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The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification

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Abstract

Homeostasis of multicellular organisms is critically dependent on the correct interpretation of the plethora of signals which cells are exposed to during their lifespan. Various soluble factors regulate the activation state of cellular receptors which are coupled to a complex signal transduction network that ultimately generates signals defining the required biological response. The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases represents both key regulators of normal cellular development as well as critical players in a variety of pathophysiological phenomena. The aim of this review is to give a broad overview of signal transduction networks that are controlled by the EGFR superfamily of receptors in health and disease and its application for target-selective therapeutic intervention. Since the EGFR and HER2 were recently identified as critical players in the transduction of signals by a variety of cell surface receptors, such as G-protein-coupled receptors and integrins, our special focus is the mechanisms and significance of the interconnectivity between heterologous signalling systems.

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Introduction

Cell surface receptors integrate a multitude of extracellular signals such as environmental stresses, growth factors, neuropeptides or hormones, thus regulating a large diversity of signalling pathways and cell responses. Receptor tyrosine kinases (RTKs) are a subgroup of transmembrane proteins with an intrinsic tyrosine kinase activity which determines various cellular functions as diverse as growth, differentiation, cell motility or survival (reviewed in Van der Geer *et al.* 1994). The epidermal growth factor receptor (EGFR) family of RTKs consists of four members: EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4. The EGFR which was the first cell surface signalling protein and protooncogene product to be characterised by molecular genetic methods (Ullrich *et al.* 1984) exemplified prototypical features of a receptor molecule. All EGFR family members are characterised by a modular structure consisting of an extracellular ligand-binding domain, a single hydrophobic transmembrane region, and the intracellular part harbouring the highly conserved tyrosine kinase domain. Ligand binding induces the formation of homo- or heterodimers which subsequently trigger the autophosphorylation of cytoplasmic tyrosine residues (reviewed in Ullrich & Schlessinger 1990, Heldin 1995, Alroy & Yarden 1997). These phosphorylated amino acids represent docking sites for a variety of signal transducers which regulate membrane-proximal steps of a complex signalling network ultimately defining the biological response to a given signal. Deregulation of this tightly controlled system of hormone–receptor and receptor–receptor interactions by overexpression, amplification or mutations of critical pathway elements and/or autocrine stimulation through aberrant growth factor loops is frequently linked to hyperproliferative diseases such as cancer (reviewed in Huang & Harari 1999, Olayioye *et al.* 2000, Zwick *et al.* 2000). More recently, the EGFR and HER2 have been identified as critical pathway elements in the signalling from G-protein-coupled receptors (GPCRs), cytokines, RTKs and integrins to a variety of cellular responses such as mitogen activated protein (MAP) kinase activation, gene transcription and proliferation. This growing field of interreceptor cross-talk has received much attention during the past four years and the elucidation of the molecular mechanisms will provide new insights into a signalling network of increasing complexity.

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The EGF receptor family signalling network

The EGF receptor family signalling network employs several modes of regulation which couple receptor activation to a highly diverse repertoire of cellular signalling pathways. Various EGF-like ligands are released following proteolytic cleavage of their transmembrane precursors. Subsequently, the mature growth factors activate four related receptors which are able to form homo- and heterodimers thus regulating a distinct subset of intracellular signalling pathways. In addition to these classical layers of diversity the role of receptor endocytosis for signal generation and attenuation has recently received increasing attention.

EGF-like ligands and neuregulins

EGFR family members are activated by a large group of EGF-related growth factors. Depending on their receptor affinities and specificities these ligands can be subdivided into four different categories. While EGF, amphiregulin (AR) and transforming growth factor α (TGFα) specifically bind the EGFR, betacellulin (BTC), heparin binding EGF-like growth factor (HB-EGF) and epiregulin bind the EGFR and HER4. Neuregulins (NRGs) or Neu differentiation factors (NDFs) which exist in several alternatively spliced isoforms directly bind and activate HER3 and HER4. Interestingly, despite the overlapping receptor specificity of the NRG1 and NRG2 isoforms they exhibit distinct biological activities depending on the cellular context (Sweeney Crovello *et al.* 1998). In line with this, earlier studies by Yarden and co-workers revealed isoform-specific functional differences of the two major NRG subtypes (Pinkas-Kramarski *et al.* 1996*a*). More recently, NRG3 and NRG4 have been cloned and identified as ligands which exclusively bind HER4 (Zhang *et al.* 1997, Harari *et al.* 1999).

Common to all these growth factors is the EGF domain with six conserved cysteine residues characteristically spaced to form three intramolecular disulphide bridges. In general, EGF-like ligands are synthesised as glycosylated transmembrane precursors which are proteolytically cleaved from the cell surface to yield the mature growth factor (reviewed in Massague & Pandiella 1993). Since 1991 when the prototypical cleavage of proTGFα had been found to be stimulated by serum factors, tetradecanoylphorbolacetate (TPA) and calcium ionophores (Pandiella & Massague 1991*a*,*b*), structural determinants for this regulatory step within the C-terminus and the juxtamembrane domain of proTGFα (Bosenberg *et al.* 1992, Arribas *et al.* 1997) and within the EGF and HB-EGF precursors (Dong & Wiley 2000) have been identified. In contrast to the TGFα and NRG precursors (Liu *et al.* 1998) which require the cytoplasmic domains for efficient proteolytic processing, proHB-EGF shedding as well as the proteolytic release of amphiregulin have been shown to be independent of their cytoplasmic moieties (Thorne & Plowman 1994, Dethlefsen *et al.* 1998, Vecchi *et al.* 1998). Besides these structural requirements, and based on studies with broad spectrum inhibitors, serine proteases (Pandiella *et al.* 1992) and metalloproteases (Arribas *et al.* 1996, Dempsey *et al.* 1997, Brown *et al.* 1998) have been identified as potential mediators of ectodomain shedding. More recently, data obtained from transgenic animals lacking the ADAM17 (TACE) zinc-dependent transmembrane metalloprotease, revealed a critical contribution to proTGFα processing (Peschon *et al.* 1998). Interestingly, phenotypical changes of TACE knock-out mice closely resemble those obtained from animals lacking TGFα (Luetteke *et al.* 1993) or the EGFR (Sibilia & Wagner 1995, Threadgill *et al.* 1995), suggesting a general role of this enzyme in EGFR signalling (Werb & Yan 1998). The involvement of another family member, ADAM9/MDC9, in TPA-induced proHB-EGF shedding further underlines the critical role of this metalloprotease family in the generation of mature EGF-like ligands (Izumi *et al.* 1998). However, considering that there are more than fifteen ADAMs with an active metalloprotease function the identity of the metalloproteases involved in the processing of most of the EGF-like growth factor precursors and the regulatory mechanisms governing this important process are still unknown.

Ectodomain cleavage affects not only ligands of the EGF family but also some of their receptors (Vecchi *et al.* 1996, reviewed in Blobel 2000). Proteolytic processing of HER2 and HER4 in response to pervanadate, a potent inhibitor of tyrosine phosphatases, has been shown to be mediated by metalloproteases, namely ADAM17 for HER4 (Vecchi & Carpenter 1997, Vecchi *et al.* 1998, Codony-Servat *et al.* 1999, Rio *et al.* 2000). Taken together, the generation of mature ligands from growth factor precursors and the regulated cleavage of transmembrane receptors represents a further mode of EGFR family signal regulation which is far from being understood at the molecular level.

