Signal characteristics of G protein-transactivated EGF receptor

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The epidermal growth factor receptor (EGFR) tyrosine kinase recently was identified as providing a link to mitogen-activated protein kinase (MAPK) in response to G protein-coupled receptor (GPCR) agonists in Rat-1 fibroblasts. This cross-talk pathway is also established in other cell types such as HaCaT keratinocytes, primary mouse astrocytes and COS-7 cells. Transient expression of either G_q- or G_i-coupled receptors in COS-7 cells allowed GPCR agonist-induced EGFR transactivation, and lysophosphatidic acid (LPA)-generated signals involved the docking protein Gab1. The increase in SHC tyrosine phosphorylation and MAPK stimulation through both G_q- and G_i-coupled receptors was reduced strongly upon selective inhibition of EGFR function. Inhibition of phosphoinositide 3-kinase did not affect GPCR-induced stimulation of EGFR tyrosine phosphorylation, but inhibited MAPK stimulation, upon treatment with both GPCR agonists and low doses of EGF. Furthermore, the Src tyrosine kinase inhibitor PP1 strongly interfered with LPA- and EGF-induced tyrosine phosphorylation and MAPK activation downstream of EGFR. Our results demonstrate an essential role for EGFR function in signaling through both G_q- and G_i-coupled receptors and provide novel insights into signal transmission downstream of EGFR for efficient activation of the Ras/MAPK pathway.
Keywords: EGF receptor/G protein-coupled receptors/ MAP kinase/PI3-kinase/Src

Introduction

Definition of cellular responses such as proliferation or differentiation by a variety of external stimuli involves the regulation of transcriptional events in eukaryotic cells through intracellular signaling cascades, including signaling pathways that activate kinases of the mitogen-activated protein kinase (MAPK) family (Treisman, 1996). The extensively studied MAPKs of the ERK subfamily become activated in response to growth factors either through receptor tyrosine kinase (RTK)- or through G protein-coupled receptor (GPCR)-triggered signals (Pagès *et al.*, 1993; Faure *et al.*, 1994; Malarkey *et al.*, 1995; van Biesen *et al.*, 1996b). Transmission of these signals requires the formation of a complex between the Grb2 adaptor protein with the guanine nucleotide exchange factor Sos, which upon recruitment to the plasma membrane allows activation of the small G protein Ras, resulting in subsequent activation of the MAPK pathway (Egan *et al.*, 1993; van Biesen *et al.*, 1995; Dikic *et al.*, 1996). The critical function of Ras in MAPK signal transduction triggered by GPCRs which are coupled either to heterotrimeric G_i or G_q proteins (Crespo *et al.*, 1994) was shown to be sensitive to genistein, suggesting that a tyrosine kinase was involved in this process (van Corven *et al.*, 1993; Sadoshima and Izumo, 1996). Moreover, stimulation of various GPCRs, such as those for lysophosphatidic acid (LPA) (van Biesen *et al.*, 1995), thyrotropin-releasing hormone (Ohmichi *et al.*, 1994), endothelin-1 (ET-1) (Cazaubon *et al.*, 1994), bradykinin (Lev *et al.*, 1995) or thrombin (Chen *et al.*, 1996), rapidly induces tyrosine phosphorylation of the adaptor protein SHC and SHC–Grb2 complex formation, steps that couple both GPCRs and RTKs to Ras (Bonfini *et al.*, 1996; Chen *et al.*, 1996; van der Geer *et al.*, 1996). In addition, a recent report implicated an unidentified 100 kDa tyrosine-phosphorylated, Grb2-binding protein in LPA-induced MAPK activation (Kranenburg *et al.*, 1997).

Recently, we identified the epidermal growth factor receptor (EGFR) as an essential link in the GPCR-mediated MAPK activation pathway in Rat-1 fibroblasts treated with the GPCR agonists ET-1, LPA or thrombin (Daub *et al.*, 1996). Moreover, angiotensin II-induced platelet-derived growth factor receptor β (βPDGFR) and thrombin-stimulated insulin-like growth factor-1 receptor (IGF-1R) tyrosine phosphorylation were reported in primary rat smooth muscle cells (Linseman *et al.*, 1995; Rao *et al.*, 1995), suggesting that transactivation of distinct RTKs might contribute in a cell-type specific manner to GPCR-mediated mitogenic signaling. In addition to GPCR activation, a variety of stimuli including cellular stresses such as UV irradiation or calcium-dependent responses in PC12 cells result in ligand-independent RTK transactivation (Knebel *et al.*, 1996; Rosen and Greenberg, 1996; Weiss *et al.*, 1997). Interestingly, cytoplasmic kinases of the Src family were also implicated in signal generation by similar stimuli (Devary *et al.*, 1992; Rusanescu *et al.*, 1995). In particular, GPCR-mediated activation of the Ras/MAPK pathway was reported in some recent studies to involve Src function (Dikic *et al.*, 1996; Luttrell *et al.*, 1996; Sadoshima *et al.*, 1996; Schieffer *et al.*, 1996), which raised the possibility that concerted action of RTKs and Src family kinases might be required to initiate intracellular signaling cascades.

In the present study, we show that ligand-independent EGFR transactivation occurs in diverse cell types. Employing COS-7 cells, we demonstrate that G_q- and G_i-coupled receptors can mediate stimulation of EGFR

tyrosine phosphorylation and, moreover, that for both GPCR subfamilies this event is essential for the stimulation of SHC tyrosine phosphorylation and MAPK activation. In addition, we present evidence for phosphoinositide 3-kinase (PI3-K) function downstream of the EGFR and address the question of whether other tyrosine kinases might be involved in EGFR transactivation and signal transduction.

Results

### EGFR transactivation in diverse cell types

We have shown previously that treatment of Rat-1 fibroblasts with the GPCR ligands ET-1, LPA and thrombin results in rapid induction of EGFR tyrosine phosphorylation. To substantiate these findings further, we investigated this mechanism in different cell lines. As shown in Figure 1A, stimulation of human HaCaT keratinocytes with thrombin or LPA resulted in enhanced tyrosine phosphorylation of endogenous EGFR. In addition, thrombin or extracellularly applied ATP triggered a comparable response in primary mouse astrocytes (Figure 1B). Although it is presently unclear whether ATP acts through GPCRs or ATP-gated ion channels (Barnard *et al.*, 1994; Salter and Hicks, 1995), EGFR transactivation might represent an important signaling element in ATP-evoked responses in astrocytes. Thrombin and LPA were also effective in COS-7 cells, where these GPCR ligands stimulated tyrosine phosphorylation of endogenous EGFR to a similar extent as 1–3 ng/ml EGF (Figure 1C). Thrombin receptor peptide (TRP) was able to mimic the thrombin response, confirming specific activation of the thrombin GPCR in COS-7 cells. The LPA vehicle bovine serum albumin (BSA), included as a negative control, showed no effect. For further characterization of the tyrosine-phosphorylated 170 kDa protein co-migrating with the EGFR, immunoprecipitations with different anti-EGFR antibodies were performed and the precipitates were treated with glycosidase. In all these control experiments, the 170 kDa band behaved exactly as the EGFR (data not shown). Moreover, time course experiments revealed that LPA rapidly induced EGFR tyrosine

