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**13. ABSTRACT** *(Maximum 200 Words)*

Antiestrogens are the most effective and widely administered therapy for the management of breast cancer. Their efficacy has been attributed to their ability to antagonize the estrogen receptor, and the presence of ER in breast tumor biopsy specimens correlates well with responsiveness to antiestrogen therapy. Still, one in four patients with ER-positive tumors do not respond to antiestrogens, while one in six patients with ER-negative breast tumors undergo objective tumor regression following antiestrogen therapy (Witliff, 1984). These clinical observations suggest that alternative mechanisms of estrogen action may regulate the growth and survival of breast tumors. We have provided evidence that estrogen acts independently of the known estrogen receptors, ERα and ERβ, via the G-protein coupled receptor, GPR30, to regulate the EGFR-to-MAP K signaling axis (Filardo et al, 2000; reviewed in Filardo, 2001; Filardo et al, 2001). Moreover, we have shown that the antiestrogens, tamoxifen and faslodex (ICI 182, 780), also trigger GPR30-dependent regulation of this HB-EGF autocrine loop.

Dysregulation of the EGFR-to-MAP K signaling axis is a common occurrence in breast cancer (Slamon et al, 1989, Sivaraman et al, 1997). The subject of this DOD award is to investigate the relationship between GPR30 expression and MAP K activity in breast tumor biopsy specimens obtained at first diagnosis or following antiestrogen or other adjuvant therapies. The results of these studies may lead to a further refinement in assessing responsiveness to antiestrogen therapy.

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## Table of Contents.

## Introduction.

Tamoxifen is the most effective and widely administered drug for the treatment of breast cancer, providing improved disease-free and overall survival for approximately 40% of breast cancer patients (Gradishar and Jordan, 1998). More recently, it has been shown that tamoxifen also serves as a chemopreventive agent for certain women at high risk for developing breast cancer (Fisher et al, 1998). Its value as a therapeutic agent is presumed to be associated with its ability to function as an ER antagonist (Katzenellenbogen et al, 1997), and accordingly, the presence of ER in breast tumor biopsy specimens provides some degree of certainty in terms of predicting responsiveness to tamoxifen therapy (Witliff, 1984). However, one in four patients with ER-positive tumors (15% of all breast tumors) for whom tamoxifen is indicated, fail tamoxifen therapy; and one in six patients with ER-negative tumors (5 % of all breast tumors), for whom tamoxifen is not currently indicated, exhibit objective tumor regression (Witliff, 1984). Moreover, it is not uncommon for breast carcinomas which initially respond to tamoxifen to acquire tamoxifen resistance, and eventually fail treatment (Katzenellenbogen et al, 1997). Thus, there is an urgent need to identify patients capable of responding to tamoxifen therapy from those who require alternative treatment modalities. This is particularly true for patients with ER-negative tumors since the majority of these patients do not respond to tamoxifen, and consequently, they have fewer treatment options.

We have provided data that GPR30, an orphan G-protein coupled receptor, transmits intracellular signals that regulate the mitogen-activated protein kinases, Erk-1 and Erk-2 in breast cancer cells following tamoxifen treatment (Filardo et al, 2000; reviewed in Filardo et al, 2001; Filardo et al 2001). Erk-1/-2 hold particular significance for breast cancer because these kinases are commonly hyperactivated in breast carcinoma (Sivaraman et al, 1997); and they are key signaling intermediaries for both estrogen and growth factor-dependent pathways.

## Body.

Our hypothesis is that low levels of GPR30 expression, or somatic mutations within GPR30, may compromise the ability of tamoxifen to regulate Erk-1/-2 activity, and correlate with nonresponsiveness to tamoxifen therapy. In the work supported by this award, we are testing this hypothesis by comparing the expression of GPR30 and Erk-1/-2 activity in archival breast biopsy specimens obtained at first diagnosis (prior to treatment) or subsequent to adjuvant therapy.

*P-Erk expression in infiltrating ductal carcinoma of the breast at first diagnosis.* As represented in Figure 1 (appendix), we have found that constitutive Erk-1/-2 activation is commonly observed in primary breast carcinomas prior to tamoxifen therapy. Our preliminary data indicates that hyperactivated Erk-1/-2 (P-erk) occurs in greater than 70% of all breast carcinomas harvested at first diagnosis. Furthermore, expression of hyperactive Erk-1/-2 in these tumors does not appear to correlate with the expression of ERα or amplification of the EGFR family member, HER-2/neu (Table 1). As outlined in

the statement of work, we will increase the number of cases of tumors examined at first diagnosis (no adjuvant therapy) and also begin to examine P-Erk expression in breast tumors obtained from patients following tamoxifen therapy or other adjuvant therapies. During the next 12 months, serial sections from these tumors (with or without adjuvant therapy) will be further stained for GPR30 expression as outlined below.

**Table I. Clinical and molecular characteristics of patients with infiltrating carcinoma at first diagnosis.**

| Patient # | Age[a] | Size (cm)[b] | LN met[c] | ER[d] | HER2/neu[e] | Ph-Erk[f] |
|---|---|---|---|---|---|---|
| 1 | 36 | 2.0 | yes | pos | neg | pos |
| 2 | 75 | 1.5 | yes | pos | neg | neg |
| 3 | 67 | 1.7 | no | pos | neg | pos |
| 4 | 66 | 1.3 | yes | pos | neg | pos |
| 5 | 69 | 1.5 | yes | pos | pos | pos |
| 6 | 72 | 2.0 | yes | pos | neg | neg |
| 7 | 60 | 1.5 | no | pos | neg | neg |
| 8 | 67 | 1.5 | no | neg | neg | neg |
| 9 | 75 | 1.5 | no | pos | neg | pos |
| 10 | 74 | 1.2 | yes | pos | neg | pos |
| 11 | 60 | 1.7 | no | pos | neg | pos |
| 12 | 74 | 3.0. | yes | pos | neg | pos |
| 13 | 75 | 0.5 | no | pos | neg | pos |
| 14 | 49 | 1.3 | yes | neg | neg | pos |
| 15 | 72 | 1.8 | no | pos | neg | pos |
| 16 | 59 | 1.6 | yes | pos | neg | pos |
| 17 | 46 | 0.6 | yes | pos | neg | pos |
| 18 | ND | ND | ND | neg | ND | pos |
| 19 | ND | ND | ND | neg | ND | pos |
| 20 | ND | ND | ND | neg | ND | pos |
| 21 | ND | ND | ND | neg | ND | pos |
| 22 | ND | ND | ND | neg | ND | neg |
| 23 | ND | ND | ND | neg | ND | neg |
| 24 | ND | ND | ND | neg | ND | pos |
| 25 | ND | ND | ND | neg | ND | pos |
| 26 | ND | ND | ND | neg | ND | pos |
| 27 | ND | ND | ND | neg | ND | neg |
| | | | | 15/27 | 1/17 | 20/27 |

[a]Patient age at diagnosis. Mean patient age = 64.5 years, median patient age = 67 years.
[b]Tumor size in transverse diameter.
[c]Evidence of lymph node metastases
[d] ER status, [e] HER2/neu positivity, and [f] phosphoerk expression were assessed by immunohistochemical staining.
ND = not determined.

Numbers below each column represent the fraction of biopsies positive for each marker.

*GPR30 expression in normal mammary epithelium and ductal carcinoma of the breast.*
To evaluate whether GPR30 serves as an indicator for assessing responsiveness to tamoxifen therapy, we have raised antibodies in rabbits to a C-terminal peptide derived from the deduced amino acid sequence of GPR30. These peptide antibodies recognize a

single 38-kDA band that is abundant in MCF-7 and SKBR3 breast cancer cells and in MDA-MB-231 cells that have been transfected with GPR30 cDNA but is barely detected in vector-transfected MDA-MB-231 cells. The apparent molecular mass of the 38-kDA band closely approximates the predicted molecular mass of the mature 351 amino acid GPR30 polypeptide (Filardo et al, 2000). Prior to submission of this proposal, we determined that these antibodies were suitable for immunochemical analysis by demonstrating that they specifically reacted with formalin-fixed GPR30-transfected MDA-MB-231 cells relative to vector-transfected MDA-MB-231 cells. In order to develop uniformity within our immunohistochemical staining technique throughout the course of this project, we generated a single large pool (pool# D01 = 150mls) of rabbit GPR30 C-ter peptide antibodies. To generate these antibodies, a single positive rabbit (D) was boosted and serum was collected from this animal for three successive weeks. GPR30 antibodies in these sera were quantified by ELISA using immobilized C-terminal peptide as a capture antigen. The titer of GPR30 antibodies from each of these bleeds were approximately similar, and were therefore, pooled. IgG antibodies within this pool were enriched by protein G-sepharose chromatography. By competitive ELISA, we have demonstrated the specificity of these GPR30 antibodies for immobilized C-terminal peptide that the ability of these GPR30 C-ter peptide antibodies to recognize immobilized C-ter peptide. While soluble C-ter peptide effectively competes for GPR30 antibodies against immobilized C-ter peptide, no inhibition is observed with soluble peptide derived from the N-terminus of GPR30 (figure 2, appendix). Employing these rabbit GPR30 C-ter peptide antibodies, we have established a set of optimal conditions for immunohistochemical detection of GPR30 protein in archival, paraffin-embedded human breast biopsy specimens (figure 3, appendix). We find that the GPR30 C-ter peptide antibodies react specifically with normal and malignant mammary epithelia (figures 3, 4 and table II) but do not react with normal kidney (figure 3D) nor vascular endothelium and smooth muscle (figure 4A). Consistent with structural data that suggests GPR30 is a transmembrane receptor, we find that the GPR30 C-ter antibodies concentrate at the membrane of normal and malignant mammary ductal epithelium (figures 4A and 4C).

As presented in table II (below), we find that GPR30 is expressed in 9 of the 12 breast carcinomas that we have examined to date. We have observed some differences in the level of GPR30 expressed in tumors that we have scored as positive. For example, patient 37 expressed high levels of GPR30 protein relative to the other tumors that we have examined so far. We do not have complete information regarding ER status in these samples, however, we have observed that among the 5 known ER negative tumors in this group, GPR30 is expressed in each of these tumors. By comparison, we detected GPR30 in 2 of the 4 ER-positive tumors GPR30. Previous evaluation of GPR30 expression in primary tumors has been accomplished by RT-PCR (Carmeci et al, 1997). In contrast to our preliminary results, these investigators found that GPR30 expression correlated with ER status, and that GPR30 was not often expressed in ER-negative tumors. Although further study is required, it is possible that the GPR30 C-ter antibodies may provide a more effective means for determining GPR30 expression. As we continue to use the GPR30 C-ter antibodies to further evaluate GPR30 expression in breast tumors, we will define the relationship between ER status and GPR30 expression.

As outlined in our statement of work, and mentioned previously, during the next 12 months, serial sections from breast tumors (with or without adjuvant therapy) will be evaluated for GPR30, ER, PR, HER-2/neu, and P-Erk expression.

**Table II. Expression of GPR30, HER-2/neu and ER in human biopsy specimens containing normal and malignant mammary epithelium.**

*Malignant*

| Patient # | GPR30[b] | ER[d] | PR | HER2/neu[e] | Ph-Erk[f] |
|---|---|---|---|---|---|
| 28 | pos | neg | ND | ND | ND |
| 29 | pos | neg | ND | ND | ND |
| 30 | pos | pos | ND | ND | ND |
| 31 | pos | neg | neg | neg | ND |
| 32 | pos | neg | ND | ND | ND |
| 33 | pos | neg | neg | neg | poa |
| 34 | neg | pos | pos | neg | pos |
| 35* | pos | ND | ND | pos | pos |
| 36 | neg | ND | ND | ND | pos |
| 37# | pos | ND | ND | pos | pos |
| 38* | pos | pos | neg | ND | ND |
| 39 | neg | pos | pos | neg | pos |
| | 9/ 12 | 4/ 9 | 2/ 5 | 2/ 6 | 6/ 6 |

*Normal*

| Patient # | GPR30[a] | ER[b] | PR[c] | HER2/neu[d] | Ph-Erk[e] |
|---|---|---|---|---|---|
| 1 | pos | ND | ND | ND | neg |
| 2 | pos | ND | ND | ND | neg |
| 3 | pos | ND | ND | ND | neg |
| 4 | pos | ND | ND | ND | neg |
| | 4/ 4 | | | | 0/ 4 |

[a] GPR30, [b]ER, [c]PR, [d] HER2/neu, and [e]phosphoErk expression were assessed by immunohistochemical staining. For the purpose of reporting this preliminary data samples have been scored as positive or negative, however, it is clear that degrees of positivity are represented in GPR30, ER , HER2/neu, and P-Erk staining, and in future studies standards will be established to accurately account for these differences.
#Patient 37 expressed high levels of GPR30.

ND = not determined.
*These patients received chemotherapy.

Numbers below each column represent the fraction of biopsies positive for each marker.

## Key Research Accomplishments.

1. Within the past year (first year of this award), we have generated two manuscripts and one review article which support a role for GPR30 in breast cancer (see Reportable Outcomes below).

2. As represented in Figure 1, human biopsy specimens from patients with ER-positive and –negative breast cancer (obtained prior to first diagnosis) have been immunostained with antibodies specific for HER2/neu and phosphorylated Erk-1/-2. We have found that greater than 70% of these breast tumors express elevated levels of phosphorylated Erk-1/-2. Moreover, in the 27 tumors that we have examined to date, we find no correlation between phosphorylated Erk expression and either ER expression or amplification of HER2/neu (table I).

3. We have optimized, and standardized, conditions for immunohistochemical (IHC) detection of GPR30 protein in archival, paraffin-embedded human breast biopsy specimens (see figure 3).

4. To ensure reproducibility within our immunochemical staining procedure over the course of this project (3 years), we have generated and quality-tested a large pool (pool#D01=150 mls) of rabbit C-terminal GPR30 peptide IgG antibodies suitable for IHC. Under our operating conditions for IHC, we are able to use these antibodies at a dilution of 1:1,000.

5. By competitive ELISA, we have demonstrated that these rabbit C-terminal GPR30 peptide IgG antibodies (pool#1) specifically recognize immobilized C-terminal peptide (see figure 2).

6. Further evidence of the specificity of these C-terminal GPR30 antibodies is provided by the observation that they react with normal and malignant mammary epithelia (figures 3, 4 and table II) but do not react with kidney (figure 3D) nor vascular endothelium and vascular smooth muscle (figure 4A). Speaking further to the specificity of the GPR30 C-terminal peptide antibodies, and consistent with its role as a heptahelical, G-protein-coupled receptor, the GPR30 C-ter antibodies generate a membrane-associated staining pattern in IHC (figures 3A and 3C).

7. Employing these GPR30 C-ter peptide antibodies, we have demonstrated that GPR30 protein can be detected by IHC in ER-negative breast tumors (figure 4B).

8. These antibodies have been requested by a number of collaborators and have been shown to detect GPR30 protein and react in immunochemical analyses in cells demonstrated to express GPR30 protein.

## Reportable outcomes.

During the past year (first year of this award), we have generated two manuscripts and one review article which support a role for GPR30 as an alternative estrogen receptor expressed in breast cancer cells.

**Manuscripts (2):**

**Filardo, EJ**, Quinn, JA, KI Bland, and Frackelton, AR, Jr. (2000). Estrogen-induced Activation of Erk-1 and Erk-2 Requires the G-Protein-Coupled Receptor Homologue, GPR30, and Occurs via Transactivation of the EGF Receptor Through Release of HB-EGF (Molec Endocrinol. **14 (10):** 1649-1660.

**Filardo, EJ,** Quinn, JA, Frackelton, AR, Jr. and KI Bland. Estrogen action via the G-protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the EGFR-to-MAP K signaling axis. (under review, Molec Endocrinol).

**Review article:**

**Filardo, E.J.** (2001). Epidermal Growth Factor Receptor (EGFR) Transactivation by Estrogen via the G-Protein_Coupled receptor, GPR30: a Novel Signaling Pathway with Potential Significance for Breast Cancer. J. Steroid Biochem & Molec Biol. (in Press).

## Conclusions.

At present, the known estrogen receptors, ERα and ERβ, are the best prognostic indicators for determining responsiveness to antiestrogen therapy. Still, one in four patients with ER-positive tumors do not respond favorably to anti-estrogens, while one in six patients with ER-negative tumors exhibit objective tumor regression following antiestrogen therapy (Witliff, 1984). These clinical findings, in conjunction with data demonstrating that antiestrogens trigger rapid signaling events typically not associated with known ERs (Aronica et al, 1994; Lee et al, 2000; Filardo et al, 2000), raises the possibility that antiestrogens may, in part, exert their antitumor effects via non ER-dependent mechanisms.

It has long been suspected that other receptors, distinct from the ER, may participate in estrogen signaling. However, until recently the physical identity of these receptors has remained unknown. Within the past two years, we have provided data demonstrating that the G-protein coupled receptor, GPR30, acts independently of known ERs to transmit intracellular signals that regulate the EGFR-to-MAP K signaling axis (Filardo et al, 2000; reviewed in Filardo, 2001; Filardo et al, 2001). This signaling axis holds particular significance for breast cancer in that it is frequently hyperactivated in breast cancer. Since antiestrogens also act as GPR30 agonists that regulate EGFR-to-MAP K signaling, the studies designed here will enable us to determine whether there is a link between GPR30 expression, Erk hyperactivation and antiestrogen responsiveness.

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Sivaraman VS, Wang H-Y, Nuovo GJ, Malbon CC (1997) Hyperexpression of mitogen-activated protein kinase in human breast cancer. J Clin Invest **99:** 1478-1483.

Wittliff JL (1984) Steroid-hormone receptors in breast cancer. Cancer **53:** 630- 643

## Appendices.

## A. Figures

**Figure 1.** Hyperexpression of activated Erk-1/-2 in infiltrating ductal carcinoma of the breast.

**Figure 2.** Specificity of rabbit antibodies generated against a peptide from the carboxyl terminus of GPR30.

**Figure 3.** Expression of GPR30 in normal mammary ductal epithelium.

**Figure 4.** Expression of GPR30 in infiltrating ductal carcinoma of the breast.

## B. Manuscripts

Filardo, EJ, Quinn, JA, Bland KI, and Frackelton, AR, Jr. (2000). Estrogen-induced Activation of Erk-1 and Erk-2 Requires the G-Protein-Coupled Receptor Homologue, GPR30, and Occurs via Transactivation of the EGF Receptor Through Release of HB-EGF. Molec Endocrinol. **14:** 1649-1660.

Filardo EJ. (2001). Epidermal Growth Factor Receptor (EGFR) Transactivation by Estrogen via the G-Protein_Coupled receptor, GPR30: a Novel Signaling Pathway with Potential Significance for Breast Cancer. J. Steroid Biochem & Molec Biol. (in Press).

Filardo EJ, Quinn JA, Frackelton AR, Jr, and Bland KI. (2001) Estrogen action via the G-protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the EGFR-to-MAP K signaling axis. (under review, Molec Endocrinol).

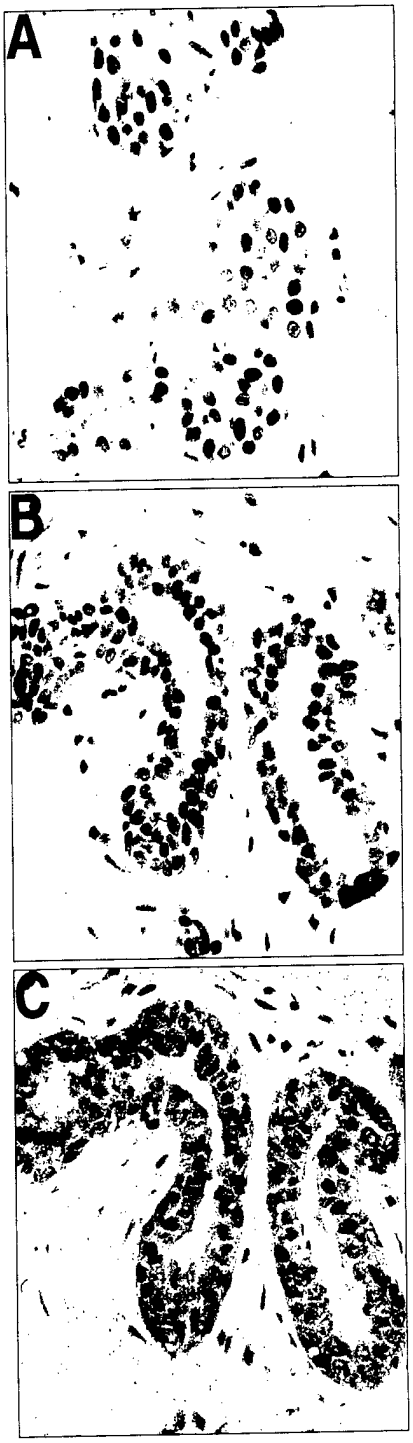


**Figure 1.Hyperexpression of activated Erk-1/-2 in infiltrating ductal carcinoma of the breast.** Archival, paraffin-embedded biopsy specimens containing **(A)** infiltrating carcinoma of the breast or **(B)** normal mammary epithelium were immunostained with rabbit antibodies specific for phosphorylated (active) Erk-1 and -2. Rabbit antibodies were visualized using a standard ABC immunoperoxidase technique and diaminobenzidine as a substrate. Positive cells appear brown and phosphorylated (active) Erk-1/-2 is observed localized within the nucleus of these cells. Nuclei were counterstained with hematoxylin (blue). **(C)** Adjacent serial section of **(B)** was immunostained with phosphorylation state-independent Erk-1/-2 antibodies.

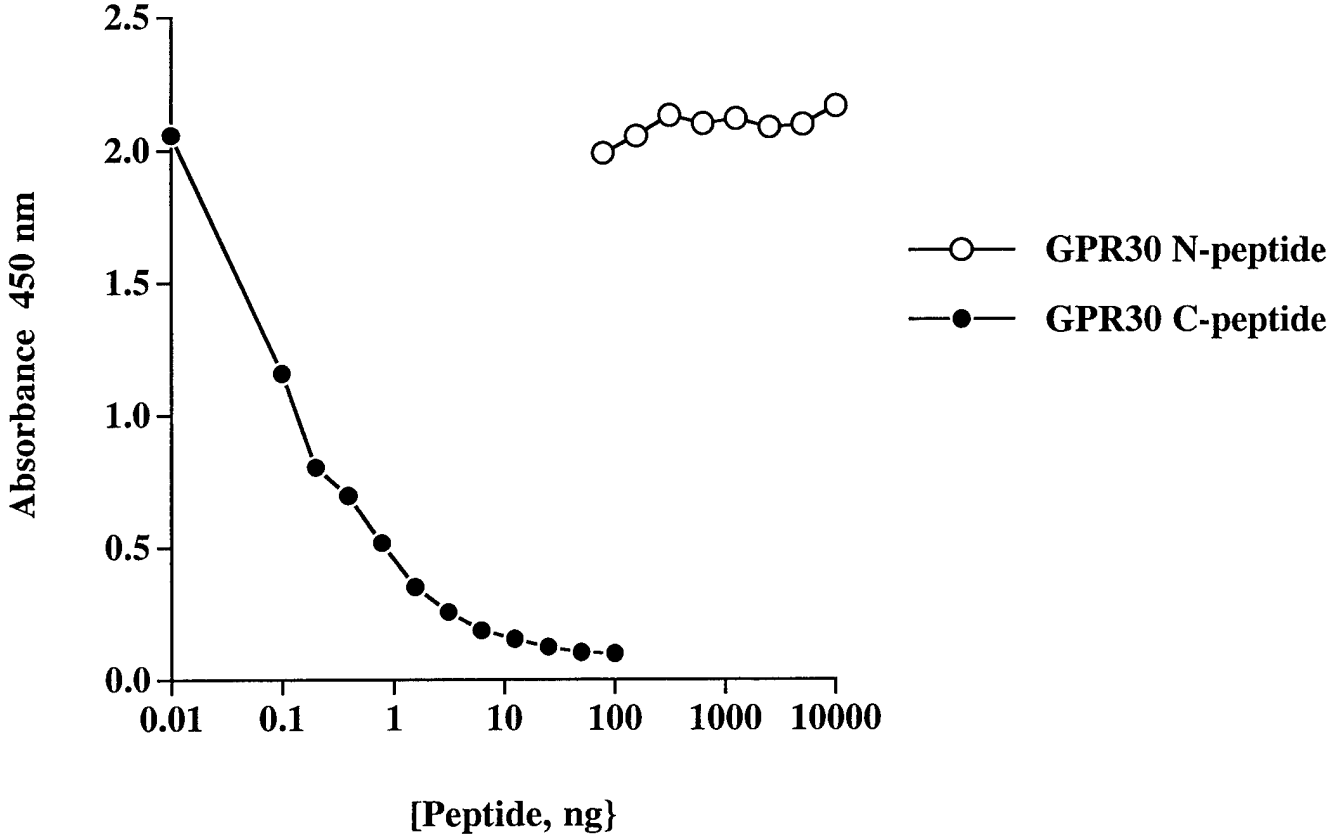


**Figure 2. Specificity of rabbit antibodies generated against a peptide from the C-terminus of GPR30.** Increasing concentrations of soluble specific (C-ter) or irrelevant (N-ter) peptide were tested for their ability to compete the reactivity of rabbit GPR30 C-ter peptide IgG antibodies (pool #D01) for immobilized C-ter peptide in a competitive ELISA. Each data point was determined in duplicate.