Homo- and heterodimerisation of EGFR family members

The EGFR family of RTKs consists of four closely related type I transmembrane receptors: the EGFR (Ullrich *et al.* 1984), HER2 (ErbB2/neu) (Coussens *et al.* 1985), HER3 (ErbB3) (Kraus *et al.* 1989, Guy *et al.* 1994) and HER4 (ErbB4) (Plowman *et al.* 1993). To study the physiological functions of these receptors, cell model systems have been established which ectopically express one individual RTK or several in combination (Riese *et al.* 1995, Olayioye *et al.* 1998). Investigations based on these systems soon established the unique role of HER2 as a receptor with high

transforming activity (Di Fiore *et al.* 1987, Hudziak *et al.* 1987) which can be transphosphorylated through heterodimerisation with the ligand-occupied EGFR (Stern & Kamps 1988, Goldman *et al.* 1990) and which acts synergistically with the EGFR (Kokai *et al.* 1989) or HER3 (Alimandi *et al.* 1995, Zhang *et al.* 1996) in transforming NIH3T3 cells. Moreover, homo- and heterocomplexes formed in response to NRG and EGF were characterised by a complex but hierarchical network of interreceptor interactions (Riese *et al.* 1995, Wallasch *et al.* 1995, Pinkas-Kramarski *et al.* 1996*b*, Tzahar *et al.* 1996) (Table 1). In this context the orphan HER2 was shown as the preferred heterodimerisation partner within the EGFR family as it decreases ligand dissociation from the receptor heterodimer thus enhancing and prolonging the activation of the MAP kinase signalling pathway (Graus-Porta *et al.* 1995, 1997, Karunagaran *et al.* 1996). Heterodimers, particularly those containing HER2 were generally found to induce signals with the strongest biological activity. Generally, ligand-induced signalling must be initiated by high affinity binding of EGF-like ligands to the EGFR and HER4 (Beerli & Hynes 1996, Riese *et al.* 1996*a*,*b*, 1998) or neuregulin binding to HER3 or HER4 (Sliwkowski *et al.* 1994, Pinkas-Kramarski *et al.* 1998) followed by activation in trans via homo- or heterodimerisation. Mechanistical studies of ligand–receptor complex formation support a bivalence model of ligands carrying a high and a low affinity binding site within each terminus of the protein (Barbacci *et al.* 1995, Tzahar *et al.* 1997). This model readily explains EGF-induced EGFR homodimerisation (Summerfield *et al.* 1996, Lemmon *et al.* 1997) as well as NRG-induced heterodimerisation with HER2 preferentially bound via the low affinity binding site of the ligand (Tzahar *et al.* 1997). Complexity within the EGFR family network is further increased since EGF and betacellulin, which directly bind to the EGFR or HER4, mediate signalling through co-expressed HER2 and HER3 in the absence of the EGFR (Alimandi *et al.* 1997, Pinkas-Kramarski *et al.* 1998).

Attenuation of EGFR signalling by ligand-induced endocytosis

After growth factor binding, homo- and heterodimers between the four EGFR family members are targeted to clathrin-coated pits which are internalised in a process termed ligand-induced endocytosis (reviewed in Carpenter 2000*a*, Ceresa & Schmid 2000). Investigations with chimeric receptors and reconstitution systems revealed that the ligand-occupied EGFR is rapidly internalised and degraded, while all other RTKs of this family are not (Baulida *et al.* 1996). In agreement with this, Pinkas-Kramarski *et al.* (1996*b*) showed that the rate of EGF uptake through the EGFR was significantly higher than the HER3–mediated endocytosis of NRG. Intracellular routing of EGF receptors to the mildly acidic early endosome is followed by the decision whether the ligand–receptor complex dissociates, with the receptor being recycled or not. Complexes which remain stable under these conditions, such as the EGF/EGFR complex, are efficiently degraded in lysosomal compartments thus reducing the cell surface content of the receptor and its signal capacity. Interestingly, TGFα or NRG binding to receptors is disrupted within the early endosomes, which favours receptor recycling and results in more potent mitogenic signalling (Waterman *et al.* 1998). EGF-induced mitogenic responses through the EGFR were shown to be potentiated by HER2 co-expression to the level achieved by TGFα stimulation due to enhanced recycling of heterodimers in contrast to EGF-bound EGFR homodimers (Lenferink *et al.* 1998). Together with the finding that increased expression of HER2 significantly reduces HER2 downregulation and lysosomal targetting of the EGFR (Wang *et al.* 1999, Worthylake *et al.* 1999) these studies could further explain the transforming potential of HER2. Specific structural features regulate the sorting of internalised receptors. While the c-terminal domain of HER3 accounts for receptor recycling to the cell membrane (Waterman *et al.* 1999), the EGFR recruits the ubiquitin-protein ligase c-Cbl which

Table 1 Signalling network of EGFR family members. The table shows multiple EGF-like ligands with specificity to distinct heterodimers and their cytoplasmic substrates. Note that only some of the known EGF-like ligands are represented

Heterodimer	Ligands	Substrates										
EGFR/HER2	EGF/TGFα β-Cellulin		Src,	ras-GAP,	PLCγ,	Shc,	Grb-2,	Grb-7,	Crk,	c-Cbl,	c-Abl,	Shp2
EGFR/HER3	EGF/TGFα β-Cellulin		Src,	ras-GAP,	PLCγ,	Shc,	Grb-2,	Grb-7,	Crk,	c-Cbl,	c-Abl,	
	NRG α/β	PI3K,	Src,	ras-GAP,	PLCγ,	Shc,	Grb-2,	Grb-7,	Crk,			
EGFR/HER4	EGF/TGFα β-Cellulin		Src,	ras-GAP,	PLCγ,	Shc,	Grb-2,	Grb-7,	Crk,	c-Cbl,	c-Abl,	
	NRG α/β	PI3K,	Src,	ras-GAP,	PLCγ,	Shc,	Grb-2,	Grb-7,	Crk,			
HER 2/HER3	NRG α/β	PI3K,	Src,	ras-GAP,	PLCγ,	Shc,	Grb-2,	Grb-7,	Crk,			Shp2
HER 2/HER4	NRG α/β	PI3K,	Src,	ras-GAP,	PLCγ,	Shc,	Grb-2,	Grb-7,				Shp2
HER 3/HER4	NRG α/β	PI3K,				Shc,						

determines the lysosomal degradation of the receptor after polyubiquitination (Joazeiro *et al.* 1999, Levkowitz *et al.* 1999). In addition to the receptor's intrinsic kinase activity, c-terminal residues of the EGFR were shown to be critical for its lysosomal targeting (Kornilova *et al.* 1996). EGFR modifications through protein kinase C (PKC)-mediated threonine phosphorylation prevented the receptor from polyubiquitination and allowed sorting to the recycling endosome (Bao *et al.* 2000).

EGFR signalling

Multiple ligands and various combinations of homo- and heterodimerisation within the EGFR family couple to a complex and diverse set of biochemical pathways. The following summary highlights some basic features and recent advances on EGFR signal transduction.