Fig. 1. EGFR transactivation in different cell lines. (**A**) Quiescent human HaCaT keratinocytes were treated for the indicated times with 2 U/ml thrombin, 10 μM LPA or LPA vehicle BSA. After lysis, EGFR was immunoprecipitated with anti-EGFR mAb 108.1. Following gel electrophoresis, tyrosine-phosphorylated EGFR was detected by immunoblotting with monoclonal anti-phosphotyrosine (αPY) antibody (upper panel). The amount of EGFR in immunoprecipitates was analyzed further by reprobing of the same filter with anti-EGFR antibody (lower panel). (**B**) Serum-starved primary mouse astrocytes were incubated for 2 min with 2 U/ml thrombin or 100 μM ATP, lysed and EGFR was immunoprecipitated using polyclonal anti-EGFR antibody. Immunoblotting was done with αPY mAb (upper panel), followed by reprobing with anti-EGFR antibody (lower panel). (**C**) Quiescent COS-7 cells were stimulated with 2 U/ml thrombin, 100 μM thrombin receptor peptide (TRP, peptide sequence: SFLLRNPNDKYEPF; van Corven *et al.*, 1993), LPA vehicle BSA, 10 μM LPA or the indicated concentrations of EGF for 5 min and lysed. (**D**) Quiescent COS-7 cells were treated for the indicated times with 10 μM LPA or 3 ng/ml EGF prior to lysis. The immunoprecipitations in (C) and (D) were performed with anti-EGFR mAb 108.1, followed by immunoblotting with monoclonal anti-phosphotyrosine antibody (upper panel) and subsequent reprobing with polyclonal anti-EGFR antibody (lower panel). Molecular size is indicated on the right.
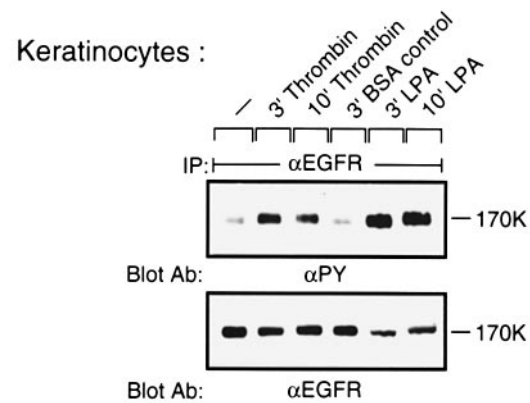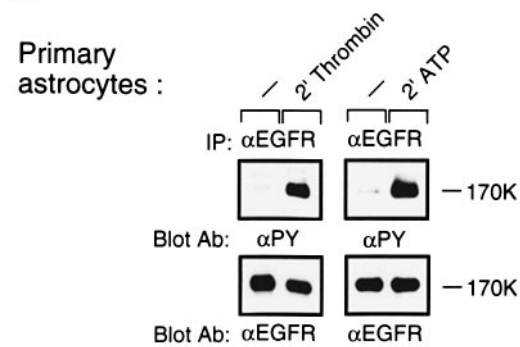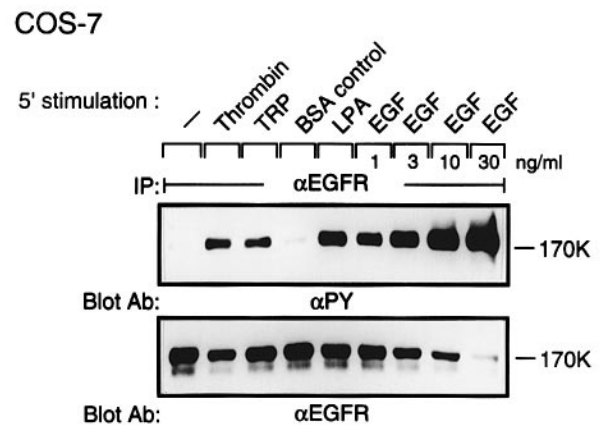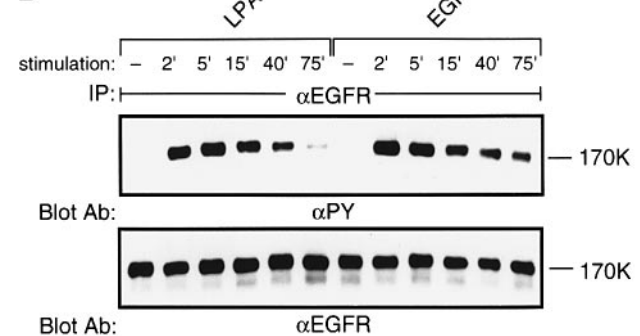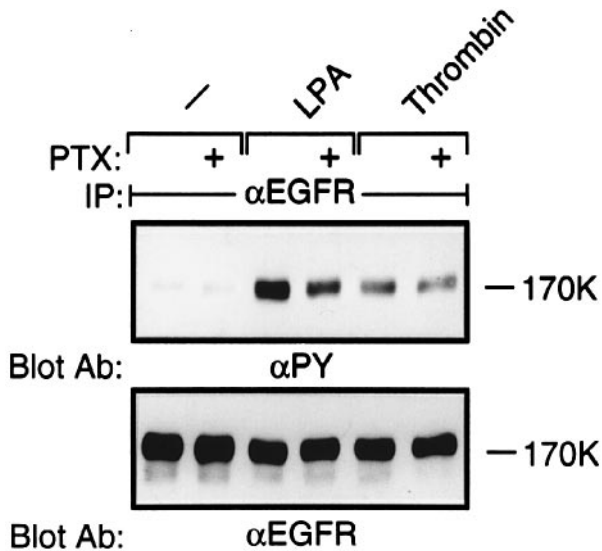
Fig. 2. Effect of PTX treatment on LPA- and thrombin-induced EGFR tyrosine phosphorylation in COS-7 cells. Serum-starved COS-7 cells were stimulated with 2 U/ml thrombin or 10 μM LPA for 5 min and lysed. Tyrosine phosphorylation of EGFR was assayed by immunoprecipitation with mAb 108.1 and immunoblotting with anti-phosphotyrosine antibody (upper panel), followed by reprobing with anti-EGFR antibodies (lower panel). Where indicated, cells were pre-treated with 100 ng/ml PTX for 18 h. Molecular weight is indicated on the right.

phosphorylation, only slightly lagging behind that induced by 3 ng/ml EGF. The EGF-induced EGFR tyrosine phosphorylation was more prolonged than that induced by LPA, possibly due to accelerated receptor down-regulation mediated by LPA-specific cellular responses (Figure 1D). Taken together, these experiments demonstrate that GPCR-mediated EGFR transactivation is not restricted to Rat-1 fibroblasts but occurs in various cell types from different species.
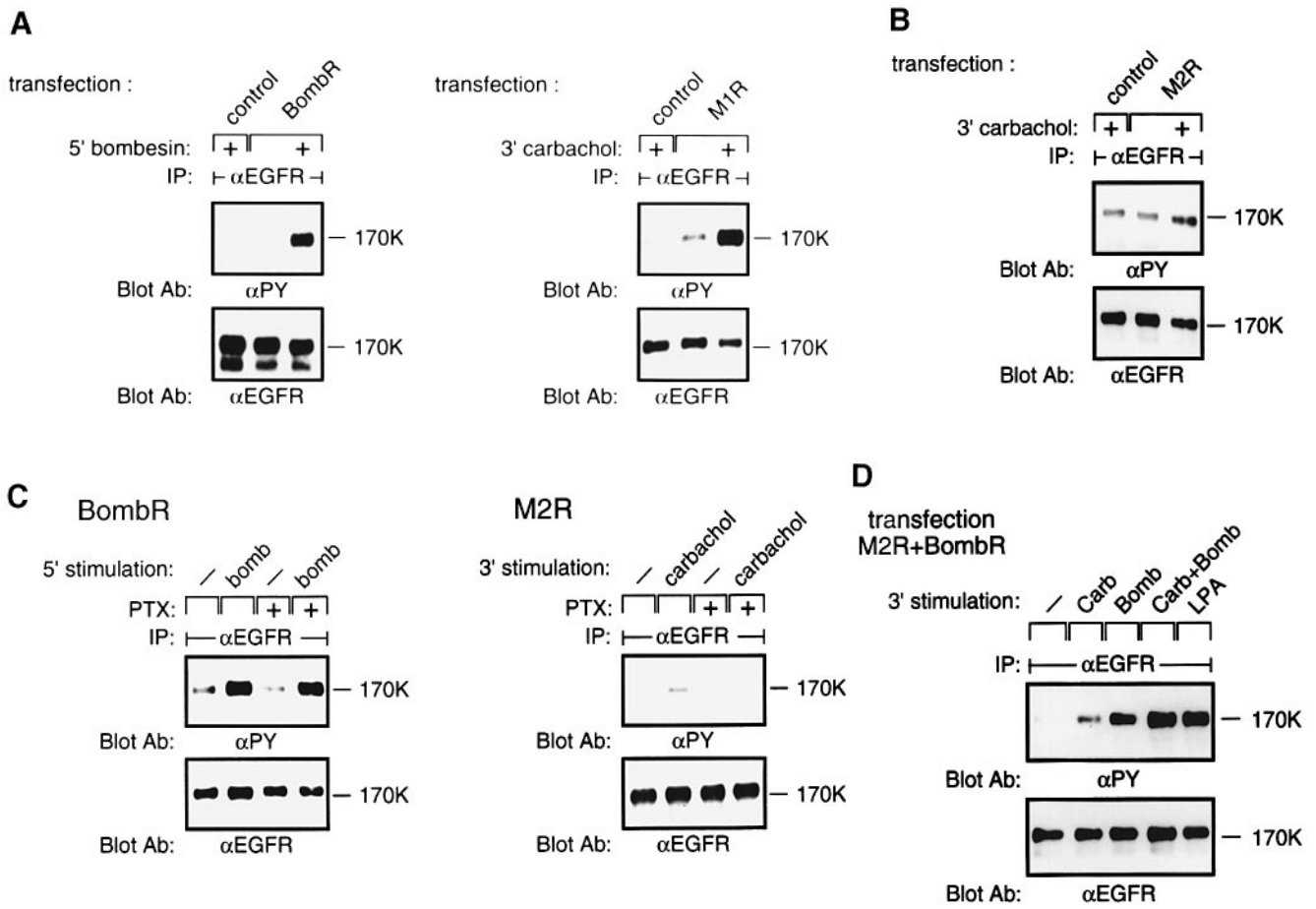
Stimulation of EGFR tyrosine phosphorylation by G_q- and G_i-coupled receptors in COS-7 cells

To examine the elements involved in EGFR transactivation further, we selected COS-7 cells, since this cell line has been used extensively to study GPCR signaling and, moreover, serves as a model system to investigate GPCR-mediated activation of the MAPK pathway (Crespo *et al.*, 1994; Hawes *et al.*, 1995; van Biesen *et al.*, 1995). First, we analyzed the effect of pertussis toxin (PTX) on EGFR transactivation. PTX catalyzes ADP ribosylation of α subunits in the G_i and G_o subfamilies of heterotrimeric G proteins, a covalent modification which leads to inactivation. Treatment of cells with PTX partially attenuated LPA-induced EGFR tyrosine phosphorylation, while the thrombin response was not significantly affected (Figure 2). Notably, PTX reduces the LPA-stimulated EGFR transactivation to a level similar to that observed after thrombin treatment. Since G_o proteins are not expressed in COS-7 cells (van Biesen *et al.*, 1996a), this indicates that LPA signals through G_i and through PTX-insensitive G proteins, while thrombin only utilizes PTX-insensitive pathways. To assess whether PTX-insensitive G proteins of the G_q subclass are able to mediate EGFR transactivation in COS-7 cells, we transiently expressed GPCRs known to be coupled predominantly to G_q proteins (Crespo *et al.*,

