Review
Tyrosine kinase signalling in breast cancer
Epidermal growth factor receptor: convergence point for signal integration and diversification

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Abstract

Cross-communication between different signalling systems is critical for the integration of multiple and changing environmental influences on individual cells. The epidermal growth factor receptor (EGFR) has been identified as a key element in the complex signalling network that is utilized by various classes of cell-surface receptors. This nonclassical mode of signalling system cross-talk, in distinction to receptor activation induced by cognate ligands, has been termed 'signal transactivation'. With the EGFR as the convergence point and distribution focus, this scenario may involve signals emitted by other members of the tyrosine kinase family, cytokine receptors, ion channels, G-protein-coupled receptors and integrins.

Keywords: cytokines, epidermal growth factor receptor, G-protein-coupled receptors, integrins, metalloproteinases, Ras–mitogen-activated protein kinase, transactivation

Introduction

In complex organisms individual cells communicate through a variety of molecular messengers and actions, such as growth factors, hormones, cytokines, neuropeptides or cell–cell contact, which are recognized by diverse signal-generating cell-surface receptors and thereby regulate cellular growth, differentiation and survival. One important membrane-spanning protein that integrates the information flux from multiple sources is the EGFR, which was the first mammalian signalling protein to be fully characterized [1]. The EGFR belongs to a family of four closely related receptor tyrosine kinases (RTKs): EGFR/ErbB1, HER2/ErbB2/neu, HER3/ErbB3 and HER4/ErbB4. These RTKs are able to form homodimers or heterodimers, and regulate a large diversity of biological processes [2,3]. Deregulation of this tightly controlled signalling network by receptor overexpression, autocrine ligand stimulation or activating mutations has been frequently implicated in several types of human cancers, especially of the breast, ovary, lung and prostate [4,5].

Generally, ligands for the EGFR such as EGF, transforming growth factor-α or heparin-binding EGF-like growth factor (HB-EGF) are synthesized as transmembrane precursors and are proteolytically cleaved by metalloproteinases to yield the mature growth factor, which subsequently activates receptors on the same or on adjacent cells [6]. Following ligand binding, the EGFR dimerizes and becomes tyrosine phosphorylated on distinct residues by the intrinsic kinase activity of the receptor [7]. These residues represent docking sites for first stage signal transducers with enzymatic activity, such as phospholipase Cγ, and adapter

EGFR = epidermal growth factor receptor; GPCR = G-protein-coupled receptor; HB-EGF = heparin-binding epidermal growth factor-like growth factor; LPA = lysophosphatidic acid; MAPK = mitogen-activated protein kinase; PI-3K = phosphatidyl inositol-3 kinase; PDGF = platelet-derived growth factor; PKC = protein kinase C; RTK = receptor tyrosine kinase.

proteins, such as Shc, Grb2 and Nck, which couple receptor activation to downstream pathways, calcium metabolism, and protein kinase C (PKC) signalling, transcription and phospholipid turnover. Apart from these established functions, the EGFR has been found to be a critical downstream element of signalling systems, including those employed by G-protein-coupled receptors (GPCRs), cytokine receptors, integrins and membrane-depolarizing or stress-inducing agents [8–11].

The present review addresses aspects of cross-communication between cell-surface receptors and the EGFR, and the implications of these cellular signalling network connections for signal generation and diversification.