Ligand-induced receptor dimerisation and subsequent autophosphorylation of distinct tyrosine residues creates docking sites for various membrane-targeted proteins. Cytoplasmic mediators which bind to EGFR phosphotyrosine residues through SH2– or PTB-domains may either be adaptor proteins or enzymes. Adaptors such as Shc, Grb2, Crk or the recently characterised Dok-R protein (Jones & Dumont 1999) show a modular structure containing protein–protein interaction domains and putative phosphorylation sites and act as signalling platforms which extend the receptor's repertoire of activated intracellular pathways. Shc exists in three different isoforms, p46shc, p52shc and p66shc which are tyrosine phosphorylated upon EGF stimulation and bind to the activated EGFR and Grb2. Interestingly, while the 46 and 52 kDa isoforms increase mitogenic signalling after EGF stimulation and are able to transform NIH3T3 cells (Pelicci *et al.* 1992), p66shc has no transforming potential and negatively influences EGF-induced c-fos transcription (Migliaccio *et al.* 1997). Furthermore, enzymes such as phospholipase Cγ (PLCγ), which hydrolyzes PIP2 thus generating diacylglycerol and inositol-trisphosphate, or the cytoplasmic tyrosine kinase c-src link EGFR activation to second messenger generation and calcium metabolism or mitogenic signalling cascades respectively. Among the multitude of signalling pathways activated by the EGFR, the highly conserved MAP kinase pathway is currently the best understood.

Activation of the MAP kinase cascade

So far, several distinct MAP kinases have been identified as targets of the EGFR, among them the extracellular regulated kinases (Erks) 1 and 2, jun N-terminal kinases (Jnks), p38 and Erk5. Since most of the key components of the Erk1/2 cascade have been characterised, EGF-induced activation of these serine/threonine kinases serves as a paradigm for signal transmission from cell surface receptors to the nucleus.

Following membrane-proximal steps such as Shc and Grb2 recruitment to the tyrosine phosphorylated receptor, the small G-protein Ras is activated through the Grb2–bound exchange factor Sos. Subsequent induction of the serine/threonine kinase Raf and the dual specificity kinase MEK1 finally activates Erk1/2 which ultimately regulates transcription factors such as Elk-1 and c-fos (Fig. 1). Recently, characterisation of heterologous positive and negative regulators of the Erk pathway generates a more complete picture from this simplified model. While the adaptors and EGFR binding proteins p66Shc and Dok-R have been shown to attenuate EGF-induced Erk activation (Okada *et al.* 1997, Jones & Dumont 1999), the Abl interactor Abi-1 interferes with MAP kinase signalling through binding to the exchange factor Sos (Fan & Goff 2000). In contrast, overexpression of SUR-8, a scaffolding protein which complexes with Ras and Raf enhances MAP kinase activation following EGF treatment as does the SH2 domain containing and Gab1–interacting protein tyrosine phosphatase Shp-2 (Li *et al.* 2000, Shi *et al.* 2000).

Less well characterised is the EGF-induced activation of Jnk via the small G-proteins Rac1 and Cdc42 (Coso *et al.* 1995, Minden *et al.* 1995). Recent reports position the STE20 related serine/threonine kinase JIK and the adaptor protein Crk upstream of the small GTPases (Dolfi *et al.* 1998, Tassi *et al.* 1999). In contrast to Crk which is critical for Jnk activation following EGF treatment of transfected COS7 cells, JIK activity is downregulated by the EGFR and overexpression of this negatively regulating kinase diminishes the EGF-induced Jnk activation. Crosstalk between different RTK families was shown since platelet-derived growth factor (PDGF) stimulation of Rat-1 fibroblasts induces inhibition of EGFR-mediated Jnk activation while leaving the Erk signal unaffected (Bagowski *et al.* 1999).

Lee and colleages revealed the critical contribution of Erk5 for EGF-induced cell proliferation and cell cycle progression. Distinct from Erk1/2 activation, this signalling pathway does not require Ras activity and is controlled by the MAP kinase kinase MEK5 (Kato *et al.* 1998).

Activation of phosphatidylinositol-3–kinase (PI3K)

Stimulation with EGF results in the activation of the lipid kinase PI3K which consists of the regulatory subunit p85 and the catalytic subunit p110 that phosphorylates PIP2 generating the second messenger PIP3 (Bjorge *et al.* 1990). Since the EGFR has no binding site for the SH2–domain of PI3K, EGF-induced activation of PI3K is relatively weak compared with other RTKs. In contrast to the EGFR, HER3 contains six putative binding sites for PI3K and potently activates this enzyme (Soltoff *et al.* 1994).

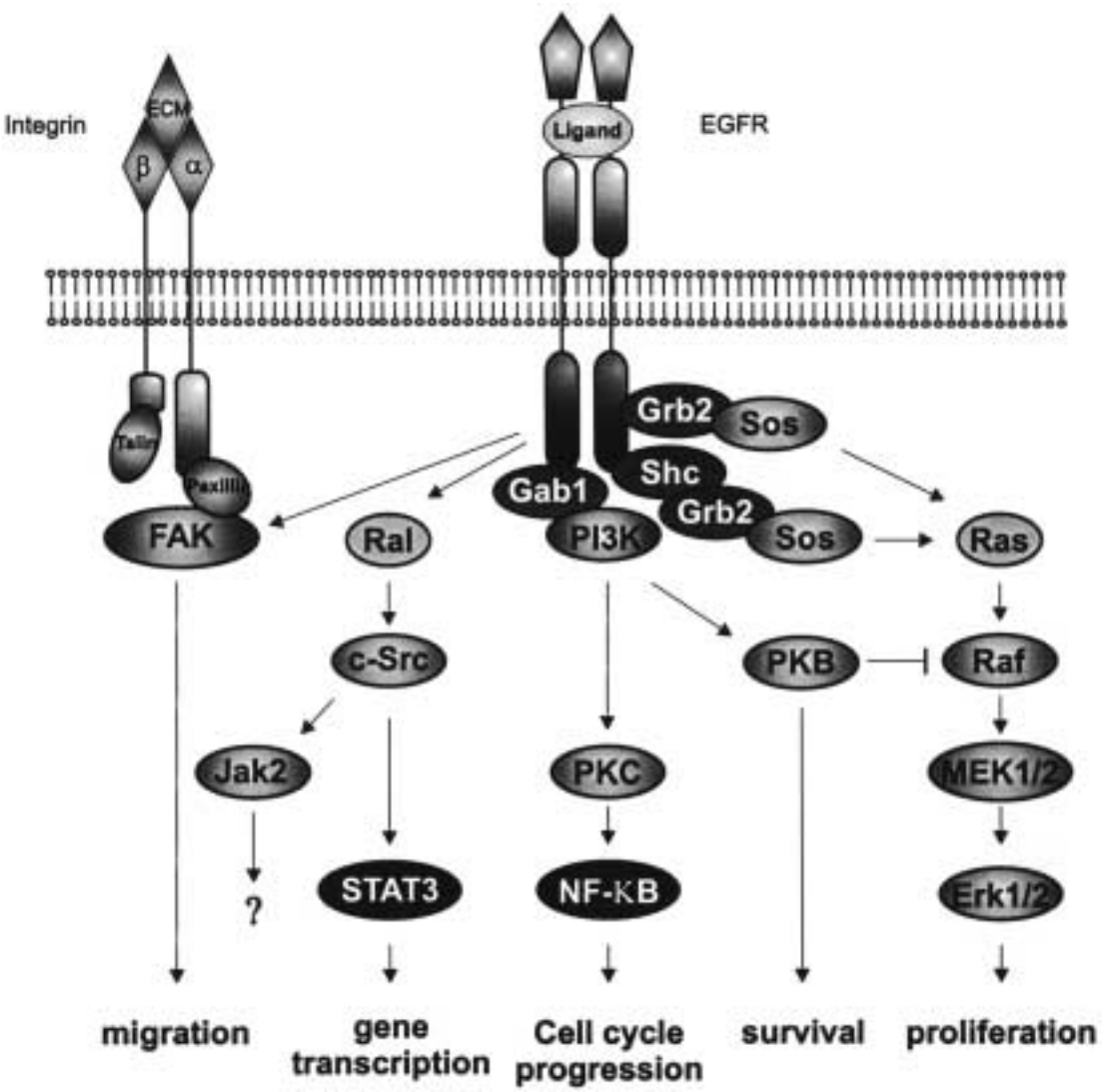

Figure 1 EGFR signalling. Ligand-induced receptor dimerisation and subsequent autophosphorylation of distinct tyrosine residues creates docking sites for SH2 or PTB domain containing effector proteins. The resulting signalling network initiates diverse cellular pathways leading to proliferation, migration, gene transcription, cell cycle progression and cell survival. ECM, extracellular matrix.

