was assessed by stripping these membranes and reprobing with ErbB1-specific antibodies. B, MDA-MB-231 or SKBR3 cells were treated with 17$\beta$-estradiol (1 nM), ICI 182,780 (1 $\mu$M), or EGF (10 ng/ml) for the indicated times (minutes) and lysed in ice-cold RIPA buffer. EGFR tyrosine phosphorylation and recovery were measured as described in A.

precursor protein (34). Therefore, to determine whether estrogen-induced activation of the EGFR and Erk may occur through a similar mechanism, we measured EGFR tyrosine phosphorylation and Erk-1/-2 phosphorylation in MDA-MB-231 (GPR30) cells that had been treated with HB-EGF-neutralizing antibodies or control rabbit antibodies before stimulation with ICI 182,780. Pretreatment with anti-HB-

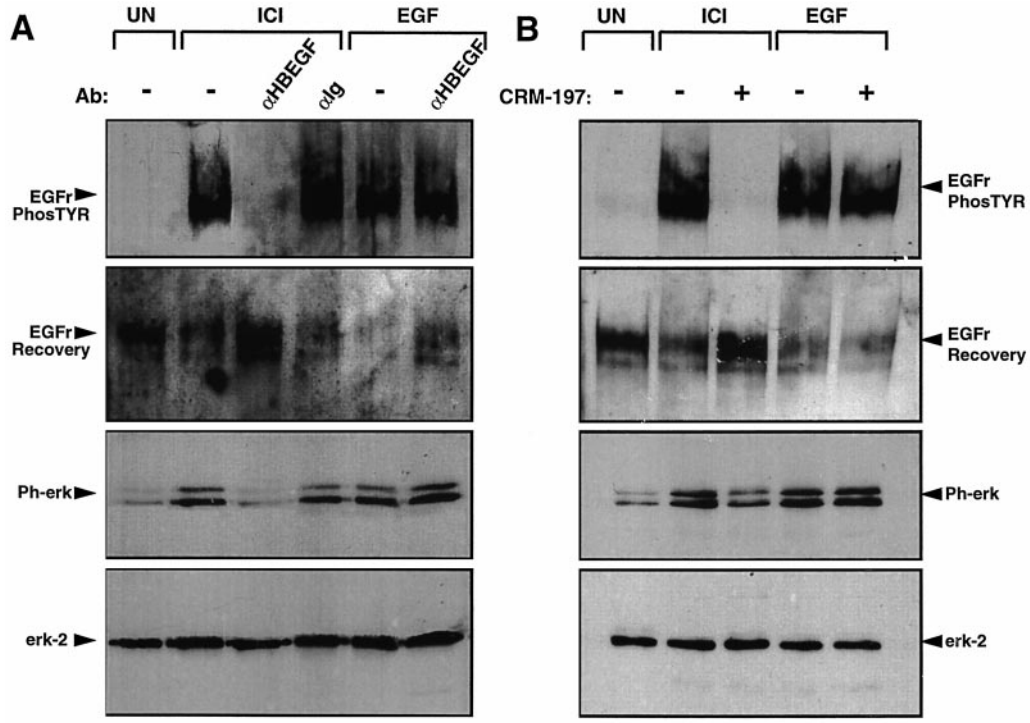


**Fig. 5.** GPR30-Mediated EGFR *Trans*-Activation by Estrogen Requires HB-EGF

MDA-MB-231 (GPR30) cells were preincubated with rabbit anti-HB-EGF or control antibodies (3–6 ng/ml; A) or pretreated with the diphtheria toxin mutant, CRM-197 (200 ng/ml for 1 h; B) before stimulation for 5 min with ICI 182,780 (1 $\mu$M) or EGF (10 ng/ml). EGFR tyrosine phosphorylation, EGFR recovery, and phospho-Erk and Erk-2 expression were determined as previously described.

EGF antibodies specifically inhibited ICI 182,780-induced EGFR tyrosine phosphorylation and Erk-1/-2 phosphorylation, but had no effect on the ability of exogenous EGF to activate EGFR or Erk-1/-2 (Fig. 5A).