G-protein-coupled receptor-induced epidermal growth factor receptor transactivation
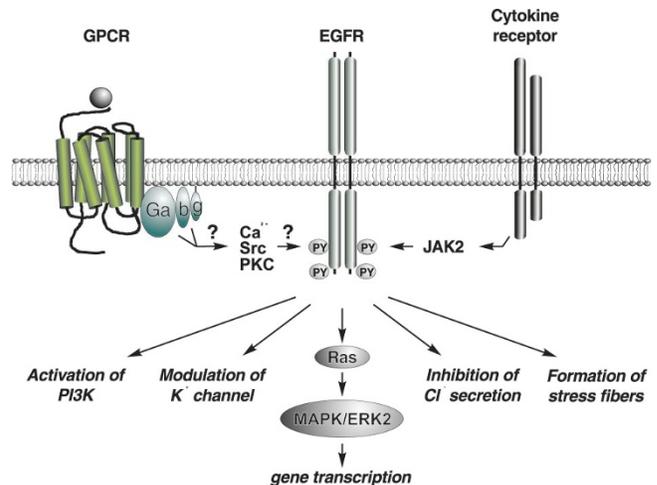### Historical background
The classical functions of GPCRs are the generation of second messengers such as cAMP, diacylglycerol and inositol trisphosphate, and the modulation of ion channel function [12]. In addition, GPCR agonists such as lysophosphatidic acid (LPA), thrombin, endothelin-1, carbachol or bombesin regulate cellular growth, differentiation and gene transcription through the Ras–mitogen-activated protein kinase (MAPK) signalling pathway [13]. Because activating mutations in GPCRs or G-proteins have been associated with cellular transformation [14,15], human endocrine tumours [16] and thyroid adenomas [17], understanding the mechanisms of G-protein-mediated growth control is essential for targeted disease intervention strategies. As an early point of convergence with RTK-induced mitogenic signalling events, GPCRs stimulate the formation and membrane recruitment of protein complexes that involve the adapter proteins Shc, Grb2 and the guanine nucleotide exchange factor Sos, which results in the activation of the small GTPase Ras [18,19]. Because these processes are sensitive to the nonspecific tyrosine kinase inhibitor genistein, the upstream activation of kinases was proposed to be essential for G-protein-mediated MAPK activation [20]. More recent studies have identified the EGFR tyrosine kinase [21], as well as other candidate mediators, including the cytoplasmic tyrosine kinase Pyk2 [22] and members of the Src family [22,23]. It is generally accepted that, depending on the expression pattern, cell type and the activated receptor, these kinases appear to contribute with varying extent to the activation of the Ras–MAPK pathway by GPCRs. Less well understood are the molecular mechanisms of how these tyrosine kinases can be activated by heterotrimeric G proteins after GPCR stimulation.

General characteristics of the transactivated epidermal growth factor receptor
Since Daub et al [21] identified the EGFR to be essential for endothelin-1, thrombin and LPA-induced MAPK activa-

Figure 1

EGFR transactivation by GPCRs and cytokine receptors. Various signalling stimuli induce EGFR tyrosine phosphorylation to activate different downstream signalling pathways. Apart from the activation of the classical Ras–MAP kinase pathway, GPCR–EGFR cross-talk is critical for G-protein-mediated stress fibre formation, ion channel modulation or PI-3K-stimulated phospholipid turnover. In contrast to cytokine-induced EGFR tyrosine phosphorylation by janus kinase (Jak)2, GPCR-induced EGFR transactivation is dependent on the intrinsic kinase activity of the receptor, and several cytoplasmic mediators have been controversially discussed in the literature.

tion and c-*fos* gene transcription in 1996, various studies [24•,25,26] have demonstrated the critical role of this inter-receptor cross-talk for mitogenic G-protein signalling (Fig. 1). Common to all these reports is the observation that GPCR-induced tyrosine phosphorylation of the EGFR is very rapid, transient and comparable to stimulation with low amounts of EGF [21,24•,25,26]. Furthermore, the same signalling capacity of the transactivated and EGF-activated EGFR [24•], carbachol-induced EGFR dimerization [27] and the critical dependence on the intrinsic tyrosine kinase activity of the EGFR [21,24•,25–29] points to an analogous mechanism of EGFR activation by G-proteins or exogenous EGF. However, the speed of transactivation and the absence of detectable amounts of EGF-like ligands after G-protein activation [25,27] led to the conclusion that GPCR-induced EGFR activation is exclusively mediated by intracellular elements [8–11].

