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Investigation on the EGFR signal transactivation by G protein coupled receptors in cancer cells

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Erklärung

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Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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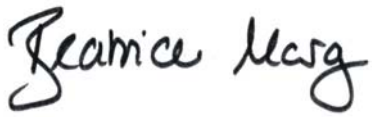

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Für meine Eltern

On ne voit bien qu’avec le cœur. L'essentiel est invisible pour les yeux.

Antoine de Saint Exupéry

Contents

1 Introduction

The most important characteristic common to all higher organisms is the capability to coordinate the functions of different cells types. This is accomplished by a complex signaling network processing incoming and outgoing signals, between different cells or different compartments within a cell.

A central role plays the binding of ligands to their respective receptors and the subsequent intracellular transmission of this signal leading to different biochemical processes like proliferation, differentiation, migration, adhesion or the programmed cell death, so-called apoptosis. Without the exact balance of the various signaling networks neither the development nor the survival of a higher organism would be possible. As more and more signaling components and pathways are discovered nowadays, it becomes apparent that signaling cascades are not separated linearly. On the contrary, they are highly interconnected and form complex networks (Downward, 2001). Thus, the regulation of these signaling networks is susceptible to disturbance and deregulated signal transmission can cause various diseases such as cancer, diabetes, immune deficiencies or cardiovascular diseases as well as many other (Hanahan and Weinberg, 2000; Schlessinger, 2000; Shawver et al., 2002).

The Human Genome Project has identified approximately 20% of the human genome to encode proteins of signaling pathways, including transmembrane proteins, guanine-nucleotide binding proteins (G proteins), kinases, phosphatases and proteases. The identification of 518 putative protein kinase genes and 130 protein phosphatases (Blume-Jensen and Hunter, 2001; Shawver et al., 2002) gives rise to the assumption that reversible protein phosphorylation is the central regulatory element of most cellular functions (Cohen, 2002a). Phosphorylation and dephosphorylation by the combined action of a protein kinase and a protein phosphatase can alter the activity as well as the stability of a protein, target it for degradation, influence its subcellular localization or change a protein's affinity towards interaction partners. According to their localization and their substrate specificity, both protein kinases and phosphatases can be subdivided into cellular and transmembrane molecules and into tyrosine or serine/threonine-specific kinases and phosphatases. The flexibility and reversibility of protein phosphorylation in combination with the availability of ATP as a phosphoryl donor can explain the preference of eukaryotic cells for this mechanism as the most common regulatory element.

Two important families of membrane-bound receptors are the G-protein coupled receptors (GPCRs) and the Receptor tyrosine kinases (RTKs). Up to date, GPCRs represent the largest family of signaling molecules known. They are integral membrane proteins without internal enzymatic activity. After ligand binding they couple to heterotrimeric G proteins (Coughlin, 1994; Gutkind, 1998) which are dynamically phosphorylated and thus regulate the levels of intracellular second messengers. RTKs have an intrinsic tyrosine kinase activity and are reversibly phosphorylated upon ligand binding which leads to the recruitment of docking and other signaling proteins (van der Geer et al., 1994).
Examples of counterparts of these receptors are protein tyrosine phosphatases (PTPs) for the RTKs and G protein coupled receptor kinases (GPKs) for the GPCRs. They control the activity of these receptors by dephosphorylation and thereby act as regulatory components in order to terminate a signal after prolonged or repeated exposure to the ligand. Another way to control the activity of membrane-bound receptors is the internalization of receptors followed by their degradations or the recycling of receptors.
Because numerous human diseases are connected to altered GPCR and RTK signaling pathways, numerous efforts have been taken to elucidate their signaling pathways in order to develop suitable drugs for their treatment.

1.1 Protein tyrosine kinases

Protein tyrosine kinases are important regulators of intracellular signal transduction pathways mediating aspects of multicellular communication and development (Cohen, 2002b) acting by catalyzing the transfer of the γ-phosphate of ATP to hydroxyl groups of tyrosines on target proteins (Hunter, 1998). Tyrosine kinases play a critical role in the control of many fundamental cellular processes including the cell cycle, migration, metabolism and survival as well as proliferation and differentiation. Among the 518 putative kinase genes 90 tyrosine kinase genes have been identified. 58 genes encode transmembrane receptor tyrosine kinases (RTKs) distributed into 20 subfamilies classified according to their structural characteristics and 32 encode cytoplasmic non-receptor tyrosine kinases (NRTKs) divided into ten subfamilies.