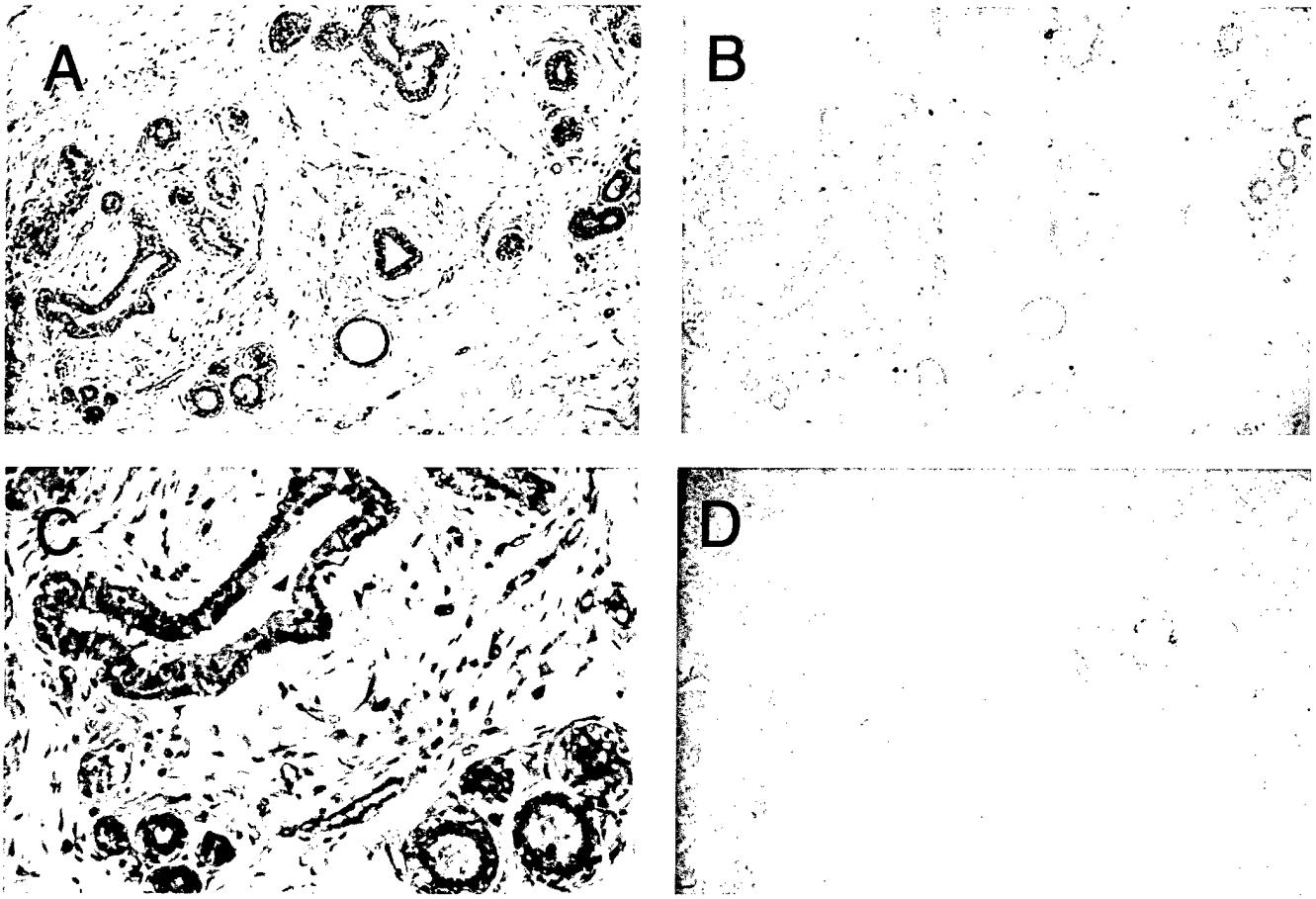


**Figure 3. Expression of GPR30 in normal mammary ductal epithelium.** Adjacent serial sections cut from paraffin-embedded normal breast tissue were immunostained with either **(A)** rabbit IgG antibodies specific for the carboxyl terminus of GPR30 (pool D-0601 C-ter GPR30) or **(B)** preimmune rabbit IgG antibodies. A higher magnification of **(A)** is presented in panel **(C)**. Note the localization of GPR30 antibodies at the plasma membrane. **(D)** GPR30 antibodies did not react with paraffin-embedded normal kidney tissue.

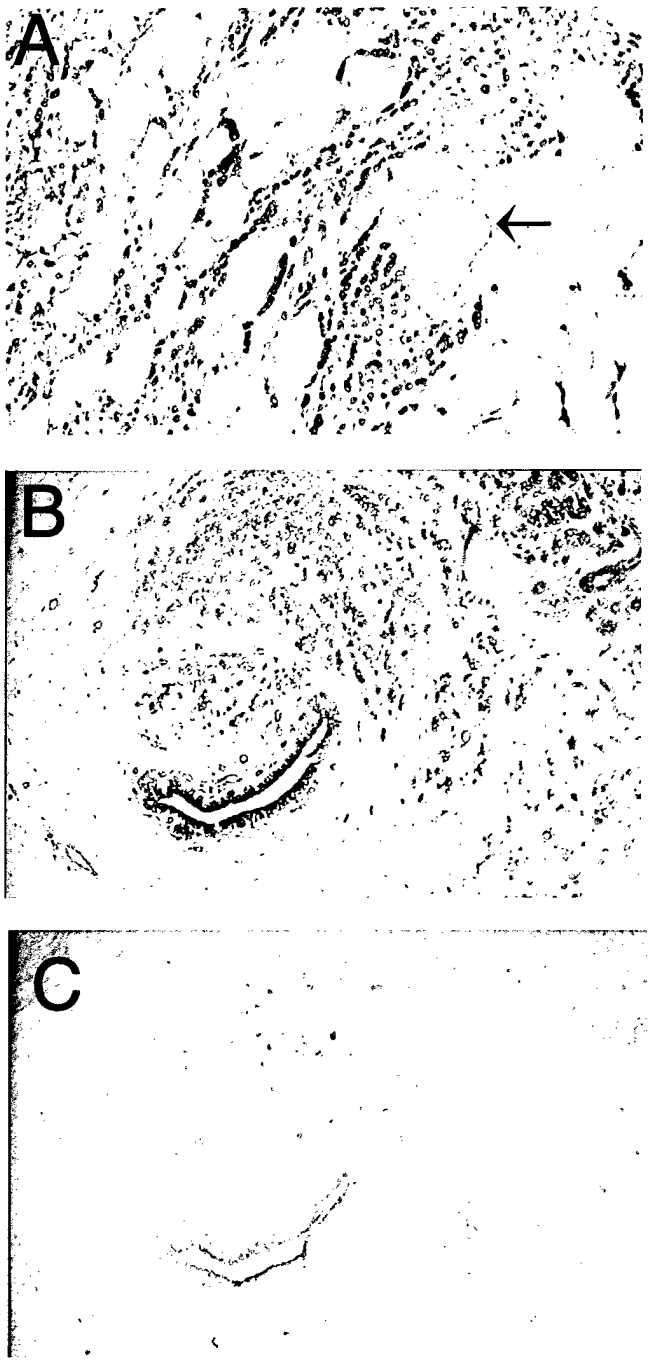


**Figure 4. Expression of GPR30 in infiltrating ductal carcinoma of the breast.** Archival paraffin-embedded breast biopsy specimens derived from patients with ER-positive **(A)** or –negative **(B)** infiltrating ductal carcinoma immunostained with C-ter GPR30 IgG antibodies (pool D-01). Infiltrating breast tumor cells are positive for GPR30 yet, detectable GPR30 protein is not observed in the vascular endothelial and smooth muscle cells from the artery in panel (A) (arrow). **(C)** Adjacent serial section of **(B)** immunostained with phospho Erk-specific antibodies.

# 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)**

0888-8809/00/$3.00/0
Molecular Endocrinology 14(10): 1649–1660


## 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 ERα and ERβ facilitate this rapid estrogen-stimulated signaling and activation of Erk-1/-2 (5, 11, 12). However, ERα and ERβ proteins lack known functional motifs that would allow for nongenomic mechanisms of estrogen action (13). Further questions regarding the roles of ERα and ERβ 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 ERα or ERβ, 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 ERα or ERβ is required for this mechanism of estrogen action. To determine whether estrogen-induced Erk activation is associated with expression of ERα or ERβ, 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 17β-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 ERα and ERβ protein (21) (Fig. 1). Surprisingly, however, 17β-estradiol induced a similar rapid increase in Erk-1/-2 in SKBR3 cells (Fig. 1) that express neither ERα nor ERβ messenger RNA (22). Erk-1 and -2 activation in these cell types could also be achieved using 1 μM of the pure anti-estrogen ICI 182,780, a concentration that blunts both ERα and ERβ (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, 17β-estradiol and ICI 182,780 each failed to activate Erk-1/-2 in MDA-MB-231 cells, which express only ERβ (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.

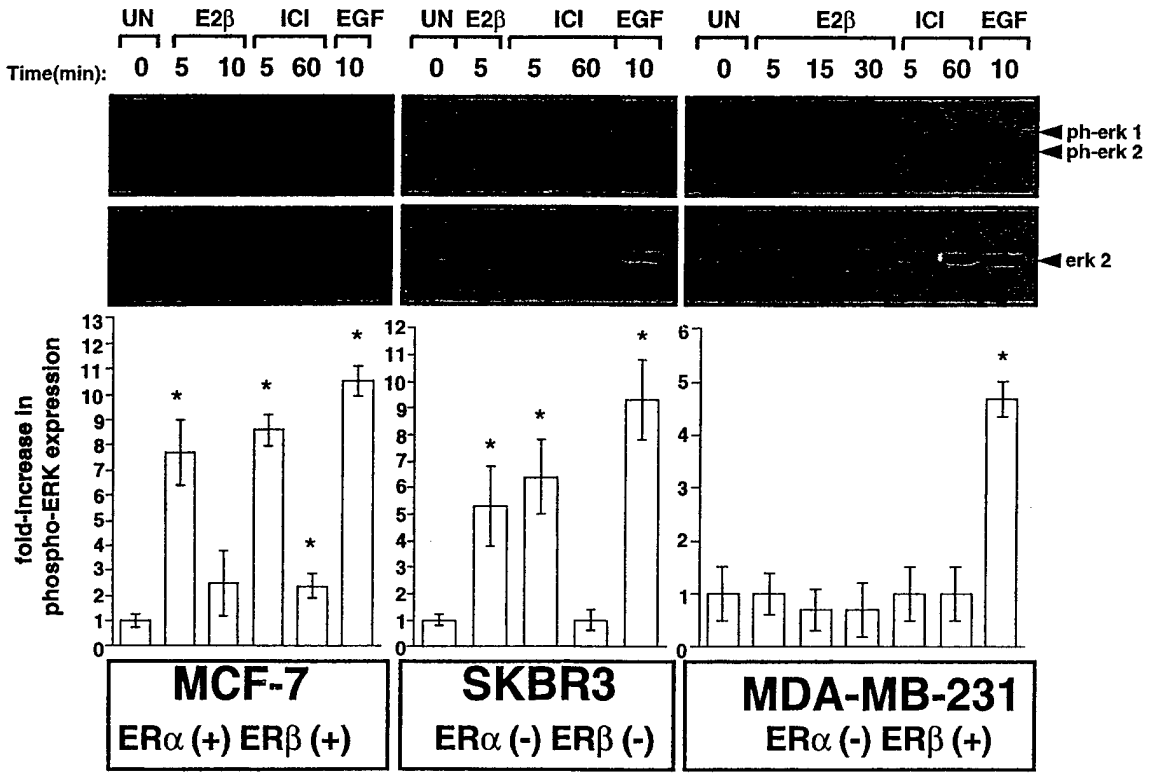


**Fig. 1.** Activation of Erk-1/-2 by Estrogens or Antiestrogens Does Not Correlate with ER Expression

Human MCF-7, SKBR3, or MDA-MB-231 breast carcinoma cells, untreated or exposed to 1 nM 17β-estradiol, 1 μM 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 μg) 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 ERα 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 ERβ protein was detected in lysates from MCF-7 and MDA-MB-231 cells; however, no detectable ERβ 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 17β-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 μM tamoxifen (partial agonist), but not by the inactive estrogen isomer, 17α-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 Gβγ, 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

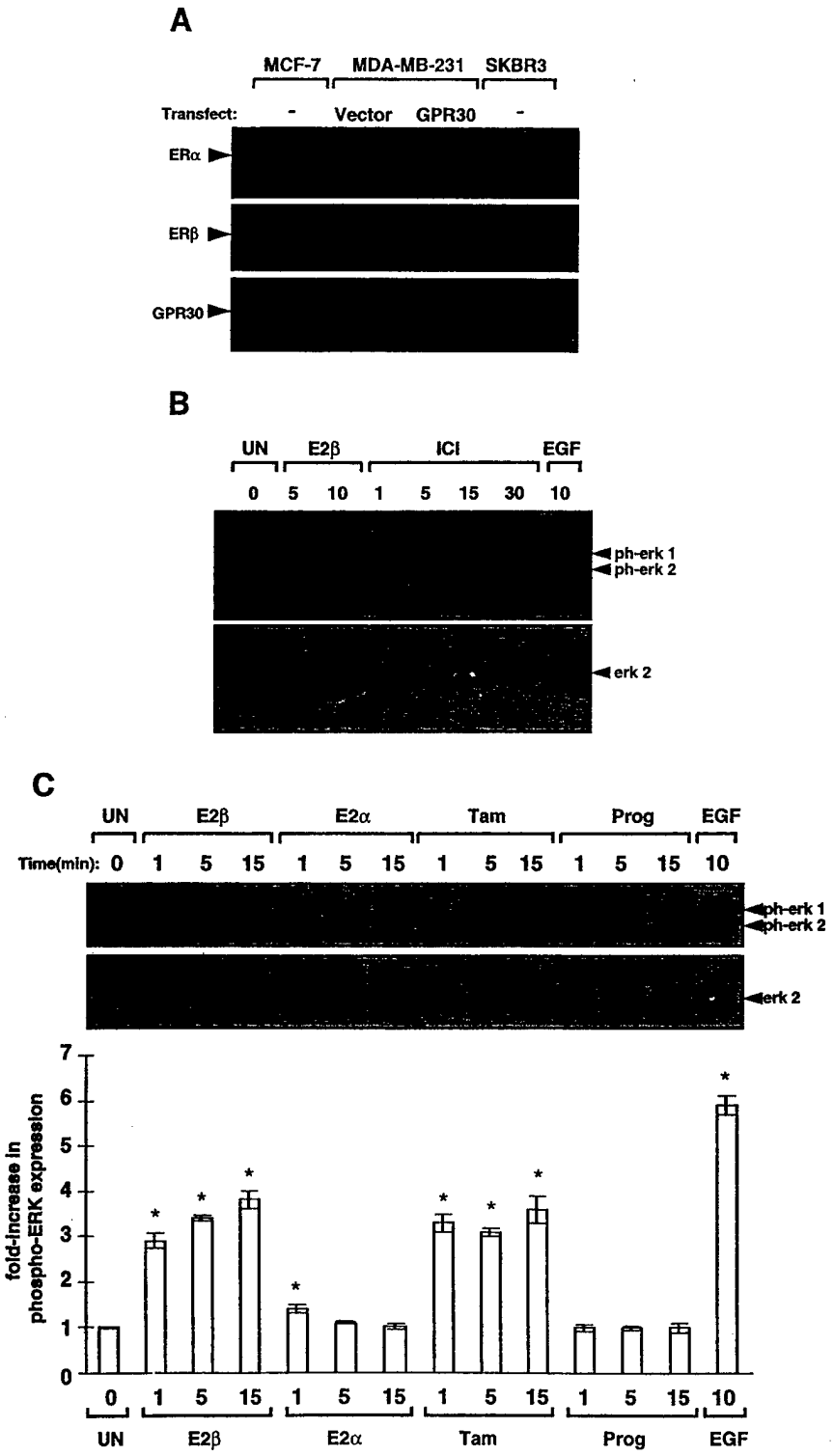


**Fig. 2.** Estrogen-Induced Activation of Erk-1/2 Requires Cellular Expression of the G Protein Receptor Homolog, GPR30

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 Gα-subunit protein dissociates from the heterotrimeric Gαβγ complex. Both free Gα and the remaining Gβγ 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 17β-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 Gβγ-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 Gβγ-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 β-adrenergic receptor kinase (βark), dominant negative Shc (shcY317F) or control vector, pcDNA3.1Zeo. Phospho-Erk and total Erk-2 protein expression were assessed in MDA-MB-231 (GPR30/βark), 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 βark 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 17β-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/βark-transfected MDA-MB-231 cells that had been either untreated or treated with 17β-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 βark. 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, Gβγ-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 17β-estradiol, the pure antiestrogen ICI 182,780, or EGF. Immunoblot analysis showed (Fig. 4A) that tyrphostin AG-1478 blocked EGF-induced as well as 17β-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 17β-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). 17β-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

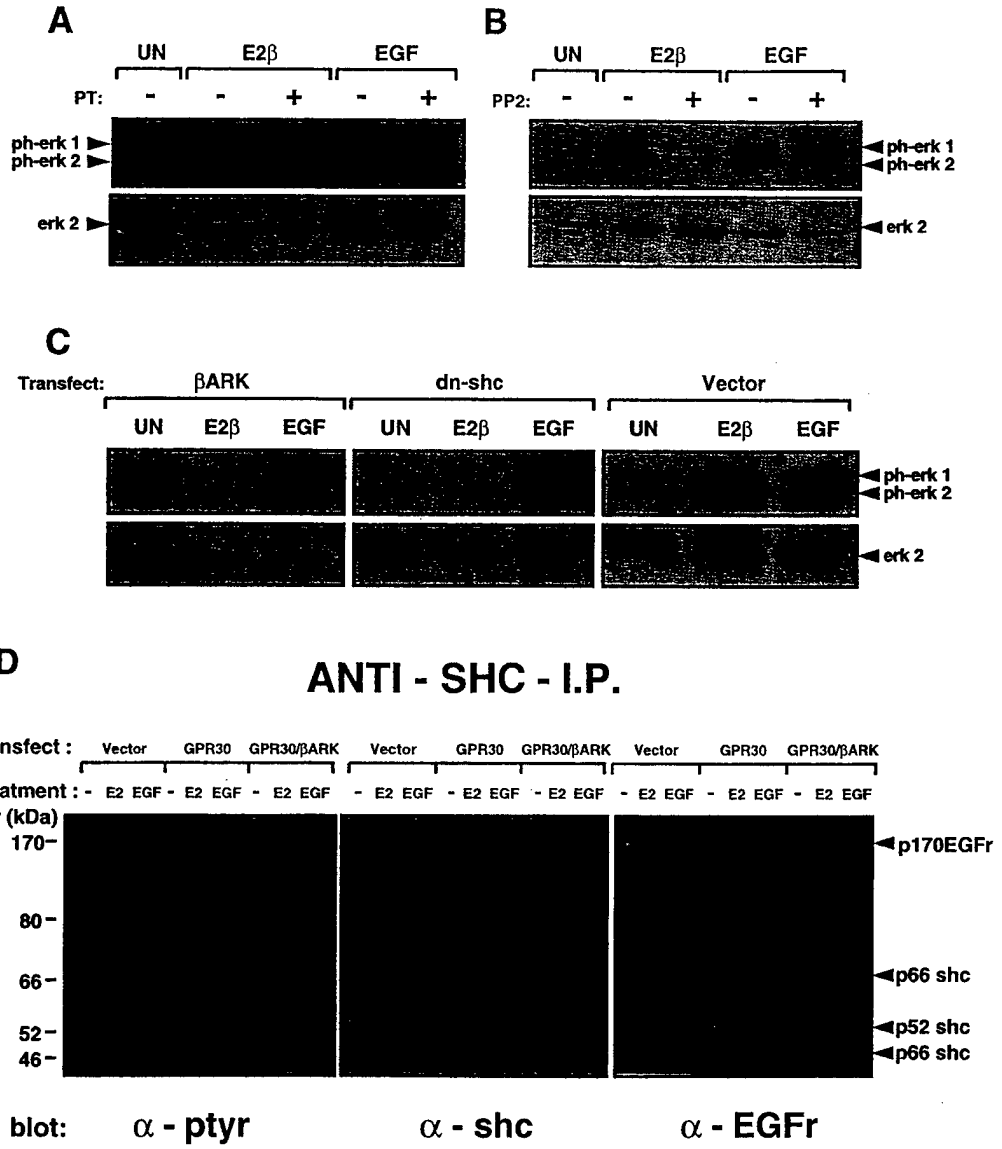


**Fig. 3.** Estrogen-Mediated, GPR30-Dependent Erk-1/-2 Activation Occurs via a Gβγ-Subunit Protein-Src-Shc Pathway and Results in Shc-EGFR Association

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 μM; 15 min), were stimulated with 17β-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 Gβγ sequestrant peptide, βark; dominant negative Shc, GSTShc317Y/F; or control vector, pcDNA3.1Zeo were assessed for their ability to phosphorylate Erk-1 or -2 after stimulation with 17β-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 (GPR30βark) cells, untreated or stimulated for 5 min with 17β-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)