The adaptor protein Gab1 was reported as a candidate for mediating the activation of PI3K by the EGFR and a recent report demonstrates a positive feedback loop in EGFR signalling through Gab1 and PI3K (Rodrigues *et al.* 2000). EGF-induced tyrosine phosphorylation of Gab1 activates PI3K, and the subsequent generation of PIP3 results in enhanced membrane recruitment and further increased activation of Gab1. PTEN, a phosphatase hydrolyzing PIP3, modulates this feedback by decreasing membrane targeting of Gab1. An important downstream target of PI3K, protein kinase B (PKB)/Akt is phosphorylated after membrane recruitment and activation of the threonine kinase PDK1 (reviewed in Alessi & Cohen 1998). Activation of PKB exerts antiapoptotic effects involving the transcription factor NF-κB, and recently the EGF-induced activation of NF-κB via PI3K and PKC has been demonstrated to be crucial for cell cycle progression in estrogen receptor-negative breast cancer cells (Biswas *et al.* 2000) (Fig. 1). However, PKB is not only a target of the EGFR/PI3K pathway, it is also involved in the regulation of the Ras-MAP kinase pathway by phosphorylating Raf and thereby inhibiting its kinase activity (Zimmermann & Moelling 1999).

Activation of Src

The cytoplasmic tyrosine kinase c-Src is involved in important cellular processes such as mitogenic signalling or cytoskeletal organisation (reviewed in Belsches *et al.* 1997). Substrates of Src upon EGF stimulation include the EGFR itself, transcription factors of the signal transducer and activator of transcription (STAT) family, Shc, cytoskeletal components and proteins of the endocytic machinery such as dynamin and clathrin (Wilde *et al.* 1999).

Co-expression and synergistic function of the EGFR and c-Src for cellular proliferation, invasiveness and tumour formation point to a close functional connection of these tyrosine kinases (Maa *et al.* 1995). In this context, Src inhibition was shown to reverse the transformed phenotype of either EGFR- or HER2-overexpressing cells (Karni *et al.* 1999). Parsons and co-workers showed that c-Src directly binds the EGFR and phosphorylates two tyrosine residues distinct from the known autophosphorylation sites, one of them being critical for EGF-induced mitogenic responses in murine fibroblasts (Biscardi *et al.* 1999, Tice *et al.* 1999). However, the indirect activation of Src by the EGFR via the GTPase Ral leading to STAT3 but not Erk activation has been reported (Goi *et al.* 2000). Another study demonstrated Src-dependent activation of Jak2 and STAT proteins following stimulation with EGF (Olayioye *et al.* 1999).

EGFR and cell adhesion

Growth factor receptors like the PDGFR or the EGFR interact with integrins which mediate cell–cell adhesion, cell–matrix association and intracellular signalling (Miyamato *et al.* 1996, Schneller *et al.* 1997). In a recent report, the focal adhesion kinase (FAK) has been demonstrated to link EGFR and integrin signalling pathways, thereby promoting EGF-induced cell migration independent of FAK's intrinsic kinase activity (Sieg *et al.* 2000). Together with its relative Pyk2, FAK has been shown to be important for EGFR and integrin signalling in neurons where it is essential for neurite outgrowth, demonstrating the necessity of a collaboration of growth factor–receptor signalling and integrin engagement for efficient signal generation (Ivankovic-Dikic *et al.* 2000).

EGFR family and cancer

Members of the EGFR family have frequently been implicated in various forms of human cancers and serve both as prognostic markers or as therapeutic targets. Several phenomena are responsible for abnormal activation of these receptors in tumours, including overexpression, amplification, constitutive activation of mutant receptors or autocrine growth factor loops (reviewed in Voldborg *et al.* 1997, Zwick *et al.* 2000). Recently, Herceptin, an antibody against HER2, was applied in the treatment of breast cancer patients overexpressing this RTK, and inhibitors which target the EGFR are in clinical trials.

EGFR and HER2 gene aberrations in human cancers

Elevated expression and/or amplification of the EGFR and HER2 have been found in a variety of human cancers (Table 2) underlying the critical role of RTKs in human tumour growth. In most of these cases receptor overexpression is caused by gene amplification.

In human breast cancer HER2 gene amplification was correlated with a shorter overall survival and relapse-free survival (Slamon *et al.* 1987). Furthermore, Watanabe and co-workers described a correlation between HER2 overexpression and the absence of estrogen receptors in breast carcinomas which is consistent with the non-responsiveness of HER2–positive tumours to endocrine therapy (Watanabe *et al.* 1993, Nicholson *et al.* 1994, Wright *et al.* 1989, 1992).

Receptor mutations

EGFR mutations

Several deletions in the extra- and intracellular domain of the EGFR have been found in glioblastomas (Ekstrand *et al.* 1992), non-small-cell lung carcinomas (Garcia de Pallazzo *et al.* 1993), breast cancer (Wikstrand *et al.* 1995) and ovarian carcinomas (Moscatello *et al.* 1995). Genetic alterations were predominantly observed in human glioblastomas with EGFR gene amplification; furthermore, in this tumour type multiple variants of EGFR mutations could be detected within individual tumours (Frederick *et al.* 2000).

The EGFRvI deletion, which lacks the extracellular domain, resembles the v-erb-B oncoprotein and has been observed in xenografts derived from a malignant human glioma (Bigner *et al.* 1990, Sugawa *et al.* 1990). EGFRvII, found in gliomas with amplified EGFR genes (Humphrey *et al.* 1991), contains an 83 amino acid deletion, spanning the region between the ligand binding and the transmembrane domain. EGFRvII responds in a similar manner to the wildtype receptor and is still capable of mediating EGF-induced cell proliferation and invasiveness (Humphrey *et al.* 1991). The best characterised and most common EGFR mutant in human cancer is EGFRvIII. The receptor lacks amino acids 6–273 (801 bp) and arises from intragene rearrangements or from alternative mRNA splicing, resulting in the insertion of a glycine residue at the deletion point (Wong *et al.* 1992). This deletion has been observed in malignant gliomas, breast carcinomas (Moscatello *et al.* 1995, Wikstrand *et al.* 1995), non-small-cell lung carcinomas (Garcia de Pallazzo *et al.*

