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Estrogen transactivates EGFR via the sphingosine 1-phosphate receptor Edg-3: the role of sphingosine kinase-1

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The transactivation of enhanced growth factor receptor (EGFR) by G protein–coupled receptor (GPCR) ligands is recognized as an important signaling mechanism in the regulation of complex biological processes, such as cancer development. Estrogen (E2), which is a steroid hormone that is intimately implicated in breast cancer, has also been suggested to function via EGFR transactivation. In this study, we demonstrate that E2-induced EGFR transactivation in human breast cancer cells is driven via a novel signaling system controlled by the lipid kinase sphingosine kinase-1 (SphK1). We show that E2 stimulates SphK1 activation and the release of sphingosine 1-phosphate (S1P), by which E2 is capable of activating the S1P receptor Edg-3, resulting in the EGFR transactivation in a matrix metalloprotease–dependent manner. Thus, these findings reveal a key role for SphK1 in the coupling of the signals between three membrane-spanning events induced by E2, S1P, and EGF. They also suggest a new signal transduction model across three individual ligand-receptor systems, i.e., "criss-cross" transactivation.

Introduction

Distinct signaling pathways have been demonstrated to mediate estrogen (E2) action and to directly affect its function. Examples include the regulation of normal mammary development and breast cancer growth. E2 is known to be coupled with growth factor–signaling networks to promote enhanced cell growth in human breast cancer. Several growth factors and their receptors are known to participate in E2 signaling, amongst which the EGF receptor (EGFR) family of receptor tyrosine kinases are of particular interest because of their critical involvement in human cancer (Bange et al., 2001; Levin, 2003). Indeed, aberrant expression and activation of EGFR is frequently observed in various tumors, especially of the breast and ovary, where it correlates with a poorer patient prognosis (Keen and Davidson, 2003; Roskoski, 2004). In addition, up-regulation of EGFR signaling is thought to be an important mechanism that confers antiestrogen resistance of breast cancer, resulting in a failure of endocrine therapy (Ali and Coombes, 2002; Nicholson et al., 2003).

Multiple lines of evidence have suggested that the interaction of EGFR with E2 signaling can occur at various levels. E2 primarily acts on nuclear estrogen receptors (ERs), leading to regulation of gene expression, which was traditionally deemed the genotropic action of E2. Many E2-responsive genes are indeed key signaling molecules that participate in EGFR signaling (for review see Levin, 2003). Alternatively, a cell membrane–associated form of ER (mER) has been reported to couple with and activate various G proteins, and thereby mediate the EGFR transactivation, serving as a nongenotropic effect of the ER (Levin, 2003; Razandi et al., 2003). More recently, an orphan G protein–coupled receptor (GPCR), GPR30, has been suggested to be an intracellular receptor of E2 that specifically binds E2 with a high affinity and promotes various rapid E2 signaling events, such as Ca^{2+} mobilization and activation of Akt cascades (Revankar et al., 2005; Thomas et al., 2005). In addition, Filardo et al. (2000) reported that E2-induced EGFR

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Abbreviations used in this paper: AO, antisense oligonucleotide; CM, conditioned media; EGFR, EGF receptor; ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein–coupled receptor; HB-EGF, heparin-binding EGF; mER, membrane-associated ER; MMP, matrix metalloprotease; p-EGFR, phosphorylated EGFR; p-ERK1/2, phosphorylated ERK1/2; PTX, pertussis toxin; S1P, sphingosine 1-phosphate; SphK, sphingosine kinase.

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transactivation was mediated via GPR30, suggesting a model of EGFR transactivation by E2 similar to that induced by other well documented GPCR ligands (Gschwind et al., 2001). However, as GPR30 was found to be uniquely localized to the endoplasmic reticulum (Revankar et al., 2005), whether this intracellular receptor coupled with G proteins can directly transactivate EGFR and the physiological function of GPR30 remains to be investigated.

Since first described by Prenzel et al. (1999), the transactivation of EGFR by GPCR ligands has been considered an important model of cellular signal transduction. Several GPCR ligands, including lysophosphatidic acid, thrombin, angiotensin II, and endothelin-1, have been documented to transactivate EGFR, leading to activation of survival or mitogenic pathways (Gschwind et al., 2001). Sphingosine 1-phosphate (S1P), which is a recently identified GPCR ligand (Hla et al., 2001), has also been shown to induce EGFR transactivation through S1P receptors (Kim et al., 2000; Tanimoto et al., 2004). We recently demonstrated that E2 serves as a potent activator of sphingosine kinase-1 (SphK1), which is a key enzyme that catalyzes the formation of S1P (Sukocheva et al., 2003). We also demonstrated that the activation of SphK1–S1P signaling participates in the nongenomic action of E2, including intracellular Ca^{2+} mobilization and ERK1/2 activation (Sukocheva et al., 2003). Moreover, SphK1 activity has been shown to regulate neoplastic cell growth of breast cancer in response to E2 stimulation at both an in vitro and an in vivo level (Nava et al., 2002; Sukocheva et al., 2003), suggesting an important role of SphK1 in the transmission of E2 signaling in breast cancer cells.

In this study, we provide evidence that not only demonstrates the capacity of S1P to stimulate EGFR transactivation in its own right through the S1P receptors in breast cancer cells but also reveals a critical role for SphK1 in mediating E2-induced EGFR transactivation in an S1P receptor–dependent manner. Furthermore, these findings illustrate a novel signaling mechanism, called criss-cross transactivation, which is triggered by SphK1 activation that signals between three individual ligand-receptor systems (i.e., E2, S1P, and EGF).

Results

S1P stimulates activation of EGFR in MCF-7 cells

Treatment of MCF-7 cells with S1P resulted in significant increases in tyrosine phosphorylation of EGFR in a concentration-dependent manner (Fig. 1). A significant response to S1P was commenced at 1 nmol/liter and peaked at ~100 nmol/liter, which fits well within the range of reported binding affinities to S1P receptors (Hla et al., 2001). In parallel, S1P treatment caused a significant increase in ERK1/2 phosphorylation, which is a key downstream signaling event of EGFR activation, in a similar concentration-dependent pattern to the S1P-induced EGFR phosphorylation (Fig. 1). Time course studies showed that S1P induced both EGFR and ERK1/2 phosphorylation that peaked at 10–15 min and decreased thereafter, but was still evident at 240 min after stimulation (Fig. 1). Collectively, these results demonstrate an ability of S1P to induce