1994; Offermanns *et al.*, 1994). As shown in Figure 3A, exposure of cells transfected with bombesin receptor (BombR) or M1 muscarinic acetylcholine receptor (M1R) to the respective GPCR ligands resulted in increased EGFR tyrosine phosphorylation, which was not observed upon stimulation of control-transfected cells. In cells transfected with the G_i-coupled M2 muscarinic acetylcholine receptor (M2R), GPCR agonist-enhanced EGFR tyrosine phosphorylation was detectable, although this effect was weaker than that obtained upon G_q-coupled receptor stimulation (Figure 3B). Moreover, PTX did not affect bombesin-induced EGFR transactivation, while the carbachol-induced response mediated by M2R was blocked upon PTX pre-incubation (Figure 3C). These experiments show that EGFR transactivation may occur through the engagement of both PTX-insensitive and -sensitive pathways. The above experiment, which showed that, in the absence of PTX, LPA induces stronger EGFR tyrosine phosphorylation than thrombin while their responses become similar upon PTX pre-treatment, suggests that G_i-mediated and PTX-insensitive pathways might exert additive effects on EGFR transactivation in COS-7 cells. To address this question, we co-transfected G_i-coupled M2R and G_q-coupled BombR and stimulated either each receptor alone or both simultaneously. Co-stimulation with carbachol and bombesin resulted in a more pronounced induction of EGFR tyrosine phosphorylation than observed with either ligand alone, providing evidence for the cooperativity of G_i- and G_q-mediated pathways. Moreover, the combined effect on EGFR transactivation was similar to the response observed upon LPA treatment (Figure 3D).

Identification of Gab1 as Grb2-interacting protein potentially involved in GPCR-mediated signaling

We previously reported that several tyrosine-phosphorylated proteins including the EGFR, SHC and an unidentified 110 kDa protein associate with glutathione *S*-transferase (GST)–Grb2 fusion protein upon ET-1, LPA and thrombin stimulation in Rat-1 fibroblasts (Daub *et al.*, 1996). Specific association of a similar set of tyrosine-phosphorylated proteins was also detected when we stimulated COS-7 cells with LPA or 3 ng/ml EGF, which induces EGFR tyrosine phosphorylation comparable with the LPA response, and subsequently incubated lysates with GST–Grb2 fusion protein (Figure 4A). Pre-incubation with the EGFR-specific inhibitor AG1478 prevented LPA-induced association of tyrosine-phosphorylated proteins (Levizki and Gazit, 1995), indicating that, in addition to SHC, the 110 kDa protein represents an additional substrate for transactivated EGFR in COS-7 cells. Moreover, a Grb2-associating protein of similar molecular weight recently was implicated in LPA-induced signaling to the Ras/MAPK pathway, but was not identified (Kranenburg *et al.*, 1997). Utilizing an immunodepletion strategy, we were able to identify the 110 kDa protein as Gab1 (Holgado-Madruga *et al.*, 1996), since the tyrosine-phosphorylated 110 kDa protein upon LPA treatment was specifically and quantitatively removed when lysates were pre-cleared with polyclonal anti-Gab1 antiserum prior to *in vitro* association with GST–Grb2 (Figure 4B). When we performed immunoprecipitations with anti-Gab1 antiserum, LPA-induced stimulation of Gab1 tyrosine phosphorylation and

Fig. 3. Stimulation of endogenous EGFR tyrosine phosphorylation through transiently expressed G_q- and G_i-coupled receptors in COS-7 cells. COS-7 cells were transiently transfected in 6-well dishes with cDNA encoding BombR, M1R or M2R (0.5 µg each) or control-transfected as described in Materials and methods. Cells transfected with G_q-coupled receptors (**A**) were stimulated with 200 nM bombesin (BombR) or 1 mM carbachol (M1R) for the indicated times. Cell transiently expressing G_i-coupled M2 receptor (**B**) were treated with 1 mM carbachol as indicated. Control-transfected cells were subjected to the same agonist stimulation (A and B). BombR- or M2R-transfected cells were pre-treated for 18 h with 100 ng/ml PTX before agonist stimulation (**C**). Cells transiently co-expressing M2R and BombR were treated for 3 min with 1 mM carbachol and 200 nM bombesin, either alone or in combination, or 10 µM LPA for 3 min (**D**). After cell lysis, endogenous EGFR was immunoprecipitated with mAb 108.1 followed by immunoblotting with anti-phosphotyrosine antibody (upper panels). Filters were reprobed with anti-EGFR antibodies (lower panels). Molecular size is indicated on the right.

Gab1–Grb2 complex formation were clearly detectable in the absence but not in the presence of the GST–Gab1-N fusion protein used for antibody generation (Figure 4C). Moreover, both signaling events were abrogated when cells were pre-incubated with AG1478 in order to block EGFR function (Figure 4D), indicating that Gab1 functions downstream of EGFR in LPA signaling.

G_q- and G_i-coupled receptor-mediated SHC tyrosine phosphorylation requires functional EGFR

In order to establish that EGFR function represents a general prerequisite for GPCR-mediated tyrosine phosphorylation of proteins such as SHC, we transfected either the G_q-coupled BombR or the G_i-coupled M2R into COS-7 cells and determined SHC tyrosine phosphorylation and association of immunoprecipitated SHC with the adaptor protein Grb2 upon stimulation with the respective GPCR ligands. Bombesin, acting through its ectopically expressed receptor, stimulated SHC tyrosine phosphorylation as well as SHC–Grb2 complex formation. Both signaling events were blocked when the dominant-negative EGFR mutant HER-CD533 was co-transfected (Kashless et al., 1991;

Redemann et al., 1992), while an analogous truncation mutant of the PDGFR (βPDGFR-CD504) had no inhibitory effect (Figure 5A), although both RTK mutants were expressed at comparable levels (data not shown). Furthermore, the EGFR-specific inhibitor AG1478 suppressed SHC tyrosine phosphorylation and complex formation with Grb2 upon stimulation of the G_i-coupled M2R (Figure 5B). Both approaches to inhibit EGFR function appeared to be specific, since SHC tyrosine phosphorylation and association with Grb2 induced by c-Src overexpression in COS-7 cells or upon nerve growth factor treatment of PC12 cells were unaffected (data not shown). Thus G_i- and G_q-mediated pathways depend on a functional EGFR to trigger SHC tyrosine phosphorylation, a signaling step considered to be critical for subsequent activation of Ras-dependent pathways.

### EGFR inhibition interferes with MAPK activation by G_q- and G_i-coupled receptors

We previously demonstrated a functional role for EGFR in the GPCR-mediated activation of the MAPK pathway of Rat-1 cells upon stimulation with agonists such as

LPA. To establish the COS-7 cell system that allows the investigation of MAPK pathway control elements, we employed a transiently expressed hemagglutinin (HA) epitope-tagged form of the MAPK ERK2 (HA-ERK2) and measured phosphorylation of myelin basic protein (MBP). The system was calibrated with 2 ng/ml EGF, which induces EGFR tyrosine phosphorylation comparable
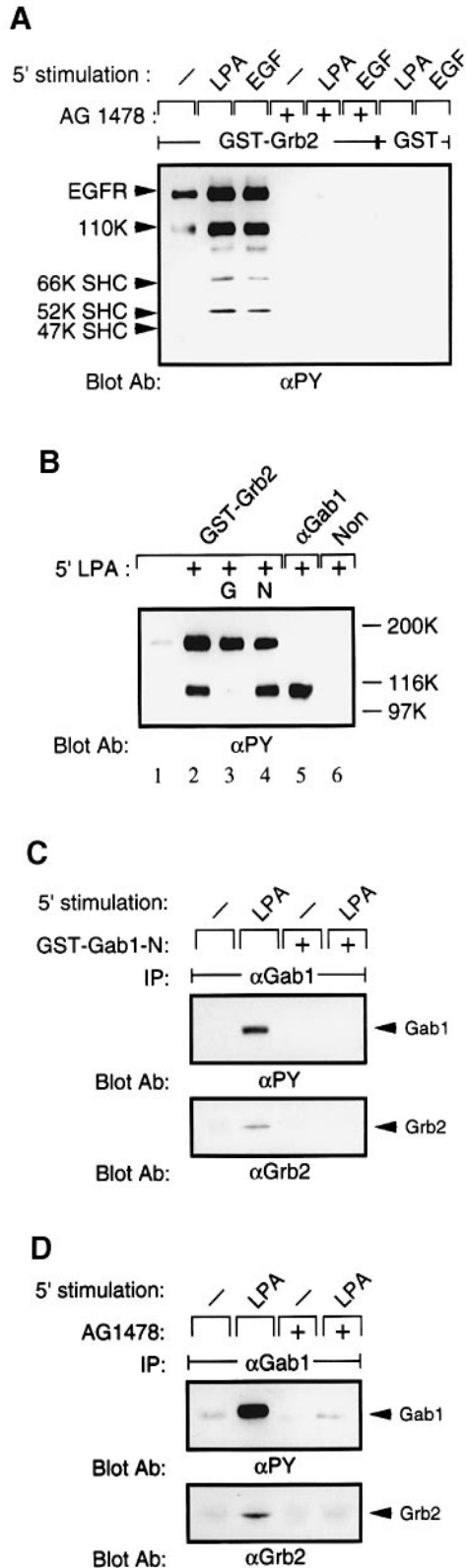
with that obtained with LPA and resulted in MAPK activation which was, as expected, inhibited upon co-transfection of HER-CD533, while the dominant-negative βPDGFR-CD504 mutant had no effect (Figure 6A, left panel). Conversely, MAPK activation through βPDGFR was unaffected by HER-CD533 but abrogated by βPDGFR-CD504 co-expression (Figure 6A, middle panel). These controls confirmed the selective inhibition of EGFR and βPDGFR signaling by HER-CD533 and βPDGFR-CD504, respectively. Under these conditions, LPA-induced MAPK activation was reduced by nearly 80% upon HER-CD533 co-expression, while βPDGFR-CD504 showed no significant effect (Figure 6A, right panel). Co-transfection of either of the dominant-negatives RTK mutants, which were expressed at comparable levels as shown by [^{35}S]methionine labeling of cells transfected in parallel followed by nearly quantitative immunoprecipitation (Figure 6B), did not affect expression of HA-ERK2 (Figure 6A, lower panel). These data clearly confirm our previous Rat-1 cell experiments, which demonstrated that EGFR transactivation represents an essential step in the signaling cascade that mediates LPA-induced activation of the MAPK pathway. Using this tightly controlled transient transfection system, we investigated the role of EGFR in G_q- and G_i-mediated MAPK activation. As shown in Figure 6C, the dominant-negative EGFR mutant nearly abolished MAPK activity stimulated by the G_q-coupled BombR and inhibited MAPK activation through the G_i-coupled M2R by 50% (Figure 6C). Comparable results were obtained upon pre-treatment of cells with AG1478 to block specifically EGFR kinase function (Figure 6D), while pre-incubation with this tyrphostin did not significantly affect MAPK activation mediated either by co-transfected IGF-1R or induced by expression of v-Abl, an oncogenic variant of the tyrosine kinase c-Abl. The selectivity of AG1478 was demonstrated using EGFR$^{-/-}$ primary fibroblasts, in which even higher concentrations of the inhibitor did not affect GPCR-mediated MAPK activation (E.Zwick *et al.*, in preparation). Thus EGFR