1.1.1 Receptor tyrosine kinases (RTKs)

RTKs are type I transmembrane proteins containing an intrinsic tyrosine kinase activity. They are composed of a glycosylated extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic domain (Hubbard and Till, 2000). The cytoplasmic domain contains a highly conserved protein tyrosine kinase core and additional regulatory sequences that are subjected to autophosphorylation and phosphorylation by heterologous protein kinases. The structural diversity of RTK is due to the presence of one or several copies of immunoglobulin-like domains, fibronectin type III-like domains, EGF (epidermal growth factor)-like domains, cysteine-rich domains or other domains within the extracellular domains (Figure 1).

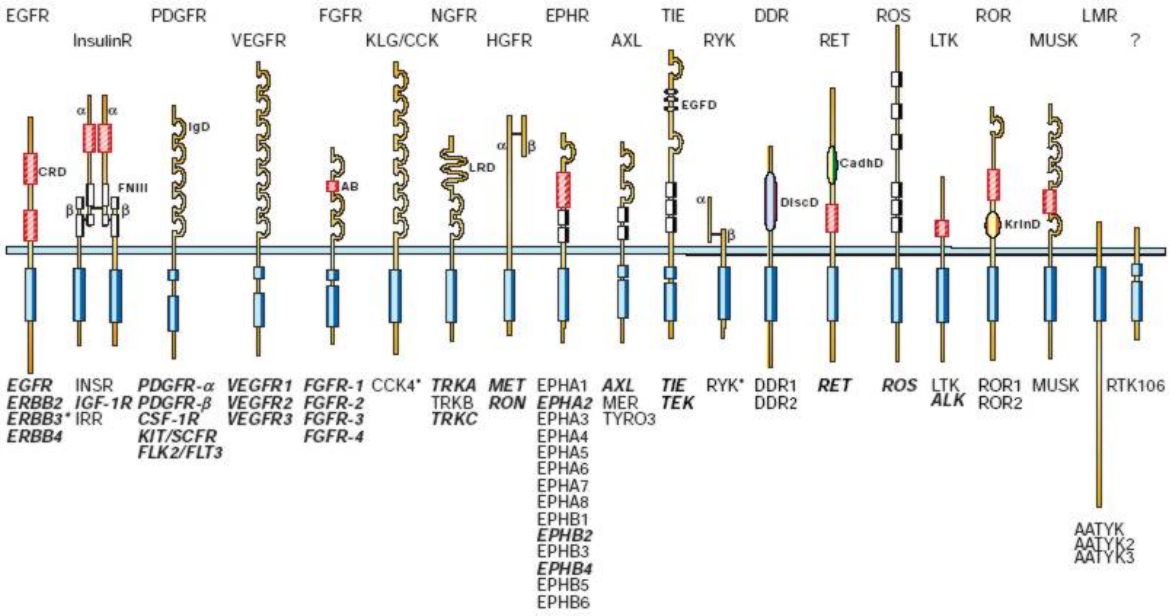

Fig. 1. Subfamilies of receptor tyrosine kinases. Abbreviations: AB, acidic box; CadhD, cadherin-like domain; CRD, cysteine-rich domain; DiscD, discoidin-like domain; EGFD, epidermal growth factor-like domain; FNIII, fibronectin type III-like domain; IgD, immunoglobulin-like domain; KrinD, kringle-like domain; LRD, leucine-rich domain. The symbols α and β denote distinct RTK subunits (Blume-Jensen and Hunter, 2001).

Activation of RTKs occurs via ligand-induced dimerisation followed by transphosphorylation of selected tyrosine residues within the cytoplasmic domain thereby generating docking sites for intracellular signal transducers with phosphotyrosine interaction domains.

