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GPCR-induced migration of breast carcinoma cells depends on both EGFR signal transactivation and EGFR-independent pathways*

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Abstract

The epidermal growth factor receptor (EGFR) plays a key role in the regulation of important cellular processes under normal and pathophysiological conditions such as cancer. In human mammary carcinomas the EGFR is involved in regulating cell growth, survival, migration and metastasis and its activation correlates with the lack of response in hormone therapy. Here, we demonstrate in oestrogen receptor-positive and -negative human breast cancer cells and primary mammary epithelial cells a cross-communication between G protein-coupled receptors (GPCRs) and the EGFR. We present evidence that specific inhibition of ADAM15 or TACE blocks GPCR-induced and proHB-EGF-mediated EGFR tyrosine phosphorylation, downstream mitogenic signalling and cell migration. Notably, activation of the PI3K downstream mediator PKB/Akt by GPCR ligands involves the activity of sphingosine kinase (SPHK) and is independent of EGFR signal transactivation. We conclude that GPCR-induced chemotaxis of breast cancer cells is mediated by EGFR-dependent and -independent signalling pathways, with both parallel pathways having to act in concert to achieve a complete migratory response.

Keywords: ADAM; breast cancer; cross-talk; HB-EGF; shedding; sphingosine kinase (SPHK).

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Introduction

Breast cancer is the most common form of malignant neoplasm among women in Western countries and is the second leading cause of cancer-related deaths in women in the United States (Landis et al., 1999). The most successful therapies to date inhibit peptide growth factor and steroid hormone receptor signalling pathways. The epidermal growth factor receptor (EGFR) and HER2 are members of the tyrosine kinase growth factor receptor family and are most commonly associated with the progression of human tumours, including breast tumours (Harris et al., 1992). Various studies have revealed that expression of EGFR or HER2 is indicative of poor prognosis originating from the lack of response to endocrine therapy in recurrent breast cancer (Klijn et al., 1992; Nicholson et al., 1994). In breast tumours, EGFR is involved in cell survival, proliferation and cell migration, but EGFR activation has also been shown to influence the migration of normal mammary epithelial cells (Wells, 1999, 2000; Navolanic et al., 2003).

G protein-coupled receptor (GPCR) agonists such as the phospholipids lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are involved in the regulation of a wide variety of physiological functions, such as cell proliferation, migration and cell survival (Pyne and Pyne, 2000; Mills and Moolenaar, 2003). Aberrant concentrations of phospholipids or dysregulated signalling contribute to the development and progression of hyperproliferative disorders such as cancer (Mills and Moolenaar, 2003). Moreover, high expression of the thrombin receptor, a member of the protease-activated receptor family of GPCRs, is preferentially found in metastatic human breast carcinoma cell lines (Even-Ram et al., 1998).

Inter-receptor cross-talk between GPCRs and the EGFR occurs in a wide variety of transformed and non-transformed cells and represents the paradigm for receptor cross-communication (Zwick et al., 1997; Eguchi et al., 1998; Adomeit et al., 1999; Fischer et al., 2003). Treatment of cells with GPCR agonists leads to tyrosine phosphorylation of the EGFR and generation of characteristic intracellular signalling (Daub et al., 1997). This mechanistic concept of EGFR signal transactivation involves the processing of transmembrane growth factor precursors by metalloproteases, which have been recently identified as members of the ADAM (a disintegrin and metalloprotease) family of zinc-dependent proteases (Blobel, 2005). It has been demonstrated that EGFR signal transactivation has broad relevance in pathophysiological diseases such as cystic fibrosis (Lemjabbar and Basbaum, 2002), cardiovascular diseases (Asakura et al., 2002) and in the development and progression of human cancer (Fischer et al., 2003). ADAM protease-mediated

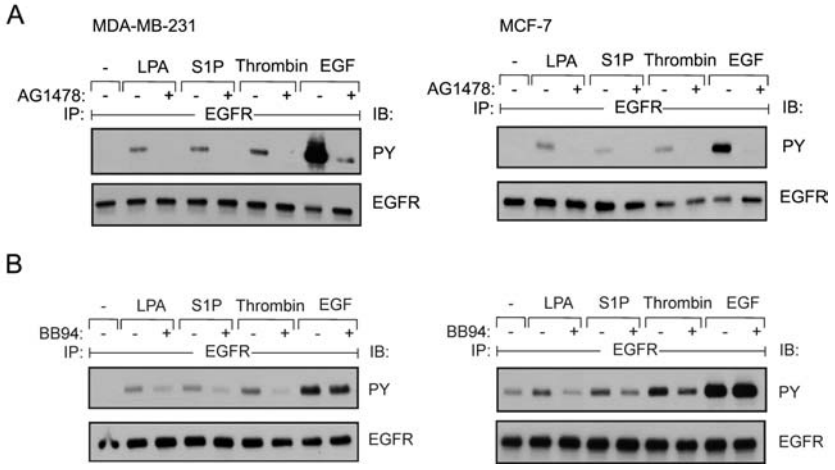


Figure 1 EGFR signal transactivation requires EGFR tyrosine kinase activity and a metalloprotease activity.
(A) Serum-starved MDA-MB-231 cells and MCF-7 cells were preincubated with EGFR-specific tyrphostin AG1478 (250 nM, 20 min) or vehicle (DMSO) and treated with LPA (10 μM), S1P (1 μM), thrombin (1 U/ml) and EGF (5 ng/ml) for 3 min. After lysis, EGFR was immunoprecipitated (IP) using anti-EGFR antibody. Tyrosine-phosphorylated EGFR was detected by immunoblotting (IB) with anti-phosphotyrosine (αPY) antibody, followed by reprobing of the same filter with anti-EGFR antibody. (B) Serum-starved MDA-MB-231 cells and MCF-7 cells were preincubated with the metalloprotease inhibitor batimastat (BB94, 5 μM, 20 min) or vehicle (DMSO), stimulated for 3 min and analysed as described in panel (A).

shedding events have been implicated in the regulation of EGFR signalling in breast cancer cells (Borrell-Pages et al., 2003). Interestingly, in oestrogen receptor-positive and -negative breast cancer cells, estrogens transactivate the EGFR via GPCR-dependent HB-EGF shedding (Filardo et al., 2002).

Therefore, we investigated whether EGFR transactivation induced by GPCR agonists such as phospholipids or thrombin is established in normal mammary epithelial cells and human breast cancer cell lines, as well as the biological significance of this cross-talk.