Table 2 Overexpression of the EGFR and HER2 in human cancers

Type of tumour	Receptor	Over-expression (%)	Reference
Mammary	EGFR	14–91	Klijn *et al.* (1992); Beckmann *et al.* (1996); Walker & Dearing (1999)
	HER2	21	Paik *et al.* (1990)
Bladder	EGFR	31–48	Salomon *et al.* (1995); Chow *et al.* (1997)
	HER2	36	Sauter *et al.* (1993)
Colon	EGFR	25–77	Salomon *et al.* (1995); Messa *et al.* (1998)
	HER2	50	Caruso & Valentini (1996)
Glioma	EGFR	40–50	Ekstrand *et al.* (1991); Salomon *et al.* (1995); Rieske *et al.* (1998)
Non-small-cell lung	EGFR	40–80	Salomon *et al.* (1995); Fujino *et al.* (1996); Rusch *et al.* (1997); Fontanini *et al.* (1998)
Pancreatic	EGFR	30–50	Salomon *et al* (1995); Uegaki *et al.* (1997)
Ovarian	EGFR	35–70	Salomon *et al.* (1995); Bartlett *et al.* (1996); Fischer-Colbrie *et al.* (1997)
	HER2	32	Berchuck *et al.* (1990)
Gastric	HER2	26	Lemoine *et al.* (1991)
Lung	HER2	28	Tateishi *et al.* (1991)
Salivary	HER2	32	Stenman *et al.* (1991)
Head and neck	EGFR	80–100	Salomon *et al.* (1995); Grandis *et al.* (1996)

1993) and ovarian tumours (Moscatello *et al.* 1995). The EGFRvIII mutation causes a receptor with a constitutively active kinase function thus stimulating cellular proliferation in the absence of ligands (Ekstrand *et al.* 1994). EGFRvIII is able to transform NIH3T3 cells (Moscatello *et al.* 1996) and strongly enhances the tumorigenicity of human glioma cells in nude mice (Nishikawa *et al.* 1994). However, the inserted glycine residue which creates a new epitope at the splice site and the tumour-selective expression of EGFRvIII qualify this mutant as a target for specific inhibitory antibodies (Humphrey *et al.* 1990, Hills *et al.* 1995, Lorimer *et al.* 1995). Moreover, the expression of EGFRvIII in gliomas leads to resistance to cisplatin, a commonly utilised chemotherapeutic agent (Nagane *et al.* 1998), suggesting the need for specific EGFRvIII inhibition in combination with chemotherapy.

HER2 mutations

In tumours from MMTV/Neu mice a deletion was found in the extracellular region of Neu, the rat HER2 homologue, resulting in a constitutively activated receptor capable of transforming Rat-1 fibroblasts (Bouchard *et al.* 1989, Guy *et al.* 1992, 1996). Furthermore, Bargmann and co-workers (1986) found a point mutation (V664E) in the transmembrane domain of the neu oncogene in chemically induced rat neuroblastomas causing increased tyrosine phosphorylation of the receptor (Bargmann *et al.* 1986). Nevertheless, no similar mutations have been described in human tumours to date. Distinct from this, a polymorphism in the transmembrane domain of human HER2 has been reported in healthy individuals as well as in neuroectodermal tumours (Papewalis *et al.* 1991). Recently, this mutation has been correlated with a decreased risk to develop breast cancer (Xie *et al.* 2000).

Autocrine growth factor loops

While the transforming capacity of overexpressed HER2 was shown as a ligand-independent process, constitutive EGFR activation has often been associated with autocrine growth factor loops. In colon cancer cell lines amphiregulin acts as a potent autocrine factor (Damstrup *et al.* 1999). TGFα is the most prominent EGFR ligand which is frequently co-expressed with the EGFR in non-small-cell lung cancers (Hsieh *et al.* 2000), prostate cancer (Seth *et al.* 1999) and gastrointestinal stromal tumours (Cai *et al.* 1999). In human mammary epithelial cells the critical contribution of metalloproteases in generating biologically active TGFα could be shown since batimastat, a metalloprotease inhibitor, is able to inhibit cell proliferation and migration (Dong *et al.* 1999).

EGFR family members as therapeutic targets

Due to their frequent overexpression and high signalling capacity the EGFR and HER2 are promising targets for therapeutic intervention in human cancer. Recent studies linked these receptors and their ligands to drug resistance (Wosikowski *et al.* 1997), and receptor overexpression has also been correlated with tumour resistance to cytotoxic agents including radiation. Therefore, selective drugs may contribute to improved cancer therapy and various approaches based on the biochemical properties of these cell surface receptors are under investigation (Fig. 2).