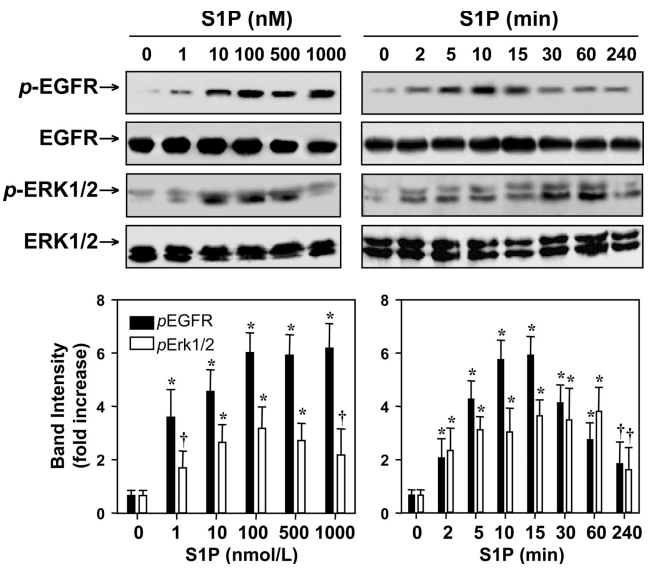

Figure 1. **S1P transactivates EGFR.** MCF-7 cells were stimulated with an increasing concentration of S1P for 15 min (left) or 1 μmol/liter S1P for the indicated time (right). Cell lysates were subjected to SDS-PAGE, and phosphorylation of EGFR and ERK1/2 was assayed by using antibodies specific for phosphorylated-EGFR (p-EGFR) and p-ERK1/2, respectively. The histograms represent band intensities that were normalized by total EGFR or ERK1/2 levels and expressed as the mean ± SD. $n = 5$. *, $P < 0.01$; †, $P < 0.05$, versus nil.

EGFR activation in MCF-7 cells, which was consistent with the observations previously reported in vascular smooth muscle cells (Tanimoto et al., 2004) and fibroblasts (Kim et al., 2000).

E2- and S1P-induced EGFR transactivation through a common signaling pathway

We have recently reported that E2 was capable of inducing SphK1 activation and S1P formation that participated in E2 nongenomic signaling (Sukocheva et al., 2003). The ability of E2 to induce EGFR transactivation has been previously demonstrated (Filardo et al., 2000; Razandi et al., 2003). We sought to determine whether S1P could mimic E2 to stimulate EGFR transactivation. Treatment of MCF-7 cells with E2 resulted in a rapid tyrosine phosphorylation of EGFR and ERK1/2 phosphorylation similar to that observed in the S1P-treated cells (Fig. 2 A). Both E2- and S1P-induced activation of EGFR and ERK1/2 were blocked by pertussis toxin (PTX), which is a Gi-specific inhibitor (Fig. 2 A). In contrast, PTX had no effect on EGF-stimulated phosphorylation of EGFR and ERK1/2 (Fig. 2 A).

As transactivation of EGFR relies on its internal tyrosine kinase activity (Prenzel et al., 1999), we examined whether the tyrosine kinase activity is required for E2- or S1P-induced EGFR transactivation. In the presence of AG1478, which is a specific EGFR tyrosine kinase inhibitor, both E2- and S1P-induced activation of EGFR and ERK1/2 were abolished completely (Fig. 2 A). Serving as a control, EGF-stimulated autophosphorylation of EGFR was completely inhibited by AG1478, supporting its specific effect on EGFR activity in MCF-7 cells.

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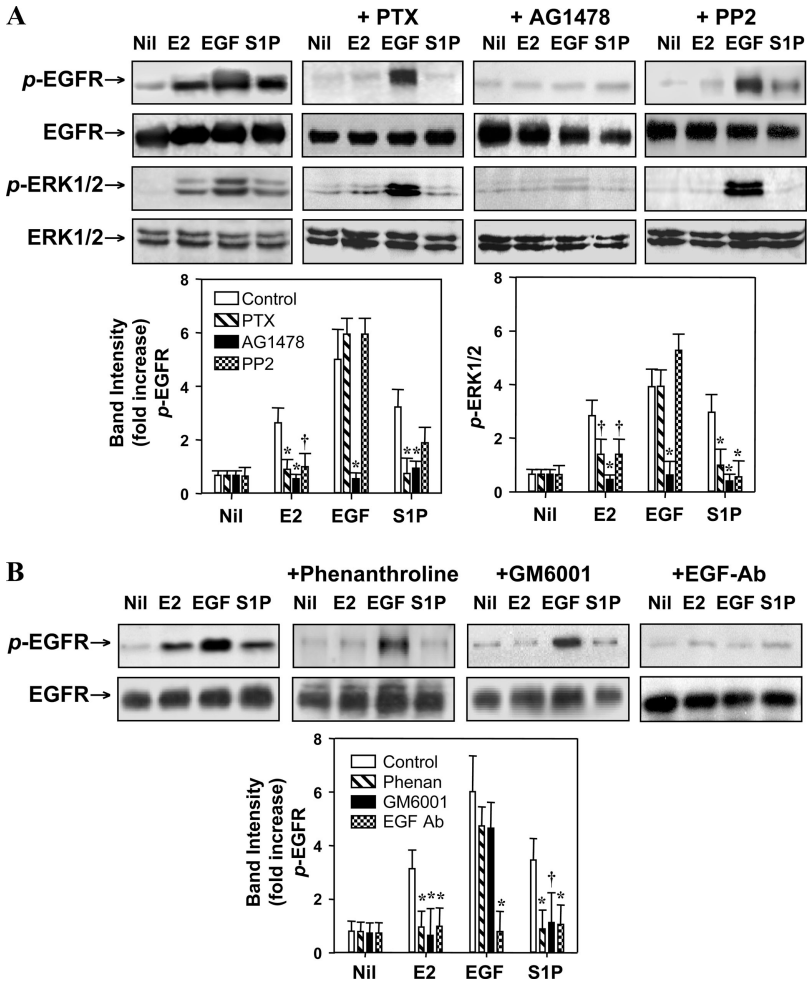

Figure 2. **S1P mimics E2 to induce EGFR transactivation.** Serum-starved MCF-7 cells were preincubated for 16 h with 100 ng/ml PTX or for 60 min with 50 μmol/liter AG1478 and 10 μmol/liter PP2 (A), or the cells were pretreated for 60 min with the 20-μmol/liter MMP inhibitor phenanthroline, 50 nmol/liter GM6001, or 15 μg/ml of the EGF-neutralizing antibodies (EGF-Ab; B). This was followed by stimulation for 15 min with 10 nmol/liter E2, 1 μmol/liter S1P, or 25 ng/ml EGF. The p-EGFR and p-ERK1/2 were then analyzed and quantified as described in Fig. 1. Data are the mean ± SD from three independent experiments. *, $P < 0.01$; †, $P < 0.05$, pretreated versus nonpretreated.

The Src family of kinases has been reported to play a signaling role in GPCR-mediated transactivation of EGFR (Gschwind et al., 2001). Src was also suggested to be required for E2-induced EGFR transactivation (Filardo et al., 2000; Razandi et al., 2003). Consistent with these previous studies, both E2- and S1P-stimulated activation of EGFR and ERK1/2 were significantly inhibited by the Src-specific inhibitor PP2 (Fig. 2 A), supporting a role for Src in mediating either E2- or S1P-induced transactivation of EGFR.