Our results demonstrate that phospholipids and thrombin are able to phosphorylate the EGFR in mammary epithelial cells, as well as in oestrogen receptor-positive and -negative breast cancer cells. Furthermore, phospholipid and thrombin stimulation leads to proHB-EGF- and tumour necrosis factor-α converting enzyme (TACE)- and/or ADAM15-mediated EGFR specific intracellular signalling such as phosphorylation of the adaptor protein SHC and activation of extracellular signal-regulated kinase (ERK) 1/2. Interestingly, treatment of MDA-MB-231 with phospholipids, but not with thrombin, induced cell migration. Notably, GPCR agonist-induced activation of protein kinase B or Akt (PKB/Akt) involves the activity of sphingosine kinase (SPHK) and is independent of EGFR signal transactivation. Taken together, GPCR-induced chemotaxis of breast cancer cells depends on both EGFR-dependent and -independent signalling pathways, which have to act in concert to achieve a complete migratory response.

Results

EGFR signal transactivation in human breast cancer cells by GPCR agonists involves a metalloprotease activity

To investigate the functional role of EGFR transactivation in oestrogen receptor-positive and -negative breast cancer cells, we tested MDA-MB-231 and MCF-7 cells for their responsiveness to the GPCR ligands LPA (10 μM), S1P (1 μM) and thrombin (1 U/ml). After pre-treatment of the cells with the EGFR kinase specific inhibitor AG1478 (250 nM) and stimulation of the cells with the GPCR agonists and EGF (5 ng/ml), the resulting EGFR tyrosine phosphorylation levels were monitored by immunoblot analysis. As shown in Figure 1A, multiple GPCR agonists rapidly induced EGFR activation within 3 min. Preincubation of the cells with the metalloprotease inhibitor batimastat (BB94) or the EGFR-specific inhibitor AG1478 prevented EGFR tyrosine phosphorylation in response to phospholipid and thrombin stimulation (Figure 1A,B). Taken together, these experiments demonstrate that GPCR agonists rapidly induce EGFR signal transactivation in a metalloprotease-dependent manner in different human breast-cancer cell lines.

SHC tyrosine phosphorylation and MAPK activation, but not PBK/Akt activation after GPCR agonist treatment, is EGFR kinase- and metalloprotease-dependent

We raised the question as to whether treatment of breast cancer cells with GPCR agonists leads to EGFR specific downstream signalling events. One key downstream event of the mitogenic EGFR signalling cascade is the tyrosine phosphorylation of adaptor proteins such as SHC (Prenzel et al., 2001). Figure 2A demonstrates that treating MDA-MB-231 cells with LPA, S1P and thrombin resulted in tyrosine phosphorylation of SHC. Furthermore, pre-treatment with batimastat or AG1478 completely blocked GPCR-induced tyrosine phosphorylation of SHC. We further analysed ERK1/2 activation of MDA-MB-231 cells by immunoblot using activation state-specific mitogen-activated protein kinase (MAPK) antibodies. As shown in Figure 2B, treatment of cells with GPCR agonists for 7 min activated ERK1/2. Preincubation with

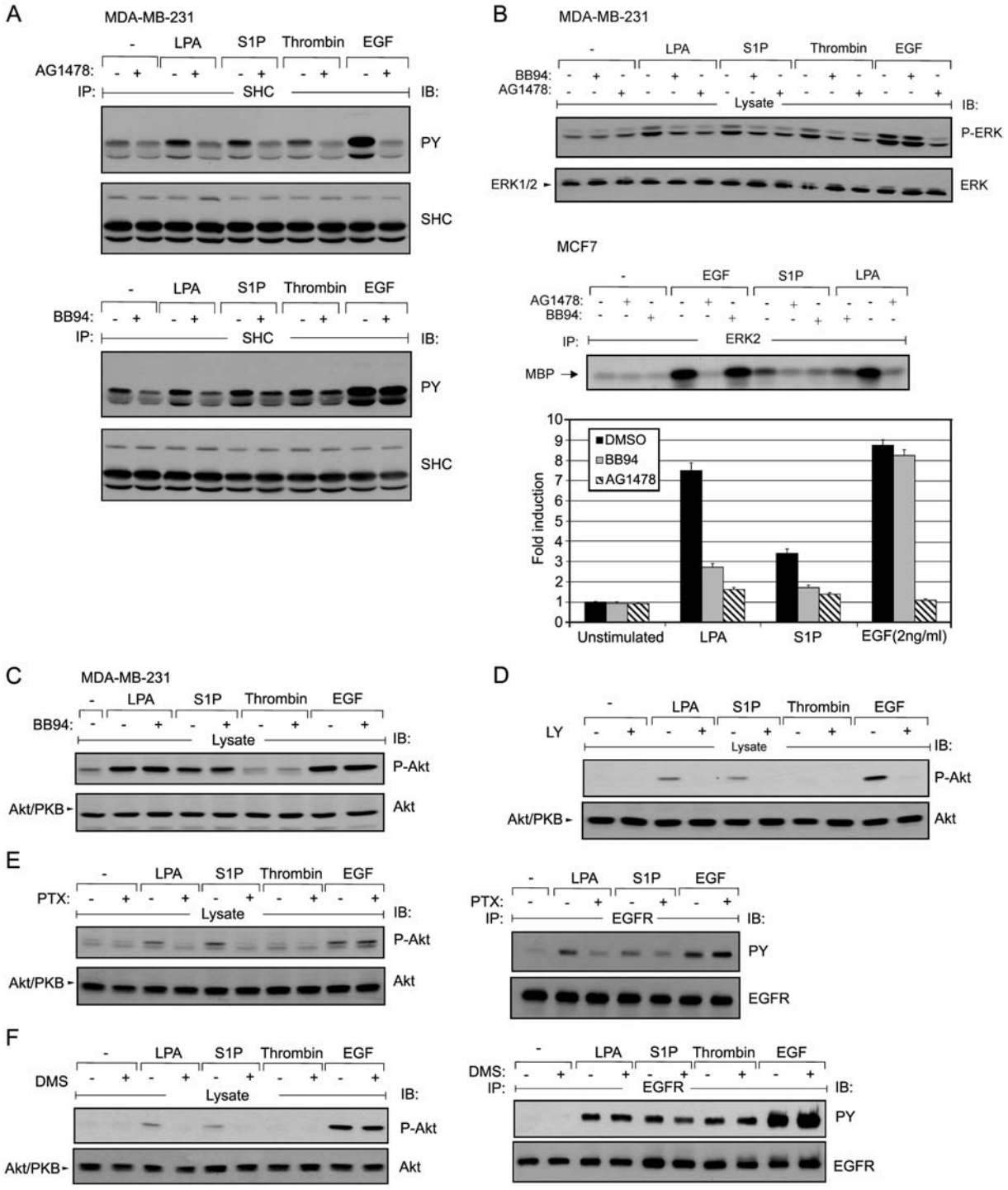


Figure 2 Phospholipid- and thrombin-induced SHC tyrosine phosphorylation and activation of ERK1/2 pathways is EGFR- and metalloprotease-dependent.