1.1.2 EGFR family

The epidermal growth factor receptor (EGFR) family consists of four RTKs: EGFR, HER2/neu for which no ligand has been described so far (Goldman et al., 1990), HER3 being kinase-inactive and HER4 (Ullrich and Schlessinger, 1990).
The EGFR is the most prominent RTK and is the first cell surface signaling protein and protooncogene product to be characterized by molecular genetic methods and exhibits prototypical features of RTKs (Downward et al., 1984; Ullrich et al., 1984). Due to its functional role as a proto-oncogene in viruses, the pathophysiological effects of EGFR mutants and its overexpression in several types of cancer, the EGFR is implicated in cancer development. Examples of EGFR related cancer forms are colon (Damstrup et al., 1999), lung and prostate (Seth et al., 1999) as well as epithelial cancer (Dong et al., 1999).
HER2 is the preferred dimerisation partner for the EGFR, HER3 and HER4 and shows a high transforming activity (Beerli et al., 1995; Graus-Porta et al., 1997). The HER2 gene amplification for example has been linked to breast cancer (Slamon et al., 1989).

1.1.3 EGF-like ligand induced activation of RTKs

Activation of the EGFR family of RTKs is generally controlled by spatial and temporal expression of their ligands (Peles and Yarden, 1993). So far eight ligands have been shown to directly activate the EGFR: EGF, transforming growth factor alpha (TGFα), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BC), epiregulin (Epi) (Riese and Stern, 1998), cripto (Salomon et al., 1999) and epigen (Strachan et al., 2001). Various neuregulin (NRG) isoforms act as ligands for HER3 and HER4.
Despite having distinct receptor binding specificity, all ligands share a common motif of 30-50 amino acids in the active peptide, the so-called EGF structural unit which contains six conserved cysteine residues, restraining the peptide in a tertiary structure containing three disulfide bonded loops (Wingens et al., 2003).
EGF-like ligands are synthesized as transmembrane precursors which are subject to proteolytic cleavage at the cell surface to produce the soluble and diffusible growth factors (Massague and Pandiella, 1993). Subsequently, the mature ligands activate RTKs of the EGFR family by autocrine or paracrine stimulation. In addition, several studies indicate

that the membrane-anchored precursors may be biologically active via juxtacrine stimulation (Brachmann et al., 1989; Wong et al., 1989).

Biophysical investigations revealed a 2:2 stoichiometry for ligand-receptor complexes generated from stable 1:1 ligand-receptor intermediates. As recent studies support, ligand binding leads to conformational changes in the receptor and therefore to subsequent formation of various homo- and heterodimers rather than to the bridging of receptor monomers by ligand molecules (Jorissen et al., 2003; Schlessinger, 2002). Receptor dimerisation was shown to occur entirely receptor-mediated by a motif termed the "dimerisation-loop" which is unique to the EGFR family of RTKs since deletions or mutations in this motif entirely abrogate ligand-induced EGFR activation (Garrett et al., 2002; Ogiso et al., 2002). The dimerisation arm only becomes exposed after ligand binding as a consequence of a domain rearrangement in the extracellular portion of the EGFR thereby regulating receptor function. HER2, which has no ligand of its own, has been shown to constitutively adopt an extended configuration with an exposed dimerisation arm (Cho et al., 2003; Garrett et al., 2003). Together with further biophysical studies indicating only weak homodimeric interaction of HER2 receptor molecules, this observation suggests that HER2 mainly forms heterodimers with the remaining ligand-activated EGFR family members (Garrett et al., 2002), thus providing an additional platform for recruitment of intracellular signaling pathways. Figure 2 shows an overview over the preferred binding specificities of the EGF-like ligands and of HER2.

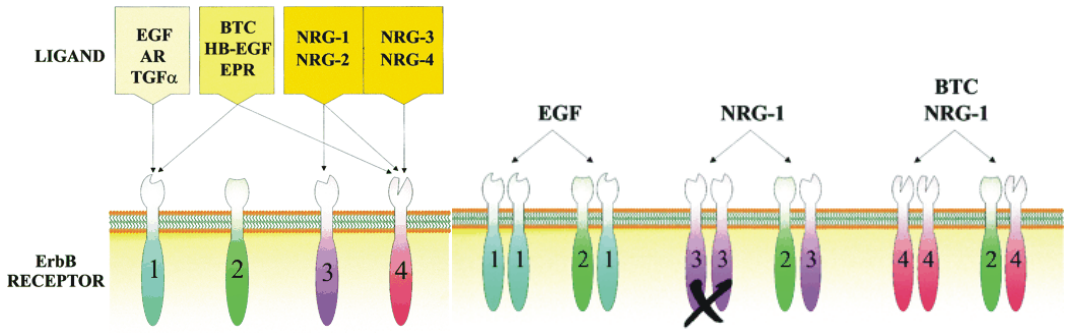

Fig. 2. Binding specificities of the EGF-related peptide growth factors. There are four categories of ligands that bind ErbB family receptors. EGF, AR and TGFα bind ErbB1; BTC, HB-EGF and EPR bind ErbB1 and ErbB4; NRG-1 and NRG-2 bind ErbB3 and ErbB4; and NRG-3 and NRG-4 bind ErbB4.