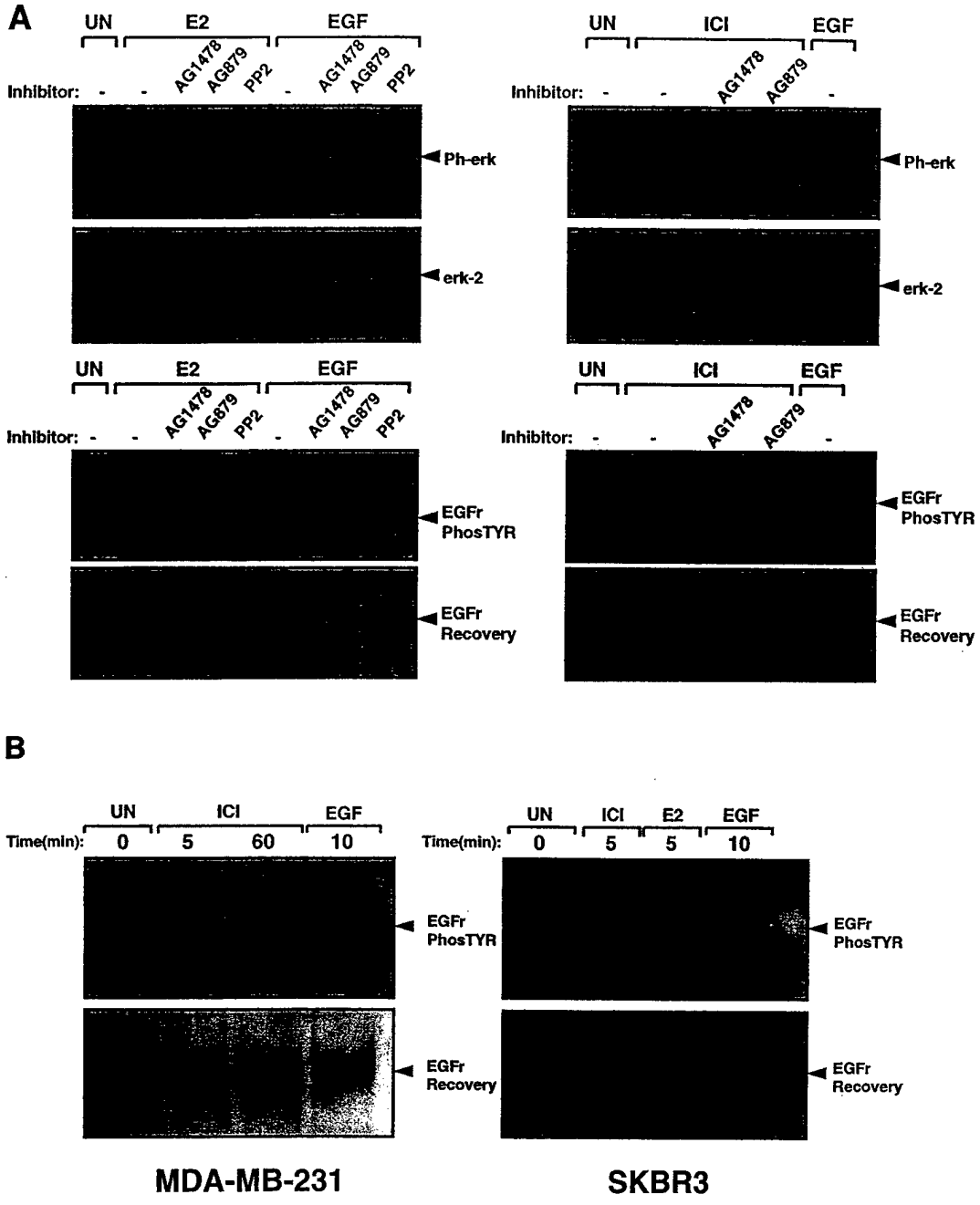


**Fig. 4.** EGF Receptor Kinase Activity Is Required for GPR30-Mediated Erk-1/-2 Activation

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-

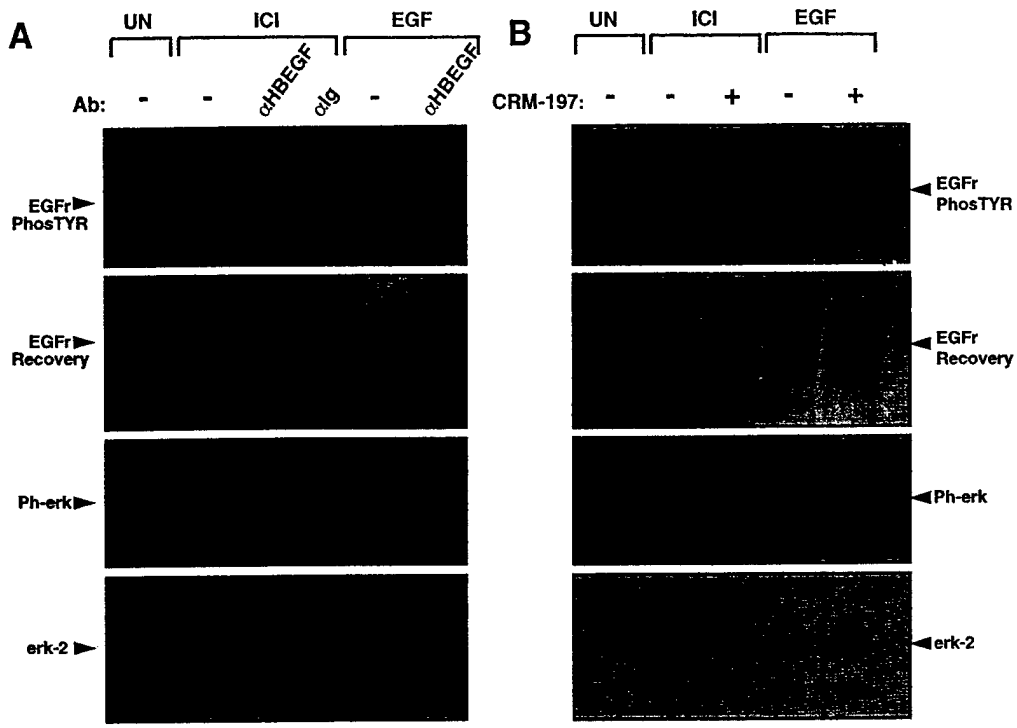


**Fig. 5.** GPR30-Mediated EGFR *Trans*-Activation by Estrogen Requires HB-EGF

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 ERα or ERβ 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 ERα and ERβ, 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 β-adrenergic receptor kinase, βark-1, which is known to function as a Gβγ-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 Gβγ-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 Gαβγ heterotrimers with the same GPCR (40).

Although it has been known for some time that Gβγ 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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# Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer.

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## Abstract

The biological and biochemical effects of estrogen have been ascribed to its known receptors, which function as ligand-inducible transcription factors. However, estrogen also triggers rapid activation of classical second messengers (cAMP, calcium, and inositol triphosphate) and stimulation of intracellular signaling cascades (MAP K, PI3K and eNOS). These latter events are commonly activated by membrane receptors that either possess intrinsic tyrosine kinase activity or couple to heterotrimeric G proteins. We have shown that estrogen transactivates the EGFR-to-MAPK signaling axis via the G-protein-coupled receptor (GPCR), GPR30, through the release of surface-bound proHB-EGF from ER-negative human breast cancer cells (Filardo et al, Molec Endocrinology **14**: 1649-1660, 2000). This finding is consistent with a growing body of evidence suggesting that transactivation of EGFRs by GPCRs is a recurrent theme in cell signaling. GPCR-mediated transactivation of EGFRs by estrogen provides a previously unappreciated mechanism of cross-talk between estrogen and serum growth factors, and explains prior data reporting the EGF-like effects of estrogen. This novel mechanism by which estrogen activates growth factor-dependent signaling and its implications for breast cancer biology are discussed further in this review.

## Introduction

Estrogens induce diverse physiological effects. Their actions are required for normal development and growth of female reproductive tissues and in certain cases, promote the growth of tumors that arise from these tissues. In addition to their impact on female reproductive tissue, estrogens regulate bone integrity (Termine and Wong, 1998), cardiovascular function (Guzzo, 2000) and the central nervous system (Hurn and Macrae, 2000). These physiological and pathophysiological responses are manifested by specific receptors whose identity has important implications for human health and disease. However, the fact that estrogens promote a multitude of biochemical actions, some which occur within seconds, others which are measured over several hours, indicates that more than one class of receptor may participate in estrogen signaling.

The first known receptor for estrogen, termed ER, was described based on its specific binding activity in extracts prepared from rat uterus and vagina (Jenkins and Desombre, 1973). Since then its protein sequence has been determined (Greene et al, 1986) and its

three-dimensional molecular structure resolved (Brzozowski et al, 1997). Based on its homology to receptors for other steroid hormones, the ER is classified as a member of the steroid hormone receptor (SHR) superfamily, which collectively function as hormone-inducible transcription factors (Beato and Klug, 2000). Transcriptional activity of the ER is regulated by allosteric alterations in its structure induced by estrogen and cofactors that associate with the ER. The molecular details concerning *cis* and *trans* regulation of ER functionality have been reviewed elsewhere (Mangelsdorf et al, 1995; Beato et al, 1996). Further complexity regarding ER signaling has been provided by the discovery of ER-related proteins. The first of these to be described, ERβ, was isolated from human prostate tissue and has also been shown to facilitate estrogen-mediated gene transcription (Mosselman et al, 1996). Last year, a third, more distantly related member of the ER family, ERγ, was cloned in teleosts (Hawkins et al, 2000). This newest member of the ER family exhibits an expression pattern distinct from that observed for ERα and ERβ (Hawkins et al, 2000). These findings provide evidence that at least three SHRs may act in concert to promote the effects of estrogen.

It has long been suspected that other receptors, distinct from the ER, may participate in estrogen signaling. This theory is borne from the observation that, in addition to its ability to promote gene transcription, estrogen stimulates classical second messengers, including cAMP (Nakhla et al, 1990; Aronica et al, 1994), inositol phosphate (Le Mallay et al 1997), and calcium (Morley et al, 1992; Audy et al, 1996). More recently, it has been shown that estrogen also triggers signaling cascades typically linked to membrane receptors that possess tyrosine kinase activity or couple to heterotrimeric G proteins, such as mitogen-activated protein kinase (MAP K) (Migliaccio et al, 1996; Endoh et al, 1997; Watters et al, 1997), phosphatidylinositiol 3-OH kinase and AKT/protein kinase B (Chambliss et al, 2000; Haynes et al, 2000; Martin et al, 2000 ). These latter effects of estrogen occur more rapidly (within seconds to minutes) than gene transcription events that are attributed to the ER (over the course of several hours). Moreover, unlike ER-mediated gene transcription, estrogen-induced second messenger signaling is insensitive to inhibitors of gene transcription. Due to the fact that heterotrimeric G proteins have been shown to be required for estrogen-induced second messenger activation, others have proposed that estrrogen may signal via a GPCR (Le Mallay et al, 1999; Nakhla et al, 1999; Benten et al, 2001). Still others have provided evidence that ER-related proteins are associated with rapid estrogen signaling from the plasma membrane and this topic has been reviewed elsewhere (Mendelsohn, 2000; Shaul, 2000).

## Transactivation of the EGFR by estrogen.

*Background and historical perspective.*

Both estrogen and EGF are required for the growth and survival of estrogen responsive tissues. While these extracellular stimuli are structurally distinct they exert physiological effects that overlap. For instance, both estrogen and EGF act as potent mitogens for cells from mammary epithelia and uterine endometrium (Dickson and Lippman, 1998). However, the receptors that mediate the effects of estrogen and EGF utilize seemingly

divergent signaling mechanisms. The proliferative effects of estrogen are primarily mediated by the ER and have been linked to its ability to induce gene transcription in these tissues (Katzenellenbogen and Katzenellenbogen, 1996). In contrast, the biological effects of EGF are transmitted through transmembrane receptor tyrosine kinases (RTKs), known as EGFRs, which signal via their ability to recruit intracellular signaling cascades.

EGFR (erbB1/HER-1) is the prototypical member of a family of four structurally -related RTKs. The other members include: erbB2/HER-2, erbB3/HER-3, and erbB4/HER4 and together they have been shown to play an integral role in the development and growth of the mammary gland and uterus (reviewed in Stern, 2000; Ebert et al, 2000). Individual EGFRs recognize members of a family of small polypeptide ligands that are homologous to EGF (amphiregulin; betacellulin; heparan- bound EGF, HB-EGF; neuregulins; and transforming growth factor alpha, TGF-$\alpha$). The exception to this rule is erbB2/HER-2 for which no known physiological ligand exists. It should be noted that while EGFR ligands are found in serum, they are synthesized as nascent, inactive membrane-anchored precursors that must be cleaved and released by metalloproteinases to generate the active, mature form of the growth factor (Massague and Pandiella, 1993). Upon binding their cognate ligands, EGFRs form homodimers and heterodimers which results in the activation of their intrinsic kinase activity and autophosphorylation of specific tyrosine residues within their cytoplasmic domains (reviewed in Schlessinger, 2000). These phosphotyrosine residues, in turn, serve as nucleation sites for the recruitment of signal transduction complexes. Coupling of these complexes to the activated EGFR is mediated by phosphotyrosine binding motifs, known as SH2 domains. Primary signal transducers may link directly to the activated EGFR, as is the case for phospholipase C$\gamma$. Alternatively, effectors with enzymatic activity may be bridged to the activated EGFRs via adaptor proteins, such as Shc, Grb-2, the p85 subunit of PI3K, and Gab-1. Activated EGFR has been shown to recruit molecular signaling complexes that stimulate MAP K, PI3K, AKT/protein kinase B.

Data showing that estrogen activates intracellular signaling events similar to those activated by EGF suggests these ostensibly divergent signaling mechanisms may cross-communicate. For example, estrogen activates the mitogen-activated protein kinases, Erk-1 and Erk-2, signaling intermediaries that lies downstream of the EGFR. MAP K as a signaling node utilized by estrogen and EGF is supported by prior data that showed that the ability of EGF to augment estrogen-induced cellular proliferation is linked to MAP K-mediated phosphorylation of the ER (Arnold et al, 1995; Kato et al, 1995). More recent work has shown that estrogen also activates a variety of signaling networks that are coupled to EGFRs, including phosphatidylinositol 3-OH, AKT/protein kinase B, and endothelial nitric oxide synthase (Caulin-Glaser et al, 1997; Lantin-Hermoso et al, 1997; Chambliss et al, 2000; Haynes et al, 2000). These findings support earlier work that indicated interplay between estrogen and EGF. *In vivo* administration of estrogen had been shown to upregulate EGFR expression (Das et al, 1994; Yarden et al, 1996). While this was shown to be the consequence of ER-mediated gene transcription, other reports indicated that estrogen could promote rapid EGF-like effects. For example, intrauterine injection of estrogen increased the local concentration of EGF (Di Augustine et al, 1988) and induced tyrosine phosphorylation of the EGFR (Mukku and Stancel, 1985). In this

latter study it was also shown that angiotensin II induced EGFR autophosphorylation *in vitro*. This finding was considered a novelty at this time since it was already known that neither estrogen nor angiotensin II serves as a ligand for the EGFR. Recently, it has been demonstrated that angiotensin II transactivates the EGFR through intracellular signals that are transduced via its GPCR (reviewed in Saito and Berk, 2001). Cross-talk between GPCRs and EGFRs is not unique to angiotensin II. In fact, many ligands that employ GPCRs, namely endothelin, thrombin, carbachol, and lysophosphatidic acid, in part, transmit intracellular signals via their ability to transactivate EGFRs (Prenzel et al, 1999).

Heterotrimeric G proteins have been implicated in second messenger signaling by estrogen (Le Mallay et al, 1999; Filardo et al, 2000; Benten et al, 2001) and thus, GPCRs serve as likely candidates to facilitate estrogen-induced second messenger signaling. GPCRs transduce their signals via G protein heterotrimers ($\alpha\beta\gamma$) that dissociate into free G$\alpha$ subunit protein and G$\beta\gamma$-subunit protein complexes following ligand stimulation (reviewed in Gether, 2000). Classical second messenger signaling is initiated by membrane-associated enzymes and ion channels that are regulated by G$\alpha$-proteins, and for these reasons, they have been implicated in rapid estrogen signaling (Le Mallay et al, 1999; Nakhla et al, 1999; Benten et al 2001). In contrast, GPCR-mediated EGFR transactivation often occurs via G$\beta\gamma$- subunit signaling (Luttrell et al, 1999). In that a single GPCR agonist can simultaneously promote both G$\alpha$- and G$\beta\gamma$-dependent signaling (Crespo et al, 1995), the hypothesis that a GPCR may participate in rapid estrogen signaling is particularly attractive since it provides a singular mechanism by which both second messenger signaling and EGF-like effects occur.

*A role for the G-protein coupled receptor homologue, GPR30, in EGFR cross-talk.*

GPR30, has been cloned by several laboratories (Owman et al, 1996; Carmeci et al, 1997; Feng and Gregor, 1997; Kvingedal and Smeland, 1997; O'Dowd et al, 1997; Takada et al, 1997) and has been referred to as FEG-1, CMKRL2, CEPR, and LyGPR. Its deduced amino acid sequence indicates that it exhibits a serpentine, heptahelical structure that is characteristic of the GPCR superfamily. By structural homology, GPR30 most closely resembles receptors for angiotensin II, chemokines and other peptide ligands. Due to this homology, prior studies suggested that the ligand for this receptor homologue is possibly a peptide. An assortment of chemotactic peptides, including; IL-8, GRO-$\alpha$, MCP-1, MCP-3, MIP-1a, C3a, C5a, RANTES, LTB-4 and other peptide ligands, such as angiotensin II and angiotensin IV, have been screened and shown not to bind to GPR30 (Owman et al, 1996; Feng and Gregor, 1997). GPR30 is widely expressed and its mRNA is found in breast, heart, leukocytes, brain and vascular endothelium (Owman et al, 1996; Carmeci et al, 1997; Feng and Gregor, 1997; Kvingedal and Smeland, 1997; O'Dowd et al, 1997; Takada et al, 1997). These tissues are responsive to the effects of estrogen and it has been noted that this expression pattern is consistent with the ability of GPR30 to function as a hormone or neurotransmitter.

Based on the observations that GPR30 is preferentially expressed in ER positive relative to ER negative breast tumor cell lines (Carmeci et al, 1997) and that inhibitors of G-protein signaling block second messenger signaling by estrogen (Le Mallay et al, 1997;

Nakhla et al, 1999 ), we queried whether GPR30 may participate in rapid signaling by estrogen. We found this possibility particularly intriguing because others had previously demonstrated that estrogen induced adenylyl cyclase activity in MCF-7 breast cancer cells that express GPR30 mRNA but did not stimulate this activity in MDA-MB-231 cells that express little GPR30 mRNA (Aronica et al, 1994). A similar response pattern is observed in these two breast cancer cell lines regarding their ability to activate MAP K following exposure to estrogen. MCF-7 cells undergo estrogen-induced MAP K activation, while MDA-MB-231 cells do not (Filardo et al, 2000). Others have concluded that the ability to trigger estrogen-induced Erk activity in MCF-7 and other cell types is dependent upon ER-like proteins (Migliaccio et al, 1996; Improta-Brears et al, 1999). However, we find that estrogen-induced Erk activation occurs in human SKBR3 breast cancer cells that fail to express either ERα or ERβ (Vladusic et al, 2000) but make GPR30 protein (Filardo et al, 2000). Yet this response is not measured in MDA-MB-231 cells that express ERβ but little GPR30 protein. Upon transfection with a GPR30 cDNA, MDA-MB-231 cells overexpress GPR30 protein and acquire the capacity to promote Erk activation in response to 17β-estradiol. GPR30-dependent Erk activation is also induced by ER antagonists, including ICI 182, 780, but not by 17α-estradiol or progesterone (Filardo et al, 2000). This result provides further evidence that this estrogen action occurs independently of the ER. Moreover, this finding is consistent with data by others demonstrating that pure antiestrogens, such as ICI 164, 384, function as agonists with regards to their ability to stimulate adenylyl cyclase activity in MCF-7 cells (Aronica et al, 1994). In contrast, others have shown that antiestrogens block estrogen -induced Erk activation (Migliaccio et al, 1996; Improta-Brears et al, 1999). One likely source of this discrepancy is the timing between the addition of the antiestrogen and estrogen and the measurement of Erk activity. We find that simultaneous presentation of estrogen and anti-estrogen does not inhibit Erk activation. In contrast, cells previously exposed to anti-estrogen become refractory to other stimuli that activate Erk-1/-2, including estrogen or EGF (Filardo, EJ and Quinn, JA unpublished data).

Unlike RTKs, GPCRs signal to Erk via a number of distinct signaling pathways, some of which require monomeric GTPases, such as Ras or Rap, others activate Raf or Mek directly (reviewed by Luttrell et al, 1997). In some instances, GPCR stimulation leads to the activation of Src-related tyrosine kinases and the assembly of Grb-2/Sos/Shc complexes on the EGFR. In conjunction with the finding that Src can directly phosphorylate the EGFR (Biscardi et al, 1999), these observations suggest the possibility that GPCRs may activate EGFRs via Src-mediated phosphorylation of the EGFR cytoplasmic tail. Consistent with this idea, we have shown that GPR30-dependent, estrogen-induced Erk activation occurs via Gβγ-subunit protein signaling and downstream activation of Src-related tyrosine kinases (Filardo et al, 2000; see figure 1). This is evidenced by the fact that pertussis toxin, Gβγ-subunit sequestrant peptides, and Src-related tyrosine kinase inhibitors all act to blunt estrogen-induced activation of Erk. In contrast, none of these inhibitors adversely affect EGF-stimulated Erk activity in breast cells (Filardo et al, 2000). While this result suggests that Gβγ-subunit proteins and EGFRs utilize distinct mechanisms to stimulate Erk, both signal via Ras-dependent Erk activation (van Biesen et al, 1995). More recent data indicates that the conversion point between Gβγ-subunit protein and EGFR signaling lies upstream of Ras at the level of the

EGFR (reviewed in Luttrell et al, 1999; Prenzel et al, 2000). Recently, it has been shown that some GPCRs mediate trans-phosphorylation of the EGFR and downstream signaling via metalloproteinase-dependent cleavage and release of heparan-bound EGF (Prenzel et al, 1999). Similarly, we have shown that estrogen signaling to Erk is dependent upon transactivation of the EGFR via the release of surface-associated HB-EGF (Filardo et al, 2000). In support of this concept, we find that estrogen signaling to Erk can be blocked by: i) specific inhibitors of EGFR tyrosine kinase, ii) neutralizing HB-EGF antibodies, and iii) downmodulation of pro-HB-EGF from the cell surface by the diphtheria toxin mutant, CRM-197. The fact that these inhibitors completely abrogate estrogen-induced EGFR receptor tyrosine phosphorylation indicates that Src must act upstream of HB-EGF release and can not directly phosphorylate the EGFR. Activation of an HB-EGF autocrine loop via GPR30-dependent, estrogen signaling provides a novel mechanism by which estrogen may promote EGF-like effects.

More than one GPCR may promote rapid estrogen signaling. The membrane receptor for sex hormone binding globulin (SHBG), a plasma protein that binds estrogen, provides one such possibility. Via the SHBG receptor, estrogen promotes Gαs-mediated activation of adenylyl cyclase (reviewed in Rosner et al, 1999). However, the SHBG receptor appears to be distinct from GPR30. First, exogenous SHBG is not required for GPR30-dependent activation of the EGFR-Erk signaling axis (Filardo et al, 2000). Secondly, while antiestrogens promote GPR30-dependent transactivation of the EGFR, they do not function as agonists for SHBG-mediated stimulation of adenylyl cyclase. While these observations indicate that more than one GPCR may participate in rapid estrogen signaling, it is likely that further complexity in estrogen-mediated GPCR signaling may occur due to coupling of different G protein heterotrimers with the same receptor. For example, while Gαq-, Gαs-, and Gαi- coupled receptors signal to Erk-1/-2, they may also promote signals in parallel that are independent of Erk-1/-2 activation (reviewed in Luttrell et al, 1997). For example, estrogen regulates inositol phosphate (Le Mallay et al, 1997), as well as calcium mobilization (Improta-Brears et al, 1999) or influx (Benten et al, 2001). Others have noted that mobilization of intracellular calcium precedes MAP K with no apparent increase in inositol triphosphate in human MCF-7 breast cancer cells (Improta-Brears et al, 1999). We did not measure whether or not calcium was required for estrogen-induced transactivation of the EGFR or downstream activation of Erk. Evidence exists indicating that via its GPCR, angiotensin II, may promote either calcium-dependent (Eguchi et al, 1998) or calcium-independent (Ushio-Fukai et al, 2001) EGFR transactivation in different cell types. Thus, it is important to consider whether GPR30 - mediated EGFR transactivation and second messenger signaling may be cell context specific. The observation that cell activation status promotes differential coupling of heterotrimers to the same receptor (Daaka et al, 1997), further suggests this possibility.