The shedding of heparin-binding EGF (HB-EGF) upon matrix metalloprotease (MMP) activation has also been recognized as an important mechanism in mediating EGFR transactivation by GPCR ligands (Prenzel et al., 1999) or by E2 (Filardo et al., 2000; Razandi et al., 2003). Therefore, we examined whether HB-EGF shedding was involved in E2- or S1P-induced EGFR transactivation. We subjected MCF-7 cells to an acid-wash step, to reduce background autocrine stimulation, and pretreated the cells with the MMP inhibitors *o*-phenanthroline or GM6001. Both E2- and S1P-induced EGFR and ERK1/2 activation were blocked by these two MMP inhibitors (Fig. 2 B). In contrast, the MMP inhibitors had no effect on EGF-induced autophosphorylation of EGFR. Furthermore, depletion of HB-EGF production from the culture media by EGF-neutralizing antibodies resulted in a significant inhibition of both E2- and S1P-induced EGFR activation (Fig. 2 B). Efficiency and specificity of the neutralizing antibodies were demonstrated by the inhibition of EGF-stimulated EGFR tyrosine phosphorylation. These data suggest that HB-EGF shedding and release are required for both E2- and S1P-induced EGFR transactivation.

SphK1 activation is involved in E2-induced EGFR transactivation

As S1P was able to mimic the effect of E2-stimulated EGFR transactivation, and E2 was capable of stimulating S1P production upon SphK1 activation, we hypothesized that the E2-induced EGFR transactivation could be mediated by SphK1 activation. To test this hypothesis, we used stably transfected MCF-7 cell lines overexpressing wild-type SphK1 ($SphK1^{WT}$), dominant-negative SphK1 ($SphK1^{G82D}$), or empty vector alone. Previously, we demonstrated that the baseline SphK activity in $SphK1^{WT}$-transfected cells was ∼10-fold higher than in control cells (Sukocheva et al., 2003). E2 stimulation resulted in a rapid increase in SphK activity of approximately twofold more than the basal level in both $SphK1^{WT}$-transfected and control MCF-7 cells (Sukocheva et al., 2003). In contrast, the $SphK1^{G82D}$-transfected cells had a similar basal SphK activity to the control cells, whereas E2-stimulated SphK activity was completely abolished (Sukocheva et al., 2003).

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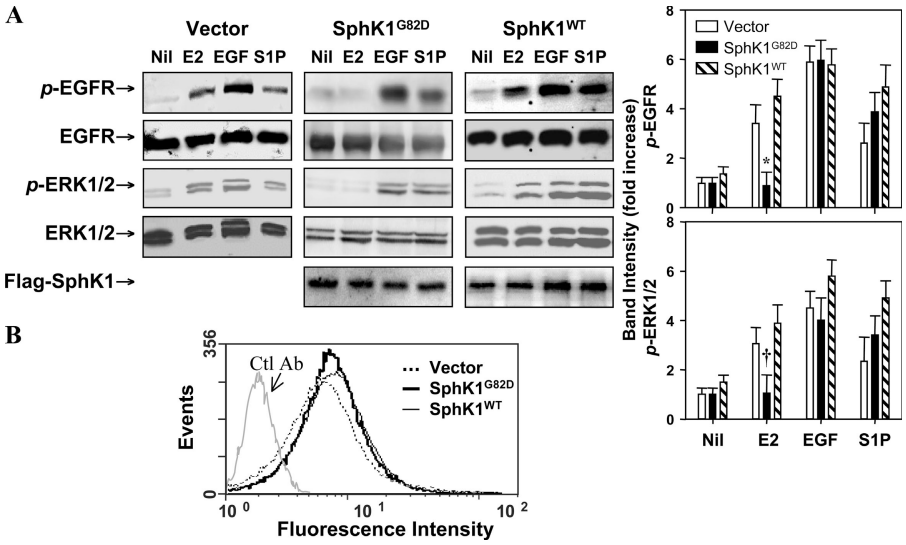

Figure 3. **Effect of SphK on E2-induced EGFR transactivation.** (A) Stably transfected MCF-7 cells overexpressing SphK1^{WT}, SphK1^{G82D}, or vector alone were stimulated with 10 nmol/liter E2, 1 μmol/liter S1P, or 25 ng/ml EGF for 15 min. The p-EGFR and p-ERK1/2 were analyzed and quantified as described in Fig. 1. Levels of SphK1^{WT} and SphK1^{G82D} expression in the transfected cells are shown in the bottom blots. Data are mean ± SD from more than three independent experiments. *, $P < 0.01$; †, $P < 0.05$, SphK1^{WT} or SphK1^{G82D} versus vector alone. (B) Flow cytometry profiles show the cell-surface expression levels of EGFR in the transfected MCF-7 cell lines. A profile using control antibodies is indicated (Ctl Ab).

Interestingly, although E2-stimulated tyrosine phosphorylation of EGFR and ERK1/2 phosphorylation were enhanced in SphK1^{WT}-transfected cells, the stimulatory effect of E2 was abrogated in the SphK1^{G82D} transfectants (Fig. 3 A). There were no significant differences in total EGFR and their cell-surface expression levels between these transfected cell lines (Fig. 3, A and B). In contrast, neither EGF nor S1P-stimulated EGFR phosphorylation was significantly influenced by SphK1^{G82D}. Thus, these results suggest a specific role for SphK activity in the E2-induced EGFR transactivation.

Two human SphK isoforms, SphK1 and SphK2, have been identified, and both isoforms account for total cellular SphK activity (Kohama et al., 1998; Liu et al., 2000). To define which isoform (if not both) is responsible for the transactivation of EGFR, as well as the role of endogenous SphK, we used an siRNA strategy to down-regulate each isoform's expression levels in MCF-7 cells. Endogenous SphK1 and SphK2 levels were reduced by 86 and 67%, respectively, after treatment with SphK1- or SphK2-specific siRNA, compared with cells treated with a scrambled siRNA (Fig. 4 A). The specificity of these siRNAs was demonstrated by their inability to inhibit the alternative isoform of SphK and the control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Both SphK1- and SphK2-siRNA decreased the baseline levels of SphK activity by ~50%. Whereas SphK1-siRNA significantly attenuated SphK activity in response to E2 stimulation, the extent of the E2-induced increases in SphK activity was not changed by SphK2-siRNA (Fig. 4, A and B), suggesting that SphK1 is the key isoform responsible for the E2-induced SphK activity. Furthermore, by down-regulating SphK1, SphK1-siRNA significantly attenuated the E2-induced EGFR and ERK1/2 phosphorylation to an extent that was similar to SphK1^{G82D}-transfected cells (Fig. 4 C). In contrast, SphK2-siRNA had no effect on the E2-induced phosphorylation of EGFR and ERK1/2. Again, serving as controls, neither EGF- nor S1P-induced EGFR and ERK1/2 phosphorylation were inhibited by SphK1- or SphK2-siRNA (Fig. 4 C). Collectively, these data suggest a critical role for endogenous SphK1, but not for SphK2, in mediating E2-stimulated transactivation of EGFR.