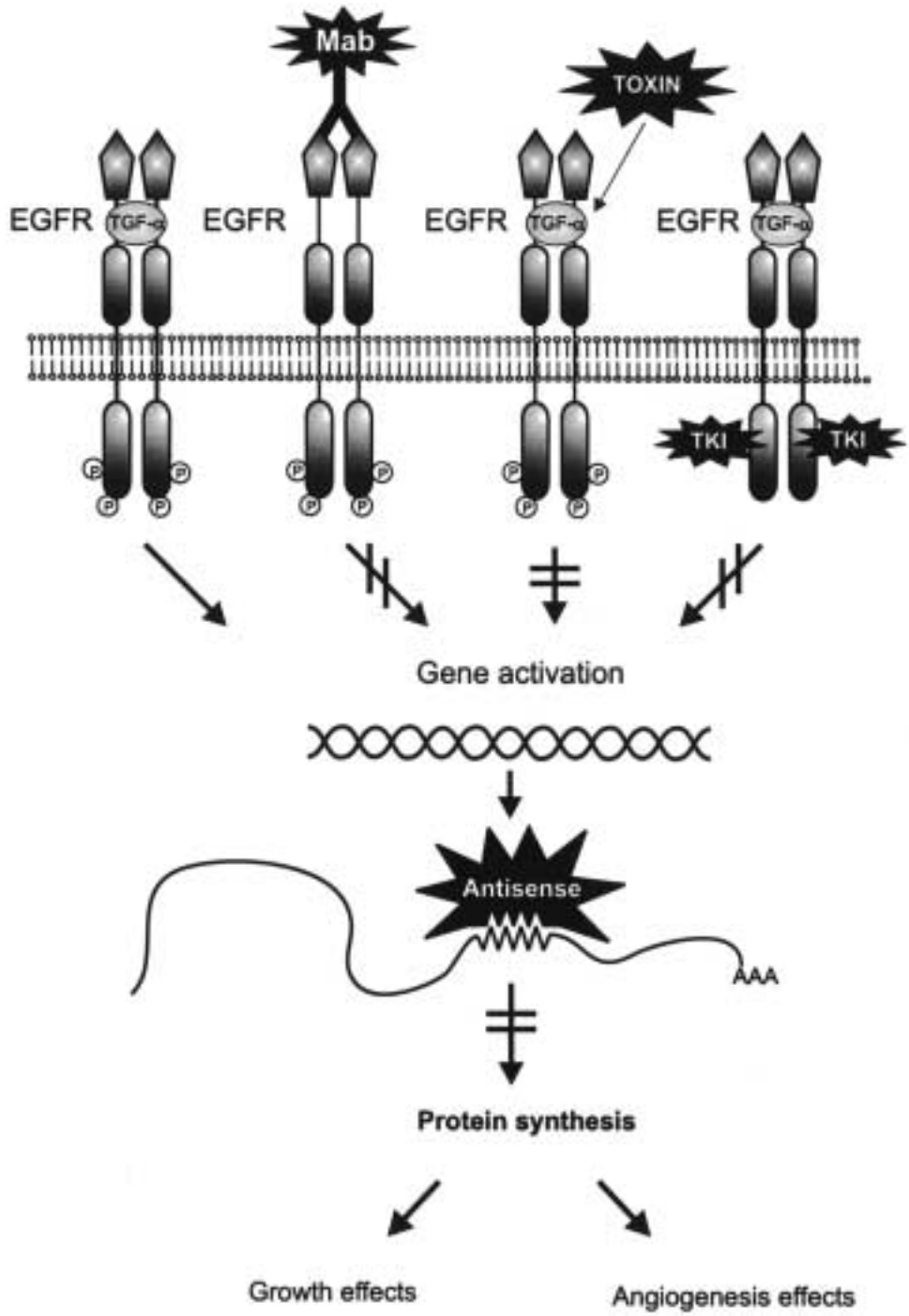

Figure 2 A schematic representation of the EGFR as a target for anti-cancer therapies. Four different strategies for inhibiting EGFR expression or activity are shown: monoclonal antibodies (Mab) against the extracellular ligand-binding domain; antisense oligonucleotides against EGFR mRNA; ligand–toxin conjugates which kill target cells following endocytosis; small molecular tyrosine kinase inhibitors (TKI) which inhibit ligand-induced EGFR activation.

Monoclonal antibodies

Monoclonal antibodies raised against several epitopes of the EGFR or HER2, as well as EGF and TGFα blocking antibodies are now in clinical trials, either alone or in combination with other therapeutic agents. The prototypical therapeutic antibody Herceptin (Trastuzumab, rhu4D5) which targets HER2 recognises the receptor's extracellular domain (Fendly *et al.* 1990). Herceptin binding leads to a decreased kinase activity combined with the inhibition of proliferation and the hypersensitivity to both pro-apoptotic tumour necrosis factor α (TNFα) as well as cytotoxic which targets HER2 recognises the receptor's extracellular domain (Fendly *et al.* 1990). Herceptin binding leads to a decreased kinase activity combined with the inhibition of proliferation and the hypersensitivity to both pro-apoptotic tumour necrosis factor α (TNFα) as well as cytotoxic cisplatin, paclitaxel and doxyrubicin in HER2 overexpressing cells (Hudziak *et al.* 1989, Hancock *et al.* 1991). In clinical trials, Herceptin was well tolerated in women with metastatic breast cancer overexpressing HER2 and produced durable objective responses (Baselga *et al.* 1996, Cobleigh *et al.* 1999). Recently, Park and co-workers (2000) developed a structure-based 1.5 kDa peptide mimic functionally similar to Herceptin that is able to reduce HER2 signalling *in vitro* and *in vivo* which could be a viable candidate for use in clinical trials. However, Herceptin which was approved by the Food and Drug Administration in 1998 is the first target-selective drug raised against an oncogenic cell surface receptor and therefore represents the first example of a new era of anti-cancer therapy.

Cetuximab (C225), a monoclonal antibody directed against the extracellular domain of the EGFR, efficiently competes with EGFR ligand binding due to its high affinity for the receptor (Goldstein *et al.* 1995). Furthermore, Cetuximab-like antibodies were shown to induce receptor internalisation and therefore downregulate EGFR signalling (Sunada *et al.* 1986). The general antitumour effect of C225 has been shown in human ovarian, breast, colon and renal carcinoma cell lines (Prewett *et al.* 1998, Ciardiello *et al.* 1999). In combination with radiotherapy, C225 increases tumour cell terminal differentiation and inhibits tumour angiogenesis thus enhancing the response to radiation, as shown in xenografts from epidermoid carcinomas and head and neck squamous cell carcinomas (Huang & Harari 2000, Milas *et al.* 2000). Finally, treatment of ovarian cancer cells with both C225 and Herceptin results in an additive anti-proliferative effect, suggesting a potential synergistic effect for anti-cancer treatment (Ye *et al.* 1999).

Ligand–toxin and immunotoxin conjugates

Ligand–toxin fusion proteins against the EGFR are constructed using NRG, EGF or TGFα conjugated to various truncated forms of *Pseudomonas* exotoxin A and other less immunogenic cellular toxins (reviewed in Noonberg & Benz 2000). These ligand–toxin conjugates enter the cells through receptor-mediated endocytosis and kill the target cell through inhibition of protein synthesis (Fitzgerald 1996, reviewed in Noonberg & Benz 2000). Based on a similar principle, approaches with immunotoxins which contain the EGF ligand fused to genistein, a broad spectrum tyrosine kinase inhibitor, are currently under investigation (Uckun *et al.* 1998*a*,*b*, Shao *et al.* 1998).

Antisense oligonucleotides

Another strategy to inhibit the receptor activity are antisense oligonucleotides, short pieces of synthetic DNA or RNA which interact with the mRNA and therefore efficiently block transcription of specific cellular target proteins (Marcusson *et al.* 1999). Growth inhibition using oligonucleotides directed against the EGFR or TGFα has been shown in cancer cell lines such as ovarian carcinoma, breast, prostate cancer and head and neck squamous cell carcinoma cell lines (Grandis *et al.* 1998, Rubenstein *et al.* 1998, Witters *et al.* 1999).

Tyrosine kinase inhibitors

Taking advantage of structure-based drug design and the variability of combinatorial chemistry, small molecule drugs developed against RTK kinase domains represent another promising approach in cancer therapy (reviewed in Levitzki 1999). ATP analogues of the Tyrphostin family have been developed to show specificity towards the ATP-binding sites of the EGFR and HER2 thereby inhibiting these kinases (Moyer *et al.* 1997, reviewed in Levitzki 1992, Gazit *et al.* 1991, Osherov *et al.* 1993). The EGFR-specific AG1478 was shown to prevent primary glioblastoma cells from invading brain aggregates (Penar *et al.* 1997) and to abrogate proliferation of prostate cancer cells (Kondapaka & Reddy 1996). In addition, AG1478 inhibits EGFR as well as HER2 signalling in MMTV/Neu+MMTV/TGFα bigenic mice and suppresses mammary tumorigenicity (Lenferink *et al.* 2000). Another promising EGFR inhibitor, ZD-1839, which shows an antiproliferative effect in ovarian, breast and colon cancer cells is under clinical development (Ciardiello *et al.* 2000).

Transactivation of EGF receptor family members

Cross-communication between heterologous signalling systems is essential to integrate the variety of extracellular stimuli into a limited number of signalling pathways. The EGFR and HER2 have been identified as critical elements in signal transduction networks utilised by G-protein-coupled receptors, cytokine receptors, integrins, ion channels and RTKs (reviewed in Gschwind *et al.* 2001, Carpenter 2000*b*,

Prenzel *et al.* 2000). To distinguish this non-classical mode of activation from receptor activation by its cognate ligands this process has been termed EGFR transactivation.

RTK transactivation by cellular stress

Since 1989 when Schlessinger and colleagues found that the EGFR and its relative HER2 become tyrosine phosphorylated in response to hyperosmotic shock (King *et al.* 1989), various other non-physiological stimuli such as UV and gamma radiation, membrane depolarizing agents and several oxidants have been shown to regulate the activation state of RTKs (Sachsenmaier *et al.* 1994, Coffer *et al.* 1995, Huang *et al.* 1996, Knebel *et al.* 1996, reviewed in Weiss *et al.* 1997 and Carpenter 1999). Furthermore, the association of the EGFR with Shc and Grb2 adapter proteins or with phospholipase Cγ following UV treatment underlined the functional importance of this ligand-independent RTK transactivation (Huang *et al.* 1996, Knebel *et al.* 1996). Based on the findings which revealed the critical involvement of RTK antagonistic protein tyrosine phosphatases (PTPases) in UV-induced effects (Knebel *et al.* 1996), PTPases together with generated reactive oxygen intermediates (ROIs) are thought to be key modulators of the RTK activation state.