In addition to its role as a growth factor precursor, pro-HB-EGF is known to serve as the primary attachment site for diphtheria toxin (35). The diphtheria toxin mutant, CRM-197, inhibits the mitogenic activity of HB-EGF (36), and this is related to its ability to sequester or down-modulate surface-expressed pro-HB-EGF (37). Therefore, to test further the hypothesis that estrogen acts through GPR30 to mediate HB-EGF-dependent activation of the EGFR and downstream activation of Erk-1/-2, we measured EGFR and Erk-1/-2 phosphorylation in MDA-MB-231 (GPR30) cells that had been pretreated with CRM-197 before stimulation with ICI 182,780 or exogenous EGF. CRM-197 markedly reduced EGFR and Erk-1/-2 activation promoted by ICI 182,780, but showed no effect on the ability of exogenous EGF to activate either EGFR or MAPK (Fig. 5B). Similarly, pretreatment with CRM-197 specifically abrogated estradiol-mediated activation of the EGFR and MAPK in both MDA-MB-231 cells (GPR30) and MCF-7 cells (data not shown).

Thus, these data support the model that estrogen-mediated MAPK activation requires GPR30 and is mediated via the activation of a G$\beta\gamma$-subunit-Src-Shc pathway that results in *trans*-activation of the EGFR and downstream signaling to the MAPKs, Erk-1/-2, through the release of pro-HB-EGF.

## DISCUSSION

Other investigators have concluded that estrogen-induced MAPK activation is promoted by ER$\alpha$ or ER$\beta$ (5, 8, 11, 12). Their studies have suggested that in addition to functioning as ligand-activated transcription factors, these ERs may promote nongenomic signaling events by estrogen. Although this conclusion is possible, several issues regarding the capacity of ERs to mediate nongenomic signaling exist. The structure of the ER, a member of the steroid hormone receptor superfamily, is well studied, and there are no known functional motifs within its structure that promote second messenger signaling (13). Moreover, studies investigating rapid MAPK activation by estrogen have employed cell lines derived from tissues known to be estrogen responsive, including MCF-7 breast cancer cells (5, 8), osteosarcoma cells (9), and neuroblastoma cells (10), but these studies have not directly addressed the roles of ER$\alpha$ and ER$\beta$ in promoting estrogen-induced Erk activation. To test this hypothesis, we examined estrogen-induced Erk activation in

breast cancer cell lines that have various patterns of ER expression. We found no correlation between the expression of either ER$\alpha$ or ER$\beta$ and the ability of estrogen to activate Erk-1/-2 in these cells (Fig. 1). In fact, we demonstrate that either estrogen or the pure antiestrogen ICI 182,780 activates Erk-1/-2 in human SKBR3 breast carcinoma cells, which we and others (22) have demonstrated lack ER$\alpha$ and ER$\beta$, protein, and messenger RNA, strongly suggesting that the ER is not involved.

Experiments conducted in other cell types have led to suggestions that membrane-associated ER-like receptors and G proteins may be responsible for nongenomic estrogen signaling (7, 11, 38). Strongly supporting such an idea, our evidence indicate that cellular expression of the orphan receptor, GPR30, is sufficient for estrogen-induced activation of Erk-1/-2. Employing GPR30 peptide antibodies raised in our laboratory, we found that human MCF-7 and SKBR3 breast cancer cell lines that expressed elevated GPR30 protein were capable of activating Erk-1/-2 in response to estrogen. Moreover, GPR30-deficient MDA-MB-231 breast cancer cells, which are normally nonresponsive to estrogen-induced Erk-1/-2 activation, can be converted to a responsive phenotype by overexpression of GPR30 protein (Fig. 2). Based on our results with breast cancer cell lines, it is tempting to speculate that GPR30 may in part define the sensitivity of other tissues to estrogen. Studies by others (20, 39) indicate that GPR30 has a restricted expression pattern, with abundant levels in placenta, bone, and brain, tissues that are considered to be estrogen responsive. Although our data strongly suggest that GPR30 participates in rapid estrogen signaling to Erk-1/-2, whether GPR30 acts alone or functions as a subunit of a receptor complex remains to be determined.