Involvement of Src kinases
Potential candidates as mediators of GPCR-induced EGFR transactivation are members of the Src family of cytoplasmic tyrosine kinases [30]. In COS-7 cells, Src was reported to induce EGFR tyrosine phosphorylation after stimulation of the G_i-coupled LPA and α_{2A}-adrenergic receptors or overexpression of Gβγ subunits, independently of the intrinsic kinase activity of EGFR [31]. In the same cell line, however, the Src kinase inhibitor PP1 only

slightly reduced LPA-induced EGFR transactivation [24•], and recent data [32] suggest Src as a point of convergence downstream of RTK transactivation and G-protein-mediated focal adhesion complex assembly. A number of reports have demonstrated a critical function of Src in GPCR-induced Shc and Gab1 tyrosine phosphorylation [24•], MAPK activation by GPCR agonists [24•,31,33] and agonist-dependent α_2-adrenergic receptor endocytosis [34] as an early step in G-protein-mediated signal generation. Furthermore, the association of Src with Shc, Grb2 and the EGFR after angiotensin II stimulation [25], together with previously described Src-mediated phosphorylation of tyrosine residues Y845 and Y1101 of the EGFR [35,36], suggest an additional means of receptor activation utilized by GPCRs that is distinct from EGFR autophosphorylation. Although the functional connection between Src and the EGFR is still unclear, EGFR tyrosine phosphorylation by Src was shown to be crucial for EGF-induced and LPA-induced mitogenic responses [37]. This confirms previous studies [38] that implicated EGFR–Src complex formation in malignant transformation of breast cancer cell lines.

Calcium-dependent epidermal growth factor transactivation
Elevation of the intracellular calcium level and the activation of the Ser/Thr PKC were both shown to be required for G_q-coupled receptors to induce EGFR transactivation. Although EGFR transactivation in response to carbachol or uridine triphosphate stimulation of PC12 cells is sensitive to PKC inhibition [27,39], bradykinin-stimulated EGFR tyrosine phosphorylation in COS-7 cells was shown to be PKC-independent, but bradykinin treatment increased PKC activity in parallel [40]. Nevertheless, in this case both pathways critically contribute to G_q-mediated MAPK activation.

Recently, calcium influx has been shown to be sufficient to transactivate the EGFR and is necessary for various GPCRs to increase the tyrosine phosphorylation of the EGFR [25,28,41]. In PC12 cells [28,39], rat vascular smooth muscle cells [25] or rat cardiac fibroblasts [42], overexpression of the dominant-negative EGFR mutant HER-CD533 or inhibition with the EGFR selective tyrphostin AG1478 strongly interfered with GPCR-induced MAPK activation or mitogenic responses. Although in PC12 cells the presence of extracellular calcium is necessary for bradykinin-induced EGFR tyrosine phosphorylation, complexation of intracellular calcium upon treatment with the chelating agent BAPTA-AM was sufficient to inhibit angiotensin II-induced EGFR activation in cardiac fibroblasts.

Because elevation in intracellular calcium or GPCR stimulation can activate the PYK2 tyrosine kinase [39,43,44•], its possible role as a link between GPCRs and the EGFR was considered [39]. Nevertheless, in PC12 cells tetracycline-inducible expression of a kinase-inactive PYK2 mutant did not effect EGFR transactivation by bradykinin or membrane-depolarization [44•]. Interestingly, pharmacological inhibition of calcium/calmodulin-dependent protein kinases completely abrogates EGFR tyrosine phosphorylation in response to membrane depolarization or angiotensin II stimulation. This finding led to the identification of these Ser/Thr kinases as possible mediators of EGFR transactivation, at least in some cell types [42,44•].