Transactivated EGFRs recruit signaling complexes other than the Ras-to-Erk pathway. For example, the EGFR tyrosine kinase inhibitor, AG-1478, abrogates the ability of lysophosphatidic acid to mediate Gβγ-subunit protein-dependent activation of PI3 K (Daub et al, 1997). In this regard, it is noteworthy that estrogen-induced activation of PI3 K has been observed in HUVECs (Haynes et al, 2000) and that these cells express GPR30 (Takada et al, 1997). Nitric oxide production by HUVECs has been associated

with a signaling pathway that involves PI3 K and AKT -mediated phosphorylation of eNOS (Haynes et al, 2000). It is important to point out, however, that PI3 K does not necessarily lie downstream of the EGFR and that both Gαi- and Gαq-coupled receptors have been shown to signal directly to PI3 K (Hawes et al, 1996; Lopez-Ilasaca et al, 1997). Heterodimerization of EGFRs provides another means to extend the effects of GPCR-mediated transactivation because it is known that each EGFR-family member has unique signaling properties. ErbB3 is a particularly interesting example, since this receptor encodes multiple binding sites for the regulatory subunit of PI3 K and thus, recruits PI3 K efficiently yet this receptor lacks tyrosine kinase activity (Kim et al, 1994). In contrast, canonical binding sites for the regulatory subunit of PI3 K are not found on ErbB1, however, this receptor stimulates PI3 K (Bjorge et al, 1990). Upon presentation with EGF ligands, cells that coexpress ErbB1 and ErbB3, form heterodimers which efficiently recruit PI3 K (Soltoff et al, 1994 ). More recently, it has been appreciated that ErbB1 employs the docking protein Gab-1 to recruit PI3 K and thereby provides a mechanism to recruit this signaling enzyme in cells that lack ErbB3 (Laffargue et al, 1999; Rodrigues et al, 2000). Additionally, the ErbB1 adapter, Shc, recruits PI3 K by assembly of a Shc-Grb2-Gab2-PI3 K complex (Gu et al, 2000 ).

**Significance for breast cancer biology:**

Estrogen induces EGF-like effects *in vivo* (reviewed in Dickson and Lippmann, 1995) and prior data has indicated that the EGFR may be a vehicle for estrogen action. Approximately a decade ago, it was demonstrated that intrauterine administration of estradiol resulted in increased concentrations of EGF (Di Augustine et al, 1988) and EGFR autophosphorylation (Mukku and Stancel, 1985). Further evidence of a relationship between the EGFR and estrogen was provided by data showing that neutralizing antibodies to EGF inhibited estrogen-mediated proliferation in the uterus (Nelson et al, 1991). Our data indicates that estrogen-mediated transactivation of the EGFR occurs independently of the ER and requires GPR30 (Filardo et al, 2000). This novel mechanism of estrogen action may have profound implications with regards to our understanding of the biology and treatment of breast cancer.

Amplification of EGFR-family members is the most common genetic alteration associated with breast cancer. Overexpression of HER-2/neu occurs in approximately one-third of all breast tumors, the majority of which fail to express ER (Press et al, 1990). However, its importance in breast cancer may occur early in disease, as indicated by the fact that elevated HER-2/neu is observed in roughly 60% of all cases of ductal carcinoma *in situ* (DCIS) (Ross and Fletcher, 1998). Since the majority of DCIS occurs in women prior to menopause, it has been speculated that the expansion of precancerous cells is the result of mitogenic coupling between EGF and estrogen. In that overamplified EGFRs undergo ligand-dependent signaling responses this hypothesis is particularly interesting. Especially, in light of the fact that elevated concentrations of EGF ligands and matrix metalloproteinases (Duffy et al, 2000) have been detected in breast cancer. In this regard, it is tempting to speculate that estrogen may promote EGFR transactivation events *in vivo*. Based on the fact that EGFRs extend their signaling capacity due to their ability to

form heterodimers, and that all four EGFRs are expressed in mammary epithelia, GPR30-mediated transactivation of the EGFR may have particular significance for breast cancer. ErbB2 provides an interesting example of possible cross signaling between EGFR members. ErbB2, is an orphan receptor that has no known physiological ligand. However, ErbB2 is strongly activated through its interactions with other EGFRs and it is favored over other heterodimers or homodimers (Tzahar et al, 1996). HER-2 has been shown to increase both the amplitude and duration of MAP K activation by EGF ligands (Graus-Porta et al, 1995). In this regard, recruitment of HER-2 by GPR30 provides a possible mechanism by which estrogen couples to the EGFR-to-MAP K signaling pathway. Frequent detection of elevated levels of phosphorylated MAP K in breast cancer is consistent with the hypothesis that hyperactivation of the EGFR-to-MAP K signaling axis is achieved by both estrogen-dependent and -independent mechanisms (Sivaraman et al, 1997).

Full activation of ER transcriptional activity requires MAP K-mediated phosphorylation at serine118 within the ATF-I domain of the ER. Phosphorylation at this site is promoted by EGF (Arnold et al, 1995; Kato et al, 1995 ) and estradiol (Joel et al, 1995). Prior observations indicate that estrogen promotes, in parallel, second messengers, activation of intracellular signaling enzymes, and gene transcription . Our data indicates that via GPR30, estrogen may, in turn, regulate or "prime" the transcriptional activity of the ER. Constitutive Erk activation achieved as a result of mutation or excessive growth factor stimulation may result in chronic phosphorylation of serine 118 of the ER, thereby facilitating hyperactive estrogen- dependent tumor cell proliferation. In this regard, it is interesting to consider that breast tumors that express low levels of ER, yet maintain GPR30, may maintain some ER function as a consequence of extrinsic or intrinsic events that hyperactivate Erk. EGFR transactivation via GPR30 provides a mechanism by which ER-negative tumors that maintain GPR30 expression may remain responsive to estrogen.

The presence of the estrogen receptor (ER) is the most important parameter in predicting improved disease-free survival and responsiveness to anti-estrogen therapy (Witliff, 1984). These clinical data support research studies that have demonstrated that antiestrogens act as ER antagonists by competitively blocking estrogen binding sites on the ER (Katzenellenbogen et al, 1997). Still, one in four patients with ER-positive tumors do not respond favorably to antiestrogens, while one in six patients with ER-negative tumors undergo objective tumor regression following antiestrogen therapy (Witliff, 1975). These observations indicate an alternative mechanism for estrogen action. Further support for this concept comes from the fact that prolonged tamoxifen use is associated with endometrial hyperplasia (Cano and Hermenegildo. 2000) and that this antiestrogen, and others, also behave as agonists *in vitro* (Aronica et al, 1994; Filardo et al, 2000; Lee et al, 2000; Simoncini and Genazzani, 2000). Our finding that the antiestrogens, tamoxifen and ICI 182, 780 promote GPR30-dependent transactivation of the EGFR is consistent with studies showing that steroids and their antihormones may act through membrane receptors and heterotrimeric G proteins ( Machelon et al, 1996; Ehring et al, 1998; Benten et al, 2001). Future studies will define the role of GPR30 in breast cancer biology. These efforts will also determine its value in refining our ability to predict

responsiveness to antiestrogen therapy and will determine whether GPR30 constitutes a valuable therapeutic target in breast cancer.

## Conclusions.

Estrogen triggers rapid activation of classical second messengers and intracellular signaling events that lie downstream of EGFRs. The recent recognition that GPCRs transduce signals, in parallel, which stimulate second messengers and activate EGFRs, suggests that GPCRs are well-suited as candidates to facilitate nongenomic estrogen signaling. Our data demonstrates that the orphan receptor GPR30 may serve such a role. We have reported that independent of ERα and ERβ, estrogen transactivates the EGFR-to Erk signaling axis via GPR30-dependent activation of an HB-EGF autocrine loop. Our data implies that GPR30 may have particular significance for the growth and survival of estrogen-negative breast tumors. Breast tumors that fail to express ER but maintain GPR30-dependent EGFR transactivation may remain estrogen responsive by employing growth factor dependent intracellular signaling pathways.

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**Figure legend.**

**Figure 1. Transactivation of the EGFR by estrogen.** Via the G-protein coupled receptor, GPR30, estrogen activates Gβγ-subunit/Src family kinase-dependent intracellular signals that promote the release of nascent proHB-EGF from the cell surface. Free, active HB-EGF binds to the EGFR (erbB1) and facilitates receptor dimerization and downstream activation of the mitogen-activated protein kinases, Erk-1 and –2.

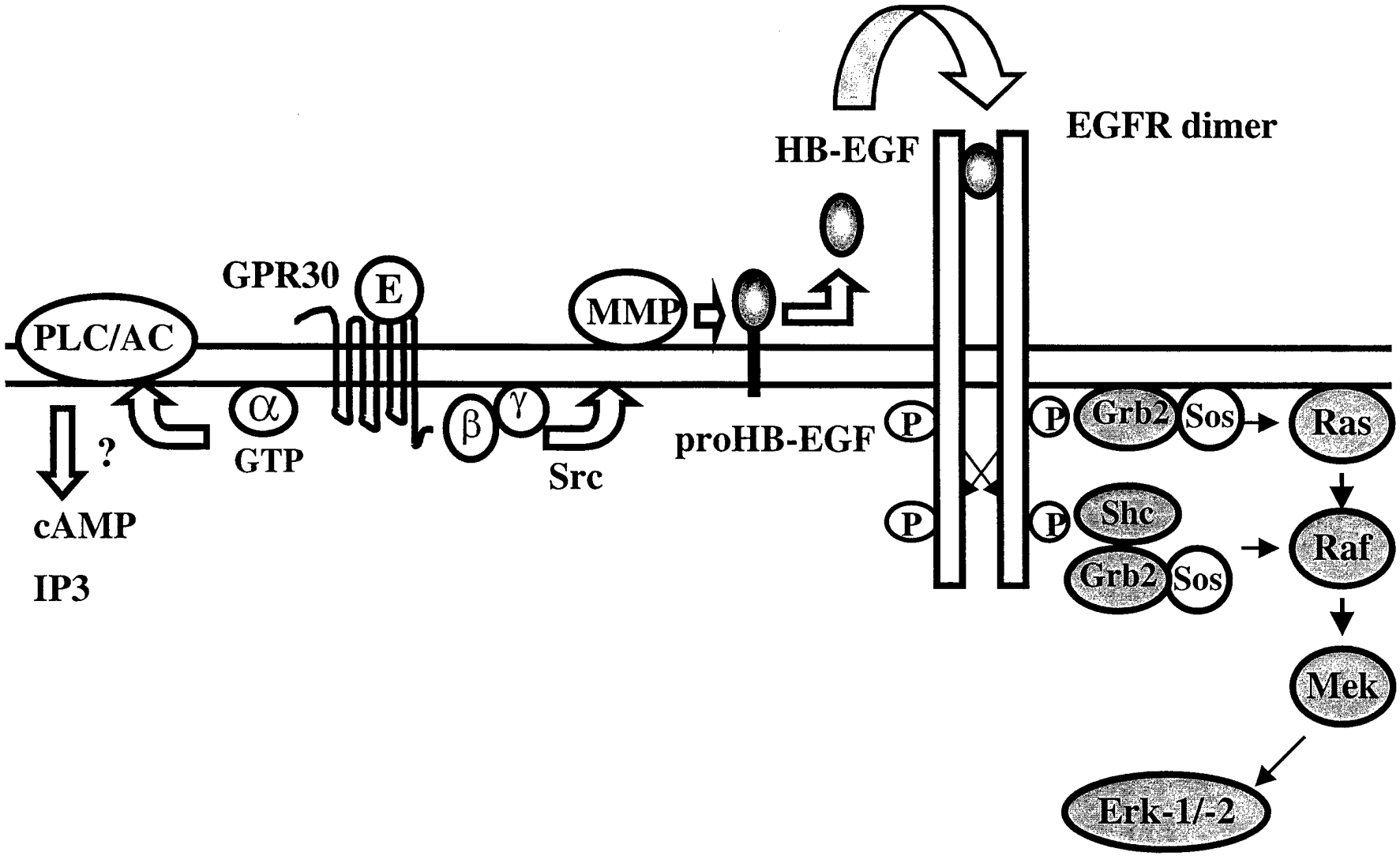


***Figure 1.*** *Transactivation of EGFR-to-MAP K pathway by estrogen.*

# Estrogen action via the G-protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the EGFR-to-MAP K signaling axis.

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Key words: estrogen / orphan receptor / signal transduction

Abbreviations used in this paper: MAP K, mitogen-activated protein kinase; Erk; extracellular signal- regulated kinase; EGF, epidermal growth factor; ER, estrogen receptor; MBP, myelin basic protein; Mek, MAP K/ ERK kinase (same as MAP kinase kinase); GPCR, G-protein coupled receptor; PKA, protein kinase A.

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## ABSTRACT.

Estrogen triggers rapid yet transient activation of the mitogen-activated protein kinases, Erk-1 and Erk-2. We have reported that this estrogen action requires the G-protein coupled receptor, GPR30, and occurs via Gβγ-subunit protein- dependent transactivation of the epidermal growth factor receptor (EGFR) through the release of proHB-EGF from the cell surface (Filardo et al, 2000). Here we investigate the mechanism by which Erk-1/ -2 activity is rapidly restored to basal levels following estrogen stimulation. Evidence is provided that attenuation of Erk-1/ -2 activity by estrogen occurs via GPR30-dependent stimulation of adenylyl cyclase and cAMP-dependent signaling. We show that 17β-estradiol represses epidermal growth factor (EGF)-induced activation of the Raf-to-Erk pathway in human breast carcinoma cells that express GPR30, including MCF-7 and SKBR3 cells which express both or neither, ER, respectively. MDA-MB-231 cells, which express ERβ, but not ERα, and low levels of GPR30 protein, are unable to stimulate adenylyl cyclase or promote estrogen-mediated blockade of EGF-induced activation of Erk-1/-2. Pretreatment of MDA-MB-231 cells with cholera toxin, which ADP-ribosylates and activates Gαs subunit proteins, results in GPCR-independent adenylyl cyclase activity and suppression of EGF-induced Erk-1/-2 activity. Transfection of GPR30 into these cells restores their ability to stimulate adenylyl cyclase and attenuate EGF-induced activation of Erk-1/-2 by estrogen. Moreover, GPR30-dependent, cAMP-mediated attenuation of EGF-induced Erk-1/-2 activity was achieved by ER antagonists such as tamoxifen or ICI 182, 780; yet not by 17α-estradiol or progesterone. Thus, our data delineate a novel mechanism, requiring GPR30 and estrogen, that acts to regulate Erk-1/-2 activity via an inhibitory signal mediated by cAMP. Coupled with our prior findings, these current data imply that estrogen balances Erk-1/-2 activity through a single GPCR via two distinct G-protein-dependent signaling pathways that have opposing effects on the EGFR to MAP K pathway.

# INTRODUCTION

Epidermal growth factor receptor (EGFR) belongs to a family of transmembrane tyrosine kinase receptors (EGFR/erbB1, HER2/erbB2, HER3/erbB3, and HER4/erbB4) that are involved in diverse cellular responses, including proliferation, differentiation and migration (reviewed in Stern, 2000). In general, EGFRs dictate various cellular responses based on their ability to activate intracellular signaling cascades that effect biochemical events necessary to alter cell structure and function. The mitogen-activated protein kinases, p42/44 MAP K (also known as Erk-1/-2 ) are key downstream mediators of EGFR function since they phosphorylate, and thereby, modify the function of numerous proteins which collectively regulate polymerization of the actin cytoskeleton, mobilization of myosin, cell cycle checkpoints, and gene transcription (Chang and Karin, 2001).

Stimulation of the EGFR-to-MAP K pathway is initiated by the specific binding of cognate ligands, such as EGF, TGFα, heregulin and HB-EGF, to specific EGFRs. This interaction results in the formation of EGFR homo- and heterodimers and autophosphorylation of tyrosyl residues within their cytoplasmic domains. Specific recognition of these phosphotyrosines by the adapter proteins Grb-2 and/or Shc, and guanine nucleotide exchange factors, such as Sos, serves to link activated EGFR to MAP K via the monomeric GTPase, p21Ras. Thus activated, Ras is capable of recruiting the serine-threonine kinase Raf-1, which in turn promotes cascade phosphorylation and activation of Mek-1 and its dedicated substrates Erk-1 and Erk-2 (English et al, 1999). Under conditions of normal growth and behavior, activation of the EGFR to MAP K pathway is transient, and attenuated by a variety of control mechanisms, which prevent downstream activation of Erk-1/-2 (Pearson et al, 2001), as well as by phosphatases, which dephosphorylate, and thereby inactivate Erk-1/-2 (Keyse, 2000). In contrast, constitutive activation of the EGFR to MAP K pathway results in dysregulated cellular behaviors associated with carcinogenesis (Marshall, 1995; Klemke et al, 1997).

Several lines of evidence suggest that dysregulation of the EGFR to MAP K pathway may have particular significance for breast carcinogenesis. First, overexpression of the EGFR family

member, HER2, is a common event in breast tumors (Slamon et al, 1989). Second, Erk-1- mediated phosphorylation of serine residue 118 of the estrogen receptor (ER) enhances its gene activation function (Kato et al, 1995; Arnold et al, 1995). Third, estrogen stimulates activation of Erk-1/-2 (Migliaccio et al, 1996; Watters et al, 1997; Endoh et al, 1997; Improta-Brears et al, 1999). Thus, Erk-1/2 may be of particular significance to breast carcinogenesis because hyperactive Erk-1/2 activity initiates dysregulated cellular behaviors exhibited by estrogen- independent tumors; Erk-1/2 may also provide a mechanism whereby hyperactive growth factor signaling may activate estrogen-dependent tumor growth. The association of increased Erk-1/-2 activity with invasive breast cancer suggests this hypothesis (Sivaraman et al, 1997).

Aside from receptor tyrosine kinases (RTKs), as represented by the EGFRs, GPCRs comprise a second major class of transmembrane receptors that signal via Erk-1/-2. Unlike EGFRs, GPCRs activate Erk-1/-2 through several distinct mechanisms, some of which couple via the monomeric GTPases, Ras or Rap, others activate Raf or Mek directly (reviewed in Luttrell et al, 1999). In some instances, GPCR stimulation leads to the activation of Src-related tyrosine kinases and the assembly of Grb-2/Sos/Shc complexes on the cytoplasmic domain of EGFRs (Luttrell et al, 1997). In conjunction with the finding that Src can directly phosphorylate the EGFR (Biscardi et al, 1999), these observations suggest that GPCRs may activate EGFRs via Src-mediated phosphorylation of the EGFR cytoplasmic tail. More recently, ligands for some GPCRs, including endothelin, bombesin, and lysophosphatidic acid have been shown to transactivate the EGFR through their ability to cleave and release surface-associated precursors of EGF-related polypeptides (Prenzel et al, 1999). These findings parallel observations that other receptors that lack intrinsic enzymatic function, such as integrins (Moro et al, 1998) and cytokine receptors (Qiu et al, 1998), also transactivate the EGFR. The fact that may different receptors transactivate the EGFR to MAP K pathway suggests that coordinated signaling is required to regulate the activity of this commonly utilized signaling axis.

Recently, we have shown that the G-protein coupled receptor, GPR30, is required for estrogen-induced activation of the mitogen-activated protein kinases, Erk-1 and Erk-2 (Filardo et al,

2000). This activation response is rapid and occurs via Gβγ-subunit protein- dependent release of surface-associated heparan bound- epidermal growth factor (HB-EGF) and transactivation of the EGF receptor. The Erk-1/-2 activation that we observe is transient, however, rapidly returning to basal levels 10-15 minutes after initial exposure to estrogen. This rapid inactivation of Erk-1/-2 implies the existence of a tightly controlled regulatory mechanism. Others have shown that estrogen (Aronica et al, 1994; Nahkla et al, 1994; Fortunati et al, 1999) also promotes stimulation of adenylyl cyclase activity and production of intracellular cAMP. Since adenylyl cyclases are commonly linked to GPCRs (Sunahara et al, 1996; Schoneberg et al, 1999) and cAMP agonists are potent inhibitors of Erk-1/-2 activity (Cook and McCormick, 1993; Wu et al, 1993), we questioned whether GPR30 may promote restoration of Erk-1/-2 activity to basal levels following estrogen stimulation. Here, we show that GPR30 is required for estrogen-induced stimulation of adenylyl cyclase and cAMP-mediated inhibition of Erk-1/-2. Moreover, we demonstrate that ER antagonists, including the anti-estrogens tamoxifen and ICI 182,780, can also induce these same GPR30-dependent rapid signaling events. Our results suggest that estrogens and anti-estrogens signal via GPR30-mediated stimulation of adenylyl cyclase to inhibit the EGFR to MAP K pathway.

## RESULTS.

### Inhibition of Protein Kinase A Prolongs Estrogen-induced Erk-1 and Erk-2 Activity.

Because the MAP K cascade can be inhibited by cAMP-dependent signaling mechanisms (Cook and McCormick, 1993; Wu et al, 1993), we investigated whether the restoration of Erk-1/ -2 from peak activity levels to basal levels was associated with 17β-estradiol-induced cAMP-dependent inhibition of Erk-1/ -2. To test this possibility, we made use of a human MDA-MB-231 breast carcinoma cell line that had been transfected with GPR30. These cells undergo rapid yet transient Erk-1/-2 activation upon exposure to estrogen, while parental MDA-MB-231 cells that lack GPR30 fail to activate Erk-1/-2 in the presence of estradiol (Filardo et al, 2000). Using GPR30-transfected MDA-MB-231 cells, we determined the effects of a cell permeant cAMP congener that inhibits cAMP-dependent protein kinase A, KT5720, on the kinetics of estrogen-mediated Erk-1/-2 activation. Following stimulation, detergent lysates were prepared and Erk-1/ -2 activity and expression was determined by immunoblotting using phosphorylation state-dependent and –independent antibodies. As previously reported (Filardo et al, 2000), 17β-estradiol-induced a rapid increase in the phosphorylation state of Erk-1 and Erk-2 in these cells. However, as observed in Figure 1, the duration of this response is transient. Increases in Erk-1/-2 phosphorylation were detected as early as 1 minute after exposure to 17β-estradiol. Peak Erk-1/ -2 phosphorylation levels occurred at 5 minutes (three to four fold increase) with Erk-1/ -2 activity returning to baseline levels by 30 to 60 minutes. Cells exposed to KT5720 for two hours exhibited reduced basal levels of Erk-1/ -2 activity relative to untreated control cells. However, following estrogen stimulation, the rate and amplitude of the Erk-1/ -2 activation response in KT5720 pretreated cells was similar to that observed in control cells with peak activity observed within 5 minutes. In contrast to untreated control cells, KT5720-treated cells maintained elevated levels of Erk-1/ -2 activity for an extended period of time (greater than 1 hour) following estrogen stimulation (figure 1). This observation

suggests that activation of cAMP-dependent PKA is required to restore estrogen-induced Erk-1/ -2 activity to basal levels.