Consistent with GPR30 promoting G protein-dependent activation of Erk-1/-2, estrogen-induced Erk-1/-2 activation is inhibited by agents that block G protein signaling. For example, Erk-1/-2 activity induced by estrogen in GPR30-expressing breast cancer cells is blunted by pertussis toxin as well as the Src family-specific tyrosine kinase inhibitor, PP2 (Fig. 3). In addition, cellular expression of the carboxyl-terminus of the $\beta$-adrenergic receptor kinase, $\beta$ark-1, which is known to function as a G$\beta\gamma$-subunit protein sequestrant peptide (31), specifically blocks estrogen-dependent Erk activation in these cells. A similar inhibitory effect on estrogen-mediated Erk-1/-2 activity was observed upon transfection of a dominant negative Shc protein (Fig. 3). Thus, our results indicate that estrogen-induced activation of Erk-1/-2 occurs via a G$\beta\gamma$-subunit protein complex-dependent signaling mechanism that requires both Src and Shc. This mechanism of Erk-1/-2 activation is used by a number of other GPCRs and is typically Ras dependent (24, 25). Although we did not test the role of Ras in estrogen-induced Erk-1/-2 signaling, increases in GTP-bound Ras have been reported after exposure of MCF-7 cells to estrogen (8). However, estrogen stimulation of these cells by others did not result in phosphorylation of Raf-1 protein (5). These results may indicate that estrogen-induced activation of Ras does not require Raf-1. Alternatively, different G protein-dependent signaling pathways leading to Erk-1/-2 activation may be used depending on the activation state of MCF-7 cells before estrogen stimulation. This later explanation is supported by the finding that intracellular signals have been shown to determine the coupling of distinct G$\alpha\beta\gamma$ heterotrimers with the same GPCR (40).

Although it has been known for some time that G$\beta\gamma$ complexes use Src family nonreceptor tyrosine kinases and Shc to promote intracellular activation of receptor tyrosine kinases, it has been demonstrated only recently that many GPCRs activate metalloproteinases that release pro-HB-EGF from the cell surface. The cleaved HB-EGF, in turn, activates EGFR signaling pathways (34). Similarly, our data suggest that estrogen activates Erk-1/-2 by pro-HB-EGF-dependent *trans*-activation of the EGFR (Figs. 4 and 5). In this regard, our findings support prior observations that estrogen administration to rodents increases levels of local EGF (41) and stimulates EGFR kinase activity in uterine membranes (42). Moreover, estrogen-dependent *trans*-activation of the EGFR underscores the potential significance of the EGFR in the growth and survival of female reproductive tissues and breast tumors and is consistent with studies that have shown high concentrations of EGF-related proteins (43, 44) and EGFR in these tissues and tumors.

Breast tumors that fail to express ER normally do not respond favorably to antiestrogen therapy (45). These tumors are referred to as estrogen independent and are presumed to use growth factor-dependent signaling mechanisms for their growth and survival. This biological distinction is furthered by the observation that ER-negative breast tumors commonly overexpress EGFR-related proteins (46) and that simultaneous expression of elevated ER and EGFR are rarely observed in cultured breast lines (47). Consistent with this, it is interesting to note that transfection of the EGFR cDNA into ER-positive MCF-7 cells results in transient expression of EGFR that is unstable in the presence of estrogen (48). In light of our findings, ER-negative breast tumors that express GPR30 may remain estrogen responsive through their ability to promote growth factor-dependent signals. To the extent that this is true, antagonism of the EGFR may be beneficial for patients with estrogen-independent or estrogen-dependent breast tumors. Further studies regarding the expression of this GPCR in breast tumor specimens will be required to test this hypothesis.

## MATERIALS AND METHODS

### Cell Culture

Human MCF-7 (ER$\alpha^+$, ER$\beta^+$), SKBR3 (ER$\alpha^-$, ER$\beta^-$), and MDA-MB-231 (ER$\alpha^-$, ER$\beta^+$) breast carcinoma cells were obtained from American Type Culture Collection (Manassas,

VA) and were cultured in phenol red-free DMEM/Ham's F-12 medium (1:1) containing 10% FBS and 100 μg/ml of gentamicin. MDA-MB-231 transfectants were generated as described below and were maintained in the same medium supplemented with 500 μg/ml geneticin (Sigma, St. Louis, MO), 200 μg/ml Zeocin (Invitrogen, La Jolla, CA), or both.

### cDNAs and Dominant Negative Constructs

GPR-BR is a cDNA encoding the full-length human GPR30 protein subcloned into the pBK-CMV expression vector (20) and was provided by Ronald Weigel (Stanford University, Palo Alto, CA). The carboxyl-terminus of βark-1 has previously been shown to function as a Gβγ sequestrant peptide and was a gift from Robert Lefkowitz (Duke University, Durham, NC) in the RK-5 vector (31). A molecular clone encoding glutathione-*S*-transferase fused to mutant mouse Shc protein containing a tyrosine to phenylalanine substitution at residue 317, GSTShcY317F, has been demonstrated to block Shc signaling and was a gift from Dr. Kodimengalam Ravichandran (49). To generate constructs suitable for generating stable cell lines expressing either Gβγ sequestrant peptide or the dominant negative Shc, the respective *Eco*RI inserts of these clones were excised and subcloned into the *Eco*RI site of the pcDNA3.1Zeo(+) expression vector.