Role of mER and GPR30 in the SphK1-dependent transactivation of EGFR by E2

Our previous work suggested that the E2-induced SphK1 activation was likely to be mediated by mER in a G protein–dependent manner (Sukocheva et al., 2003). As GPR30, which is an orphan GPCR, has been more recently identified as an E2-specific GPCR (Revankar et al., 2005; Thomas et al., 2005), we sought to define the role of GPR30 in E2-induced SphK1 activation. To this end, we used GPR30 antisense oligonucleotides (AOs) that specifically down-regulated GPR30 expression in MCF-7 cells (Fig. 5 A). The cells treated with AO-GPR30 resulted in a significant reduction of the E2-induced increases in SphK activity (Fig. 5 B), suggesting a critical involvement of GPR30 in the E2-stimulated SphK1 activation. Serving as a control, AO-GPR30 had no effect on EGF-induced SphK activity (Fig. 5 B). Consistent with our previous study (Sukocheva et al., 2003), treatment of cells for 18 h with ICI 182780, which down-regulated ERα expression (Fig. 5 C), resulted in a reduction of the E2-induced SphK activity similar to that observed in the AO-GPR30–treated cells (not depicted). Consequently, the E2-induced EGFR and ERK1/2 phosphorylation were significantly inhibited by either AO-GPR30 or ICI 182780 (Fig. 5 C). In contrast, neither AO-GPR30 nor ICI 182780 had effects on the S1P- or EGF-induced EGFR and ERK1/2 phosphorylation. Collectively, these data suggest that both GPR30 and ERα are capable of mediating SphK1 activation, and the resultant EGFR transactivation in response to E2 stimulation.

The S1P receptor Edg-3 is required for the E2-induced EGFR transactivation

The biological function of SphK1 relies on its product, S1P, which functions chiefly as a ligand for the Edg family of GPCR receptors (Hla et al., 2001; Spiegel and Milstien, 2003). Therefore, we sought to determine the role for S1P and its receptors in the SphK1-dependent EGFR transactivation induced by E2. We first examined whether S1P is released upon SphK1 activation in cells responding to E2 stimulation. As shown in Fig. 6 A, in parallel with the elevated intracellular content of S1P,

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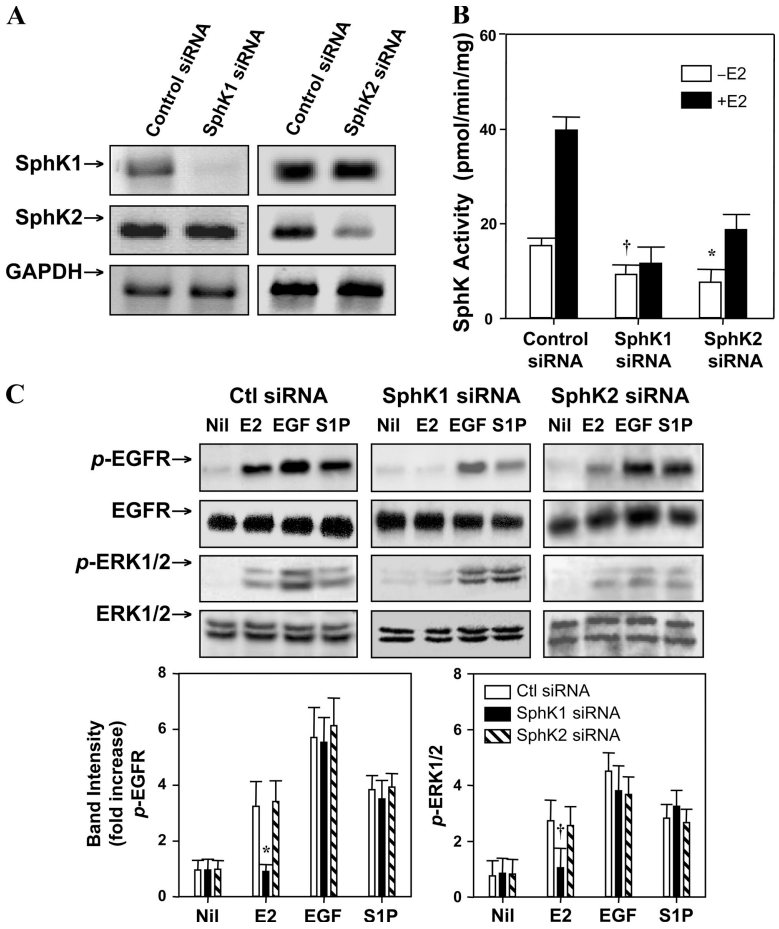

Figure 4. **SphK1, but not SphK2, is responsible for the E2-induced EGFR transactivation.** MCF-7 cells were transfected with siRNA specific for SphK1, SphK2, or scramble control siRNA, as described in Materials and methods. After a 24-h transfection, (A) mRNA levels of SphK1, SphK2, and GAPDH were determined by RT-PCR, (B) SphK activity was measured after treatment with 10 nmol/liter E2 for 15 min, and (C) p-EGFR and ERK1/2 were analyzed after the indicated stimulation, as described in Fig. 1. Data are mean ± SD from three to five independent experiments. *, $P < 0.01$; †, $P < 0.05$, SphK1- or SphK2-siRNA versus control siRNA.

S1P levels were increased by 86% ($P < 0.01$) in conditioned media (CM) collected from the E2-stimulated MCF-7 cells in comparison to that from unstimulated cells. No increase in S1P levels were detected after E2 stimulation in CM from the SphK1^{G82D}-transfected cells (Fig. 6 A), indicating that SphK1 activation is responsible for the elevated S1P production and release from the E2-stimulated cells. Correspondingly, CM derived from the E2-treated cells exhibited a substantially greater capacity to stimulate EGFR tyrosine phosphorylation compared with the CM from untreated cells (Fig. 6 B). Furthermore, treatment with CM derived from the E2-treated SphK1^{wt}-transfected cells that contained high levels of S1P (Fig. 6 A) resulted in a further increase in EGFR phosphorylation, whereas CM derived from the SphK1^{G82D} transfectants had no detectable effect on the EGFR phosphorylation (Fig. 6 B). These results suggest that the ability of the CM to stimulate EGFR activation was dependent on its cellular SphK1 activity and the amount of S1P release. To explore this notion further, we used two strategies: (a) we lipid stripped CM to remove S1P, and (b) before CM stimulation, we treated cells with PTX that has been reported to block the majority of S1P receptors (Hla et al., 2001). Either lipid-stripped CM or CM pretreated with PTX completely abolished the CM-induced EGFR activation (Fig. 6 B), supporting a critical involvement of S1P and its receptors in the E2-stimulated transactivation of EGFR.