**Estrogen-mediated Stimulation of Adenylyl Cyclase Activity is ER-independent and Requires the Expression of GPR30.**

Others have reported that estrogen stimulates intracellular cAMP production through its ability to activate adenylyl cyclase in the plasma membrane (Aronica et al, 1994; Nahkla et al, 1994). Based on the fact that estrogen-induced adenylyl cyclase activity has been measured in MCF-7 breast adenocarcinoma cells that express ERα and ERβ, this rapid action of estrogen has been linked to the ER (Aronica et al, 1994; Rosner et al, 1999). However, the mechanism by which ER stimulates adenylyl cyclase activity is unclear and, in general, adenylyl cyclase activity is regulated by receptors that couple to heterotrimeric G proteins (reviewed in Sunahara et al, 1996). To discriminate between these possibilities, we measured the ability of estrogen to stimulate cAMP production in membranes isolated from human breast cancer cell lines that lack ER protein. We found that membranes prepared from human SKBR3 breast carcinoma cells, that express neither ERα nor ERβ messenger RNA (Vladusic et al, 2000), were capable of producing cAMP in response to17β-estradiol stimulation (figure 2A). However, this activity was not promoted by the isomer, 17α-estradiol. In agreement with the observations of Aronica and colleagues (Aronica et al, 1994), demonstrating that ER antagonists can stimulate adenylyl cyclase activity in MCF-7 membranes, we found that the anti-estrogen ICI 182, 780, also stimulated cAMP production in membranes from SKBR3 cells. In contrast, as had been previously noted by others (Aronica et al, 1994), we found that membranes from MDA-MB-231 cells that express ERβ but not ERα protein (Dotzlaw et al, 1997) did not generate cAMP upon exposure to either 17β-estradiol or ER antagonists (figure 2B). Nevertheless, cholera toxin, an agonist that ADP-ribosylates and directly activates Gαs subunit proteins, stimulated a 15-fold increase in cAMP in MDA-MB-231 membranes, indicating that the MDA-MB-231 membrane preparations retained Gαs proteins capable of activating adenylyl cyclase

(figure 2B). Together these results suggest that estrogen-induced activation of adenylyl cyclase activity occur via an ER-independent mechanism.

Recent data from Rosner and colleagues have shown that estrogen-mediated activation of adenylyl cyclase activity requires Gαs-subunit-protein signaling, implying that a GPCR mechanism may be required for estrogen-induced cAMP production (Rosner et al, 1999). Because our prior study showed that the GPCR homolog, GPR30, can promote estrogen-mediated Erk-1/-2 activation and we have shown here that a cAMP congener prolongs estrogen-mediated Erk-1/-2 activity, membranes prepared from MDA-MB-231 cells expressing GPR30 were tested for their ability to produce cAMP in response to estrogen stimulation. We found that membranes isolated from GPR30-transfected MDA-MB-231 cells supported stimulation of adenylyl cyclase following exposure to either 17β-estradiol, tamoxifen, or ICI 182, 780 (figure 2C). The saturation dose for 17β-estradiol-mediated stimulation of adenylyl cyclase activity was near 1 μM, while sub-nanomolar concentrations of 17β-estradiol showed a half-maximal response. Half-maximal stimulation was achieved with 0.2 μM tamoxifen, and full activation at approximately 1 μM. No increases in cAMP production were observed in MDA-MB-231 (GPR30) membranes treated with 17α-estradiol, an isomer of 17β-estradiol which is unable to support ER function. Similarly, the sex steroid hormone progesterone, failed to elicit cAMP production from MDA-MB-231 (GPR30) membranes. Collectively, these results indicate that GPR30 has a role in promoting estrogen-mediated stimulation of adenylyl cyclase.

**Estrogen represses EGF-induced Erk-1/-2 activation via its ability to generate cAMP via GPR30.**

To further assess the mechanism by which estrogen inhibits Erk-1/-2 activity, we examined the ability of 17β-estradiol to suppress epidermal growth factor (EGF) –induced Erk-1/ -2 phosphorylation. As described previously (Filardo et al, 2000) and shown in Figure 3A, stimulation of quiescent MCF-7 cells (ERα+, ERβ+, GPR30+) with EGF induces substantial (5- 10 fold)

increases in the phosphorylation state, or activity, of Erk-1/ -2 within 15 minutes. Pretreatment of MCF-7 cells with 17β-estradiol for 30 minutes significantly inhibited EGF-induced Erk-1/-2 phosphorylation or activity (Figure 3A). This state of estradiol-induced suppression of EGF-induced Erk-1/ -2 phosphorylation could be measured in cells maintained in 17β-estradiol for as long as 120 minutes prior to EGF stimulation. Reprobing these filters with phosphorylation state-independent Erk-2 antibodies verified that these changes in Erk-1/-2 phosphorylation were not due to changes in Erk-2 protein expression. To address whether the suppressive effect of estrogen on EGF-stimulated Erk-1/-2 activity might be due to a delay of the onset of EGF-induced Erk-1/-2 activation, Erk-1/ -2 phosphorylation was measured in MCF-7 cells which were pretreated with estrogen and then stimulated with EGF for various lengths of time. Basal Erk-1/-2 phosphorylation levels were observed in cells which had been pretreated with 17β-estradiol and subsequently challenged with EGF for any of the time intervals tested (figure 3B), indicating that 17β-estradiol did not delay the onset of EGF-induced Erk-1/-2 activity in these cells. ER-antagonists, 4-hydroxytamoxifen or ICI 182, 780 behaved similarly to 17β-estradiol with regards to their ability to attenuate EGF-induced Erk-1/-2 phosphorylation (data not shown).

To determine whether the estrogen-induced suppressive effect on EGF-induced Erk-1/-2 activity also occurs via activation of cAMP-dependent protein kinase A, MCF-7 cells were incubated with KT5720 prior to exposure to tamoxifen and then stimulated with EGF. KT5720-treatment completely abrogated tamoxifen-mediated attenuation of EGF-induced phosphorylation of Erk –1/ -2 in these cells (figure 4A). No changes were observed in the expression of total Erk-2 protein in response to KT5720, while this treatment abolished tamoxifen-mediated repression of EGF-induced Erk-1/-2 activity (figure 4A). We found that estrogen suppression of EGF-induced Erk-1/-2 was also observed in ER-negative SKBR3 cells (figure 4B). Repression of EGF-induced Erk-1/-2 activity in these cells was achieved by not only 17β-estradiol but also the ER-antagonists tamoxifen and ICI 182, 780 (figure 4B). As was the case for MCF-7 cells (figure 4A), estrogen-mediated repression of EGF-induced Erk-1/-2 activation in SKBR3 cells was similarly sensitive to the cAMP congener, KT5720 (figure 4B). Because KT5720 functions as an inhibitor of cAMP-

dependent protein kinase A (PKA) (Kase et al, 1987), our data suggests that PKA-mediated, cAMP-dependent signalling is necessary for repression of Erk-1/2 activity by estrogens and anti-estrogens. These findings indicate that the ER is not required for this estrogen suppressor activity.

To determine whether GPR30 might promote this estrogen suppressor activity, we compared the effect of estrogen on EGF-induced stimulation of Erk-1/-2 activity in parental MDA-MB-231 or MDA-MB-231 cells forced to overexpress GPR30 protein. Upon exposure to EGF, serum-deprived MDA-MB-231 cells exhibited a 3 to 5 fold increase in Erk-1/-2 phosphorylation and activity (figure 5A). Prior exposure to tamoxifen (figure 5A) or 17β-estradiol (data not shown) did not inhibit EGF-induced stimulation of Erk-1/-2 phosphorylation in these cells. However, exposure of these parental MDA-MB-231 cells to either dibutyrl cAMP or the potent cAMP agonist, cholera toxin resulted in a dramatic reduction of EGF-stimulated Erk-1/-2 activity and phosphorylation (figure 5A). In contrast, GPR30-transfected MDA-MB-231 cells expressed the estrogen suppressor phenotype. These cells exhibited 20-fold less EGF-induced Erk-1/-2 phosphorylation following tamoxifen treatment than mock-transfected MDA-MB-231 cells (figure 6). A similar inhibition of EGF-induced Erk-1/-2 phosphorylation was observed for MDA-MB-231(GPR30) cells treated with 17β-estradiol (data not shown). However, attenuation of EGF-induced Erk-1/-2 phosphorylation was not inhibited in MDA-MB-231 (GPR30) cells exposed to 500 nM of either the inactive 17α-estradiol isomer or progesterone (figure 7). No differences were observed between vector- and GPR30- transfected MDA-MB-231 cells in total Erk-2 protein expression under any of these conditions (figures 6 and 7). Thus, collectively these data suggest that the cAMP-signaling pathway promoting estrogen-mediated repression of Erk-1/-2 is intact in MDA-MB-231 cells, and that these cells are unable to potentiate estrogen suppressor activity due to a defect in the pathway leading to Gαs-subunit protein activation. Overexpression of GPR30 protein reconstitutes the estrogen suppressor phenotype suggesting that GPR30 is required for estrogen-mediated suppression of the EGFR-to-MAP K signaling axis. Moreover, these data provide specificity for the GPR30-dependent responses measured here, and suggest a novel mechanism by which estrogenic hormones can regulate growth factor signaling.

**Attenuation of Estrogen-induced Erk-1 and Erk-2 Activity Does Not Effect EGFR Activation or Internalization.**

We have previously demonstrated that estrogen stimulation of GPR30-expressing breast carcinoma cells results in transactivation of the EGF receptor (EGFR) through release of surface-associated HB-EGF (Filardo et al, 2000). To determine whether attenuation of estrogen-induced Erk-1/-2 activity is associated with a decrease in EGFR activity, EGFR tyrosine phosphorylation was measured in detergent lysates prepared from MDA-MB-231(GPR30) cells which were exposed to estrogen for various periods of time. Significant EGFR tyrosine phosphorylation was observed as early as 3 minutes following exposure to 17β-estradiol (figure 8A). Comparable amounts of tyrosine phosphorylated erbB1/EGFR was observed in samples collected 30 or 60 minutes after estrogen stimulation (figure 8A), even though basal levels of phosphorylated Erk-1/-2 are present at these later time points (figure 1). To further investigate whether restoration of Erk-1/-2 to basal levels of activity following estrogen stimulation may be the consequence of EGF receptor downmodulation, surface expression of erbB1/EGFR was measured following estrogen stimulation (figure 8B). MDA-MB-231(GPR30) cells were treated with 17β-estradiol or EGF, or pretreated with 17β-estradiol for 30 minutes and then exposed to EGF. Following stimulation at 37C, cells were fixed in paraformaldehyde, immunostained with Ab-1, an erbB1/EGFR-specific monoclonal antibody directed against an epitope that maps outside the EGF-binding pocket of the receptor, and analyzed by flow cytometry. As observed in figure 8B, exposure of cells to EGF (100 ng/ml) for 15 minutes resulted in a 50% decrease in surface EGFR. In contrast, less than a 5% decrease in surface EGFR was observed in cells exposed to estrogen for 3, 10, 30, or 60 minutes. Yet, cells which were pre-exposed to 17β-estradiol, internalized 50% of their surface EGFR within 15 minutes subsequent to stimulation with EGF (figure 8B). Taken together these data imply that the restoration of estrogen-induced Erk-1/-2 activity to basal levels observed by 30 minutes following exposure to estrogen is not due to a decrease in EGFR activity or expression, and suggests that the

estrogen-induced blockade of Erk-1/-2 activity occurs downstream of the EGFR.

To further define the mechanism associated with estrogen-mediated repression of EGF-induced Erk-1/-2 activation, we measured the phosphorylation status of Mek-1 and the activity of Raf-1, which serve as intermediate components of the EGFR-to-Erk cascade. EGF stimulation of MDA-MB-231(GPR30) cells induced rapid Mek-1 phosphorylation (figure 8C) and Raf-1 activity (figure 8D). 17β-estradiol stimulation of these cells induced rapid, yet transient, Mek-1 and Raf-1 phosphorylation and activity with a kinetic response that paralleled the activation response observed for estrogen-induced Erk-1/-2 phosphorylation observed in figure 1. Both Raf-1 and Mek-1 activation by 17β-estradiol in this cell background is dependent on GPR30 expression (data not shown). Pretreatment with 17β-estradiol abrogated both EGF-induced Mek-1 phosphorylation (figure 8C) and Raf-1 activation (figure 8D), suggesting that estrogen-mediated repression of EGF-induced Erk-1/-2 activity occurs at, or upstream of Raf-1. Thus, together our data suggest that restoration of Erk-1/-2 activity to basal levels in breast carcinoma cells stimulated by estrogen or growth factor is achieved through GPR30-mediated stimulation of adenylyl cyclase, which suppresses the EGFR-to-Erk pathway through protein kinase A-dependent inhibition of Raf-1 activity. Furthermore, these data imply that breast tumors that fail to express GPR30, or produce mutant variants of this GPCR that are unable to couple to adenylyl cyclase, may no longer be able to effectively regulate the EGFR-to-Erk pathway in response to estrogens or anti-estrogens.

## DISCUSSION.

Estrogen exerts its effects on a diverse array of target tissues. At present, it is uncertain whether all of these effects are mediated by the known estrogen receptors, ERα and ERβ. It has long been appreciated that these ERs belong to the steroid hormone receptor superfamily and function as ligand-activated transcription factors (Beato et al, 1995). Over the past decade, a number of investigators have reported that estrogen (Nakhla et al, 1990; Aronica et al, 1994; Le Mallay et al, 1994; Tesarik and Mendoza, 1995; Migliaccio et al, 1996; Improta-Brears et al, 1999; Benten et al, 2001 ), and other steroid hormones (Lieberherr and Grosse, 1994; Machelon et al, 1996; Falkenstein et al, 1999; Benten et al, 1999), trigger rapid intracellular signaling events typically associated with membrane receptors that possess intrinsic tyrosine kinase activity or couple to heterotrimeric G-proteins. Previously, we have demonstrated that estrogen acts via the GPCR, GPR30, to promote rapid transactivation of the EGFR to MAP K pathway through the release of pro-HB-EGF (Filardo et al, 2000). Here, we show that through GPR30, estrogen stimulates adenylyl cyclase and inhibits Erk-1/-2 activity via a cAMP-dependent mechanism. Together these data demonstrate that estrogen signals via GPR30 to trigger opposing G-protein-dependent signaling mechanisms that act to balance Erk-1/-2 activity. This mechanism of GPCR-Erk-1/-2 regulation is consistent with prior data showing a dual regulatory effect on MAP K by a single β-adrenergic receptor (Crespo et al, 1995).

Here we provide several lines of evidence suggesting that estrogen-mediated activation of adenylyl cyclase occurs independently of known ERs but rather requires GPR30 protein. First, the anti-estrogens, tamoxifen and ICI 182, 780, do not antagonize estrogen-induced activation of adenylyl cyclase but rather act as agonists capable of stimulating adenylyl cyclase activity (figure 2). Second, we show that either antiestrogens or 17β-estradiol are able to promote activation of adenylyl cyclase activity in MCF-7 and SKBR3 human breast cancer cell lines that express both (Dotzlaw et al, 1997) or neither (Vladusic et al, 2000; Filardo et al, 2000 ) ERα and ERβ, respectively, but do express elevated levels of GPR30 protein. Conversely, we find that MDA-MB-231 cells which express ERβ, but not ERα and express only low levels of GPR30

protein are unable to stimulate adenylyl cyclase activity (figure 2) or mediate cAMP-dependent suppression of the EGFR to MAP K pathway (figure 5). However, we do show that MDA-MB-231 cells forced to overexpress GPR30 are able to regulate these activities (figures 2 and 6) in response to estrogen.

A requirement for GPR30 in stimulation of adenylyl cyclase by estrogen is consistent with studies that have implicated GPCRs and G-proteins in rapid membrane signaling events mediated by estrogen (Razandi et al, 1999; Rosner et al, 1999; Benten et al, 2001) and other steroid hormones (Machelon et al, 1996; Benten et al, 1999; Falkenstein et al, 1999 ). Our finding that antiestrogens also promote adenylyl cyclase stimulation has previously been reported by others who demonstrated that ER antagonists, namely tamoxifen and ICI 164, 384, could stimulate this activity and generate intracellular cAMP in human MCF-7 breast cells (Aronica et al, 1994). These investigators also found increased levels of cAMP in the uterus of rats injected with either estrogen or the aforementioned anti-estrogens. In this regard, it is noteworthy that prolonged tamoxifen use in women has been associated with endometrial hyperplasia (Cano and Hermenegildo. 2000) and that intrauterine injection of cholera toxin has been induces estrogen-like growth in the uterus of rats (Stewart and Webster, 1983). Others have provided evidence that estrogen induced stimulation of adenylyl cyclase may occur via a GPCR-dependent mechanism (Fortunati et al, 1999; Rosner et al, 1999). These investigators have shown that sex hormone binding globulin (SHBG), a serum protein that binds circulating estrogen and androgens with high affinity, when unliganded, specifically interacts with a membrane receptor on breast and prostate cancer cells, termed SHBGR. Upon exposure to estrogen or androgens, these preformed SHBG/ SHBGR complexes bind hormone and stimulate adenylyl cyclase activity (Rosner et al, 1999). Although the molecular nature of the SHBG receptor remains unknown, recent data demonstrating that: (i) nonhydrolyzable guanosine triphosphate analogs inhibit SHBG binding and (ii) a dominant negative Gαs subunit protein decreases estrogen-induced, SHBG-dependent cAMP signaling, indicates that this receptor may belong to the GPCR superfamily (Nakhla et al, 1999). While it is possible that GPR30 may serve as a receptor for SHBG, in our experiments, as well as those conducted by others (Aronica et

al, 1994) no exogenous factors are required to initiate estrogen-induced activation of adenylyl cyclase. Furthermore, in contrast to the findings reported for SHBG-mediated estrogen action (Nakhla et al, 1994), we find that GPR30-dependent activation of adenylyl cyclase can also be promoted by the antiestrogens, tamoxifen and ICI 182, 780 (figure 2).

We show that estrogen-mediated repression of EGF- induced activation of the Raf to Erk cascade can be reversed by the cell permeant cAMP congener, KT5720. Because this analogue irreversibly binds to the regulatory subunits of PKA, and thereby prevents its catalytic activation, our data indicate that estrogen mediated suppression of the EGFR-to-MAP K cascade via GPR30 occurs via PKA-dependent signaling. Other hormones and agonists that elevate cAMP are known to oppose activation of the EGFR-to-MAP K cascade in many other cell types. This inhibitory effect requires activation of protein kinase A and results in inhibition of Raf-1 activity (Hafner et al, 1994), however the precise mechanism by which this occurs remains unclear. While direct phosphorylation of Raf-1 by protein kinase A at serine residues 43 (Morrison et al, 1993; Wu et al, 1993) and 621 (Hafner et al, 1994; Mischak et al, 1996) have been proposed to be responsible for this inhibitory effect, others have provided evidence that PKA may act upstream of Raf-1 (Sidovar et al, 2000). Here we show that estrogen inhibits Raf-1 activity (figure 8D) and this is associated with decreased activity of Erk-1/-2 and its activating kinase Mek-1 (figure 8C). While we did not observe significant decreases in EGFR expression or tyrosine phosphorylation (figure 8A and B), the data presented here do not allow us to eliminate the possibility that estrogen is acting in these cells to uncouple signaling components that lie between the EGFR and Raf-1. In other cell types, cAMP agonists are known to promote stimulation of MAP K activity via PKA-induced phosphorylation and activation of the GTPase, Rap-1, which in turn, promotes B-Raf-mediated activation of Mek-1 and Erk-1/-2 (Vossler et al, 1997). A similar Rap-1 dependent mechanism is activated in LNCaP prostatic carcinoma cells in response to agents that elevate cAMP (Chen et al, 1999). We have found that neither dibutyrl cAMP or cholera toxin are capable of inducing rapid activation of Erk-1/-2 in MDA-MB-231 cells (data not shown), indicating that B-Raf-induced activation of Erk-1/-2 does not occur in these cells.

Estrogen-responsive cells employ both serum growth factors and estrogen for their growth and survival. Coordinated signaling between growth factor receptors and estrogen receptors is required for controlled growth and behavior of normal mammary epithelium. The discovery that these distinct extracellular stimuli utilize common intracellular signaling pathways, as exemplified by the EGFR-to-MAP K signaling axis, further emphasizes this concept. Several lines of evidence support the concept that the EGFR-MAP K signaling axis is a common pathway that is regulated by estrogen. EGF-related ligands enhance ER transcriptional activity (Ignar-Trowbridge et al, 1996), and this has been shown to result from MAP K- mediated phosphorylation of serine 118 within the activation function II (ATF-II) domain of the ER (Arnold et al, 1995; Kato et al, 1995). In this regard, these studies indicate that the ER lies downstream of the EGFR-MAP K signaling axis and may enhance ER-dependent cellular growth. Conversely, estrogen has been shown to increase EGFR expression and activity in the uterus (Mukku and Stancel, 1985; Das et al, 1994). However, it is important to note that this response is transient, and ultimately, results in the restoration of EGFR expression to levels observed prior to estrogen stimulation (Yarden et al, 1996). Studies designed to investigate the refractoriness of ER-transfected cells to undergo estrogen-dependent proliferation have demonstrated that EGFR signaling must be silenced for estrogen-dependent proliferation to occur in these cells (Briand et al, 1999). Others have shown that estrogen can inhibit serum-mediated, MAP K-dependent growth of vascular smooth muscle cells (Morey et al, 1997).

While our studies indicate that the GPCR, GPR30, may affect estrogen-mediated regulation of the EGFR-MAP K axis, others have also indicated that the ER may promote activation of MAP K (Migliaccio et al, 1996; Endoh et al, 1997; Watters et al, 1997; Improata-Brears et al, 1999). A novel functional role for the ER in rapid estrogen has also been suggested from studies which have indicated that the ER can engage and promote activation of phosphatidylinositol 3'OH kinase (Simoncini et al, 2000) and protein kinase B/AKT (Haynes et al, 2000). It is noteworthy that these downstream signaling effectors lie downstream of receptor tyrosine kinases, including the EGFR. Although the data presented here and previously (Filardo et al, 2000) strongly suggest that GPR30 participates in the regulation of the EGFR-to-MAP K signaling axis, whether or not

GPR30 acts alone or functions as part of a receptor complex remains to be determined. However, it is worth reiterating that we have demonstrated that estrogen is capable of regulating the EGFR-to-MAP K signaling axis in SKBR3 breast cancer cells that lack ERα as well as ERβ, but express GPR30 (data presented here and in Filardo et al, 2000). It is possible however, in other cell types, GPR30 may form a signaling complex with the ER, or communicate with the ER to promote rapid nongenomic estrogen signaling.

A schematic diagram depicting a likely mechanism by which GPR30 may regulate growth factor receptor and ER signal transduction pathways is shown in figure 9. We have previously shown that estrogenic hormones and GPR30 act to stimulate Gβγ-subunit protein dependent transactivation of the EGFR-to-Erk signaling axis through the release of proHB-EGF (Filardo et al, 2000). Here, we demonstrate that estrogen also stimulates adenylyl cyclase activity and cAMP-dependent protein kinase A-mediated suppression of the EGFR-Erk pathway. Our model outlines a regulatory loop comprised of opposing signals, triggered by estrogen and requiring GPR30, that serve to balance the EGFR-to-Erk pathway. Although our experiments indicate that these opposing mechanisms can be activated by estrogen *in vitro*, our results raise an interesting question regarding which one of these opposing estrogen-induced signals prevails in breast tumors *in vivo*. Amplification of EGFRs is the most common genetic alteration associated with breast cancer, and is detected in 30% of all breast tumors and primarily among those tumors that fail to express ER (Salomon et al, 1989). Likewise, dysregulated expression of MAP K has been reported to be a frequent event in breast cancer (Sivaraman et al, 1997). However, mutations in Ras genes are rarely observed (less than 5% of all breast cancer cases) even though they occur frequently in other carcinomas (Clark and Der, 1995). These data suggest that intermediate components of the EGFR-to-Erk cascade are tightly regulated in normal breast epithelial cells. In this regard, genetic alterations that affect signaling pathways that attenuate the EGFR-MAP K signaling cascade, including loss or mutation of GPR30, may be a common occurrence in breast cancer.

The existence of an alternative membrane- localized G-coupled protein receptor for estrogen would provide a new paradigm by which steroid hormone-activated signals interdigitate

with growth factor-mediated signals to regulate the cellular behavior of steroid hormone responsive cells. Finally, the identification of GPR30 as an important mediator of estrogen action may provide further insight into the molecular mechanisms by which breast carcinomas grow and survive.