(A) Serum-starved MDA-MB-231 cells were treated as described in Figure 1A. Precipitated SHC was immunoblotted with αPY antibody, followed by reprobing of the same filters with anti-SHC antibody. (B) Serum-starved MDA-MB-231 cells were preincubated with AG1478 (250 nM, 20 min) or BB94 (5 μM, 20 min), respectively, and stimulated for 7 min as indicated. Phosphorylated ERK1/2 was detected by immunoblotting with phospho-specific ERK1/2 antibody. The same filters were reprobed with anti-ERK antibody. Serum-starved MCF-7 cells were preincubated with AG1478 (250 nM, 20 min) or BB94 (5 μM, 20 min), respectively, and stimulated for 7 min as indicated. ERK1/2 activity was determined using MBP as substrate as described under materials and methods. (C–E) Serum-starved MDA-MB-231 cells were preincubated with BB94 (5 μM, 20 min), AG1478 (250 nM, 20 min) or LY-294,002 (1 μM, 20 min) and stimulated for 7 min as indicated. Phosphorylated Akt/PKB was detected by immunoblotting with phospho-specific Akt/PKB antibody. The same filters were reprobed with anti-AKT/PKB antibody. (F) Serum-starved MDA-MB-231 cells were preincubated with PTX (5 μM, 20 min) and stimulated for 7 min (Akt) or 5 min (EGFR) as indicated. Phosphorylated Akt/PKB and EGFR were detected as previously described. (G) Serum-starved MDA-MB-231 cells were preincubated with *N*,*N*-dimethylsphingosine (DMS, 5 μM, 20 min) and stimulated for 7 min (Akt) or 5 min (EGFR) as indicated. Phosphorylated Akt/PKB and EGFR were detected as previously described.

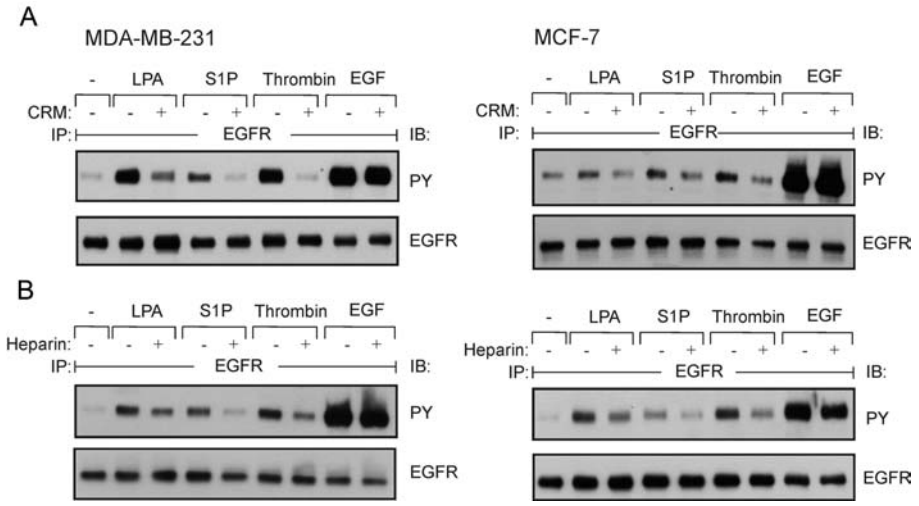


Figure 3 HB-EGF mediates GPCR-induced EGFR transactivation in breast cancer cells.
(A) Serum-starved MDA-MB-231 cells and MCF-7 cells were preincubated with heparin (100 ng/ml, 15 min) and treated with LPA (10 μM), S1P (1 μM), thrombin (1 U/ml) and EGF (5 ng/ml) for 3 min. After lysis, EGFR was immunoprecipitated (IP) using anti-EGFR antibody. Tyrosine-phosphorylated EGFR was detected by immunoblotting (IB) with anti-phosphotyrosine (αPY) antibody, followed by reprobing of the same filter with anti-EGFR antibody. (B) Serum-starved MDA-MB-231 cells and MCF-7 cells were preincubated with the diphtheria mutant CRM197 (10 μg/ml, 20 min), stimulated for 3 min and analysed as described for panel (A).

AG1478 or BB94 abrogated MAPK activation, indicating that GPCR-induced ERK1/2 activation completely depends on EGFR signal transactivation (Figure 2B). Moreover, in MCF-7 cells, ERK1/2 activation was measured *in vitro* with an immunocomplex assay using myelin basic protein (MBP) as a substrate. As shown in Figure 2B, LPA, S1P and EGF treatment increased ERK1/2 activity in MCF-7 cells, while LPA- and S1P-induced activation of ERK1/2 requires both EGFR and metalloprotease activity (Figure 2B).

In addition to the induction of mitogenic signalling, phospholipids and thrombin can act as survival factors by activating the phosphatidylinositol-3-kinase (PI3K)-dependent phosphorylation of PKB/Akt (Sautin et al., 2001). We therefore addressed the question as to whether GPCR agonists increase the activity of PKB/Akt in breast cancer cells. As shown in Figure 2C, LPA and S1P, but not thrombin, markedly induced phosphorylation of PKB/Akt in MDA-MB-231 cells. Notably, activation of PKB/Akt was not inhibited by preincubation with batimastat or AG1478. Preincubation with pertussis toxin (PTX) blocked both EGFR signal transactivation and PKB/Akt activation upon treatment with LPA and S1P (Figure 2F), indicating that both signalling events depend on PTX-sensitive G proteins of the Gi/o family. Moreover, PKB/Akt phosphorylation was sensitive to PI3K inhibition by LY294002 and was also abrogated by the SPHK inhibitor *N*,*N*-dimethylsphingosine (Figure 2E,G). On the other hand, preincubation of the cells with *N*,*N*-dimethylsphingosine did not interfere with EGFR signal transactivation (Figure 2G).