Fig. 4. Identification of Gab1 as Grb2-associated protein which becomes tyrosine phosphorylated upon LPA stimulation. (**A**) Following pre-incubation with or without 100 nM AG1478 for 10 min, quiescent COS-7 cells were stimulated for 5 min with 10 μM LPA or 3 ng/ml EGF. Lysates were subjected to *in vitro* association with GST–Grb2 fusion protein or GST alone, followed by immunoblotting with anti-phosphotyrosine antibody. The positions of tyrosine-phosphorylated EGFR, 110 kDa protein and SHC isoforms are indicated on the left. (**B**) Quiescent COS-7 cells were stimulated for 5 min with 10 μM LPA as indicated, and lysates were either control-incubated or immunoprecipitated with polyclonal anti-Gab1 antiserum (lane 5) or appropriate non-immune serum (lane 6) for 3 h at 4°C. Supernatants from these immunoprecipitations (G and N) and control-incubated lysates were then subjected to an *in vitro* association with GST–Grb2 fusion protein (lanes 1–4), followed by anti-phosphotyrosine immunoblotting. Molecular size is indicated on the right. (**C**) After stimulation with 10 μM LPA and lysis of COS-7 cells, anti-Gab1 immunoprecipitations were performed in the presence or absence of 10 μg of GST–Gab1-N fusion protein, followed by immunoblotting with anti-phosphotyrosine antibody (upper panel) or monoclonal anti-Grb2 antibody (lower panel). The positions of Gab1 and Grb2 are indicated on the right. (**D**) Following pre-incubation with or without 100 nM AG1478 for 10 min, quiescent COS-7 cells were stimulated for 5 min with 10 μM LPA. After lysis, Gab1 was immunoprecipitated using polyclonal anti-Gab1 antiserum and subsequently immunoblotted with anti-phosphotyrosine antibody (upper panel) or monoclonal anti-Grb2 antibody (lower panel). The positions of Gab1 and Grb2 are indicated on the right.

A

B
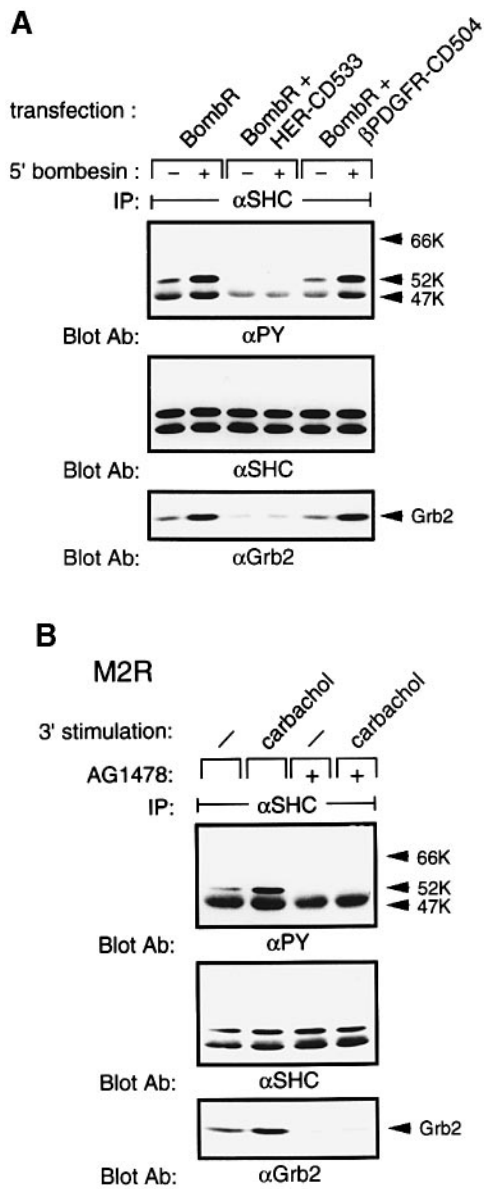
Fig. 5. Effect of EGFR inhibition on SHC tyrosine phosphorylation and SHC–Grb2 complex formation mediated through G$_q$- and G$_i$-coupled receptors. COS-7 cells were transiently transfected in 6-well dishes with cDNA encoding BombR plus either control vector or cDNA encoding HER-CD533 (0.3 μg/well) or βPDGFR-CD504 (0.5 μg/well) and stimulated with 200 nM bombesin for 5 min (**A**). Cells transfected with cDNA encoding M2R were pre-treated either with 100 nM AG1478 or an equal volume of DMSO for 10 min before stimulation with 1 mM carbachol (**B**). After lysis, SHC was immunoprecipitated using polyclonal anti-SHC antiserum and subsequently immunoblotted with anti-phosphotyrosine antibody (upper panels) or monoclonal anti-Grb2 antibody (lower panels). Filters were reprobed with polyclonal anti-SHC antibody (middle panels). The positions of SHC isoforms and Grb2 are indicated by arrowheads.

function is important for G$_q$- and G$_i$-mediated MAPK activation and, since in the case of the BombR (data not shown) and M2R (Crespo *et al.*, 1994) this event is suppressed by dominant-negative Ras, EGFR represents a pathway element upstream of Ras.

Interestingly, comparison of the time course of MAPK activation in COS-7 cells upon treatment of COS-7 cells with either LPA or 3 ng/ml EGF revealed that LPA induced

MAPK activity more rapidly (Figure 6E). This suggests that during the initial phase of LPA-mediated MAPK activation, additional LPA-triggered signals synergize with EGFR-mediated signaling, leading to a more rapid MAPK activation than upon EGFR activation alone.

PI3-K is required for MAPK activation but not EGFR transactivation

To assess the role of PI3-K function in the GPCR-mediated transactivation signal leading to EGFR tyrosine phosphorylation, we pre-treated COS-7 cells with the PI3-K inhibitor wortmannin (100 nM) prior to LPA or EGF stimulation. As shown in Figure 7A, wortmannin did not affect EGFR tyrosine phosphorylation in response to either different LPA concentrations or 3 ng/ml EGF. Similar results were obtained for M2R- or BombR-mediated EGFR transactivation. and even up to 10 μM wortmannin had no effect on the LPA response (data not shown). Thus PI3-K is not involved in cross-talk between GPCRs and the EGFR in COS-7 cells. Next, we analyzed the effect of the PI3-K inhibitors wortmannin and LY294002 (Hawes *et al.*, 1996) on GPCR-mediated MAPK activation. Both inhibitors significantly reduced MAPK activity stimulated through M2R or in response to LPA, as reported previously (Hawes *et al.*, 1996; Lopez-Ilasaca *et al.*, 1997). Moreover, we found strong reduction of BombR-mediated MAPK stimulation. Surprisingly, PI3-K inactivation also markedly reduced MAPK activation induced by low doses of EGF (3 ng/ml), while the response to constitutively active Ras was not significantly affected (Figure 7B). Similar results were obtained when we examined tyrosine phosphorylation of endogenous ERK2 (data not shown). Our findings confirm that PI3-K function plays an essential role upstream of Ras in GPCR-mediated MAPK stimulation and, furthermore, indicate an important function downstream of the EGFR. In order to identify a potential site of PI3-K action downstream of EGFR, we tested whether PI3-K might interact with GST–Grb2 fusion protein. As shown in Figure 7C, the p85α subunit specifically interacted with GST–Grb2 in unstimulated cells, consistent with previous work (Wang *et al.*, 1995). Interestingly, however, stimulation with LPA or 3 ng/ml EGF enhanced this interaction. Moreover, this response was abrogated when cells were pre-treated with EGFR-specific inhibitor AG1478, although the basal level of Grb2-associated p85α was unaffected by EGFR inactivation. Furthermore, we detected an increase in PI3-K activity associating with GST–Grb2 upon LPA treatment, which was reversed upon AG1478 pre-treatment (Figure 7D). Control incubations indicated that most of the detected PI-K activity reflected PI3-K activity specifically associated with the GST–Grb2 fusion protein. Although we did not discriminate whether PI3-K interacts either directly with Grb2 or indirectly via a linker protein such as, for example, Gab1, EGFR-mediated recruitment of functionally active PI3-K into Grb2 complexes might represent the critical, PI3-K inhibitor-sensitive step necessary for subsequent activation of the MAPK cascade.