According to Wang et al. (1999), Edg-3, which is a PTX-sensitive GPCR, is the predominantly expressed S1P receptor in MCF-7 cells. To evaluate the potential role of Edg-3 in E2-induced EGFR transactivation, we used the antisense strategy to knockdown endogenous Edg-3 expression. Cells transfected with AO-Edg3 resulted in a significant down-regulation of Edg-3 expression levels (~80%; Fig. 7 A). Correspondingly, S1P-induced EGFR tyrosine phosphorylation was also blocked by AO-Edg3 (Fig. 7 A). Moreover, treatment of MCF-7 cells with AO-Edg3 caused a significant reduction in E2-induced EGFR tyrosine phosphorylation, whereas EGF-induced EGFR autophosphorylation was retained (Fig. 7 A). Furthermore, as a functional consequence, E2-induced cell growth was significantly inhibited by AO-Edg3 to a similar extent as that observed in cells treated with the EGFR inhibitor AG1478 (Fig. 7 B). Collectively, these findings suggest that the S1P receptor Edg-3 is required for the E2-induced EGFR transactivation and cell growth in MCF-7 cells.

Discussion

The current understanding of cell signaling has grown broadly, from individual ligand-receptor systems, such as those controlled by GPCR or receptor tyrosine kinases, to an interdependent network that is capable of communicating across

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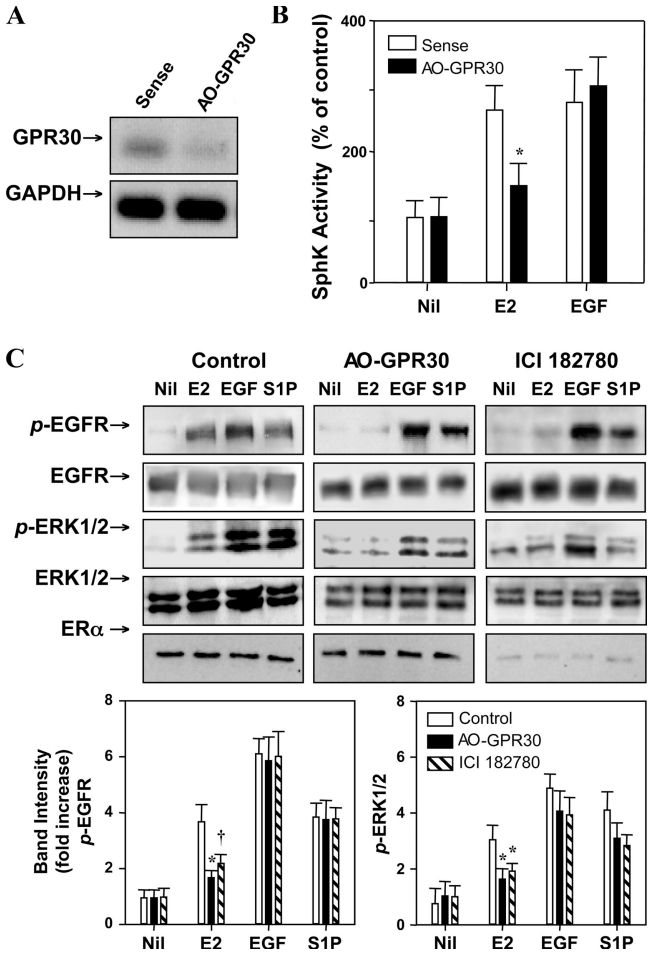

Figure 5. **Role of GPR30 and ERα in the E2-induced SphK activity and EGFR transactivation.** (A) Levels of GPR30 and GAPDH mRNA were determined by RT-PCR in MCF-7 cells transfected with GPR30 sense oligonucleotides or AOs. (B) SphK activity was measured after stimulation for 15 min with 10 nmol/liter E2 or 25 ng/ml EGF. (C) Levels of p-EGFR were analyzed after the indicated stimulation, as described in Fig. 1, in MCF-7 cells transfected with GPR30 sense oligonucleotides or AOs or pretreated with 10 μmol/liter ICI 182780 for 18 h. Expression levels of ERα are shown in the bottom blots. Data are mean ± SD from three independent experiments. *, $P < 0.01$; †, $P < 0.05$, versus the respective controls.

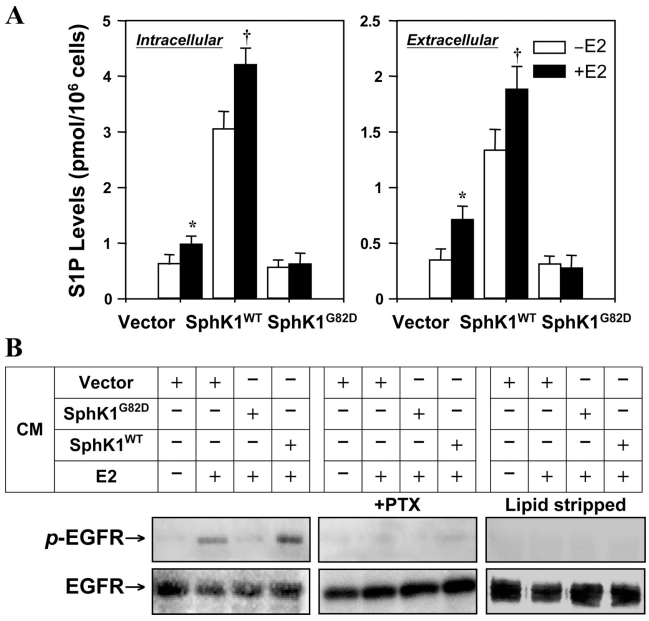

Figure 6. **E2-induced EGFR transactivation is mediated by S1P production.** (A) Intracellular and extracellular S1P levels were determined in the transfected MCF-7 cells overexpressing SphK1^{WT}, SphK1^{G82D}, or vector alone after stimulation with 10 nmol/liter E2 for 15 min. Data are mean ± SD from three independent experiments. *, $P < 0.01$; †, $P < 0.05$, E2 treated versus untreated. (B) MCF-7 cells were pretreated with or without PTX for 16 h and stimulated for 15 min with lipid-stripped or unstripped CM derived from the transfected MCF-7 cells treated with or without E2. Levels of p-EGFR were determined as described in Fig. 1.

individual signaling systems. One particular example is that of EGFR, which can be transactivated by several GPCR ligands (Gschwind et al., 2001). Although the mechanism that controls this transactivation remains largely unknown, EGFR transactivation has been recognized as an important pathway in the regulation of complex biological processes, such as cancer development. In this study, we demonstrate that E2, acting on its own receptors (GPR30 and/or mER), results in the activation of the S1P-specific receptor Edg-3 via SphK1 activation, leading to EGFR transactivation (summarized in Fig. 8). To the best of our knowledge, this is the first work to describe such a signaling phenomenon, i.e., a given GPCR ligand–mediated (S1P) EGFR transactivation is driven by another independent ligand (E2), which suggests a new model of criss-cross transactivation between three individual ligand-receptor systems.