Collectively, these data substantiate the central role of the EGFR for the GPCR-induced activation of downstream signalling events, including the adaptor protein SHC and MAPKs in human breast cancer cells. Interestingly, PKB/Akt activation is independent of the signal transactivation of the EGFR, but critically depends on SPHK activity.

EGFR signal transactivation is mediated by ectodomain shedding of proHB-EGF

Different proEGF-like ligands such as proAmphiregulin (proAR), proHB-EGF and proTGFα have been shown to be involved in GPCR-EGFR cross-talk, depending on the cellular context (Gschwind et al., 2003; Hart et al., 2004; Blobel, 2005). To investigate which ligand mediates the EGFR signal transactivation following LPA, S1P and thrombin stimulation in breast cancer cells, we preincubated the cells with heparin, which abrogates both proAR and proHB-EGF function. Heparin was able to block EGFR phosphorylation after GPCR agonist treatment of MDA-MB-231 and MCF-7 cells (Figure 3A). Furthermore, the ability of the diphtheria toxin mutant CRM197, a specific inhibitor of proHB-EGF function, to block EGFR phosphorylation in response to GPCR agonists indicates that proHB-EGF mediates EGFR transactivation in these cells (Figure 3B). Together these results show that LPA-, S1P- and thrombin-induced EGFR signal transactivation in breast cancer cells is mediated by specific proteolytic processing of proHB-EGF by a metalloprotease.

EGFR signal transactivation and proHB-EGF shedding is mediated by TACE and ADAM15

We have previously shown the critical involvement of the metalloprotease-disintegrin TACE in proAmphiregulin shedding in response to GPCR stimulation (Gschwind et al., 2003). Furthermore, TACE is involved in constitutive shedding of proEGFR ligands (Peschon et al., 1998; Sunnarborg et al., 2002) and immunoblot analysis revealed that TACE is widely expressed in human breast cancer cells (Figure 4F). To examine the functional role of TACE for GPCR-EGFR cross-talk in breast cancer cells, we used two different approaches to selectively inhibit TACE activity. Expression of endogenous TACE was blocked using a siRNA technique. ADAM10, ADAM15

and TACE protein were effectively and specifically reduced by transfecting siRNAs into MDA-MB231 cells (Figure 4B). Downregulation of TACE expression but not of the related ADAM10, ADAM12 or ADAM15 completely suppressed phospholipid-induced EGFR tyrosine phosphorylation in MDA-MB-231 cells (Figure 4A). Surprisingly, thrombin-induced EGFR phosphorylation was only partially reduced by transfection of TACE siRNAs, while ADAM15 siRNA resulted in a similar reduction of the transactivation signal induced by thrombin (Figure 4A). As expected, suppression of neither protease had an affect on signalling events induced by direct EGF stimulation.

To further substantiate these findings, we ectopically expressed dominant-negative mutants of TACE (ΔMP17) and ADAM15 (ΔMP15), which are lacking the pro- and metalloprotease domain (Salomon et al., 1999). In MDA-MB-231 and MCF-7 cells, ΔMP17 was able to interfere with phospholipid-induced EGFR transactivation (Figure 4C,E). Again, ΔMP17 and ΔMP15 reduced thrombin-induced EGFR tyrosine phosphorylation in both breast cancer cell lines (Figure 4C,E).

Taken together, these experiments demonstrate that phospholipids specifically activate TACE, which leads to proHB-EGF-mediated EGFR signal transactivation. In contrast, thrombin activates two different metalloproteases, namely ADAM15 and TACE, which process the same EGF-like growth factor precursor proHB-EGF.

Phospholipids enhance EGFR signal transactivation-dependent cell motility in MDA-MB-231 cells

Direct stimulation of the EGFR induces chemotactic responses in the metastatic human breast-cancer cell line MDA-MB-231 (Sturge et al., 2002), as well as GPCR agonists such as LPA, promoting migration in head and neck squamous carcinoma and ovarian cancer cells (Fishman et al., 2001; Gschwind et al., 2002). We therefore assumed that GPCR-induced EGFR signal transactivation might influence the migratory behaviour of breast cancer cells.

Using a modified Boyden-chamber assay, we investigated the effect of phospholipids and thrombin on the chemotactic motility of breast cancer cells. Both phospholipids and EGF, but not thrombin, were able to promote chemotactic migration in MDA-MB-231 cells (Figure 5A). Moreover, inhibition of the EGFR kinase or the metalloprotease activity abolished phospholipid- and EGF-induced migration of MDA-MB-231 cells (Figure 5A). Furthermore, we evaluated the effect of TACE siRNA and HB-EGF siRNA on the migratory behaviour of MDA-MB-231 cells. Downregulation of TACE protein efficiently blocked S1P-induced chemotactic migration, while EGF-induced migration was slightly reduced by TACE siRNA. On the other hand, knockdown of proHB-EGF completely blocked migration of MDA-MB-231 after S1P treatment (Figure 5B). Furthermore, inhibition of PI3K with LY294002 or MEK with PD98059 significantly blocked phospholipid-induced chemotaxis (Figure 5C).

These results demonstrate that physiologically relevant concentrations of phospholipids are able to promote cell migration of human breast cancer cells, thus identifying EGFR signal transactivation via TACE and HB-EGF and the EGFR-independent PI3K-pathway as the underlying molecular mechanisms.

EGFR signal transactivation in primary mammary epithelial cells

Various reports demonstrating that EGFR signal transactivation is established in a wide variety of cancer and non-tumour cell lines raised the question as to whether this GPCR-EGFR cross-talk also exists in primary cells. We prepared primary mouse mammary epithelial cells and stimulated them with the phospholipids LPA and S1P and thrombin. All three ligands phosphorylated the EGFR within 3 min (Figure 6A). Furthermore, preincubation with batimastat completely blocked tyrosine phosphorylation of the EGFR after GPCR stimulation.

In addition to EGFR activation, GPCR stimulation of mammary epithelial cells resulted in downstream activation of ERK1/2 MAPK, which is completely EGFR kinase-dependent (Figure 6B).