### Effects of the Src tyrosine kinase inhibitor PP1

Since cytoplasmic tyrosine kinases of the Src family have been implicated recently in GPCR-mediated MAPK activation (Dikic *et al.*, 1996; Luttrell *et al.*, 1996;
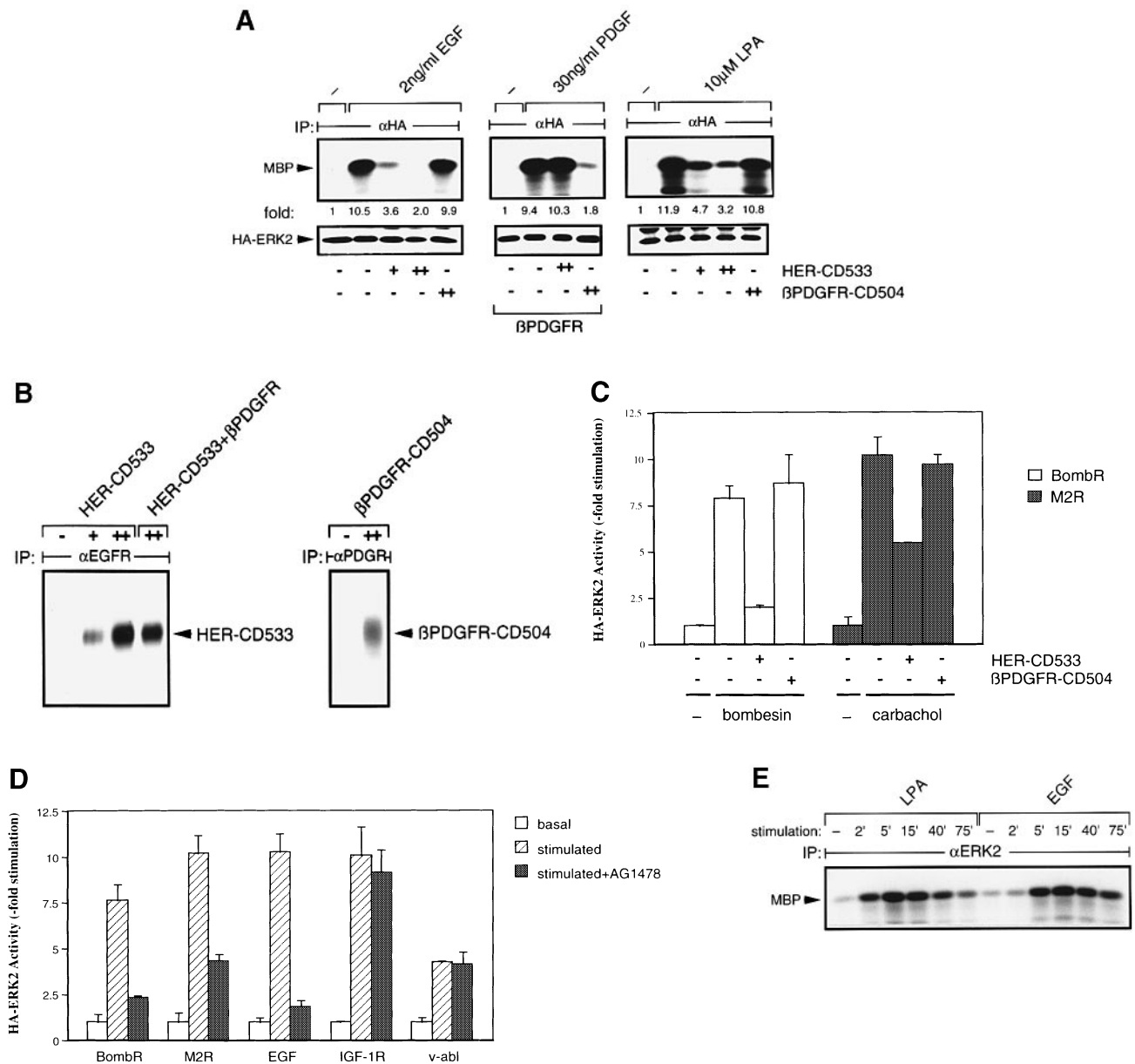
Fig. 6. Effect of EGFR inhibition on stimulation of MAPK activity. (**A**) COS-7 cells were transiently transfected with expression plasmid encoding HA-ERK2 (100 ng/well) plus either empty expression vector or plasmid encoding βPDGFR (25 ng/well). Where indicated, plasmids encoding HER-CD533 (+: 50 ng/well, ++: 150 ng/well) or βPDGFR-CD504 (++: 250 ng/well) were co-transfected. Following serum starvation for 24 h, cells were treated for 7 min with EGF (2 ng/ml), PDGF B/B (30 ng/ml) or LPA (10 μM), lysed and HA-ERK2 activity was determined using MBP as substrate as described in Materials and methods. Phosphorylated MBP was visualized by autoradiography after gel electrophoresis (upper panel) and HA-ERK2 was immunoblotted in parallel using polyclonal anti-ERK2 antibody (lower panel). For expression control, in parallel, transfected cells were labeled with [^{35}S]methionine and lysates were subjected to immunoprecipitation with monoclonal anti-EGFR antibody (**B**, left panel) or monoclonal anti-βPDGFR antibody (B, right panel). Mutant RTK expresion was visualized by autoradiography (18 h exposure). (**C**) COS-7 cells were transfected with expression plasmid encoding HA-ERK2 (100 ng/well) plus expression plasmids encoding either BombR or M2R (100 ng/well). Where indicated, plasmids encoding HER-CD533 (150 ng/well) or βPDGFR-CD504 (250 ng/well) were co-transfected. After 7 min stimulation with 200 nM bombesin or 1 mM carbachol, HA-ERK2 activity was determined. (**D**) COS-7 cells were transfected with expression plasmid encoding HA-ERK2 (100 ng/well) plus either empty expression vector or plasmids encoding BombR, M2R, IGF-1R (50 ng/well) or v-Abl (200 ng/well). Where indicated, cells were treated with 100 nM AG1478 10 min prior to stimulation with 200 nM bombesin, 1 mM carbachol, 3 ng/ml EGF or 10^{-7} M IGF-1 for 7 min and subsequent detemination of HA-ERK2 activity. (**E**) Serum-starved COS-7 cells were treated for the indicated times with 10 μM LPA or 3 ng/ml EGF prior to lysis. Immunoprecipitated endogenous ERK2 was subjected to an *in vitro* kinase assay, and phosphorylated MBP was visualized by autoradiography after gel electrophoresis. Data shown in (C) and (D) represent the mean ± SD for duplicate samples.

Sadoshima *et al.*, 1996; Schieffer *et al.*, 1996), we investigated a possible connection between Src and the EGFR. For this purpose, we utilized the tyrosine kinase inhibitor PP1, which was reported to be specific for Src-like kinases (Hanke *et al.*, 1996). Pre-treatment of cells with 10 μM PP1 attenuated tyrosine phosphorylation of endogenous