Downstream of the transactivated epidermal growth factor receptor
The signalling capacity of the transactivated EGFR is not restricted to coupling GPCRs to the Ras–MAPK pathway, but rather functions as a critical intermediate for G_q-, G_i- and $G_{12/13}$-coupled receptors to induce a multitude of biological responses. Several isoforms of phosphatidyl inositol-3 kinase (PI-3K) have been shown to be involved in $G\beta\gamma$-dependent, LPA- or noradrenaline-induced MAPK activation, but are likely to act downstream of or separate from transactivated EGFR [24•,45–47]. Moreover, inhibition of EGFR function blocks LPA-induced PI-3K activity in COS-7 cells [24•]. The complex formation of the docking protein Gab1 with the EGFR and p85, the catalytic subunit of PI-3K, was essential for LPA-stimulated PI-3K lipid product generation [48•]. Furthermore, the p70 S6 kinase, a known effector of PI-3K that is critical for cell cycle progression, is activated by endothelin-1 via EGFR-dependent pathways [49].

Another cellular event regulated by various GPCRs that depends on the EGFR function is the rearrangement of the actin cytoskeleton and subsequent stress fibre formation via $G\alpha_{13}$ but not $G\alpha_{12}$ subunits [50,51•]. Finally, the EGFR-dependent modulation of a potassium channel or regulation of chloride secretion after carbachol stimulation [26,27] represent additional responses within the complex signalling network utilized by GPCRs, with the EGFR as the major signal integrator.

Triple membrane-passing signal mechanism of epidermal growth factor receptor transactivation
Generally, GPCR-induced EGFR transactivation has been, so far, considered to be a ligand-independent process, with several cytoplasmic players controversially discussed as mediators of this inter-receptor cross-talk.

Experiments with different stable Rat-1 cell lines [52••] have revealed the critical involvement of the EGFR ligand-binding domain for EGFR transactivation, as well as the possibility of intercellular transactivation between cocultured cells in a cell-density dependent manner. This again raised the question whether ligands for the EGFR can contribute to GPCR-induced transactivation. Moreover, LPA, carbachol and bombesin induce shedding of the pro-HB-EGF growth factor precursor in COS-7 cells. Inhibition of this cleavage by the metalloproteinase inhibitor

batimastat completely abolished GPCR-induced EGFR transactivation, as well as further downstream signalling steps such as Shc and Gab1 tyrosine phosphorylation in COS-7 and HEK293 cells [52••]. Because proteolytic processing affects various transmembrane proteins, this critical role of metalloproteinases extends the repertoire of known G-protein effectors and might open a new dimension of GPCR signalling. Furthermore, the ability of HB-EGF to bind to cell-surface heparan sulphate proteoglycans prevents the immediate release of the ligand after precursor cleavage, which might explain why mature growth factors are not released into the culture medium after GPCR stimulation. Additionally, transactivation and the critical involvement of metalloproteinases for this cross-communication were shown in prostate cancer cells, which are known to regulate their growth via autocrine pathways requiring either the bombesin or the EGFR [52••]. This suggests a link between GPCR and EGFR autocrine signals, which further emphasizes that EGFR transactivation may contribute to pathophysiological disease states. Although this triple membrane-passing signal model provides new insights into mechanistic details of transactivation (Fig. 2), it raises a variety of new questions. The nature of the metalloproteinase(s) involved and its regulation by heterotrimeric G-proteins will be the key issue in the future.

Cross-talk between cytokine and epidermal growth factor receptor family members

In addition to GPCRs, growth hormone and prolactin, members of the cytokine superfamily, have been reported to increase the EGFR tyrosine phosphorylation and EGFR–Grb2 association in mouse liver *in vivo* [53••]. In this case the EGFR was directly phosphorylated by the Janus tyrosine kinase Jak2, which couples cytokine stimulation to MAPK activation and c-*fos* gene transcription via the transactivated EGFR. In contrast to GPCR-induced effects, growth hormone only induces phosphorylation of Tyr1068, the major Grb2 binding site of the EGFR, independently of the intrinsic kinase activity of the receptor. Furthermore, interleukin-6 was shown [54•] to stimulate the autophosphorylation of HER2 in prostate cancer cells, leading to increased HER2 and HER3 tyrosine phosphorylation, MAPK activation and cell proliferation. Clustering of the interleukin-6 receptor β-subunit, gp130 and HER2 was proposed to initiate these tyrosine phosphorylation events.