Discussion

Aberrant GPCR signalling has been related to the development and progression of pathophysiological disorders such as cancer (Gutkind, 1998). Several reports indicate higher expression of LPA or S1P receptors in tumour cells, including breast cancer, compared to untransformed cells (Goetzl et al., 1999a,b). Moreover, the number of thrombin receptors of human breast cancer cells has been correlated with their metastatic ability (Even-Ram et al., 1998). Increasing experimental evidence supports the concept of the EGFR as a central integrator of diverse GPCR signals (Carpenter, 1999; Marinissen and Gutkind, 2001; Prenzel et al., 2001). Therefore, we investigated the role of EGFR signal transactivation in response to GPCR agonists in oestrogen-dependent (MCF-7) and oestrogen-independent (MDA-MB-231) human breast cancer cells and in primary mouse mammary epithelial cells. Indeed, our results demonstrate that treatment of breast cancer cell lines and primary mammary epithelial cells with LPA, S1P and thrombin leads to rapid tyrosine phosphorylation of the EGFR (Figure 1 and 6).

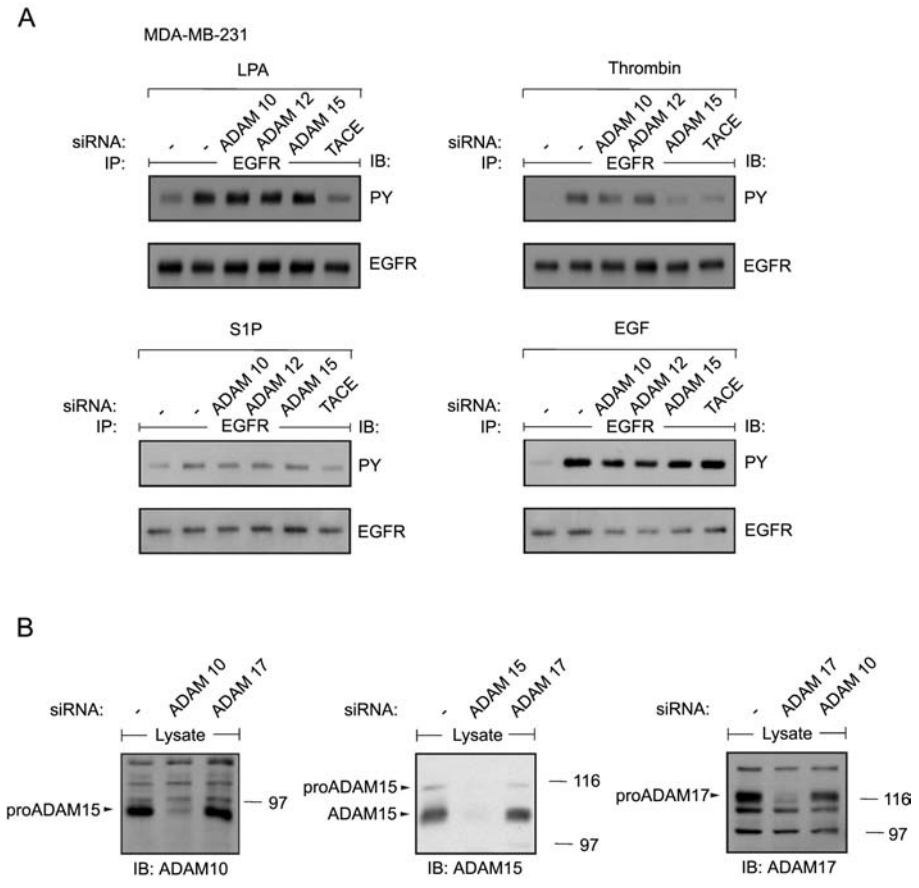
Previously, Filardo et al. (2000) showed that overexpression of the GPCR GPR30 in MDA-MB-231 cells resulted in oestrogen-induced transactivation of the EGFR through release of HB-EGF. Furthermore, osteopontin induced an Src-dependent transactivation of the EGFR in MDA-MB-231 and EGFR-transfected MCF-7 cells (Das et al., 2004). Our experimental data presented here show a batimastat-sensitive and therefore metalloprotease-mediated EGFR signal transactivation. In human breast-cancer cell lines, we demonstrate that metalloproteases mediate transactivation of the EGFR after GPCR agonist stimulation via proteolytic processing of proHB-EGF (Figures 3 and 4). Previous studies demonstrated proteolytic processing of the membrane-spanning proEGF-like growth factor proHB-EGF by a single zinc-dependent metalloprotease such as ADAM9, 10, 12, 15 or TACE, depending on the cellular context (Suzuki et al., 1997; Izumi et al., 1998; Yan et al., 2002; Hao et al., 2004; Hart et al., 2004; Schafer et al., 2004; Blobel,

2005). Our current data identify the metalloprotease TACE as a mediator of pro-HB-EGF shedding in LPA- and S1P-induced EGFR signal transactivation in human breast cancer cell lines since the expression of either a dominant-negative TACE mutant or knockdown of TACE expression by RNA interference blocked EGFR tyrosine phosphorylation (Figure 4A,C,E). Earlier observations showed that TACE is distinctly overexpressed in human breast cancer and proposed a role for TACE in EGFR activation by proteolytic cleavage of proTGFα (Borrell-Pages et al., 2003).

Interestingly, thrombin-induced EGFR signal transactivation was mediated by the action of two different metalloproteases: ADAM15 and TACE (Figure 4A,C,E). This is the first time that, within one cell line, it has been shown that one distinct GPCR agonist activates two different metalloproteases that process the same proEGF-like ligand. In contrast to Asakura et al. (2002), we did not observe downregulation of the endogenous expression of ADAM10, 15 and 17 on ectopic expression of a dominant negative mutant lacking the metalloprotease and the prodomain (data not published). In addition, transactivation induced by LPA, S1P and thrombin was sensitive to pertussis toxin, indicating that the same G proteins are involved in EGFR signal transactivation (Figure 4E); however, the mechanism by which metalloproteases of the ADAM family are activated by heterotrimeric G proteins is currently poorly understood (Tanaka et al., 2004). Concomitantly, inhibition of EGFR function or metalloprotease activity by small chemical compounds after GPCR stimulation blocked phosphorylation of SHC and activation of the ERK/MAPK in MDA-MB-231 and MCF-7 cells (Figure 2A,B). These data indicate the important role of the EGFR in transmitting mitogenic signals generated by GPCRs in breast cancer cells and primary mammary epithelial cells.