Src. The residual Src tyrosine phosphorylation is most likely due to CSK-mediated phosphorylation at Tyr527, a non-autophosphorylation site with inhibitory functions (Luttrell *et al.*, 1997). Expression of a constitutively active Src mutant lacking this tyrosine resulted in increased Src tyrosine phosphorylation which was strongly inhibited
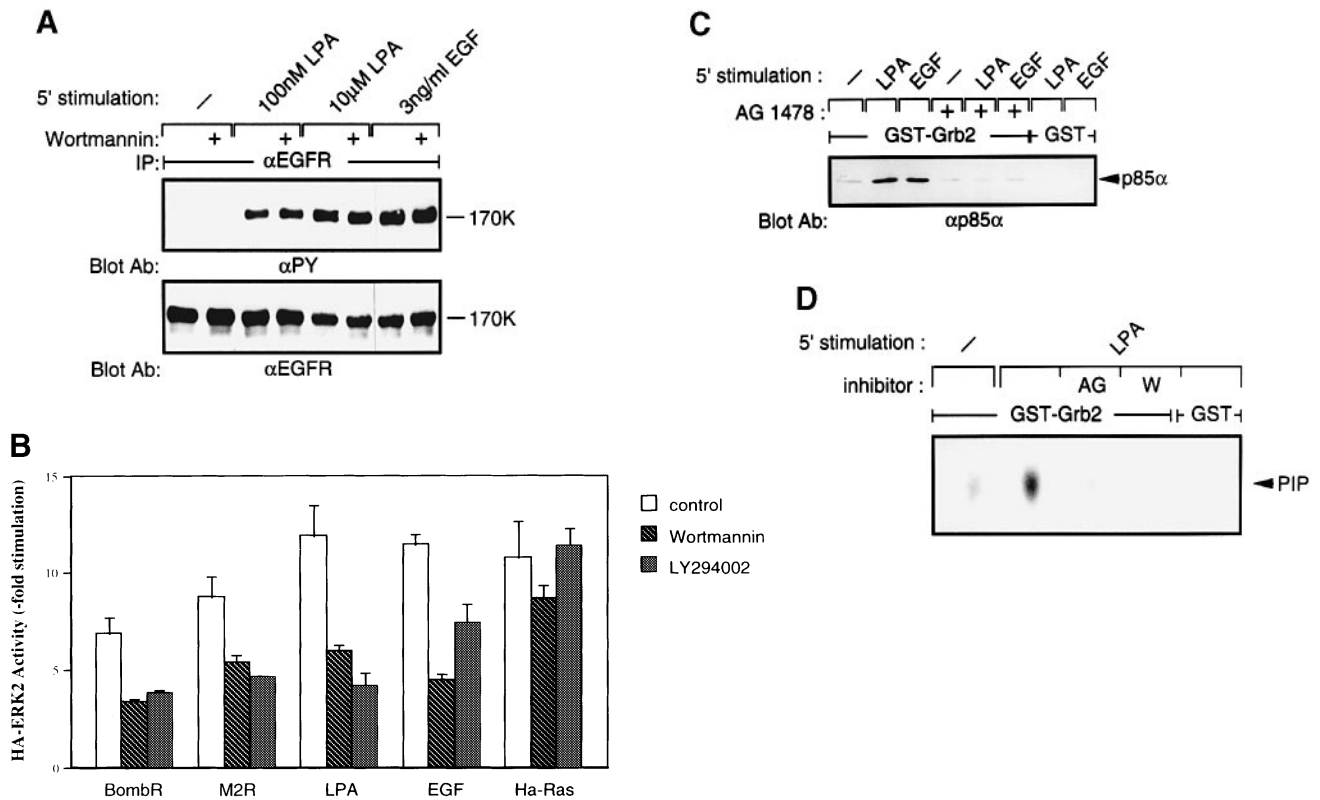
Fig. 7. Analysis of PI3-K function upon EGF or GPCR ligand stimulation in COS-7 cells. (**A**) Quiescent COS-7 cells were pre-treated either with vehicle or with 100 nM wortmannin for 10 min prior to stimulation with the indicated concentrations of LPA or EGF for 5 min. After cell lysis, endogenous EGFR was immunoprecipitated with mAb 108.1 followed by immunoblotting with anti-phosphotyrosine antibody (upper panel). Filters were reprobed with anti-EGFR antibodies (lower panel). Molecular size is indicated on the right. (**B**) COS-7 cells were transfected with expression plasmid encoding HA-ERK2 (100 ng/well) plus either empty expression vector or plasmids encoding BombR, M2R or Ha-Ras (200 ng/well). Cells were treated with vehicle, wortmannin (100 nM) or LY 294002 (25 µM) 10 min prior to stimulation with 200 nM bombesin, 1 mM carbachol, 10 µM LPA or 3 ng/ml EGF for 7 min and subsequent detemination of HA-ERK2 activity. Data shown represent the mean ± SD for duplicate samples. (**C**) Following pre-incubation with or without 100 nM AG1478 for 10 min, quiescent COS-7 cells were stimulated for 5 min with 10 µM LPA or 3 ng/ml EGF. Lysates were subjected to *in vitro* association with GST–Grb2 fusion protein or GST alone, followed by immunoblotting with anti-p85α antibody. The position of p85α is indicated on the right. (**D**) Similarly processed lysates from untreated or LPA-stimulated COS-7 cells were used for a PI3-K assay as described in Materials and methods. Where indicated, cells were pre-treated with 100 nM AG1478 (AG) or 100 nM wortmannin was included in immunoprecipitations (W). Phosphoinositide phosphate (PIP) was resolved by TLC and plates were then exposed to autoradiography.

upon PP1 pre-incubation. Furthermore, PP1 suppressed tyrosine phosphorylation of various Src substrates in COS-7 cells (Figure 8A) and led to moderately increased EGF-induced EGFR tyrosine phosphorylation, possibly due to an effect on EGFR down-regulation, while LPA-induced transactivation was somewhat inhibited (Figure 8B). This indicated that PP1 does not directly inhibit EGFR kinase and further suggests that Src-like kinases or another PP1-sensitive kinase partially contribute to EGFR transactivation upon LPA stimulation of COS-7 cells. Surprisingly, however, PP1 treatment strongly interfered with LPA- and EGF-stimulated SHC and Gab1 tyrosine phosphorylation, Gab1–Grb2 complex formation and MAPK activation (Figure 8C–E). Since PP1 did not affect MAPK activation in response to constitutively active Ras, we conclude that PP1 targets essential signaling elements upstream of Ras and downstream of the EGFR. Moreover, quantification of this data revealed that PP1-induced inhibition of Gab1 tyrosine phosphorylation was most similar to the effects observed at the level of MAPK, suggesting that this multisite docking protein represents the critical link to EGFR-dependent activation of the Ras/MAPK pathway in COS-7 cells. In general, our results

establish PP1 as an excellent pharmacological reagent capable of dissociating EGFR tyrosine phosphorylation from immediate receptor-proximal signaling steps such as SHC and Gab1 tyrosine phosphorylation.

Discussion

We have demonstrated here that ectopic expression of G_q- and G_i-coupled receptors reconstitutes GPCR agonist-induced stimulation of EGFR tyrosine phosphorylation in COS-7 cells. LPA, acting through its endogenous receptor, utilizes both PTX-insensitive and -sensitive pathways leading to EGFR transactivation. While, as mentioned above, it is very likely that heterotrimeric G_i proteins account for the PTX-sensitive contribution to the LPA response (van Biesen *et al.*, 1996b), it remains to be established whether G_q signaling, although sufficient, indeed mediates the PTX-insensitive effects on EGFR tyrosine phosphorylation or, alternatively, whether other PTX-insensitive heterotrimeric G proteins may be involved in LPA signaling. Importantly, LPA treatment or stimulation with low doses of EGF, which activated EGFR to an extent similar to LPA, resulted in comparable tyrosine
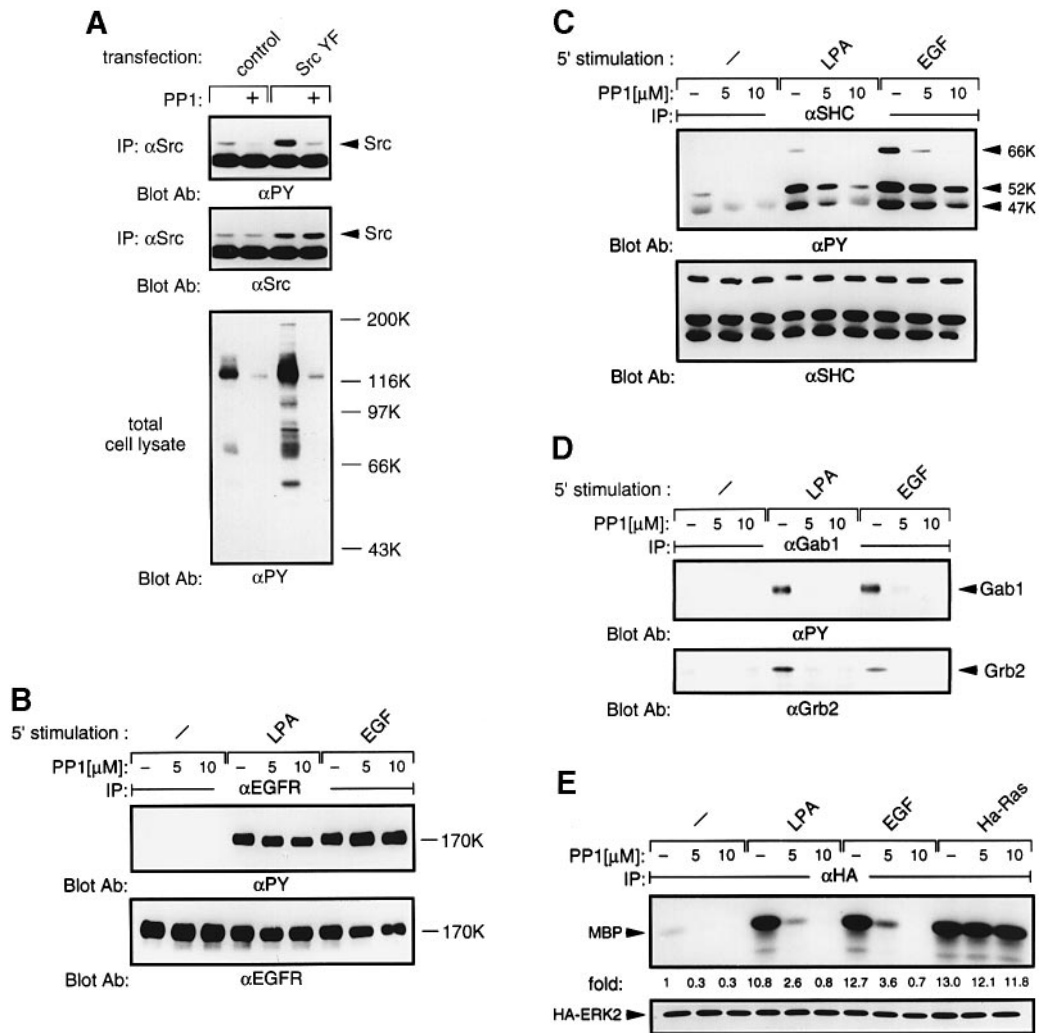
Fig. 8. Effects of PP1 on Src, LPA- and EGF-induced tyrosine phosphorylation of EGFR, SHC, Gab1 and activation of MAPK. (**A**) COS-7 cells were either control-transfected or transfected with plasmid DNA encoding Src Y527F, serum-starved and treated for 15 min with 10 μM PP1 where indicated. After lysis, Src was immunoprecipitated with monoclonal anti-Src antibody followed by immunoblotting with anti-phosphotyrosine antibody (upper panel) and reprobing with monoclonal anti-Src antibody (middle panel). Total cell lysates were immunoblotted with anti-phosphotyrosine antibody (lower panel). (B–D) Quiescent COS-7 cells were pre-treated either with vehicle or with the indicated concentrations of PP1 10 min prior to stimulation with 10 μM LPA or 3 ng/ml EGF for 5 min. Lysates were subjected to immunoprecipitation either with monoclonal anti-EGFR antibody (**B**), polyclonal anti-SHC antibody (**C**) or polyclonal anti-Gab1 antibody (**D**), followed by immunoblotting with anti-phosphotyrosine antibody (B–D, upper panel). Filters were reprobed with the respective antibodies (B and C, lower panel) or anti-Grb2 antibody (D, lower panel). Molecular weight or positions of SHC isoforms are indicated on the right. (**E**) COS-7 cells were transfected with expression plasmid encoding HA-ERK2 (100 ng/well) plus either empty expression vector or plasmid Ha-Ras (200 ng/well). Cells were treated with vehicle or the indicated concentrations of PP1 10 min prior to stimulation with 10 μM LPA or 3 ng/ml EGF for 7 min, lysed and HA-ERK2 activity was determined using MBP as substrate as described. Phosphorylated MBP was visualized by autoradiography after gel electrophoresis (upper panel) and HA-ERK2 was immunoblotted in parallel using polyclonal anti-ERK2 antidody (lower panel).