# MATERIALS AND METHODS.

## Cell culture.

Human MCF-7 (ERα+, ERβ+), MDA-MB-231 (ERα-, ERβ+), and SKBR3 (ERα-, ERβ-) breast carcinoma cell lines were obtained from the American Tissue Culture Collection (Manassas, VA). MDA-MB-231 (GPR30) cells are stable transfectants expressing GPR30 protein and have been described previously (Filardo et al, 2000) . Both MCF-7 and SKBR3 cells express elevated levels of GPR30 protein relative to MDA-MB-231 cells (Filardo et al, 2000). All cultures were grown in phenol red-free DMEM/ Ham's F12 media (1:1) supplemented with 10% fetal bovine serum and 100 μg/ ml gentamicin. MDA-MB-231 (GPR30) cells were maintained in the same medium supplemented with 500 μg/ml geneticin (Sigma, St. Louis, MO)

## Growth factors, estrogens and anti-estrogens, cAMP agonists and congeners.

Recombinant human EGF was purchased from the Upstate Biotechnology Institute (Lake Placid, NY). Water-soluble 17β-estradiol; its inactive isomer, 17α-estradiol; progesterone; 4-hydroxytamoxifen; and cholera toxin were purchased from Sigma (St. Louis, MO). The pure ER antagonist, ICI 182, 780 was obtained from Tocris Chemicals (Ballwin, MN). Dibutyrl-cAMP was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN) and the cell permeant cAMP congener, KT5720 from Calbiochem (La Jolla, CA).

## Antibodies.

The p42/44 MAPK antibody which recognizes total Erk-1 and Erk-2 protein (phosphorylation state- independent) and phospho-specific antibodies which recognize either phosphorylated Erk -1 and -2 (phospho-Erk), or phosphorylated Mek- 1 (phospho-Mek) were purchased from New England Biolabs, now Cell Signaling Technologies, Inc (Beverly, MA). The Erk-2 antibodies were also purchased from the same vendor and are also known to cross react with Erk-1. Monoclonal antibodies Ab-1 (Calbiochem, La Jolla, CA) and 29.1 (Sigma, St. Louis, MO) recognize the

ErbB1/EGFR receptor and do not cross react with ErbB2 (Her-2/Neu), ErbB3, or ErbB4. mAB 29.1 recognizes an epitope external to the ligand binding domain of the EGFR and does not interfere with EGF binding. The phosphotyrosine-specific mAB, PY20, was purchased from Transduction Laboratories, Inc., Lexington, KY). Raf-1 (C-12) antibodies raised against a peptide from the carboxyl terminus of the human Raf-1 protein were purchased from Santa Cruz Biotechnology ( Santa Cruz, CA).

**Conditions for cell stimulation.**

Breast carcinoma cells were seeded onto 90 mm Falcon tissue culture dishes in phenol-red free DMEM/ F12 medium containing 10% fetal calf serum. The following day, the cell monolayers were washed three times with phenol-red free, serum free DMEM/ F12, and exchanged for fresh phenol-red free, serum free media on each of the following three days. Stimulations of quiescent cells were carried out at 37C in serum-free medium as described in the figure legends. After stimulation, monolayers were washed twice with ice cold phosphate-buffered saline, and lysed in ice-cold RIPA buffer (150 mM NaCl, 100 mM Tris, pH 7.5, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 3.5 mM $NaVO_4$, 2 mM PMSF, 50 mM NaF, 100 mM sodium pyrophosphate plus a protease inhibitor cocktail; Complete™, Boehringer Mannheim, Indianapolis, IN). Crude lysates were clarified by centrifugation and cellular protein concentration was determined using the bichichoninic acid method according to manufacturer's suggestions (Pierce Biochemicals Inc., Rockford, IL). Detergent lysates were stores at –70 C until use.

**Western Blotting.**

Total cellular protein (50 μg) was boiled in standard Laemmli buffer with reducing agents and resolved by SDS-polyacrylamide gel electrophoresis. Proteins were electrotransferred onto nitrocellulose membranes (0.45 μM pore size; Schleicher and Schuell, Keene, NH) using a semi-dry transfer cell (CBS, Del Mar, CA) at 1 $mA/cm^2$ for 4 h. Phospho-Erk was detected by probing membranes, which were preblocked in Tris-buffered saline containing 0.1% Tween-20 and 2%

BSA (TBST-BSA), with phospho-Erk-specific antibodies diluted 1: 1,000 in TBST-BSA for 1 hour at room temperature. Rabbit antibody- antigen complexes were detected with horseradishperoxidase-coupled goat antibodies to rabbit IgG diluted 1: 5,000 in TBST-BSA and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL). Relative levels of total Erk-2 protein in each sample were determined by stripping the phospho-specific Erk rabbit antibodies from the nitrocellulose membrane and reprobing with antibodies to Erk-2. Phosphorylated Mek-1 protein was detected in much the same manner, except that filters to be probed with Phospho-Mek antibodies were blocked in TBST containing 5% nonfat dry milk and antibodies were delivered overnight in TBST-BSA. Apparent molecular weights were determined from Rainbow molecular weight standards (Amersham).

**Adenylyl cyclase activity.**

Cells (50 x $10^6$) were homogenized in 20 ml of 10 mM Tris-HCl, pH 7.4, 5 mM EDTA samples were sonicated, and sedimented twice (1,000 x g for 5 minutes and 40,000 x g for 20 minutes). The membrane pellet was resuspended at a final concentration of 3-5 mg/ ml in 75 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 mM $MgCl_2$ and stored at - 80C. 10 μg of membrane protein were added to reactions containing 1 mM ATP, 50 nM GTP, 0.2 IU pyruvate kinase, 0.1 IU myokinase, 2.5 mM phosphoenolpyruvate and 1.0 mM isobutylmethylxanthine, and treated with 17β-estradiol, 17α-estradiol, progesterone, 4-hydroxytamoxifen, or cholera toxin for 20 minutes at 37C. Reactions were terminated by precipitating the samples with ice-cold ethanol. Supernatants were dried and cAMP was measured in a competitive ELISA using rabbit cAMP-specific antisera (Cayman Biochemicals, Ann Arbor, MI).

**Detection of Erk-1/-2 and Raf-1 activity**

Erk-1/-2 activity was measured by standard immune complex assay utilizing myelin basic protein (MBP) as a substrate. Erk -1 and -2 were immunopurified from 500 μg of lysate using 2 μg/sample of p42/44 MAP K antibody plus 50 μl of a 50% slurry of protein G-agarose (Pierce Chemicals,

Rockford, IL). Erk immunoprecipitates were washed twice in 50 mM Hepes, pH 7.9, 100 mM NaCl and then resuspended in immune complex kinase buffer : 25 mM Hepes, pH 7.9, 1 mM DTT, 10 mM cold ATP and 50 μM 32Pγ-ATP (0.25 μCi) and 8 μg MBP (Upstate Biotechnology Institute, Lake Placid, NY). Following a 30 minute incubation at 30C, samples were boiled in standard Laemmli buffer and subjected to SDS-polyacrylamide gel electrophoresis. Gels were dried and exposed to Kodak XAR film for autoradiography. Raf-1 activity using a kinase cascade assay kit, essentially as described by the manufacturer (Upstate Biotech, Lake Placid, NY). Raf-1 was immunoprecipitated from 500 μg of lysate using 2 μg/sample of Raf-1 antibody plus 50 μl of a 50% slurry of protein G-agarose. Raf-1 immunoprecipitates were washed three times in assay dilution buffer ( 20 mM MOPS, pH 7.2; 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol) and then resuspended in the same buffer containing 1 mM ATP, 75 mM $MgCl_2$ and 0.4 μg of unactivated (unphosphorylated) Gst-Mek1 protein. Following a 30 minute incubation at 30C, 1.0 μg of unactivated (unphosphorylated) Gst-Erk2 was added to this kinase reaction and incubated an additional 30 minutes at the same temperature. The reaction was terminated by the addition of boiling standard Laemmli buffer. Products of the reaction were separated by SDS-gel electrophoresis and phosphorylated GST-Erk2 was detected by immunoblotting using phosphoErk specific antibodies as described above.

**Detection of Phosphotyrosyl residues on the EGFR.**

Tyrosine phosphorylation of the EGFR was assessed by immunoblotting EGFR immunoprecipitates with phosphotyrosine specific antibodies. EGFR was immunoprecipitated from 250 μg of total cell protein, extracted in RIPA buffer using 2 μg/ sample of the ErbB1-specific mAB, Ab-1. EGFR-Ab-1 complexes were precipitated with 50 μl of a 50% slurry of protein G-agarose (Pierce Chemical Co). EGFR immunoprecipitates were washed, resuspended in standard Laemmli buffer containing reducing agents and subjected to SDS-PAGE. Following electrophoresis, the immunoprecipitated material was then transferred to nitrocellulose membranes, blocked with TBST-BSA and then immunoblotted with the phosphotyrosine-specific mAB, PY20.

**EGF receptor internalization.**

Serum-deprived MDA-MB-231 (GPR30) cells were detached in Hepes-buffered saline containing 5 mM EDTA, washed twice in phenol red-free DMEM/F12 containing 0.5% BSA and resuspended at a concentration of $10^6$/ml in the same buffer in the absence of BSA. One million cells were aliquoted into flow cytometry tubes and allowed to equilibrate to 37C in a water bath for 15 minutes. Samples were either untreated, exposed to 1 nM 17β-estradiol, or 10 ng/ml of EGF for various lengths of time at 37C. Following stimulation, cells were fixed by adding an equal volume of 8% paraformaldehyde to each sample. Cells were collected by centrifugation, washed twice in PBS-containing 0.5% BSA (PBS-BSA) and resuspended in the same. Fixed cells were incubated with 5 μg/ml EGFR mAB 29.1 for 30 minutes at room temperature. Cells were then washed twice in PBS-BSA, resuspended in the same buffer containing a 1:250 dilution of fluorescein isothiocyanate-conjugated anti- mouse IgG antibodies and incubated for 30 minutes at room temperature. Cells were then centrifuged, washed and surface expression was assessed by flow cytometry using a FACScan instrument.

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## LEGENDS.

**Figure 1. Inhibition of protein kinase A activation results in prolonged estrogen mediated activation of Erk-1/-2.** Serum-deprived human MCF-7 breast adenocarcinoma cells were pretreated with the cAMP congener, KT5270, or vehicle prior to stimulation with 1 nM 17β-estradiol (17β-E2) for the indicated lengths of time (minutes) and then lysed in detergent. 50 μg of protein from each detergent lysate was electrophoresed through 15% reducing 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 position of phosphorylated Erk-1/-2 protein or total Erk-2 protein are indicated at left. The data shown are representative of at least three independent experiments.

**Figure 2. Estrogen stimulation of adenylyl cyclase activity is ER-independent and requires the expression of the G-protein coupled receptor homologue, GPR30.** Adenylyl cyclase activity was determined from membranes prepared from SKBR3 as well as vector- or GPR30-transfected MDA-MB-231 human breast cancer cells which were stimulated with either cholera toxin (CT) (1 μg/ ml) or various concentrations of 17β-estradiol, 17α-estradiol, 4-hydroxy-tamoxifen, ICI 182, 780, or progesterone. The y-axis values are on a linear scale and represent pmoles of cAMP generated per mg of membrane protein per minute. The x-axis values are expressed on a logarithmic scale as the molar concentration of hormone. Each data point represents the mean $\pm$ the standard deviation of quadruplicate samples.

**Figure 3. Attenuation of EGF-induced Erk-1/-2 activity by estrogen.** Phospho-Erk expression was determined in serum-deprived MCF-7 cells that were exposed to estrogen prior to EGF-stimulation. (A) Cells were pretreated with 1 nM 17β-estradiol for various lengths of time (0 to 120 minutes) and then stimulated with 100 ng/ml EGF for 15 minutes and lysed in detergent. (B) Cells were pretreated with 1 nM 17β-estradiol for 30 minutes and then stimulated with 100 ng/ml of EGF for various lengths of time (1 to 60 minutes) and then extracted in detergent. Expression of phosphorylated Erk-1/-2 or total Erk-2 protein was determined as described in figure 1.

**Figure 4. Attenuation of EGF-induced Erk-1/-2 activity by estrogens or anti-estrogens is abrogated by the cAMP congener, KT5720.** Following a one hour exposure to KT5720 (10 μM) or vehicle (DMSO), MCF-7 cells (A) or SKBR3 cells (B) were treated with 17β-estradiol (1 nM), 4-hydroxy-tamoxifen (1 μM), or ICI 182, 780 (1 μM) for 30 minutes and then stimulated

with EGF (100 ng/ml; 15 minutes). Detergent extracts were prepared and the expression of phosphorylated Erk -1/-2 or total Erk-2 protein were determined as described previously. *Below,* Band intensities from this experiment were quantified using NIH Image software. Results were normalized to total Erk-2 expression in each sample and plotted as Arbitrary units.

**Figure 5. Tamoxifen-mediated attenuation of EGF-induced phosphorylation of Erk-1/-2 does not occur in MDA-MB-231 breast carcinoma cells.** MDA-MB-231 (ERα- ERβ+) breast carcinoma cells were pretreated with either 1 μm 4-hydroxytamoxifen (Tam), 1 μg/ml cholera toxin (CT) or 1 mM dibutyrl cAMP (dB) for 1 hour, stimulated with 100 ng/ ml EGF for 15 minutes and detergent lysates were prepared. Expression of phospho-Erk - 1/-2 and total Erk -2 protein was determined as previously described. Erk-1/-2 activity was measured from these lysates by standard immune complex kinase assay using myelin basic protein (MBP) as an exogenous substrate.

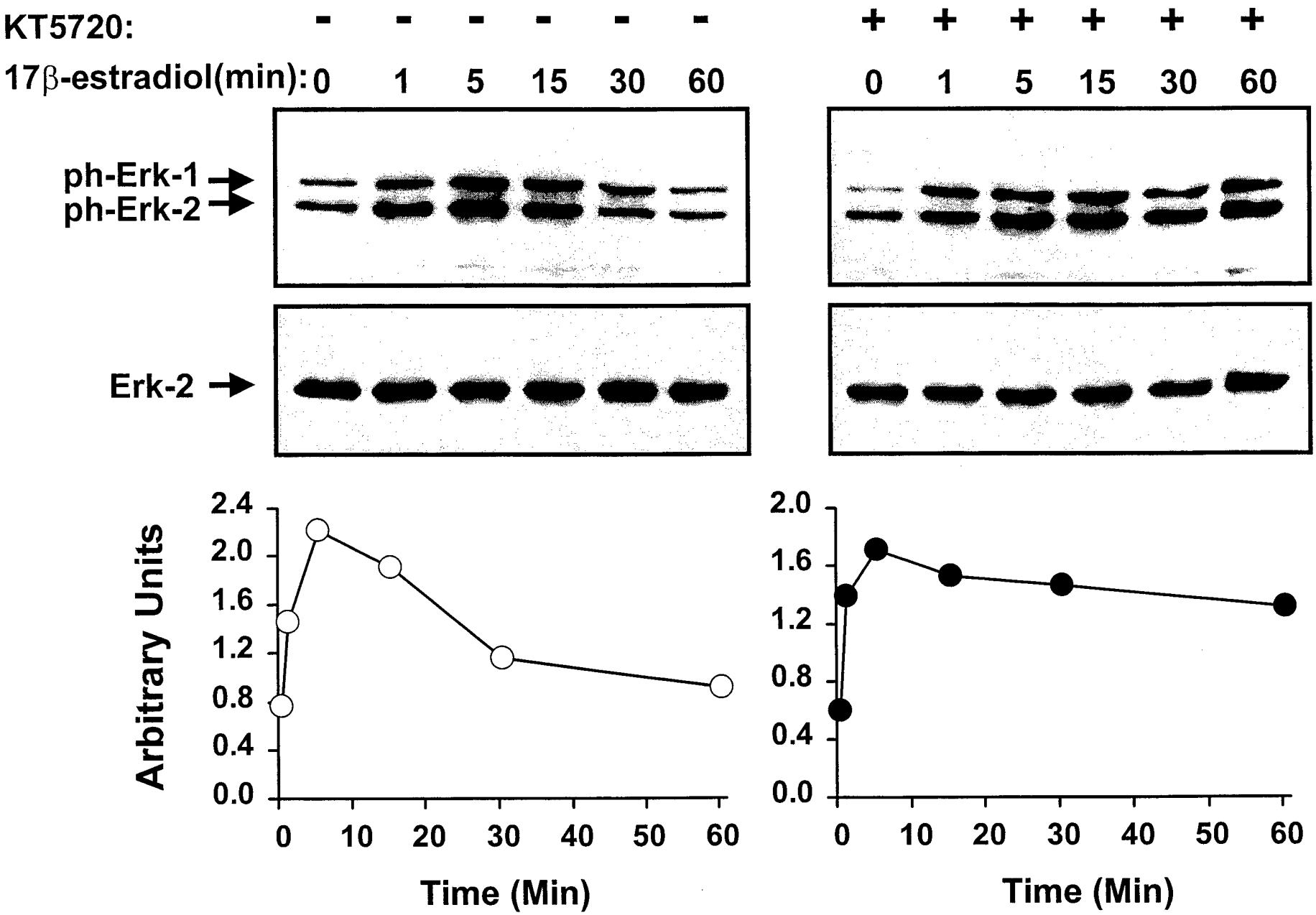
**Figure 6. Expression of GPR30 in MDA-MB-231 breast carcinoma cells restores estrogen-mediated repression of EGF-induced Erk-1/-2 phosphorylation.** Detergent lysates were prepared from vector- or GPR30-transfected MDA-MB-231 cells that were unstimulated, EGF-stimulated or pretreated with 1 μM 4-hydroxytamoxifen (Tam) for 1 hour prior to EGF stimulation. 50 μg of cellular protein was electrophoresed through SDS-polyacrylamide and transferred to nitrocellulose. Phosphorylated Erk-1/-2 proteins were detected by immunoblotting with phospho-Erk specific antibodies. The membrane was then stripped and reblotted with antibodies that detect total Erk-2 protein.

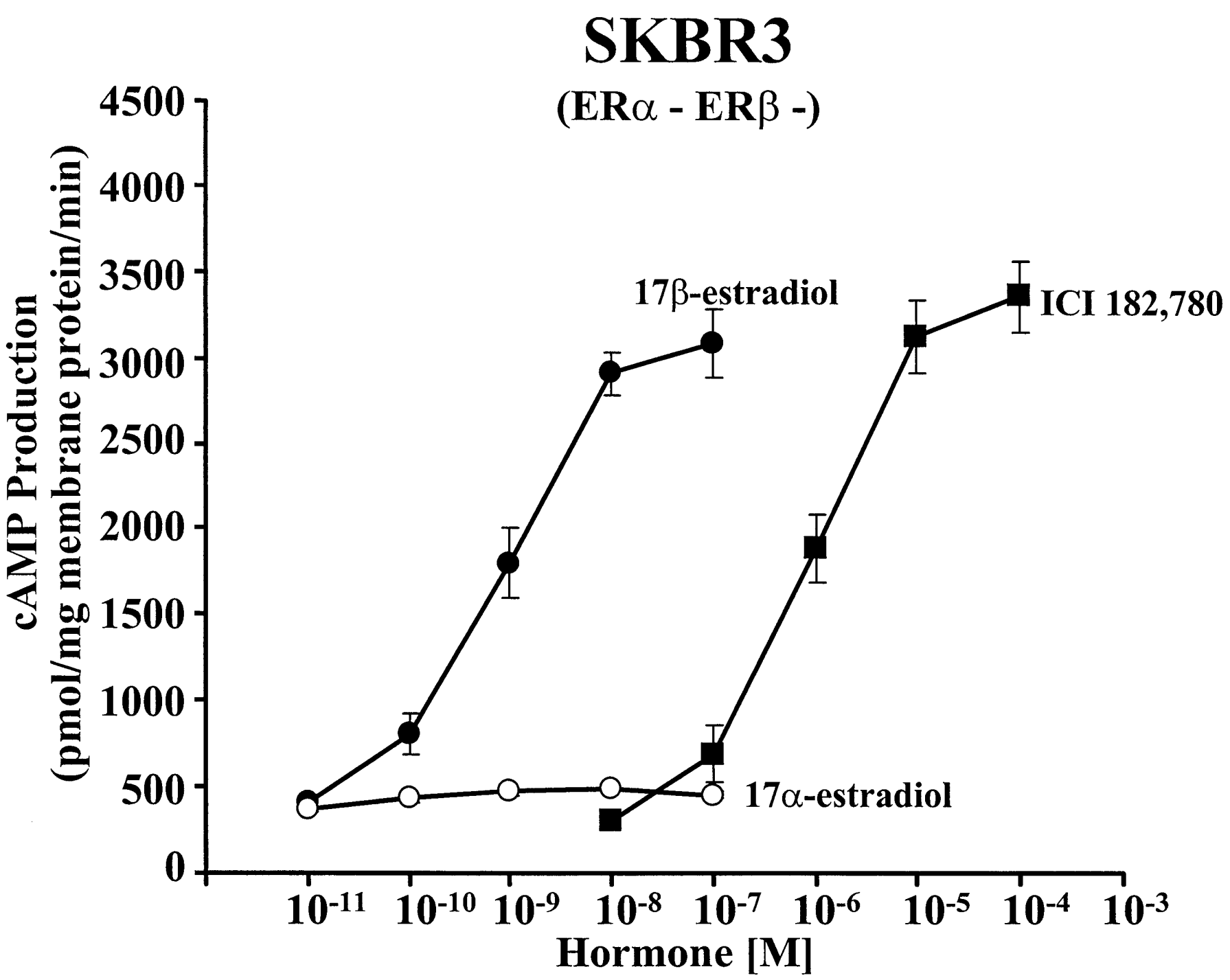
**Figure 7. GPR30-dependent attenuation of EGF-induced activation of Erk-1/-2 does not occur in cells treated with 17α-estradiol or progesterone.** Expression of phospho-Erk or total Erk - 2 protein was measured in detergent lysates prepared from GPR30-transfected MDA-MB-231 breast carcinoma cells which were pretreated with either 17β-estradiol (E2β), 17α-estradiol (E2α), or progesterone (Prog) for 1 hour prior to stimulation with 100 ng/ml EGF for 15 minutes.