The fatal consequences of breast cancer are mainly due to organ-specific metastasis, which results from the capability of the cancer cells to migrate and invade other tissues. Even-Ram et al. (1998) demonstrated that the PAR1 expression level is directly correlated with the invasiveness of primary breast specimens and established cancer cell lines. We demonstrated that LPA and S1P induced an EGFR-dependent migration of MDA-MB-231 cells (Figure 5). Previous reports demonstrated endothelial-cell wound healing after LPA and S1P treatment (Lee et al., 2000). Interestingly, stimulation of EDG-1-overexpressing HEK293 cells with nanomolar concentrations of S1P increased chemotaxis, whereas micromolar concentrations inhibited chemotaxis (Wang et al., 1999).

Blockade of TACE by either batimastat or TACE siRNA or inhibition of HB-EGF function with CRM197 or proHB-EGF siRNA abrogated the migratory behaviour after GPCR stimulation (Figure 5). TACE siRNA also reduced basal and EGF-induced migration, arguing for the existence of autocrine EGFR activation loops by TACE, which are important for chemotaxis of breast cancer cells. In contrast, thrombin, which is capable of activating the EGFR and downstream signalling events, did not induce migration of MDA-MB-231 cells. This result is in agreement with the data of Kamath et al. (2001), who showed that thrombin-induced activation of PAR1 inhibited the



migration of MDA-MB-231 cells. Interestingly, Even-Ram et al. (1998) proposed a direct correlation between thrombin receptor expression and the degree of invasiveness of breast carcinoma cell lines, while cell motility in response to thrombin stimulation was not investigated. Chemotactic responses of MDA-MB-231 induced by EGF are entirely dependent on PI3K activity (Sturge et al., 2002). This finding is further supported by our observation that PKB/Akt as a downstream target of PI3K is phosphorylated after LPA and S1P, but not after thrombin treatment of the cells (Figure 2C–G). LPA-, S1P- and HB-EGF-induced chemotaxis was dependent on PI3K activity (Figure 5C). PKB/Akt activation was not blocked by the EGFR-specific inhibitor AG1478 or the metalloprotease inhibitor BB94, suggesting that PKB/Akt is independent of EGFR signal transactivation (Figure 2C). However, PTX blocked PKB/Akt activation, as well as EGFR signal transactivation, upon LPA and S1P treatment, indicating that PTX-sensitive G proteins of the Gi/o family activate two separate signalling pathways that are indispensable for chemotaxis in breast cancer cells (Figure 2F). Very recently, Sukocheva et al. (2003) presented data showing that in breast cancer cells SPHK is activated within 5 min upon G protein activation. *N*,*N*-

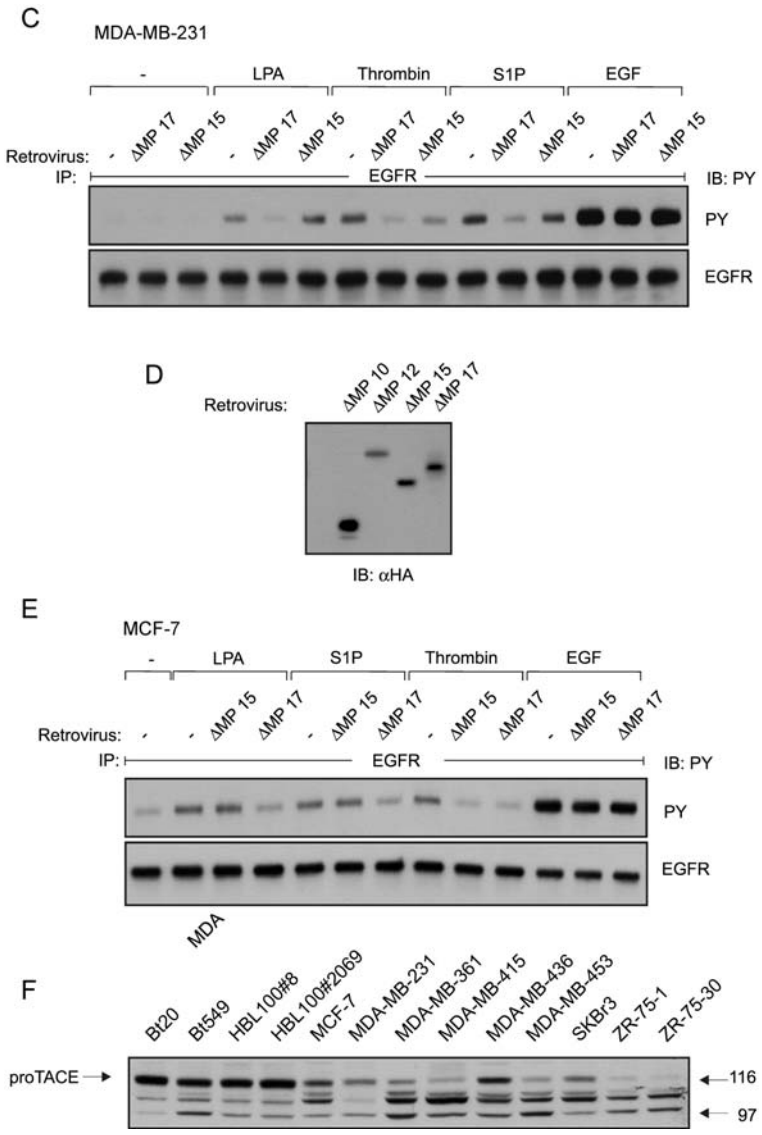


Figure 4 EGFR signal transactivation in breast cancer cells is mediated by ADAM15 and TACE.
(A) MDA-MB-231 cells were transfected with ADAM10, 12, 15 or TACE siRNA, serum-starved, stimulated for 3 min with LPA (10 μM), S1P (1 μM), thrombin (1 U/ml) and EGF (5 ng/ml) and assayed for EGFR tyrosine phosphorylation content. (B) siRNA raised against ADAM proteases blocks endogenous expression. MDA-MB-231 cells were transfected with siRNA directed against ADAM10, 12, 15 or TACE. Gene expression was analysed by immunoblotting with polyclonal ADAM antibodies. (C) After retroviral gene transfer of dominant negative ADAM15 (ΔMP15) and dominant negative TACE (ΔMP17) in MDA-MB-231 cells, cells were serum-starved, stimulated for 3 min with LPA (10 μM), S1P (1 μM), thrombin (1 U/ml) and EGF (5 ng/ml) and assayed for EGFR tyrosine phosphorylation content. (D) Expression of dominant negative ADAM15 and TACE carrying a C-terminal HA tag was confirmed by immunoblotting of total cell lysates with anti-HA antibody. (E) After retroviral gene transfer of dominant negative ADAM15 (ΔMP15) and dominant negative TACE (ΔMP17) in MCF-7 cells, cells were serum-starved, stimulated for 3 min with LPA (10 μM), S1P (1 μM), thrombin (1 U/ml) and EGF (5 ng/ml) and assayed for EGFR tyrosine phosphorylation extent. (F) TACE expression in different breast cell lines and breast cancer cell lines was detected by immunoblotting with a polyclonal TACE antibody.