As described in this study, SphK1, which is the enzyme that catalyzes S1P formation, plays an essential role in this criss-cross transactivation phenomenon. We have previously shown that E2 stimulates SphK1, resulting in both a rapid, transient response and a delayed but prolonged activation (Sukocheva et al., 2003). Although the latter response relies on ER transcriptional activity, the E2-induced rapid activation of SphK1 appears to be necessary for E2 cytoplasmic signaling, such as intracellular Ca^{2+} mobilization and ERK1/2 activation (Sukocheva et al., 2003). In addition, cellular SphK activity has been functionally linked to the E2-dependent mitogenic and carcinogenic action in human breast cancer (Nava et al., 2002; Sukocheva et al., 2003), suggesting an important signaling role of SphK1 in the biological function of E2. Indeed, SphK1, serving as an agonist-activated signaling enzyme, has been implicated in a wide spectrum of agonist-driven cellular responses, including cell survival, motility, proliferation, and differentiation (Spiegel and Milstein, 2003). This pleiotropic action of SphK1 is attributed to its product, S1P, which functions as both an intracellular second messenger and a ligand for cell-surface receptors (Hla et al., 2001; Spiegel and Milstein, 2003).

S1P receptors belong to the Edg family of GPCR, which consists of Edg-1 (also called $S1P_1$), -3 ($S1P_3$), -5 ($S1P_2$), -6 ($S1P_4$), and -8 ($S1P_5$; Hla et al., 2001). The identification of S1P as a ligand for GPCR has provoked exploration of a potential role for S1P in the transactivation of receptor tyrosine kinases. Tanimoto et al. (2004) recently reported that S1P was capable of inducing transactivation of EGFR and the platelet-derived growth factor β receptor in vascular smooth muscle cells. Consistent with this finding, we are able to confirm that

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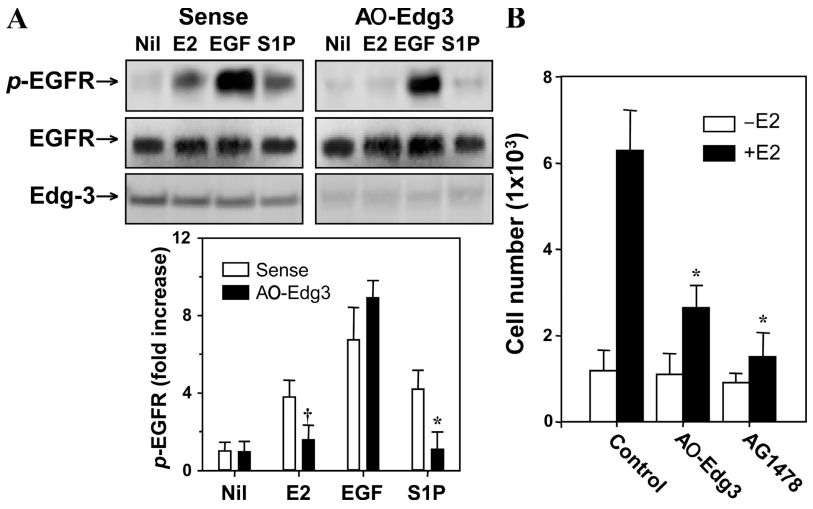

Figure 7. **Effect of Edg-3 on E2-induced EGFR transactivation.** MCF-7 cells were transfected with Edg-3 sense oligonucleotides or AOs, as described in Materials and methods. (A) p-EGFR was analyzed after the indicated stimulation, as described in Fig. 1. The expression levels of Edg-3 are shown in the bottom blots. (B) Cell number was determined in MCF-7 cells transfected with Edg-3 sense oligonucleotides or AOs or pretreated with 10 μmol/liter AG1478 in the presence or absence of 10 nmol/liter E2 for 5 d. Data are mean ± SD from three independent experiments. *, $P < 0.01$; †, $P < 0.05$, versus the control.

S1P transactivates the EGFR in MCF-7 breast cancer cells, in both a time- and dose-dependent manner (Fig. 1). S1P, like many other GPCR ligands, induces EGFR transactivation via Gi activation of the GPCR and the intrinsic kinase activity of EGFR (Gschwind et al., 2001), as demonstrated by the inhibitory effects of PTX and AG1478 on S1P-induced transactivation of EGFR (Fig. 2). The ability of GPCR ligands to activate MMP, resulting in HB-EGF shedding and release, has been demonstrated as a necessary event in GPCR-mediated transactivation of EGFR (Prenzel et al., 1999). Although we have not directly determined the MMP activities and HB-EGF production in this study, MMP activation is likely involved in S1P-induced EGFR transactivation, as the transactivation of EGFR was blocked by either the MMP inhibitors (*o*-phenanthroline or GM6001) or EGF-specific neutralizing antibodies (Fig. 2). Additional investigations are needed to determine the effect of S1P on MMP activation.

E2, which is a steroid hormone that functions primarily through its nuclear receptors (ERα and ERβ), has recently been shown to elicit a variety of rapid nongenotropic effects, including intracellular Ca^{2+} mobilization; activation of adenylyl cyclase, Raf-1, c-Src, and ERK1/2; and EGFR transactivation (Segars and Driggers, 2002; Levin, 2003). These rapid actions of E2 are believed to be mediated by its membrane-associated receptors. Studies examining the identity of these receptors are ongoing, with evidence to suggest that they may be related to nuclear ER (Razandi et al., 1999) or the orphan GPCR GPR30 (Filardo et al., 2000), or form part of a GPCR–ER complex (Razandi et al., 2003). More recently, GPR30 has been suggested as an E2-specific intracellular receptor with a high affinity and a specific binding site for E2 (Revankar et al., 2005; Thomas et al., 2005). In keeping with these findings, we found that down-regulation of GPR30 expression in MCF-7 cells by AO-GPR30 attenuated the E2-induced SphK activity (Fig. 5 B). On the other hand, we have previously reported that down-regulation of ERα in MCF-7 cells by long-term treatment with ICI 182780 resulted in a similar inhibition of E2-induced SphK activity (Sukocheva et al., 2003), suggesting the involvement of ERα in this signaling event. Consequently, E2-induced EGFR transactivation was significantly inhibited by either AO-GPR30 or ICI 182780 in MCF-7 cells (Fig. 5 C). These results suggest the capacity of both GPR30 and mER, and perhaps of their cooperative actions, to mediate E2-induced SphK1 activation and the resultant EGFR transactivation. However, it remains to be defined if and how these receptors function cooperatively in transmitting E2 signaling in breast cancer cells.