### Transfections and Selection of Stable Cell Lines Expressing Dominant Negative Constructs

MDA-MB-231 cells were transfected with either pBK-CMV vector or GPR-BR plasmid DNA using Lipofectamine Plus (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's suggestions. Three days after transfection, 500 μg/ml of geneticin (Sigma) were added to the growth medium. The resulting uncloned population of geneticin-resistant cells was propagated to generate cell lines used for further study. MDA-MB-231 (GPR30) geneticin-resistant cells were retransfected with pcDNA3.1Zeo(+) constructs expressing either Gβγ sequestrant peptide (βark) or dominant negative Shc Y317F and were selected for dual resistance in medium containing (500 μg/ml) geneticin and (200 μg/ml) Zeocin as described above.

### Growth Factors, Estrogens, and Inhibitors

Recombinant human EGF was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Water-soluble 17β-estradiol; its inactive isomer, 17α-estradiol; progesterone; and 4-hydroxytamoxifen were purchased from Sigma. The pure ER antagonist, ICI 182,780, was obtained from Tocris Chemicals (Ballwin, MO). The diphtheria toxin mutant, CRM 197, was purchased from Berna Products (Coral Gables, FL). Tyrphostins AG-879 and AG-1478 were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). The former has been shown to preferentially inhibit Her-2/neu autophosphorylation (50), and the latter has been demonstrated to be a selective inhibitor of ErbB1 (EGFR) activity (51). The Src family tyrosine kinase inhibitor PP2 (26) was purchased from Calbiochem (La Jolla, CA).

### Antibodies

Phospho-specific antibodies that recognize phosphorylated Erk-1 and Erk-2 (phospho-erk) were purchased from New England Biolabs, Inc. (Beverly, MA). The Erk-2 antibodies were also purchased from the same vendor and are also known to cross-react with Erk-1. ERα-specific antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ERβ-specific antibodies raised against a synthetic peptide representing amino acids 46–63 of human ERβ were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). GPR30-specific antibodies were generated against a synthetic peptide, CAVIPDSTEQSDVRFSSAV (Multiple Peptide Systems, San Diego, CA), derived from the carboxyl-terminus of the deduced amino acid sequence of human GPR30 polypeptide. The GPR30 peptide was covalently coupled to keyhole limpet hemocyanin using the bifunctional cross-linker, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, and injected intradermally into New Zealand White rabbits. The IgG antibody fraction of the immune serum was enriched by affinity chromatography using protein G-agarose columns. The pan-Shc antibody, which detects all Shc isoforms, and sheep EGFR antibody, which detects all ErbB family members, were purchased from Upstate Biotechnology, Inc.. The EGFR (ErbB1) monoclonal antibody (clone Ab-1) purchased from Calbiochem, recognizes an epitope within the extracellular domain of the p170 EGFR and does not react with ErbB2 (Her-2/Neu), ErbB3, or ErbB4. Phosphotyrosine-specific monoclonal antibodies, 4G10 and PY20, were purchased from Upstate Biotechnology, Inc. and Transduction Laboratories, Inc. (Lexington, KY), respectively. HB-EGF-neutralizing antibodies were purchased from R&D Systems (Minneapolis, MN).

### Conditions for Cellular Stimulation and Detergent Lysates

One million cells were seeded onto 90-mm Falcon tissue culture dishes in phenol red-free DMEM/F-12 medium containing 10% FCS. The following day, the cell monolayers were washed twice in PBS and placed in fresh phenol red-free, serum-free medium. Cells were maintained in phenol red-free medium for an additional 3 days, an interval of time that we have determined to be necessary to minimize basal levels of Erk-1/-2 activity. Stimulations of quiescent cells were carried out at 37 C in serum-free medium as described in the figure legends. Concentrations of 17β-estradiol (1 nM) and the anti-estrogen, ICI 182,780 (1 μM) were chosen from preliminary experiments to provide more than half-maximum 17β-estradiol activation of Erk-1/-2, in agreement with values determined by others (8–10). After stimulation, monolayers were lysed with ice-cold RIPA buffer consisting of 150 mM NaCl, 100 mM Tris (pH 7.5), 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 3.5 mM $Na_3VO_4$, 2 mM phenylmethylsulfonylfluoride, 50 mM NaF, 100 mM sodium pyrophosphate, plus a protease inhibitor cocktail (Complete, Roche Molecular Biochemicals, Indianapolis, IN). Crude lysates were clarified by centrifugation, and protein concentrations were determined by the bicinchoninic acid method according to the manufacturer's suggestions (Pierce Chemical Co., Rockford, IL). Detergent lysates were stored at −70 C until use.