GPCR-induced EGFR transactivation

In 1996, Daub and co-workers found that the EGFR was an essential element in coupling ligand stimulation of G-protein-coupled receptors specific for lysophosphatidic acid (LPA), thrombin and endothelin-1 (ET-1) to the Ras-MAP kinase pathway, c-fos gene transcription and cell growth (Daub *et al.* 1996). In this report, specific inhibition of the EGFR function by either the small molecule drug AG1478 or a dominant negative receptor mutant blocked GPCR-induced mitogenic signalling in Rat-1 cells. The ligand-independent mechanism described was reminiscent of stress-induced EGF receptor activation thus pointing to inactivation of PTPase activity rather than the involvement of EGF-like ligands. This initial finding was followed by a number of studies which showed the crucial contribution of the EGFR for signalling of various GPCRs via all classes of G-proteins to a variety of different cellular messengers (reviewed in Gschwind *et al.* 2001, Carpenter 1999, Zwick *et al.* 1999*a*). In general, EGFR transactivation is characterised by rapid and transient kinetics (Daub *et al.* 1997, Keely *et al.* 1998, Li *et al.* 1998) and the critical dependence on the receptor's intrinsic tyrosine kinase domain (Daub *et al.* 1996, Tsai *et al.* 1997, Zwick *et al.* 1997, Eguchi *et al.* 1998).

Mechanism of EGFR transactivation

Based on the rapid onset of GPCR-induced EGFR tyrosine phosphorylation and the absence of EGF-like ligands in the cell culture media after G-protein activation (Tsai *et al.* 1997, Eguchi *et al.* 1998), a mechanism exclusively attributed to intracellular elements was postulated (Daub *et al.* 1996, reviewed in Zwick *et al.* 1999*a*). In this context, several candidate cytoplasmic tyrosine kinases, Ser/Thr kinases and second messengers have been discussed as potential mediators of EGFR transactivation.

Involvement of Src kinases

Non-receptor tyrosine kinases of the Src family have frequently been implicated in GPCR-induced activation of the Ras-MAP kinase pathway (Luttrell *et al.* 1996). G_i-mediated and therefore pertussis toxin (PTX)-sensitive or βγ-mediated EGFR tyrosine phosphorylation and MAP kinase activation via Src-kinases was shown in COS-7 cells (Luttrell *et al.* 1997). In contrast, while LPA-induced Erk2 induction was abrogated by Src inhibition, EGFR transactivation was unaffected in the same cell line (Daub *et al.* 1997). In vascular smooth muscle cells, PP1, an inhibitor of Src-like kinases blocked angiotensin II (ATII)-induced EGFR tyrosine phosphorylation (Bokemeyer *et al.* 2000). In line with these reports GPCR ligands such as gonadotrophin-releasing hormone and neuropeptide YY (PYY) activate the MAP kinase pathway in various cell types dependent on both EGFR function and Src kinase activity (Grosse *et al.* 2000*a*, Mannon & Mele 2000). However, these newer findings did not depict the concrete position of Src acting up- or downstream of the EGFR in further detail. The idea that Src family kinases represent a point of convergence for different pathways (Della Rocca *et al.* 1999) is further supported by the existence of Src-EGFR complexes following ATII or carbachol stimulation (Eguchi *et al.* 1998, Keely *et al.* 2000) and Src-GPCR association in isoproterenol treated cells (Luttrell *et al.* 1999). The finding that ATII-induced Src recruitment to the EGFR is not sensitive to AG1478 suggested the EGFR as a scaffold for pre-activated Src (Eguchi *et al.* 1998). Remarkably, Parsons and colleagues showed that mutation of Tyr845, a Src phosphorylation site within the EGFR, is critically involved in LPA and EGF-induced mitogenic signalling without altering the receptor's intrinsic kinase activity (Tice *et al.* 1999). In conclusion, regardless of their signalling position, Src kinases act as critical players in GPCR as well as in EGFR signalling and may contribute to GPCR-induced EGFR transactivation to varying extents, depending on the cellular context and the activated receptor.

Involvement of calcium and Pyk2

Based on the observation that calcium influx is sufficient to trigger MAP kinase activation and EGFR tyrosine phosphorylation in PC12 cells (Rosen & Greenberg 1996), Zwick *et al.* (1997) revealed the functional role of the EGFR as a critical mediator of both calcium-induced and bradykinin-triggered MAP kinase activation. This cell

type-specific picture has recently been extended since several findings have demonstrated Ca^{2+} to be necessary for ET-1, ATII, UTP and carbachol-induced EGFR transactivation in several model systems (Eguchi *et al.* 1998, 1999, Matsubara *et al.* 1998, Soltoff 1998, Keely *et al.* 2000) and for the action of interleukin-8 in ovarian cancer cells (Venkatakrishnan *et al.* 2000). Due to this critical function of Ca^{2+}, the Ca^{2+}-regulated FAK family kinase Pyk2 (Lev *et al.* 1995) was discussed as a mediator of EGFR transactivation in the signalling elicited by carbachol in intestinal epithelial cells (Keely *et al.* 2000) and UTP in PC12 cells (Soltoff 1998). While Keely *et al.* (2000) reported the transactivation-dependent recruitment of Pyk2 and Src to the EGFR, the failure of AG1478 to inhibit Pyk2 tyrosine phosphorylation in response to UTP led to the speculation that Pyk2 acts upstream of the EGFR signal (Soltoff 1998). However, inducible-expression of a kinase-inactive Pyk2 mutant did not affect either GPCR- or calcium-induced transactivation suggesting a role of this kinase in parallel to the EGFR (Zwick *et al.* 1999*b*). Similar to Src kinases, Pyk2 may exert diverse biological functions depending on its restricted expression and the activating receptor or stimulus (Lev *et al.* 1995, Dikic *et al.* 1996, Della Rocca *et al.* 1999). Very recently, Pyk2 was shown as a potent mediator of ATII-induced c-Jun NH2–terminal (Jnk) kinase activation and c-jun gene expression (Murasawa *et al.* 2000), a process distinct from EGFR and Ras activation which mediate ATII-induced Erk induction (Eguchi *et al.* 1998, Murasawa *et al.* 1998).

Involvement of PKC

The serine/threonine kinase PKC has often been implicated as a mediator of EGFR transactivation especially following stimulation of Gq-coupled receptors. Nevertheless, even though both the M1 and M3 subtypes of muscarinic acetylcholine receptor couple to Gq-proteins, carbachol-induced EGFR tyrosine phosphorylation was blocked by the PKC inhibitor GF109203X only in M1R expressing HEK293 cells (Tsai *et al.* 1997, Slack 2000). Interestingly, abrogating PKC function even enhanced M3R-induced crosstalk significantly (Slack 2000), while in colonic epithelial cells, Keely and colleaques (1998) observed no prominent role of PKC in signalling from M3R to Erk activation. While EGFR transactivation was strictly PKC-dependent in gonadotrophin-releasing hormone-stimulated αT3–1 gonadotrophs and PC12 cells treated with UTP (Soltoff 1998, Grosse *et al.* 2000*b*), PKCε was found downstream of the EGFR in a gut epithelial cell line treated with the peptide GPCR agonist PYY (Mannon & Mele 2000). ATII-stimulated EGFR transactivation in rat liver epithelial cells was shown only if PKC function was blocked (Li *et al.* 1998). Finally, bradykinin stimulation of COS-7 cells was mediated by both EGFR transactivation and PKC activation via independent signalling pathways which converged at the small GTPase Ras (Adomeit *et al.* 1999).