The role of SphK1 activation in the coupling of E2-induced EGFR transactivation was further demonstrated by the

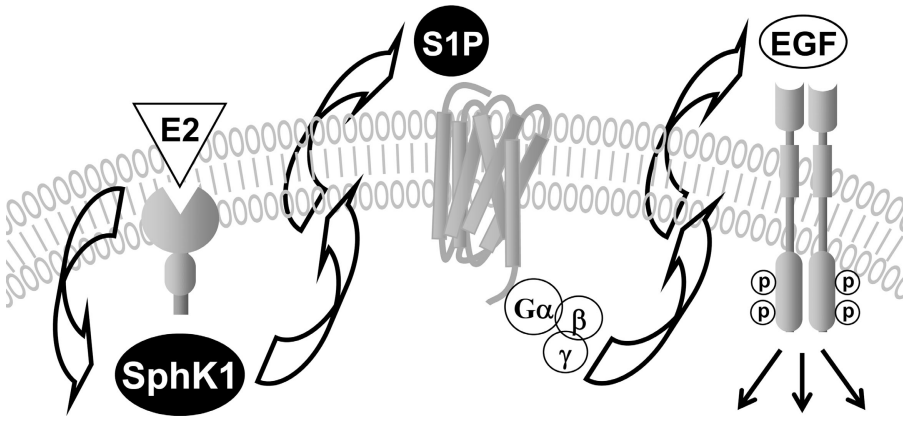

Figure 8. **Model of E2-induced criss-cross transactivation of EGFR through activation of the SphK1–S1P signaling pathway.** E2, acting on its own receptors (GPR30 and/or mER), induces the activation of SphK1 release of S1P and the consequent activation of the S1P receptor Edg-3, leading to the EGFR transactivation.

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following series of observations: (a) CM obtained from E2-stimulated cells that contained higher levels of S1P were capable of inducing EGFR activation; (b) removal of S1P from CM by either lipid stripping or the pretreatment of cells with PTX before CM stimulation completely abolished the ability of CM to stimulate EGFR phosphorylation; (c) abrogated SphK1 activation by the expression of $SphK1^{G82D}$ resulted in an attenuation of the E2-stimulated EGFR transactivation, whereas S1P-induced EGFR transactivation was preserved; (d) down-regulation of endogenous SphK1, but not of SphK2, by their specific siRNA caused a significant inhibition of both SphK1 activation and EGFR transactivation in response to E2 stimulation; and furthermore, (e) by down-regulating endogenous Edg-3, which is a specific receptor for S1P, AO-Edg3 profoundly inhibited the E2-induced EGFR transactivation. Thus, we have provided compelling evidence to suggest that an autocrine or paracrine S1P signaling loop, triggered by SphK1 activation, plays a critical role in transactivating EGFR through the S1P receptor Edg-3 in response to E2 stimulation. Despite Edg-3 being previously reported as (Wang et al., 1999), and shown to be, the predominant receptor that accounts for the receptor-dependent action of S1P in MCF-7 cells, we are unable to rule out the possibility that other members of the S1P receptor family expressed in these cells may also be subsidiarily involved in the EGFR transactivation. This requires further investigation.

It is noteworthy that by blocking E2-induced SphK1 activation without alterations in the baseline SphK activity, $SphK1^{G82D}$ attenuated the E2-stimulated EGFR transactivation. Moreover, although both SphK1- and SphK2-siRNA caused a decrease in the basal SphK activity, only SphK1-siRNA that inhibited E2-induced SphK1 activity was able to block the EGFR transactivation. In contrast, SphK2-siRNA had no effect on E2-induced SphK1 activity or EGFR transactivation. These results not only suggest a specific role for the SphK1 isoenzyme but also strongly indicate that the activation of SphK1, rather than its baseline activity, is critical for the E2-induced EGFR transactivation. In fact, the enzymatic function of SphK1 has been suggested to act at two levels: (a) the constitutive basal activity is involved in the catabolism of cellular sphingolipids and, therefore, may play a housekeeping role (Pitson et al., 2000); and (b) the agonist-induced elevated activity is fundamental for its signaling role in the regulation of many biological functions, including cell survival, proliferation, differentiation, and oncogenesis (Xia et al., 2000, 2002; Pitson et al., 2000).

Although the detailed mechanism by which E2 induces SphK1 activation is currently unknown, E2 was able to stimulate SphK1 phosphorylation in an ERK1/2-dependent manner (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200506033/DC1). This is consistent with the findings of Pitson et al. (2003) and suggests that ERK1/2-promoted phosphorylation is required for E2-induced SphK1 activation. Indeed, by inhibiting ERK1/2 activity, the ERK kinase-specific inhibitor U0126 not only blocked the E2-induced SphK1 phosphorylation but also significantly attenuated the SphK1-mediated EGFR transactivation in response to E2 stimulation (Fig. S2). Interestingly, in addition to the role of ERK1/2 in initiating SphK1 activation and the resultant EGFR transactivation, inhibition of SphK1 activity by either $SphK1^{G82D}$ or SphK1-siRNA resulted in a significant attenuation of ERK1/2 activation by E2. The E2-induced ERK1/2 activation was also inhibited by a blockade of EGFR transactivation in MCF-7 cells (Fig. 2), which is in agreement with previous studies (Filardo et al., 2000; Razandi et al., 2003). Collectively, these observations suggest that ERK1/2 could be placed upstream or downstream of the SphK1 signaling and has a dual role in the initiation and amplification of a positive-feedback signaling loop across E2, SphK1, and EGFR in breast cancer cells. However, one question that has been raised by these observations is, how does E2 induce an "initial" activation of ERK1/2? Recent studies have demonstrated that membrane ERα was able to assemble a signalsome complex with various signal molecules, such as c-Src (Migliaccio et al., 1996), the p85 subunit of phosphoinositide 3 kinase (Simoncini et al., 2000), or caveolin-1 (Chambliss et al., 2000). Whether such complexes directly initiate ERK1/2 activation and the methodology to detect the initial signal require further investigation. Nevertheless, as ERK1/2, SphK1, and EGFR all possess potent mitogenic signals, this positive-feedback loop could contribute to the aberrant signaling associated with neoplastic cell growth. Indeed, inhibition of the SphK1–S1P pathway by expression of $SphK1^{G82D}$ (Sukocheva et al., 2003) or AO-Edg3 (Fig. 7 B) resulted in a significant inhibition of breast cancer cell growth in response to E2 stimulation, similar to that previously observed in experiments with the ERK1/2- or EGFR-specific inhibitors (Bange et al., 2001; Levin, 2003).