### Western Blotting

Total cellular protein (50 μg) was boiled in standard Laemmli buffer with reducing reagents and resolved by SDS-PAGE. Proteins were electrotransferred onto nitrocellulose membranes (0.45 μm pore size; Schleicher and Schuell, Keene, NH) using a semidry transfer cell (CBS, Del Mar, CA) at 1 mA/cm$^2$ for 4 h. Phospho-Erk was detected by probing membranes, which were blocked overnight in Tris -buffered saline containing 0.1% Tween-20 and 2% BSA (TBST-BSA), with phospho-Erk-specific rabbit antibodies diluted 1:1000 in TBST-BSA for 1 h at room temperature. Rabbit antibody-antigen complexes were detected with horseradish peroxidase-coupled goat antibodies to rabbit anti-IgG diluted 1:5000 in TBST-BSA and visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Arlington Heights, IL). Relative levels of total Erk-2 protein in each sample were determined by stripping the phospho-specific rabbit antibodies from the nitrocellulose membrane and reprobing with antibodies to Erk-2. ERα and GPR30 proteins were detected on nitrocellulose membranes in the same

manner, except that filters to be probed with GPR30 peptide antibodies were blocked in TBST containing 5% nonfat dry milk. ERβ was detected using ERβ-specific peptide antibodies purchased from Upstate Biotechnology, Inc., following specifications provided by the manufacturer. In brief, membranes were blocked for 30 min in PBS containing 3% nonfat dry milk (PBS-MLK), and incubated with 1 μg/ml ERβ-specific peptide antibodies diluted in fresh (PBS-MLK). After an overnight incubation at 4 C, membranes were washed in water, and immobilized rabbit antibodies were incubated with horseradish peroxidase-coupled goat antibodies to rabbit anti-IgG diluted 1:5000 in PBS-MLK for 1.5 h at room temperature. The membrane was then rinsed in water and washed in PBS containing 0.05% Tween-20 before visualizing ERβ antibody-goat IgG horseradish peroxidase complexes by ECL (Amersham Pharmacia Biotech). Apparent mol wts were determined from Rainbow mol wt standards (Amersham Pharmacia Biotech).

### Detection of Tyrosine-Phosphorylated EGFR and Shc-Associated Tyrosine-Phosphorylated Proteins

Tyrosine phosphorylation of the EGFR was assessed by immunoblotting EGFR immunoprecipitates with phosphotyrosine-specific antibodies. EGFR was immunoprecipitated from 500 μg total cellular protein, extracted in RIPA buffer using 2 μg/sample Ab-1, a monoclonal antibody to ErbB1. Similarly, Shc-associated tyrosine-phosphorylated proteins were immunopurified from 1 mg total cellular protein, prepared in RIPA buffer, and diluted 5-fold in 1% Nonidet P-40, using 2 μg/sample pan-Shc antibodies. In either case, antigen-antibody complexes were immunoprecipitated with 50 μl of a 50% slurry of protein G-agarose (Pierce Chemical Co.). EGFR immunoprecipitates were washed, resuspended in standard Laemmli buffer containing 875 mM β-mercaptoethanol, and subjected to SDS-PAGE. Immunoprecipitated proteins were electrotransferred to nitrocellulose, blocked with TBS-BSA, and then immunoblotted with the phosphotyrosine-specific monoclonal antibodies, PY20 or 4G10, diluted 1:1,000 or 1:10,000 in TBS-BSA. Immobilized mouse antibody-antigen complexes were detected with horseradish peroxidase-coupled sheep antibodies to mouse IgG diluted 1:5,000 in TBS-BSA and visualized by ECL. Recovery of EGFR or Shc in each of these immunoprecipitates was measured by stripping the phosphotyrosine antibodies from the membrane and reprobing with EGFR or Shc antibodies, respectively.

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