Taken together, EGFR transactivation links stimulation of various G-protein-coupled receptors to a plethora of cellular phenomena such as MAP kinase activation (Cunnick *et al.* 1998, Castagliuolo *et al.* 2000), gene transcription (Daub *et al.* 1996, Vaingankar & Martins-Green 1998, Moriguchi *et al.* 1999), regulation of ion channels (Tsai *et al.* 1997), PI3K activation (Daub *et al.* 1997, Laffargue *et al.* 1999) and cytoskeletal rearrangements (Gohla *et al.* 1998, 1999).

Several cytoplasmic mediators contribute to varying extents to GPCR-induced EGFR transactivation but a general and new principle which extended the classical mechanistic view by some new and previously unexpected elements will be summarised in the following section.

Triple membrane-passing signal mechanism of GPCR-induced EGFR transactivation

As discussed above, EGFR transactivation has been considered to be mediated exclusively by intracellular elements in a ligand-independent mechanism. In contrast to this, stimuli such as TPA and Ca^{2+} ionophores which are known to induce tyrosine phosphorylation of the EGFR, and activators of heterotrimeric G-proteins, AlF^{4-} and GTPγS, were shown to induce the cleavage of EGF-like growth factor precursors thus generating mature ligands (Bosenberg *et al.* 1993, Goishi *et al.* 1995, Dethlefsen *et al.* 1998). Moreover, the finding that a chimeric receptor, consisting of the ligand-binding domain of the EGFR and the PDGF receptor's transmembrane and intracellular part, can be transactivated supported the notion that the EGFR extracellular domain is involved in the GPCR-EGFR cross-talk (Prenzel *et al.* 1999). Intercellular EGFR transactivation after carbachol stimulation of co-cultured cell lines stably expressing either the M1R or the human EGFR further underlines the thesis that a diffusible factor may mediate the effects observed. In another set of experiments we could show that several ligands for GPCRs such as LPA, thrombin or bombesin can induce the proteolytic processing of the proHB-EGF precursor, and inhibition of HB-EGF function abrogated EGFR transactivation in COS-7 and HEK 293 cells. Finally, treatment of cells with the broad-spectrum metalloprotease inhibitor Batimastat completely blocked GPCR-induced proHB-EGF shedding and subsequent EGFR and Shc tyrosine phosphorylation thus leading to the molecular model of a triple membrane-passing signal mechanism (Prenzel *et al.* 1999) (Fig. 3). Despite the identification of these previously unexpected but critical pathway elements, the nature of the metalloprotease and the mechanism of its G-protein-mediated activation are not yet clear. Very recently, however, other studies confirmed this concept of metalloprotease-mediated and therefore ligand-dependent transactivation mechanism. Thrombin-induced EGFR transactivation and cell migration in

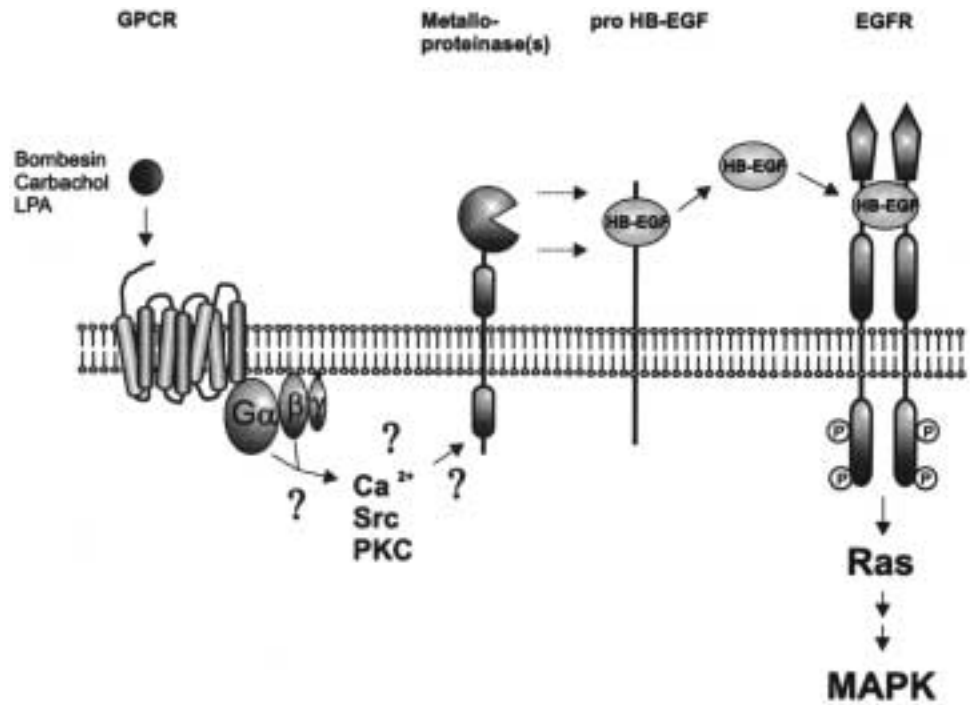

Figure 3 Triple-membrane-passing signal mechanism (TMPS) of the EGFR transactivation. GPCR-induced and metalloprotease-mediated proteolytic cleavage of EGF-like growth factor precursors leads to transactivation of the EGFR. This mechanistic signalling model for ligand-dependent interreceptor communication encloses three membrane passages and couples GPCR activation to the Ras-MAP kinase (MAPK) pathway.

smooth muscle cells was shown to be mediated by proHB-EGF shedding (Kalmes *et al.* 2000). The same ligand is critical for estrogen-induced and GPCR-mediated EGFR transactivation and Erk activation in breast cancer cell lines (Filardo *et al.* 2000). This finding, together with the observation that bombesin-induced EGFR tyrosine phosphorylation and basal EGFR activation are Batimastat sensitive in PC3 prostate cancer cells (Prenzel *et al.* 1999) underlines the putative role of the transactivated EGFR in hyperproliferative disorders such as cancer (Vacca *et al.* 2000, reviewed in Gschwind *et al.* 2001).

Conclusion

Since the initial cloning of the EGFR in 1984 and the following characterisation of HER2 and its relatives, the investigation of the EGFR family of RTKs has contributed to the understanding of fundamental cellular processes such as growth, differentiation and transformation. Despite the high complexity of signal generation with a variety of activating ligands, four related receptors and multiple intracellular effectors, numerous elegant studies have shed light on the basic mechanisms of this interconnected signalling network. Additionally, cross-communication between other cell surface receptors and the EGFR and their potential involvement in hyperproliferative diseases underlines the critical position of EGFR family members as hot spots of signalling and promising therapeutical targets. However, key network components, such as the regulators of growth factor precursor shedding and receptor-specific antagonistic phosphatases are still not identified, but the growing field of proteomics and additional information from genomics will provide tools and knowledge to unravel their identity.

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