Cross-talk between integrins and epidermal growth factor

Adhesion of cells to the extracellular matrix is mediated through the integrin family of cell-surface receptors and leads to the activation of multiple biological responses, including calcium influx, increased protein tyrosine phosphorylation and activation of the MAPK cascade [55]. Integrins, which are devoid of any intrinsic enzymatic activity, have been demonstrated [56••] to transactivate the EGFR
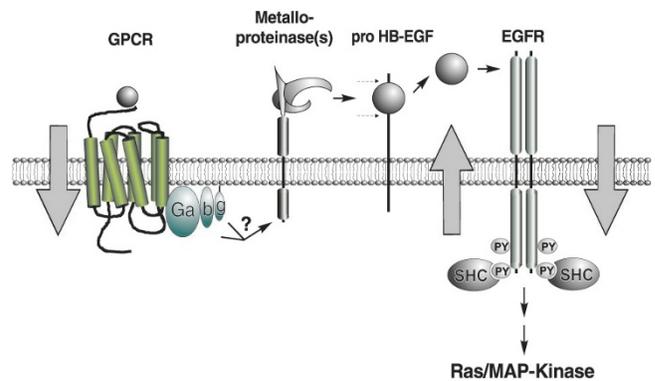
Triple membrane-passing signal mechanism of EGFR transactivation. GPCR-induced EGFR transactivation involves as key elements pro-HB-EGF, an EGF-like growth factor precursor, and a metalloproteinase activity that is induced upon G-protein activation. Therefore, this new mechanistic concept for ligand-dependent GPCR–EGFR cross-talk entails three membrane-passing signals and couples G-protein signalling to activation of the Ras–MAPK pathway.

in order to generate further cellular responses, most notably adhesion-dependent cell survival. In accordance with the first studies of the GPCR–EGFR cross-talk, those investigators suggested, on the basis of medium-transfer experiments, a ligand-independent mechanism. It would be interesting to investigate the requirement of metalloproteinases in this process.

Cross-talk between platelet-derived growth factor and epidermal growth factor receptor

Inter-receptor communication was not only demonstrated between heterologous receptor classes, but also among different members of the RTK family. The EGFR and the platelet-derived growth factor (PDGF)-β receptor are known to interact physically [57], and EGF stimulation has been shown to increase the tyrosine phosphorylation of PDGF-β receptor and subsequent recruitment of PI-3K to the PDGF receptor [58]. In contrast to tyrosine phosphorylation events, signal regulation by serine or threonine phosphorylation has been poorly characterized, but is generally thought to influence negatively the signalling capacity of RTKs such as the EGFR and its relative HER2/neu [59]. Interestingly, Bagowski *et al* [60] provided evidence that PDGF stimulates threonine phosphorylation of T654 and T669 of the EGFR, thus negatively regulating EGF-induced c-*jun* amino-terminal kinase activation, and c-*jun* transcription and cellular transformation. In this context the PKC-related Ser/Thr kinase protein kinase D has been identified as a potential mediator, but the detailed molecular mechanism of this intracellular RTK–RTK cross-talk still remains elusive.

Conclusion

Signal transduction was often considered to be organized in discrete signalling cassettes, linking receptor activation

to cell division and gene transcription in a linear manner. Recent studies have broadened this view to encompass a complex network, which allows cross-communication between separate signalling units with the EGFR as a key element for signal integration and diversification from a multitude of stimuli. We are just beginning to understand the molecular mechanisms of RTK transactivation. On the basis of identification of critical players, such as Tyr or Ser/Thr kinases, metalloproteinases and growth factor precursors, however, one must expect a general role of this cross-talk in developmental and normal physiological processes, as well as in pathophysiological disorders such as cancer.

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