In summary, we have demonstrated for the first time that SphK1 plays a prominent role in mediating E2's nongenomic signaling across three membrane-spanning events including GPR30/mER, Edg3, and EGFR. Pathways triggered by receptor tyrosine kinases have been strongly implicated in the pathogenesis and progression of a variety of cancers, such as breast cancer (Bange et al., 2001; Levin, 2003). Indeed, the retention, up-regulation, and transactivation of EGFR in endocrine-resistant or ER-negative tumors have been demonstrated to be associated with a more aggressive phenotype, high disease recurrence rates, and decreased patient survival (Keen and Davidson, 2003; Roskoski, 2004). Our previous studies have shown an oncogenic potential for SphK1 that is not only able to transform rodent fibroblasts and form tumors in nude mice (Xia et al., 2000; Pitson et al., 2005) but also able to potentiate the carcinogenic effects of an oncogene, *H-Ras* (Xia et al., 2000), in addition to that of E2 (Sukocheva et al., 2003). Thus, the findings reported here may represent a specific example of a general system in which SphK1 plays a coordinating role between multiple oncogenic signaling systems. This not only elucidates the molecular mechanism responsible for the carcinogenic potential of SphK1, but may also provide a potential target to create new therapeutic strategies for cancer treatment by blocking the SphK1 signaling pathway.

Materials and methods

Cell culture and transfection

Human MCF-7 ($ER\alpha^+/\beta^+$) breast cancer cells were obtained from the American Type Culture Collection and cultured in phenol red–free DME (CSL Biosciences) containing 10% FBS. Constructs of $SphK^{WT}$ and $SphK^{G82D}$,

and stably transfected MCF-7 cell lines overexpressing $SphK^{WT}$, $SphK^{G82D}$, or empty vector alone, were previously described (Pitson et al., 2000; Sukocheva et al., 2003).

Experiments with siRNA and AOs

Chemically synthesized siRNA duplexes with 3′-fluorescein modification were purchased from QIAGEN. The siRNA targeted sequences were as follows: AAGAGCTGCAAGGCCTTGCCC (SphK1), AACCTCATCCAGACAGAACGA (SphK2), and AATTCTCCGAACGTGTCACGT (scrambled control siRNA). The following 18-mer phosphothioate oligonucleotides were synthesized by Geneworks: Edg-3 antisense 5′-CGGGAGGGCAGTTGCCAT-3′ and sense 5′-ATGGCAACTGCCCTCCCG-3′; and GPR30 antisense 5′-TTGGGAAGTCACATCCAT-3′ and sense 5′-GATCTCAGCACGGCAAAT-3′. For transfection, Lipofectamine 2000 reagent (Invitrogen) was used, and cells were seeded into 6-well plates at a density of 50,000 cells per well 1 d before the experiment. After a 36–48-h transfection, the targeted gene expression levels were detected by RT-PCR and/or Western blot.

RT-PCR analysis

Total RNA was extracted from cell cultures using TRIzol (Invitrogen). First-strand cDNA was synthesized from 1 μg total RNA using Omniscript reverse transcriptase (QIAGEN) and oligo-dT primer (Geneworks), in a 20 μl total volume. SphK1, SphK2, and EdgG-3 were amplified on a PTC-100 programmable thermal controller (Bio-Rad Laboratories) with an internal GAPDH control. The primers used to amplify were as follows: SphK1 sense 5′-TTGAACCATTATGCTGGCTATGA and antisense 5′-GCAGGTGTCTTGGAACCC; SphK2 sense 5′-GCTCAACTGCTCACTGTTGC and antisense 5′-GCAGGTCAGACACAGAACGA; Edg-3 sense 5′-GCCCTCTCGTGGATTTTGG and antisense 5′-CGCATGGAGACGATCAGTTG; and GPR30 sense 5′-CTGGGGAGTTTCCTGTCTGA-3′ and antisense 5′-GCTTGGGAAGTCACATCCAT-3′. The amplified products were visualized by electrophoresis on 1.5% agarose stained with ethidium bromide. Images were captured on a gel documentation system (UVitec).

Immunoblot analysis

Cells were harvested and lysed by sonication in lysis buffer containing 50 mM Tris/HCl, pH 7.4, 10% glycerol, 0.05% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 2 mM Na_3VO_4, 10 mM NaF, 1 mM EDTA, and protease inhibitors (Roche). Aliquots of cell lysates were resolved by 8–12% SDS-PAGE and transferred to Hybond-P membranes (GE Healthcare). The membranes were then probed with the appropriate antibodies, according to manufacturer's standard method. The immunocomplexes were detected with an enhanced chemiluminescence PLUS kit (GE Healthcare). Densitometry was performed on a mode imager (Typhoon 9410; GE Healthcare) using the ImageQuant program (Molecular Dynamics).

Assays of SphK activity and S1P measurement

SphK activity was routinely determined by incubating the cytosolic fraction with 5 μM D-*erythro*-sphingosine dissolved in 0.1% Triton X-100 and γ-[^{32}P]ATP for 30 min at 37°C, as described previously (Xia et al., 1998). The enzyme activity was defined as the amount of S1P formation (picomoles per minute per milligram of protein). For measurement of S1P, an enzymatic method was used as previously reported (Edsall and Spiegel, 1999). After E2 stimulation for 30 min, cells and conditional media were collected separately and lipids were extracted by alkaline mixture of chloroform and methanol. The basic aqueous fractions containing S1P were incubated with alkaline phosphatase for 30 min at 37°C and lipids were extracted twice with 1 ml of acidic chloroform. Pooled organic fractions containing newly generated sphingosine were dried by vacuum-spin, resuspended in 100 μl of reaction buffer, and incubated with recombinant SphK1 and γ-[^{32}P]ATP for 30 min at 37°C. The generated [^{32}P]S1P was then resolved by TLC and quantified as described previously (Edsall and Spiegel, 1999).

Cell growth assay

MCF-7 cells were transfected with Edg-3 sense or AOs and incubated with or without 10 nM E2 in phenol red-free media containing 1% charcoal-treated FBS for 5 d. Cell number was then quantified by the MTS assay as described previously (Sukocheva et al., 2003). The absorbance intensity of the MTS product is directly proportional to the number of viable cells in culture when cell number is between 2,000 and 200,000, otherwise the exponential dependence was determined. Total cell numbers were calculated based on calibration curves.

Statistical analysis

Unpaired *t* tests were used for comparison between two groups. For multiple comparisons, results were analyzed by analysis of variance, followed by the Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

Online supplemental material

Fig. S1 shows that E2 stimulated SphK1 phosphorylation in an ERK-dependent manner. Fig. S2 shows that inhibition of ERK activity by the ERK kinase-specific inhibitor U0126 resulted in a significant attenuation of EGFR phosphorylation in response to E2 stimulation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200506033/DC1.

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