ErbB2 is the preferred dimerisation partner for the other ErbB receptors. Ligand binding to ErbB1 (EGF), ErbB3 (NRG-1) or ErbB4 (NRG-1, BTC) induces the formation of receptor homodimers and ErbB2-containing heterodimers. ErbB3 homodimers do not signal (indicated by the X), since the receptor has impaired kinase activity. Only some of the possible ligand–receptor-induced combinations are indicated in Figure 2 for the sake of simplicity (Olayioye et al., 2000).

The dimerisation of EGFR family members leads to intermolecular autophosphorylation of cytoplasmic tyrosine residues in the activation loop of the catalytic tyrosine kinase domain (Schlessinger, 2002) thereby leading to an open conformation of the activation loop. This enables access to ATP and substrate resulting in an enhanced tyrosine kinase activity and the subsequent recruitment of downstream signaling molecules.

1.1.4 Cytoplasmic tyrosine kinases

In addition to the membrane-anchored receptor tyrosine kinase, a second class of tyrosine kinase exists, the cytoplasmic non-receptor tyrosine kinase (NRTKs). The 32 NRTKs known so far are divided into 10 subfamilies based on their kinase domain sequence: Abl, Ack, Csk, Fak, Fes, Frk, Jak, Src, Tec and Syk (Blume-Jensen and Hunter, 2001; Robinson et al., 2000).

NRTKs lack receptor-like features such as an extracellular ligand binding domain and a transmembrane-spanning region. NRTKs can bind to cell surface receptors, but many are localized in different intracellular compartments including the cytoplasm, the nucleolus, mitochondria and the endoplasmic reticulum. Furthermore, they can bind to the cell membrane through amino-terminal modifications, such as myristoylation and palmitoylation (Hantschel and Superti-Furga, 2004).

The most common theme in NRTK regulation, as in RTK function, is tyrosine phosphorylation. In particular, phosphorylation of tyrosines in the activation loop of NRTKs, which occurs via *trans*-autophosphorylation or phosphorylation by a number of other NRTKs, leads to an increased enzymatic activity, whereas phosphorylation of tyrosines outside of the activation loop can negatively regulate kinase activity. In addition to the tyrosine kinase activity, NRTKs contain domains mediating protein-protein, protein-lipid and protein-DNA interactions (Hubbard and Till, 2000).

With nine members, the Src family is the largest subfamily of NRTKs and consists of Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes and Yrk (Blume-Jensen and Hunter, 2001). Src family members participate in a variety of signaling processes, including mitogenesis, T- and B-cell activation and cytoskeleton remodeling. Various *in vivo* substrates have been described for Src including the platelet derived growth factor receptor (PDGFR) and the EGFR, the NRTK focal adhesion kinase Fak, the adapter protein p130Cas, which is involved in integrin- and growth factor-mediated signaling and cortactin, an actin-binding protein essential for the proper formation of cell matrix contact sites (Hubbard and Till, 2000).

Regulation of Src catalytic activity has been extensively studied. Src and its family members contain a myristoylated amino terminus, a stretch of positively-charged residues that interact with phospholipid head groups, a short region with low sequence homology, a SH3 domain, a SH2 domain, a tyrosine kinase domain, and a short carboxy-terminal tail. Src exhibits two important regulatory tyrosine phosphorylation sites. Phosphorylation of Tyr-527 in the carboxy-terminal tail of Src by the NRTK Csk represses kinase activity. The importance of this phosphorylation site is emphasized in v-Src, an oncogenic variant of Src which is a product of the Rous sarcoma virus. Because of a carboxyterminal truncation, v-Src lacks the negative regulatory site Tyr-527 and is constitutively active, resulting in uncontrolled growth of infected cells. A second regulatory phosphorylation site in Src is Tyr-416, an autophosphorylation site in the activation loop. Maximum stimulation of kinase activity occurs when Tyr-416 is phosphorylated. Src has also been implicated in several human carcinomas, including breast, lung and colon cancer (Biscardi et al., 1999).