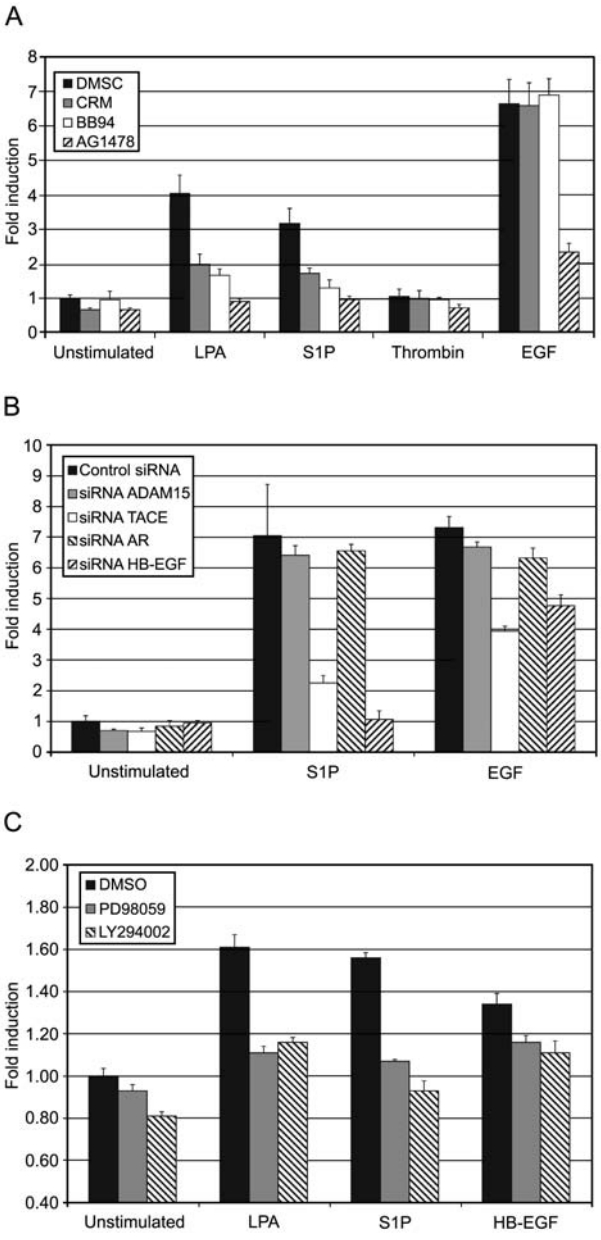


Figure 5 Phospholipid-induced migration of MDA-MB-231 cells depends on EGFR signal transactivation mediated by TACE and HB-EGF and on PI3K activity.
(A) Serum-starved MDA-MB-231 cells were treated with AG1478 (250 nM), the metalloprotease inhibitor BB94 (5 μM) and CRM197 (10 μg/ml) and analysed for trans-well migration towards LPA (10 μM), S1P (1 μM), thrombin (1 U/ml) and EGF (5 ng/ml) as chemoattractant. Each bar is the average of quadruplicate values (mean±SD). (B) siRNA-transfected and serum-starved MDA-MB-231 cells were analysed for trans-well migration towards LPA and EGF as chemoattractant. Each bar is the average of triplicate values (mean±SD). (C) Unstarved MDA-MB-231 cells were treated with the MEK inhibitor PD98059 (250 nM) and the PI3K inhibitor LY-294002 (5 μM) and analysed for trans-well migration towards LPA (10 μM), S1P (1 μM) and HB-EGF (5 ng/ml) as chemoattractant. Each bar is the average of triplicate values (mean±SD).

Dimethylsphingosine, an inhibitor of SPHK, blocked phospholipid-induced PKB/Akt activation, but did not interfere with EGFR signal transactivation (Figure 2G). In contrast to the results of Pitson et al. (2003), activation

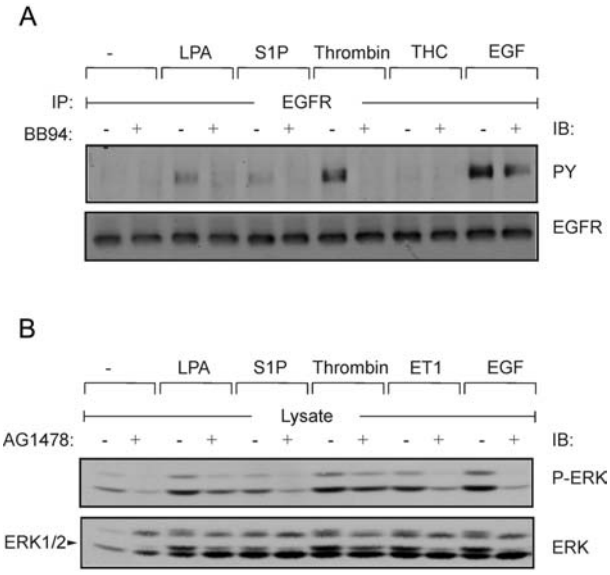


Figure 6 EGFR signal transactivation in primary mammary epithelial cells.
(A) Serum-starved mammary epithelial cells were preincubated with BB94 (5 μM, 20 min) or vehicle (DMSO) and stimulated for 3 min. After lysis, EGFR was immunoprecipitated (IP) using a polyclonal anti-mouse EGFR antibody. Tyrosine-phosphorylated EGFR was detected by immunoblotting (IB) with anti-phosphotyrosine (αPY) antibody, followed by reprobing of the same filter with anti-EGFR antibody. (B) Serum-starved mammary epithelial cells were preincubated with AG1478 (250 nM, 20 min) and stimulated for 7 min as indicated. Phosphorylated ERK1/2 was detected by immunoblotting with phospho-specific ERK1/2 antibody. The same filters were reprobed with anti-ERK antibody.

of PKB/Akt, and therefore of SPHK, was not blocked by PB98059 in our experiments (data not shown).