phosphorylation of Grb2-interacting proteins such as SHC, indicating a similar signaling capacity of either transactivated or EGF-activated EGFR in intact cells. Furthermore, LPA induced tyrosine phosphorylation of the docking protein Gab1, which quantitatively accounted for the tyrosine-phosphorylated 110 kDa protein previously described as associating with Grb2 (Holgado-Madruga *et al.*, 1996). Conversely, this result demonstrates that tyrosine-phosphorylated proteins of similar molecular weight, such as, for example, focal adhesion kinase or c-Cbl, are not present at detectable levels, although these tyrosine kinases can bind to Grb2 (Meisner *et al.*, 1995; Schlaepfer *et al.*, 1997). Moreover, since the docking protein Gab1 plays an important role in RTK-mediated definition of biological responses (Holgado-Madruga *et al.*,

1996; Weidner *et al.*, 1996), our identification of Gab1 as a substrate molecule for LPA-induced tyrosine phosphorylation suggests that this signaling molecule might also be critical for GPCR-initiated signal transmission.

Focusing on the tyrosine kinase substrate SHC for further analysis, we found induction of tyrosine phosphorylation mediated either through G_q-coupled BombR or through G_i-coupled M2R. These findings extend previous results from Lefkowitz and colleagues who attributed GPCR-mediated SHC tyrosine phoshorylation solely to G_i signaling in COS-7 cells (van Biesen *et al.*, 1995; Luttrell *et al.*, 1996). In fact, our experiments show that transiently transfected COS-7 cells are a useful model system to study SHC tyrosine phosphorylation, and our results are consistent with previous data that implicated

G_q-mediated pathways triggered by angiotensin II, bradykinin or thrombin (Cazaubon *et al.*, 1994; Ohmichi *et al.*, 1994; Lev *et al.*, 1995; Chen *et al.*, 1996; Sadoshima and Izumo, 1996) as well as activation of the G_i-coupled *N*-formyl chemoattractant receptor (Ptasznik *et al.*, 1995) in SHC tyrosine phosphorylation and SHC–Grb2 complex formation. Importantly, we found that both signaling events were completely abrogated upon BombR or M2R stimulation when EGFR function was blocked specifically. Thus, EGFR links signals emanating from G_q- and G_i- coupled receptors to the adaptor protein SHC. Although there are conflicting reports on the involvement of SHC in GPCR-mediated activation of the Ras/MAPK pathway (Chen *et al.*, 1996; Kranenburg *et al.*, 1997; Lopez-Ilasaca *et al.*, 1997), this adaptor and, perhaps even more importantly, Gab1 appear to play a significant role in the signal transmission pathway via Grb2 and Sos, resulting in activation of the Ras/MAPK signal (van Biesen *et al.*, 1995; Dikic *et al.*, 1996). Consistent with this role for the EGFR, we observed that BombR-mediated MAPK activation was nearly abolished and the M2R-mediated response was significantly reduced when we specifically inactivated endogenous EGFR either by expression of the dominant-negative EGFR truncation mutant HER-CD533 or by treatment of cells with nanomolar concentrations of the selective EGFR inhibitor AG1478. While there is a consensus that Ras is required for G_i-mediated MAPK activation, one must point out that previous studies have produced contradictory results, reporting either Ras-dependent or Ras-independent MAPK activation through G_q-coupled receptors in COS-7 cells (Crespo *et al.*, 1994; Hawes *et al.*, 1995; Pace *et al.*, 1995). In the case of BombR, expression of a dominant-negative Ras allele interferes with MAPK activation to a similar extent as EGFR inhibition, consistent with the notion that EGFR is positioned upstream of Ras. Moreover, G_q signaling is sufficient to activate Ras (Sadoshima and Izumo, 1996), and further evidence exists that signaling factors such as SHC, Grb2 and Sos, which act upstream of Ras, play essential roles in G_q-mediated MAPK activation (Chen *et al.*, 1996; Dikic *et al.*, 1996). With respect to the contradictory results about G_q signaling in COS-7 cells, one possible explanation could be that up to a certain receptor expression level Ras is required and, when expression exceeds this threshold, additional pathways are activated which allow MAPK activation via Ras-independent routes.

In experiments where we pre-treated cells with the PI3-K inhibitor wortmannin, no differences in GPCR-induced EGFR tyrosine phosphorylation were detected, thus PI3-K activation does not account for an intermediate step in this pathway. Interestingly, PI3-K inhibitors not only attenuated MAPK activation in response to GPCR stimulation, but also in response to low doses of EGF, that mimicked, for example, the LPA effect on EGFR tyrosine phosphorylation. Thus, under these conditions, PI3-K might be required downstream of the EGFR. This assumption is supported further by our finding that recruitment of endogenous, catalytically active p85α/p110 PI3-K into Grb2-containing complexes occurs downstream of the EGFR. In addition, the PI3-Kγ isoform, which is activated by βγ-subunits of heterotrimeric G proteins, was implicated recently in G_i-mediated MAPK activation in COS-7 cells

(Lopez-Ilasaca *et al.*, 1997). However, the involvement of this enzyme in BombR-mediated or EGF-stimulated MAPK activation is unlikely since it occurs in a βγ-independent manner (Faure *et al.*, 1994). Although it is presently unclear how PI3-K activity might contribute to activation of the MAPK pathway, one potentially interesting clue comes from observations that the PI3-K product phosphatidylinositol-3,4,5-trisphosphate (PIP_3) can interact directly with SH2 domains and might thereby contribute to the recruitment of SH2 domain-containing proteins such as p85, Grb2 or SHC to the plasma membrane (Rameh *et al.*, 1995). Consistent with this hypothesis, partial reduction of SHC tyrosine phosphorylation following GPCR stimulation was detected upon wortmannin treatment (Touhara *et al.*, 1995; Lopez-Ilasaca *et al.*, 1997). In addition, physical interaction of p85α/p110 PI3-K with multiprotein complexes containing Grb2, SHC and Gab1 could lead to locally increased PIP_3 levels which stabilize membrane association and thereby support further signal transmission. Moreover, the PI3-K product phosphatidylinositol-3,4-bisphosphate (PIP_2) binds to the pleckstrin homology (PH) domain of the serine/threonine kinase Akt (Franke *et al.*, 1997). While it is presently unclear whether this finding can be extended to other PH domain-containing proteins such as Sos or Gab1, it is noteworthy that, in addition to the C-terminal proline-rich part of Sos which interacts with Grb2, the N-terminal region comprising the PH domain is essential for Sos function (McCollam *et al.*, 1995; Byrne *et al.*, 1996). Further, homing of Sos to the plasma membrane might also be supported by βγ-subunits of heterotrimeric G proteins, which can interact directly with PH domain-containing proteins such as β-adrenergic receptor kinase (Shaw, 1996).