1.1.5 RTK downstream signaling and protein interaction domains

Ligand-induced RTK activation induces specific intracellular signal transduction pathways, depending on the stimulus and the cellular context. To regulate many different cellular processes, most proteins involved in intracellular signaling contain modular protein domains that specifically interact with other protein domains, lipids, and nucleic acids. These interaction domains either target proteins to a specific subcellular localization, provide means of recognition for posttranslational protein modification or chemical second messengers. Furthermore, they can control the conformation, activity and substrate specificity of enzymes (Pawson and Nash, 2003).

The most important domains in RTK signaling are those which recognize the phosphorylated tyrosine itself (Schlessinger and Lemmon, 2003). Phosphotyrosine residues are recognized by Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains with SH2 representing the most prevalent binding domain (Schlessinger, 2000). PTB domains are not restricted to bind phosphotyrosine residues, because they can also bind to non-phosphorylated peptide sequences.

WW and 14-3-3 domains bind to phosphoserine, while phosphothreonine residues are recognized by FHA and WD40 domains. The proline-rich sequence motif PXXP represents an additional binding moiety which binds specifically to SH3 domains. Pleckstrin homology (PH), phox homology (PHOX), FERM and FYVE domains bind to

phosphoinositides. Figure 3 shows an overview of different interaction domains and their binding specificities.

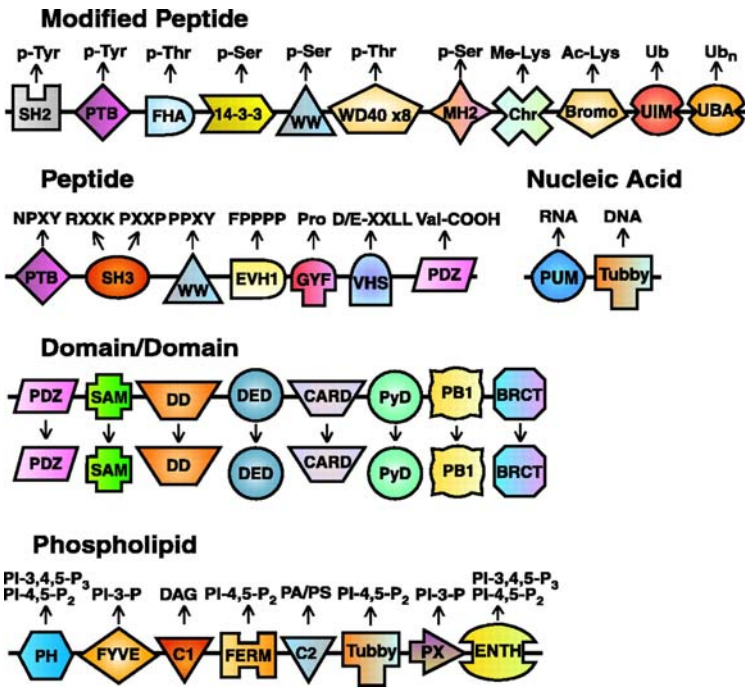

Fig. 3. Modular interaction domains in signal transduction. Interaction domains bind proteins, phospholipids, or nucleic acid. A subset of such domains is illustrated and their general binding functions are indicated (Pawson and Nash, 2003).

A wide variety of proteins possesses both, an interaction domain and enzymatic activity. In addition to their SH2 domain, Src kinases have a protein kinase activity and PLC-γ a phospholipase C activity. But some signaling proteins exclusively consist of SH2 and SH3 domains, such as Grb2, Crk and SHC linking activated RTKs to downstream signaling events such as the mitogen-activated protein kinase (MAPKs).

The ability of RTKs to recruit and activate a wide variety of adaptor proteins provides a signaling platform for the cell to regulate miscellaneous biological responses.

1.2 Mitogen-activated-protein-kinase (MAPK) pathways

Mitogen-activated protein kinases (MAPKs) are important signal transducing enzymes in the pathway connecting cell surface receptors to regulatory targets within the cell. MAPKs phosphorylate specific serine and threonine residues of target proteins and are implicated in cell migration, invasion, proliferation, differentiation, transformation and survival as

well as in angiogenesis. At least four differently regulated subgroups of MAPKs are present in mammals, extracellular-regulated kinase 1/2 (ERK1/2), Jun amino-terminal kinase (JNK1/2/3), p38 proteins (p38 α/β/γ/δ) and ERK 5 (Chang and Karin, 2001).