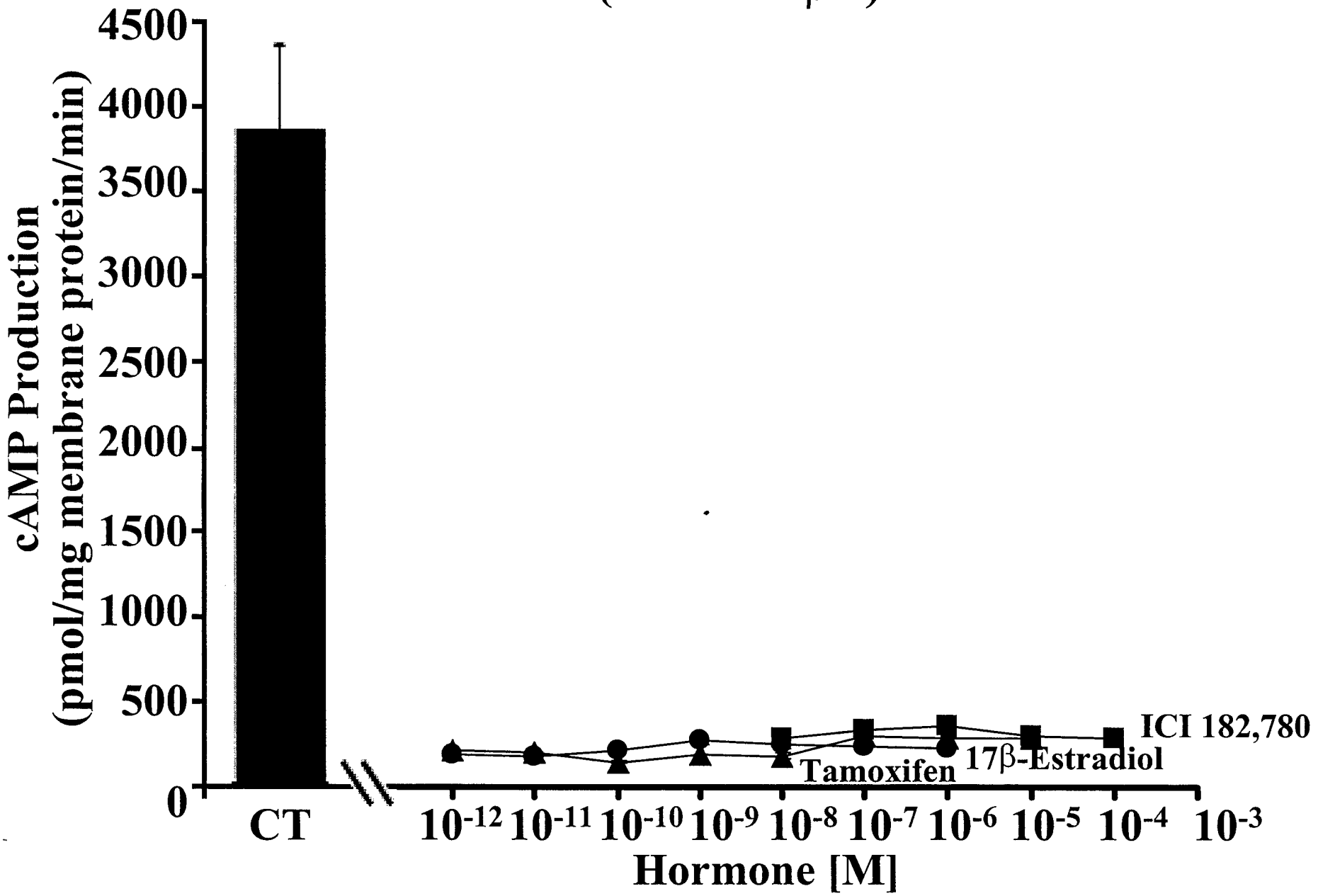
**Figure 8. Inhibition of EGF-induced Erk-1/-2 activity by estrogen occurs at the level of Raf-1.** (A) Serum-deprived MDA-MB-231(GPR30) cells which were untreated or stimulated with EGF or 17β-estradiol for the indicated lengths of time (minutes) were lysed in detergent. After immunoprecipitation with the ErbB1-specific mAB, Ab-1, tyrosine- phosphorylated EGFR was detected by immunoblotting with the phosphotyrosine-specific antibody, PY20. EGFR recovery was assessed by stripping this nitrocellulose membrane and reprobing with sheep anti-EGFR

antibodies. (B) EGFR surface expression was assessed by flow cytometry using ErbB1- specific antibodies in MDA-MB-231 (GPR30) cells which were untreated, exposed to EGF, or pretreated with 17β-estradiol prior to EGF stimulation. Cells were then fixed in paraformaldehyde and immunostained with the ErbB1-specific mAB, 29.1, which reacts with an epitope external to the EGF-ligand binding domain on the receptor. Activity of Mek-1 (C) or Raf-1 (D) were measured in detergent lysates prepared from MDA-MB-231 (GPR30) cells which were untreated, EGF stimulated or pretreated with 17β-estradiol prior to EGF stimulation. Mek-1 activity was determined from 50 μg of total cellular protein by probing immunoblots with phospho-Mek-specific antibodies. Raf-1 activity was assessed in a cascade assay using immunopurified Raf-1, GST-Mek-1 and GST-Erk-1. Erk-1 phosphorylation was detected using phospho-specific Erk-1/-2 antibodies.

**Figure 9. Proposed mechanism by which estrogen acts via GPR30 to regulate growth factor receptor and ER signal transduction pathways.** Data presented here suggests that via GPR30, estrogens as well as anti-estrogens are capable of stimulating adenylyl cyclase activity, which in turn, leads to protein kinase A-mediated suppression of EGF-induced Erk-1/-2 activity. Previously, we have shown that estrogen and anti-estrogens act via GPR30 to promote EGFR transactivation through a Gβγ-subunit protein pathway that promotes Src-mediated, metalloproteinase (MMP)-dependent cleavage and release of HB-EGF from the cell surface. Thus, via GPR30, estrogen may balance Erk-1/-2 activity by stimulating two distinct G-protein signaling pathways which have opposing effects on the EGFR-to-MAPK axis.

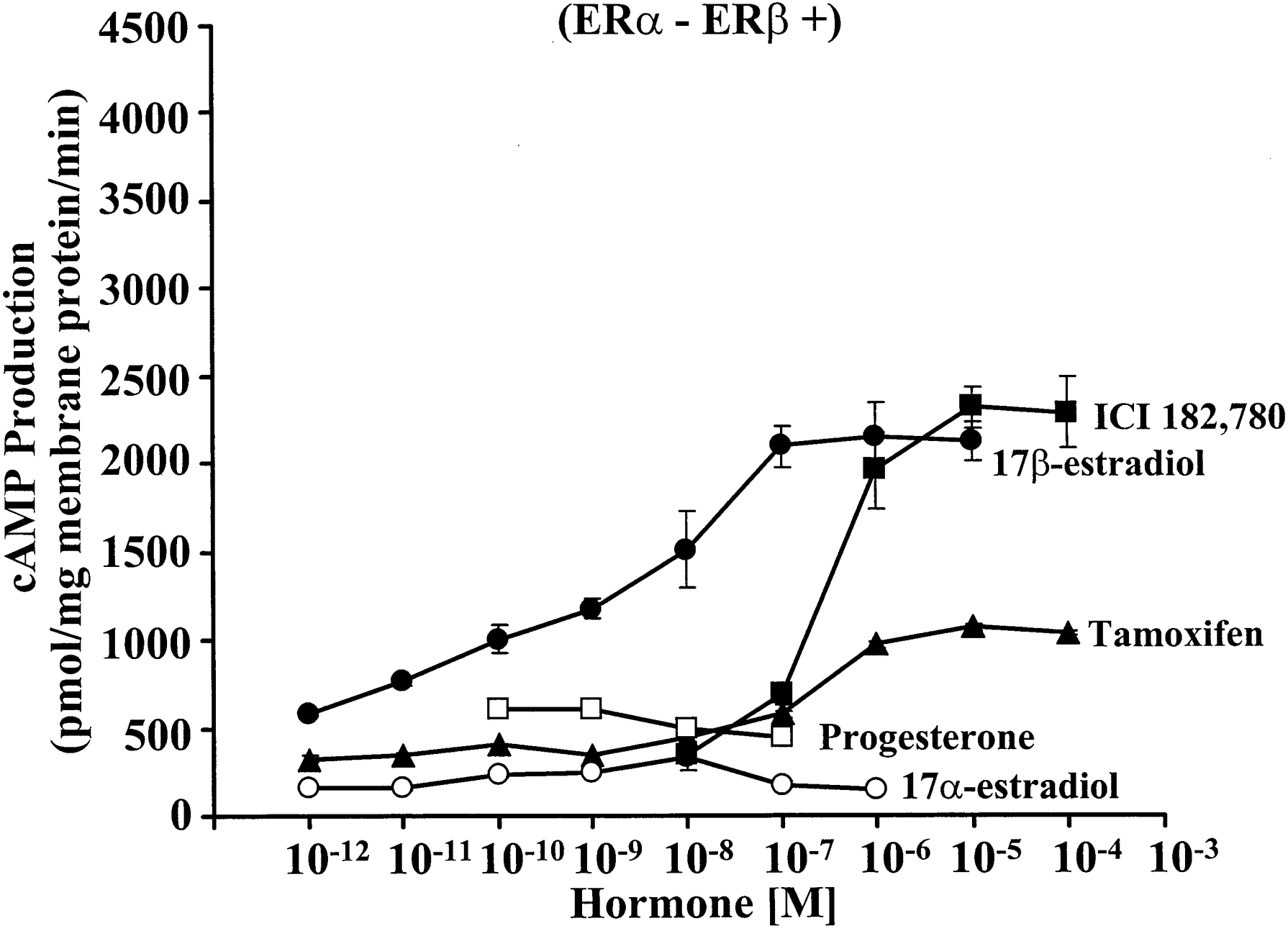

KT5720:
- - - - - -
+ + + + + +
17β-estradiol(min): 0 1 5 15 30 60
0 1 5 15 30 60
ph-Erk-1
ph-Erk-2
Erk-2
Arbitrary Units
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0 10 20 30 40 50 60
Time (Min)
2.0
1.6
1.2
0.8
0.4
0.0
0 10 20 30 40 50 60
Time (Min)

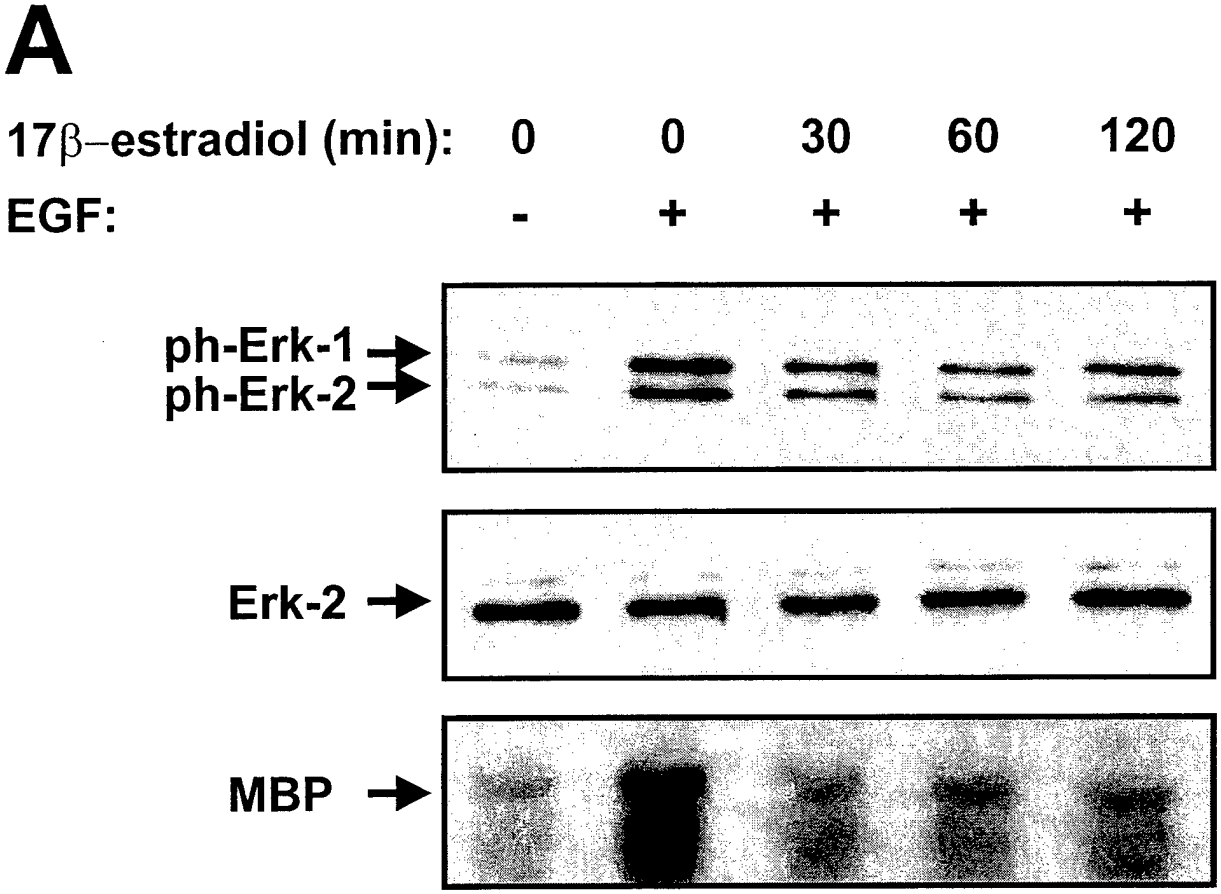

SKBR3
(ERα - ERβ -)
cAMP Production
(pmol/mg membrane protein/min)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
17β-estradiol
ICI 182,780
17α-estradiol
10-11 10-10 10-9 10-8 10-7 10-6 10-5 10-4 10-3
Hormone [M]


MDA-MB-231
(ERα - ERβ +)
cAMP Production
(pmol/mg membrane protein/min)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
CT
10-12 10-11 10-10 10-9 10-8 10-7 10-6 10-5 10-4 10-3
Hormone [M]
ICI 182,780
17β-Estradiol
Tamoxifen

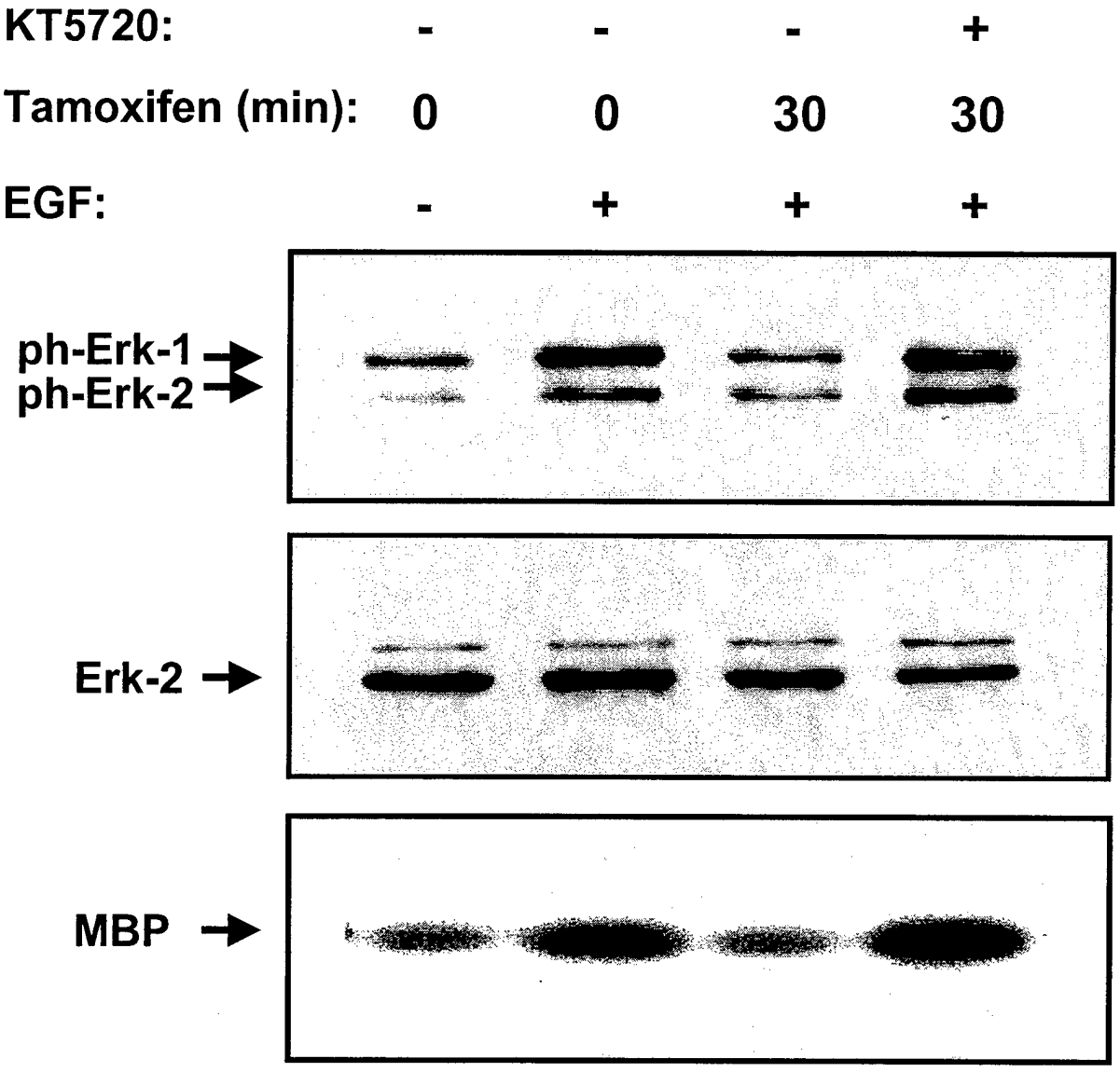
# MDA-MB-231(GPR30)

(ERα - ERβ +)



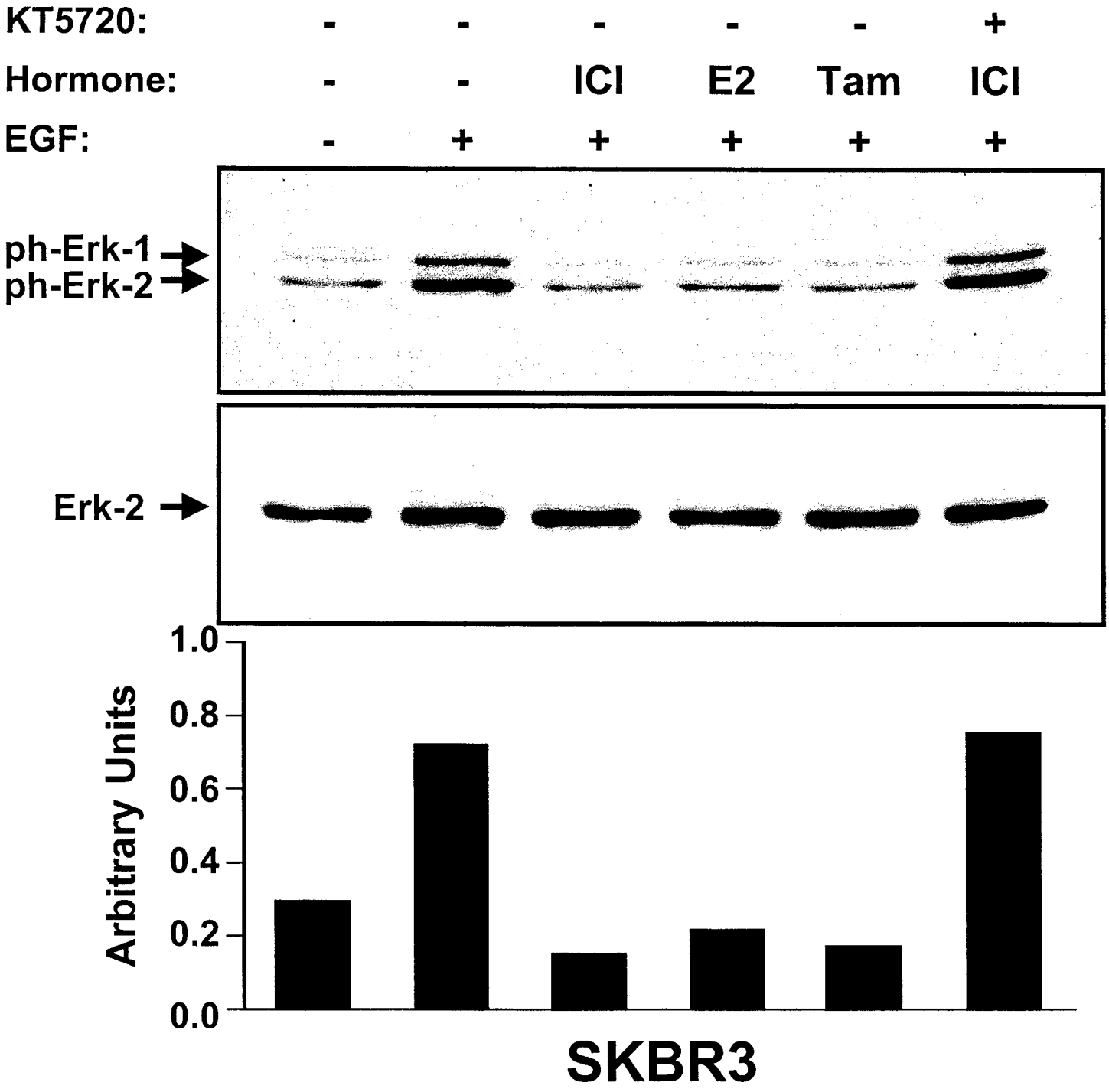

A
17β–estradiol (min): 0 0 30 60 120
EGF: - + + + +
ph-Erk-1
ph-Erk-2
Erk-2
MBP
B
17β–estradiol: - - + + + + + +
EGF (min): 0 15 0 1 5 15 30 60
ph-Erk-1
ph-Erk-2
Erk-2