Finally, employing the tyrosine kinase inhibitor PP1, which slightly increased EGF-induced and only moderately inhibited LPA-induced EGFR tyrosine phosphorylation, we provide evidence that additional signaling factors, most probably tyrosine kinases, act downstream of the EGFR and significantly contribute to activation of the Ras/MAPK pathway. Although PP1 has been reported to be selective for Src-like kinases, our data must be interpreted carefully since inhibitory effects on other tyrosine kinases such as PYK2 may be possible, which could account for the effects observed. Moreover, there are some discrepancies concerning the role of Src in GPCR-mediated activation of the Ras/MAPK pathway. This is especially true in view of rather diverging reports regarding the participation of Src in GPCR-mediated activation of the Ras/MAPK pathway (Dikic *et al.*, 1996; Luttrell *et al.*, 1996; Sadoshima *et al.*, 1996; Schieffer *et al.*, 1996; Kranenburg *et al.*, 1997). It can be anticipated, however, that the identification of PP1 target molecules will provide important insights into the Ras/MAPK activation mechanism not only in response to GPCR stimulation, but also upon direct RTK ligand treatment. It is tempting to speculate that an autophosphorylated EGFR recruits substrates such as SHC which are thereby brought into proximity with and phosphorylated by an additional, PP1-sensitive tyrosine kinase such as Src. Recently, Luttrell *et al.* (1977) reported that Src family kinases mediate PTX-sensitive or βγ-induced EGFR tyrosine phosphorylation in COS-7 cells, and suggested that this

response does not involve activation of intrinsic EGFR kinase activity because of differential use of receptor phosphorylation sites. In our study, however, both the EGFR inhibitor AG1478, which targets the EGFR kinase domain, and the EGFR truncation mutant HER-CD533, which selectively blocks endogenous EGFR through formation of inactive heterodimers, fully abrogate EGFR tyrosine phosphorylation without affecting Src-induced tyrosine phosphorylation (Daub *et al.*, 1996; and data not shown). Moreover, our findings support the conclusion that the signal generation by either transactivation of the EGFR or direct EGF binding are indistinguishable.

While the mechanistic details of GPCR-mediated EGFR transactivation remain elusive, data obtained in other ligand-independent RTK activation scenarios such as UV irradiation or oxidant-induced EGFR tyrosine phosphorylation (Knebel *et al.*, 1996) strongly support the involvement of EGFR-antagonizing phosphatases whose inhibition could account for the GPCR-mediated responses observed. Alternatively, interaction with and perhaps even covalent modification by intermediate signaling factors might lead to GPCR-induced EGFR transactivation. Although the signaling events that immediately precede EGFR transactivation await further investigation, the data presented here not only establish the general significance of EGFR function in signals of diverse GPCR subclasses in diverse cell types, but further identify additional elements of GPCR and RTK signals, thereby providing important and novel insights into the signaling network involving two major classes of cell surface receptors.

Materials and methods

Reagents, antibodies and plasmids

COS-7 cells were from Genentech Inc. Human HaCaT keratinocytes were a gift from N.E.Fusenig (Boukamp *et al.*, 1988). Primary mouse astrocytes were prepared essentially as described (Jehan *et al.*, 1995). Culture media and Lipofectamine were purchased from Gibco-BRL. Protein A–Sepharose was from Pharmacia Biotech Inc. PP1 and LY294002 were obtained from Calbiochem. PDGF B/B was from Boehringer Mannheim, and AG1478 (Levitzki and Gazit, 1995) was kindly provided by P.Hirth. All other reagents were obtained from Sigma.

Antibodies purchased were monoclonal anti-HA antibody (Boehringer Mannheim), rabbit polyclonal anti-EGFR antibody (Santa Cruz), sheep polyclonal anti-EGFR antibody (UBI), mouse monoclonal anti-Grb2 antibody (UBI), mouse monoclonal anti-PI3-K antibody (UBI), rabbit polyclonal anti-ERK2 antibody (Santa Cruz) and mouse monoclonal anti-Src antibody (Calbiochem). Endogenous EGFR from COS-7 cells was precipitated with mouse monoclonal antibody 108.1 (Seedorf *et al.*, 1994); mouse monoclonal anti-PDGFRβ antibody 128D4C10 was a gift from H.J.Bühring. Rabbit polyclonal anti-SHC antibody has been described (Seedorf *et al.*, 1994). For Gab1 antibody generation, Gab1 cDNA was cloned from a human placenta cDNA library, the amino acids 23–189 were expressed as pGEX fusion protein in *Escherichia coli* and, after purification, used as antigen in rabbits. Identity with the published sequence (Holgado-Madruga *et al.*, 1996) was verified by DNA sequencing. Respective secondary antibodies were obtained from Bio-Rad and Dianova. For immunoblot detection, the ECL system from Amersham was used. Stripping and reprobing of blots were performed according to the manufacturers' recommendations.

All cDNAs used except SrcY527F (Luttrell *et al.*, 1997), which was in a retroviral expression vector (Daub *et al.*, 1996), were in cytomegalovirus-based expression plasmids containing HER-CD533 (Redemann *et al.*, 1992), βPDGFR (Vogel *et al.*, 1993), βPDGFR-CD504 (Strawn *et al.*, 1994), IGF1-R (Schuhmacher *et al.*, 1993), Ha-Ras (Lengyel *et al.*, 1995), v-Abl (Gishinzky *et al.*, 1995), BombR (Lustig *et al.*, 1993), M1R (Crespo *et al.*, 1994) and M2R (Faure *et al.*, 1994). Mouse ERK2 (Her *et al.*, 1991) was fused to an N-terminal HA epitope as described (Meloche *et al.*, 1992).

Cell culture and transfections

HaCaT keratinocytes and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Primary astrocytes were maintained in DMEM F-12 containing 10% fetal bovine serum. COS-7 cells were transiently transfected using Lipofectamine essentially as described (Hawes *et al.*, 1995). For transfection in 12-well dishes, cells were incubated for 4 h in 0.4 ml of serum-free medium containing 4 µl of Lipofectamine and 0.8 µg of total plasmid DNA per well. For transfections in 6-well dishes, 1.0 ml of serum-free medium containing 10 µl of Lipofectamine and 2.0 µg of total plasmid DNA per well were used. The transfection mixture was then supplemented with an equal volume of medium containing 20% fetal bovine serum and, 20 h later, cells were washed and cultured for a further 24 h in serum-free medium until lysis. For [35S]methionine labeling, cells were incubated in methionine-free DMEM during starvation, and 50 µCi/ml [35S]methionine were added 16 h prior to lysis.

Cell lysis, immunoprecipitation, association with fusion proteins and immunoblotting

Prior to lysis, cells grown to 80–90% confluency were treated with inhibitors and agonists as indicated, washed once with phosphate-buffered saline and then lysed for 10 min on ice in buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride and 10 µg/ml aprotinin. Lysates were pre-cleared by centrifugation at 13 000 r.p.m. for 10 min at 4°C. Supernatants were diluted with an equal volume of HNTG buffer (Seedorf *et al.*, 1994) and subsequently immunoprecipitated using the respective antibodies and 30 µl of protein A–Sepharose for 4 h at 4°C. Alternatively, lysates were subjected to *in vitro* associations with either 3 µg of GST–Grb2 or 2 µg of GST as control pre-bound to 30 µl of gluthathione–agarose beads. Precipitates were washed three times with 0.5 ml of HNTG buffer, suspended in SDS sample buffer, boiled for 3 min and subjected to gel electrophoresis on 7.5% gels or 6–12.5% gradient gels. Following SDS–PAGE, proteins were transferred to a nitrocellulose membrane and immunoblotted.

MAPK assay

Epitope-tagged HA-ERK2 was immunoprecipitated from lysates obtained from 12-well dishes using 2.5 µg of 12CA5 antibody; endogenous ERK2 was immunoprecipitated using 0.5 µg of polyclonal anti-ERK2 antibody. Immunoprecipitates were washed three times with 0.25 ml of HNTG buffer and once with 0.4 ml of kinase buffer (20 mM HEPES pH 7.5, 10 mM MgCl$_2$, 1 mM dithiothreitol, 200 µM sodium orthovanadate). Subsequently, 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-32P]ATP for 10 min at room temperature. Reactions were stopped by addition of 30 µl of 2× SDS sample buffer and subjected to gel electrophoresis on 15% gels. The upper parts of the gels were transferred to nitrocellulose membrane and immunoblotted with anti-ERK2 antibody; labeled MBP on the lower part was quantified using a PhosphorImager (Fuji).

Phosphatidylinositol 3-kinase assay

PI3-K assays were performed essentially as described (Wallasch *et al.*, 1995), with some modifications. Briefly, GST–Grb2 *in vitro* associations were washed twice with HNTG and then three times with 1 ml of buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM EGTA, 0.2 mM sodium orthovanadate and 10% glycerol. Then 30 µl of a master mix consisting of 10 µl of 5× buffer (100 mM Tris–HCl pH 7.5, 500 mM NaCl, 25 mM Mg acetate, 25 mM MnCl$_2$, 2.5 mM EGTA), 10 µl of sonicated phosphatidylinositol (0.5 mg/ml in 20 mM HEPES pH 7.5), 5 µl of 2 mM adenosine and 5 µl of H$_2$O were added to the beads followed by a pre-incubation step for 10 min at room temperature. The kinase reaction was started by addition of 10 µl of 0.1 mM ATP containing 2.5 µCi of [γ-32P]ATP and performed for 30 min at 30°C. The reaction was stopped by addition of 100 µl of 1 M HCl, and lipids were extracted from supernatants with 200 µl of methanol:chloroform (1:1 v/v). The lipid-containing organic phase was resolved on oxalate-coated, thin-layer chromatography plates (Merck) developed in methanol:chloroform:water:glacial acetic acid:acetone (40:13:7:12:15 by vol.). The plates were then dried and exposed for PhosphorImager (Fuji) analysis.

Acknowledgements

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