Taken together, phospholipid treatment of breast cancer cells induces EGFR-dependent and EGFR-independent signalling pathways via PTX-sensitive G proteins, which have to act in concert to achieve a migratory response. Future investigations will have to focus on the impact of EGFR signal transactivation in human breast cancer, especially in oestrogen receptor-positive tumours, which are not responsive to endocrine therapy. Thus, components of the EGFR signal transactivation process represent promising targets for therapeutic intervention in combination with anti-oestrogen therapy.

Materials and methods

Cell culture

All cell lines (American Type Culture Collection, Manassas, VA, USA) were routinely grown according to the supplier's instructions. Batimastat (BB94) was kindly provided by Dr. K. Maskos (Max-Planck-Institute of Biochemistry, Martinsried, Germany). Heparin (Sigma, St. Louis, MO, USA), CRM197 (Quadratech Ltd., Epsom, UK) and AG1478 (Alexis Biochemicals, San Diego, CA, USA), as well as *N,N*-dimethylsphingosine (DMS, Sigma) and LY294002 (Sigma) were added to serum-starved cells 20 min before the respective growth factor. EGF and HB-EGF were dissolved in phosphate-buffered saline (PBS). LPA and all other chemicals were purchased from Sigma.

Full-length cDNAs encoding ADAM15 and TACE were amplified by PCR from a human placenta cDNA library and subcloned into pcDNA3 (Invitrogen, Carlsbad, CA, USA) and pLXSN vectors (Clontech, Palo Alto, CA, USA). For virus production, dominant negative protease constructs lacking the pro- and metalloprotease domains were generated as described elsewhere (Salomon et al., 1999; Asakura et al., 2002). All protease constructs included a C-terminal haemagglutinin (HA) tag, detectable with an anti-HA monoclonal antibody (Babco, Richmond, VA, USA). The amphotropic packaging cell line Phoenix was transfected with pLXSN retroviral expression plasmids by the calcium phosphate/chloroquine method as described previously (Kinsella and Nolan, 1996). At 24 h after transfection, the viral supernatant was collected and used to infect subconfluent MDA-MB-231 cells (3×10^5 cells/6-well plate) and MCF-7 cells (1×10^5 cells/6-well plate).

Protein analysis

Cells were lysed and proteins immunoprecipitated as previously described (Borrell-Pages et al., 2003). Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membranes. Western blots were performed according to standard methods. The antibodies against human EGFR (108.1) and SHC have been described before (Gschwind et al., 2003). Phosphotyrosine was detected with the 4G10 monoclonal antibody (UBI, Lake Placid, NY, USA). Polyclonal anti-phospho-p44/p42 (Thr202/Tyr204) MAPK antibody and anti-phospho-Akt (Ser473) antibody were purchased from New England Biolabs (Beverly, MA, USA). Polyclonal anti-Akt1/2 and anti-ERK2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-TACE and anti-ADAM10 antibody from Chemicon (Harrow, UK). The polyclonal antibody against ADAM15 was produced against the peptide sequence CGTKSQGPAKPPPPKPL.

RNA interference

Transfection of 21-nt-long siRNA duplexes (Dharmacon Research, Lafayette, CO, USA) for targeting of endogenous genes was carried out using Oligofectamine (Invitrogen) and 4.2 μg of siRNA duplex per 6-well plate as previously described (Borrell-Pages et al., 2003). Transfected MDA-MB-231 cells were serum-starved and assayed 3 days after transfection. Highest efficiencies in silencing target genes were obtained using mixtures of siRNA duplexes targeting different regions of the gene of interest. The siRNA sequences used were previously described (Borrell-Pages et al., 2003). Specific silencing of targeted genes was confirmed by Western blotting (ADAM10, ADAM15 and TACE).

MAPK assay

Endogenous ERK2 was immunoprecipitated from lysates obtained from 6-well dishes using 0.4 μg of anti-ERK2 antibody. Precipitates were washed three times with 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton-X-100 and washed once with kinase buffer [20 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 200 μM sodium orthovanadate]. Kinase reactions were performed in 30 μl of kinase buffer supplemented with 0.5 mg/ml MBP, 50 μM ATP, and 1 μCi of [γ-^{32}P]ATP for 10 min at room temperature. Reactions were stopped by the addition of 30 μl of Laemmli buffer and subjected to gel electrophoresis on 15% gels. Labelled MBP was quantitated using a phosphorimager (Fuji, Tokyo, Japan).

Primary epithelium cell cultures

After sacrifice of the mouse (day 14–15 of pregnancy), it was dipped entirely in isopropanol for 5 s, four glands were isolated and the lymph nodes were removed. First, the glands were set in PBS in culture dish on ice, then placed on an inverted 100-mm dish cover under a sterile hood and 300 μl of complete medium with collagenase (1 mg/ml) was added. The glands were minced with scalpel blades to an estimated fragment size of 1.0–2.0 mm^3 and were then added to 20 ml of complete medium with collagenase in a 50-ml conical tube and incubated overnight (37°C, 5% CO_2).

Next day, the tissue was dissociated with a 10-ml pipette until no tissue clumps were observed. Centrifugation was carried out at 100 *g* for 10 min, and the top fat layer and the liquid phase were discarded. The pellet was resuspended and dissociated in 5 ml of complete medium (without collagenase from this point on). After centrifugation at 100 *g* for 10 min the supernatant was removed, the pellet was resuspended in 5–10 ml of complete medium with a 19-gauge needle (twice) and centrifuged again at 100 *g* for 10 min. Finally, the resuspended pellet was plated in complete medium on a cell culture dish (one gland per 100-mm dish), followed by 48 h of incubation (37°C, 5% CO_2). Before stimulation, the cells were serum-starved for 24 h.

Migration

MDA-MB-231 cells in exponential growth (Figure 5C) or serum-starved for 24 h (Figure 5A,B) were harvested, washed and resuspended in standard medium without fetal calf serum (FCS). Cells were preincubated with either DMSO (control) or the inhibitors for 20 min. Then 2×10^5 cells were seeded into polycarbonate membrane inserts (6.5-mm diameter and 8-μm pore size) in 24-trans-well dishes. The lower chamber was filled with standard medium without FCS containing the chemoattractant. Cells were permitted to migrate for 6 h.

Following incubation, non-migrated cells were removed from the upper surface of the membranes. The cells that had migrated to the lower surface were fixed and stained with crystal violet. Evaluation of migrated cells was performed by counting the cells using a microscope.

Statistical analysis

Values are expressed as mean±SD of at least triplicate samples.

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