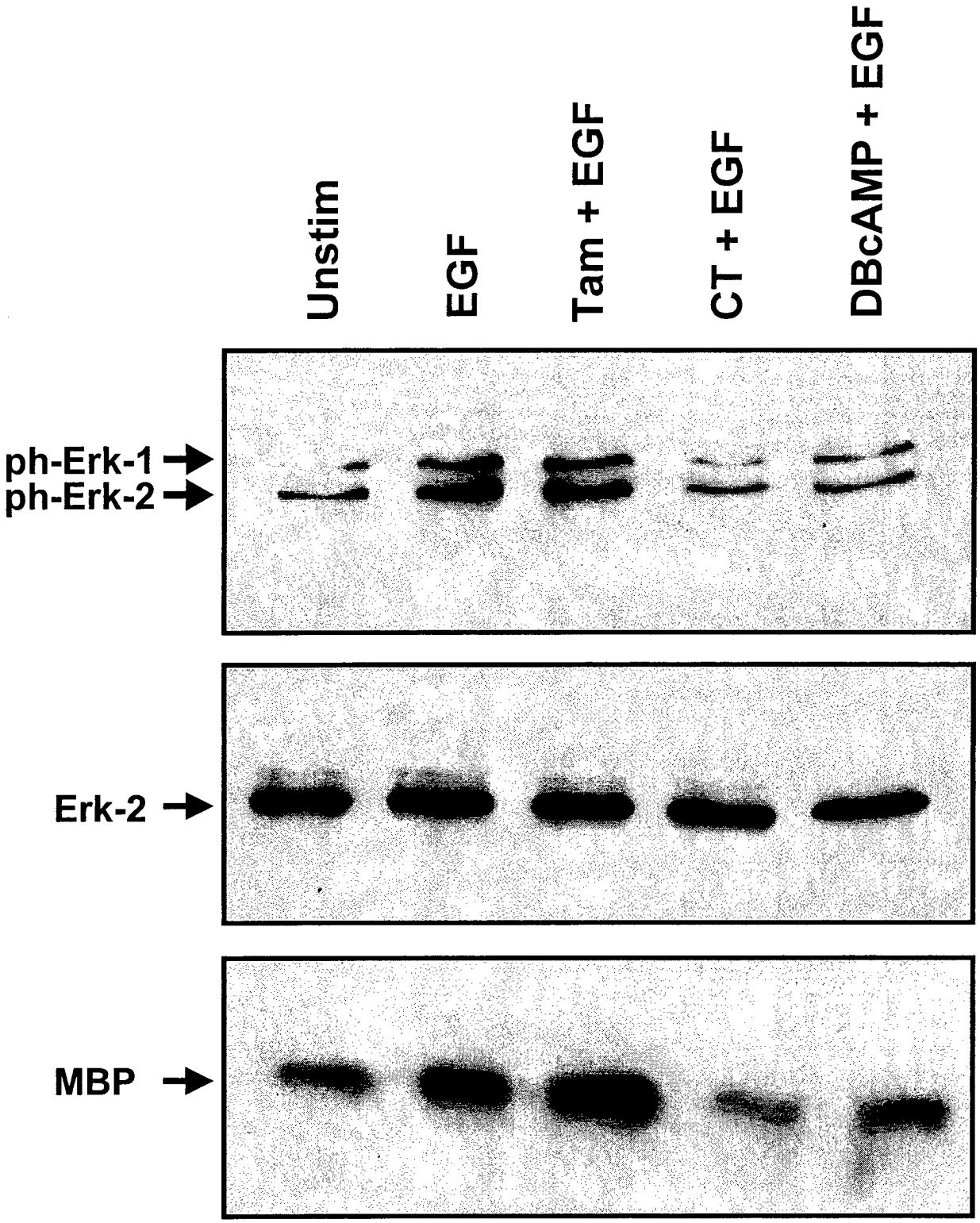
# A



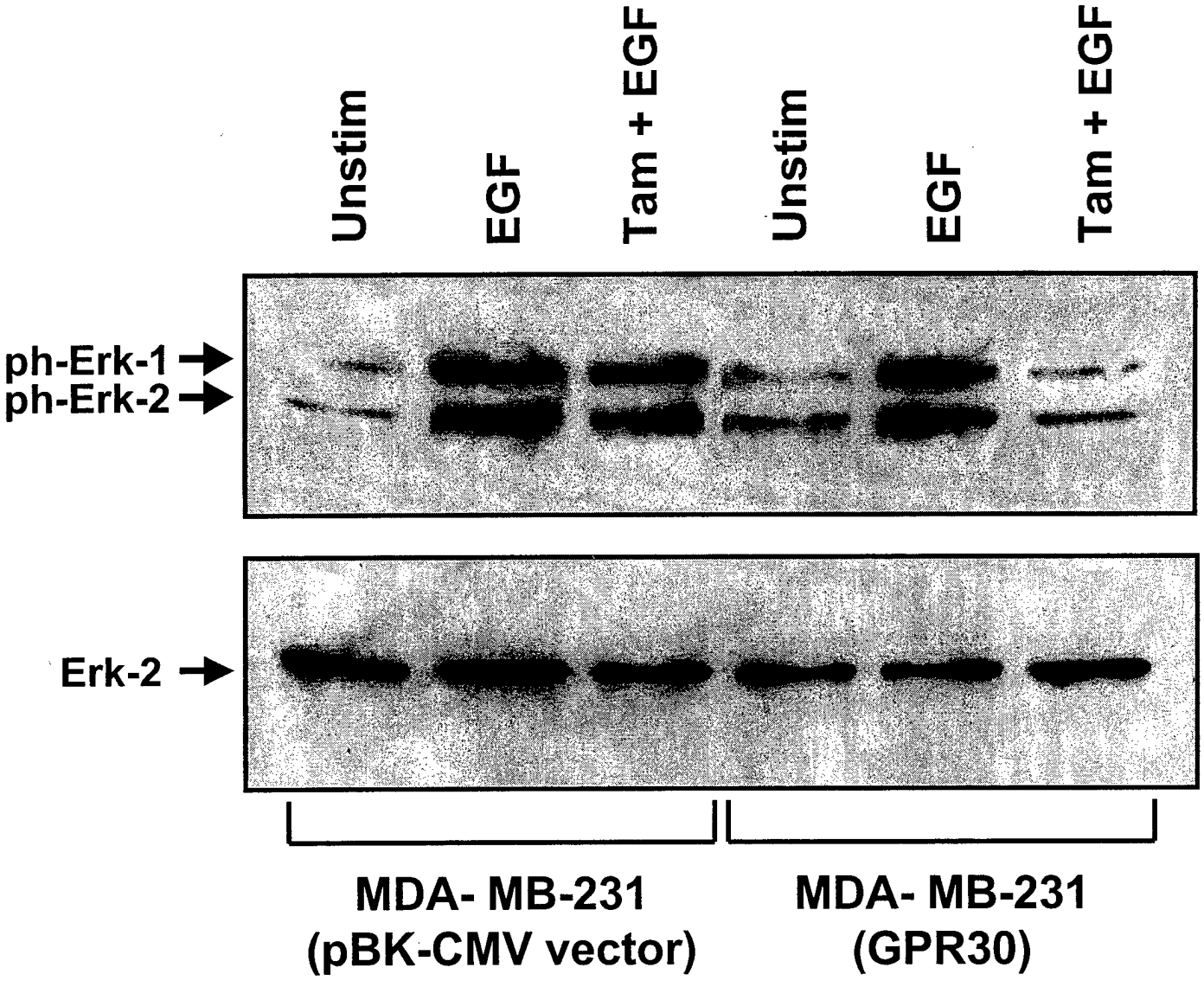

MCF-7

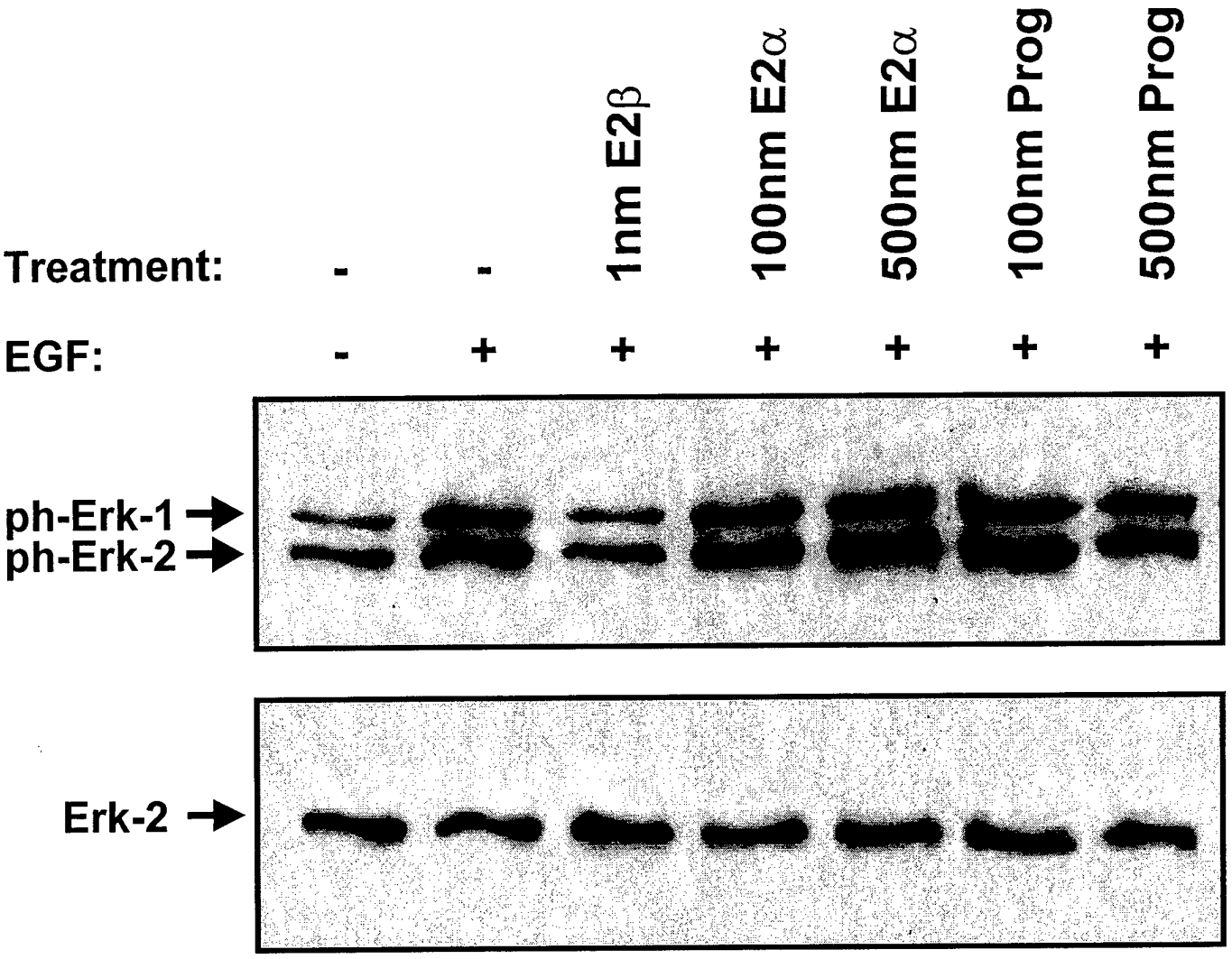
B


KT5720: - - - - - +
Hormone: - - ICI E2 Tam ICI
EGF: - + + + + +
ph-Erk-1
ph-Erk-2
Erk-2
Arbitrary Units
1.0
0.8
0.6
0.4
0.2
0.0
SKBR3



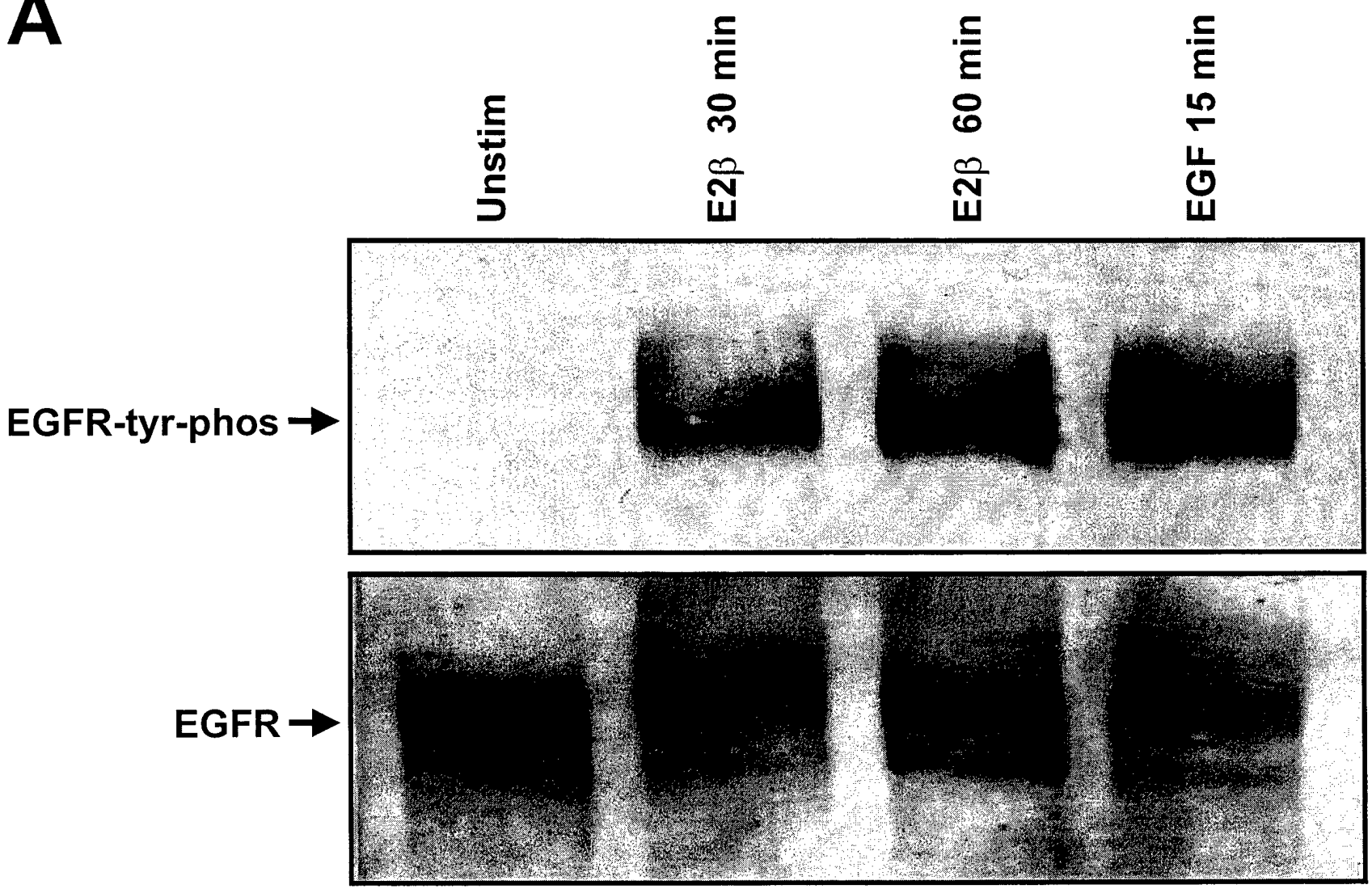

MDA-MB-231


Unstim
EGF
Tam + EGF
Unstim
EGF
Tam + EGF
ph-Erk-1
ph-Erk-2
Erk-2
MDA- MB-231
(pBK-CMV vector)
MDA- MB-231
(GPR30)

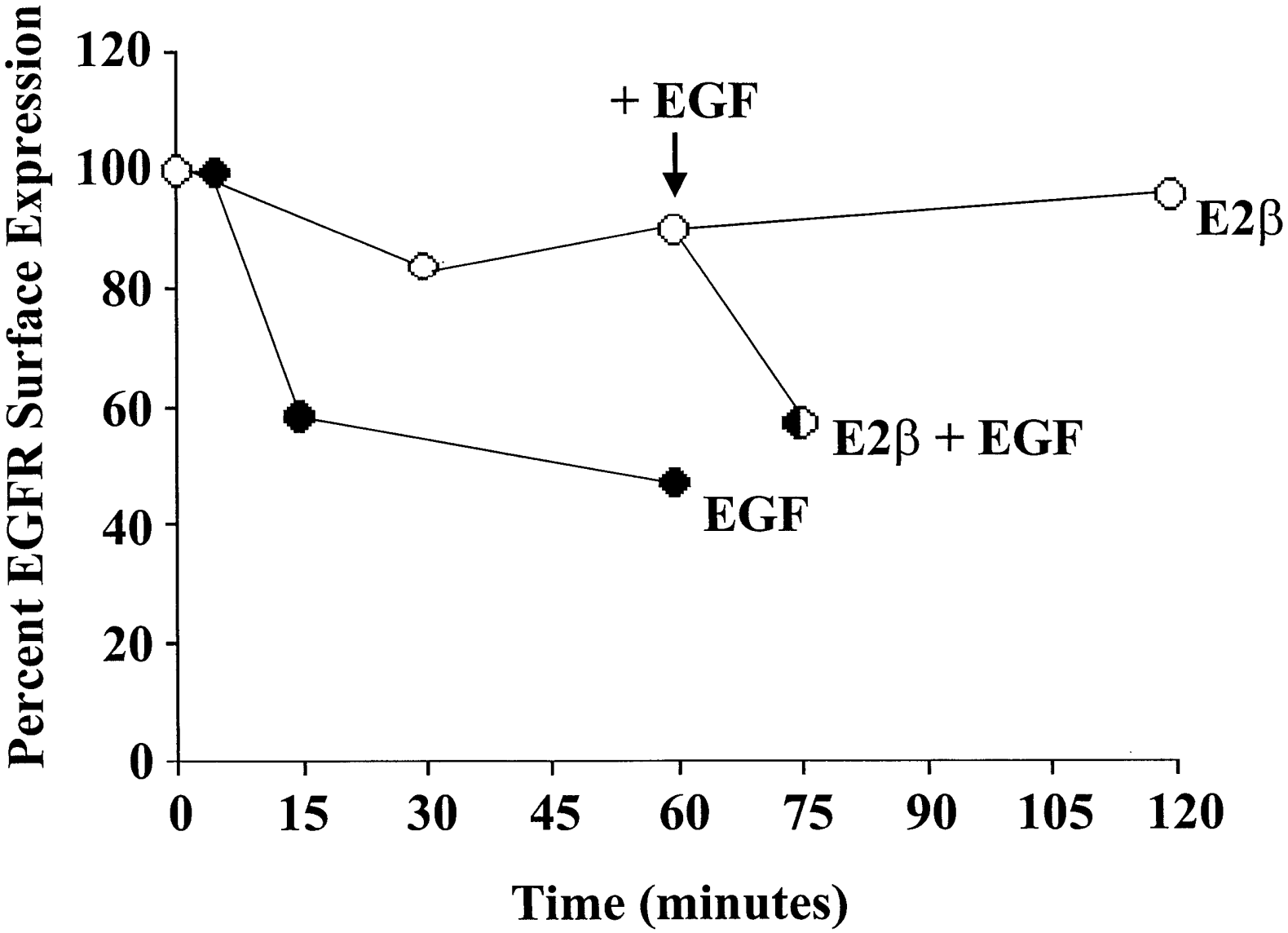



MDA-MB-231(GPR30)

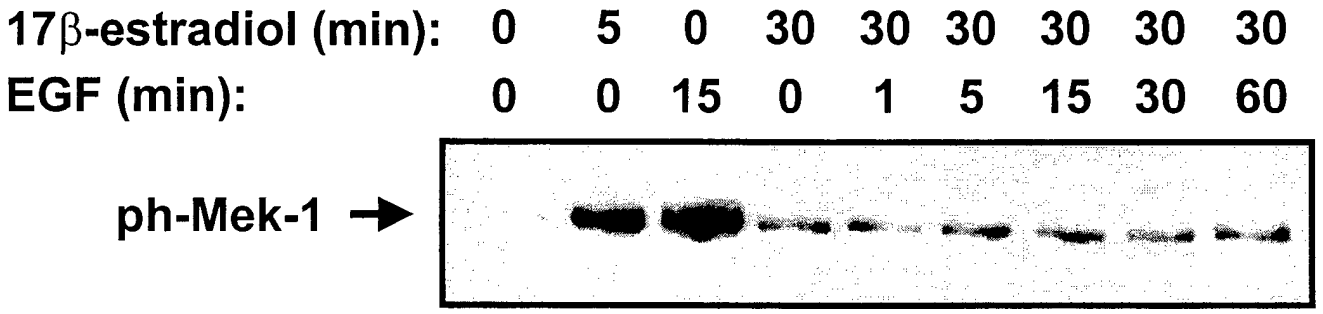
A



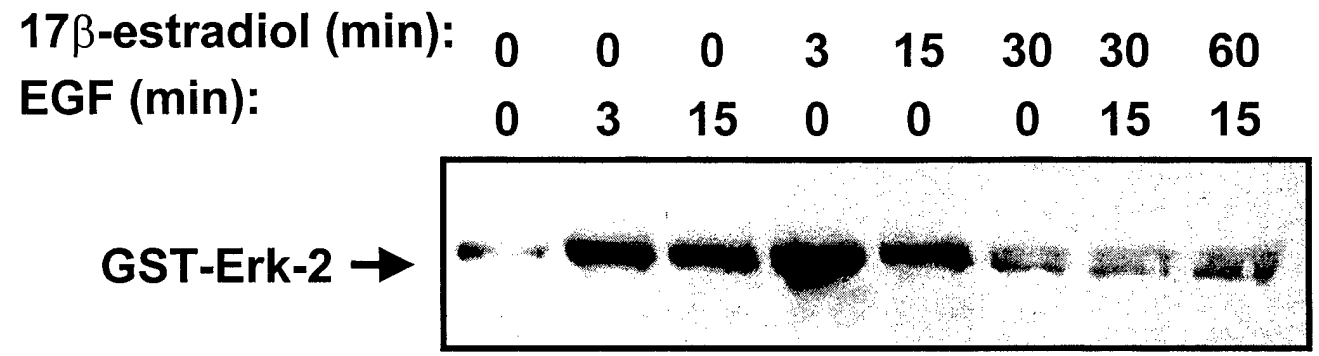
**B**

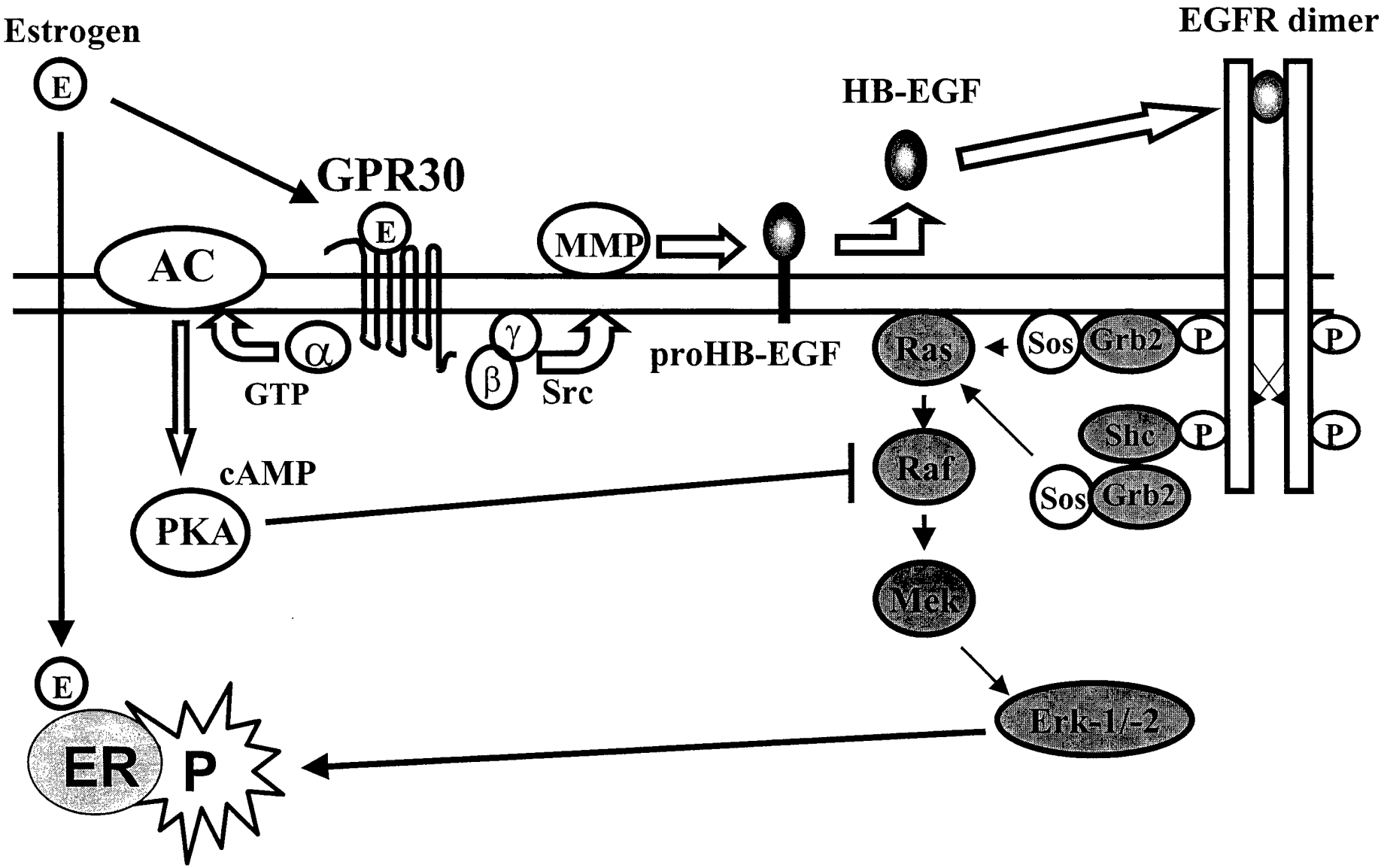

Percent EGFR Surface Expression
120
100
80
60
40
20
0
+ EGF
E2β
E2β + EGF
EGF
0
15
30
45
60
75
90
105
120
Time (minutes)

C


17β-estradiol (min): 0 5 0 30 30 30 30 30 30
EGF (min): 0 0 15 0 1 5 15 30 60
ph-Mek-1

D


17β-estradiol (min): 0 0 0 3 15 30 30 60
EGF (min): 0 3 15 0 0 0 15 15
GST-Erk-2


Estrogen
E
GPR30
E
AC
MMP
HB-EGF
EGFR dimer
α
GTP
γ
β
Src
proHB-EGF
Ras
Sos
Grb2
P
P
Shc
P
P
cAMP
PKA
Raf
Sos
Grb2
Mek
E
ER
P
Erk-1/-2