The major function of the MAPK pathway is the regulation of gene expression either by direct phosphorylation of transcription factors, target co-activators or co-repressors (Yang et al., 2003).

The most prominent MAPK pathway is the cascade leading to activation of ERK 1/2 by RTKs. Upon ligand-induced activation of RTKs, the adaptor protein Grb2 is recruited. Grb2 associates with the RAS-GEF Sos complex thereby activating it, which in turn activates membrane-associated Ras, a small monomeric GTP-binding protein (Schlessinger, 2000). Activated Ras interacts with several effector proteins such as the MAPK kinase kinase (MAPKKK) Raf-1 and PI3-K to stimulate numerous intracellular processes. Activated Raf-1 sequentially stimulates the MAPK kinases 1/2 (MAPKK, MEK 1/2) by phosphorylating a key serine residue in the activation loop. In the following, MEK 1/2 phosphorylates MAPK on threonine and tyrosine residues in regulatory TEY-motif leading to its activation (Robinson and Cobb, 1997). Activated MAPK phosphorylates a variety of cytoplasmic and membrane linked substrates. For example, it influences the transmembrane protein processing of ADAM17 by phosphorylation of the intracellular domain (Diaz-Rodriguez et al., 2002; Fan and Derynck, 1999). In addition, MAPK is rapidly translocated into the nucleus where it phosphorylates and activates transcription factors. The signaling cassette composed of MAPKKK, MAPKK, and MAPK is highly conserved in evolution and plays an important role in the control of metabolic processes, cell cycle, cell migration and cell shape as well as in cell proliferation and differentiation (Hunter, 2000).

The specificity of MAPK interactions and of the effector molecules stimulated depends largely on the MAPK subtypes involved. In particular, extracellular signal-regulated kinases (ERK1/2)/MAPKs are primarily stimulated by growth factors and modulate cell growth and differentiation, whereas c-Jun N-terminal kinases (JNKs) and p38 MAPKs are most commonly activated by stress stimuli and are involved in cell growth, differentiation, survival, apoptosis, and cytokine production (Marinissen and Gutkind, 2001).

1.3 Protein kinase B (PKB)/Akt

In mammals three isoforms, Akt 1, 2 and 3, of the serine/threonine kinase PKB/Akt are present, which are structurally closely related. They are composed of three functionally distinct regions, an N-terminal pleckstrin homology (PH) domain, a central catalytic domain and a C-terminal hydrophobic motif (HM).

Protein kinase B is activated by the phosphoinositide 3-kinase (PI3-K) pathway. Generation of PIP_3 and $PIP(3,4)P_2$ is necessary for recruitment of PKB to the membrane surface. Activation takes place by multisite phosphorylation, but the main phosphorylation site is Threonine 308. The fully activated multiphosphorylated PKB then dissociates from the plasma membrane and targets substrates located in the cytoplasm and nucleus leading to the activation of genes involved in diverse cellular processes. Furthermore, deregulated PKB activation is known to contribute to tumor development and metastasis as well as chemotherapeutic resistance (Hanada et al., 2004; Nicholson and Anderson, 2002).

1.4 G protein coupled receptors

The cell surface superfamily of G protein coupled receptors (GPCRs) forms one of the largest protein family and, with more than 1000 family members, represents up to 1% of the total genome of mammalians (Hermans, 2003). GPCRs are involved in diverse physiological functions including neurotransmission, photoreception, chemoreception, metabolism, growth and differentiation (Fukuhara et al., 2001). A diverse array of external stimuli including neurotransmitters, hormones, lipids, photons, odorants, taste ligands, nucleotides and calcium ions can act by binding to GPCRs and therefore induce diverse physiological functions as shown in Figure 4.

GPCRs can be divided into three major families, the receptors related to rhodopsin (type A), receptors related to the calcitonin receptor (type B) and the receptors related to metabotropic recptors (type C) (Gether and Kobilka, 1998).

Based on their highly conserved protein structure, GPCRs are also called heptahelical or serpentine receptors as they contain a conserved structural motif of seven α-helical membrane-spanning regions, each consisting of 20-27 amino acids. N-terminal segments, the cyto- and exoloops as well as the C-terminal segment can greatly vary in size. The amino-terminus is exposed to the extracellular environment and is believed to play a role in ligand binding for there exists a weak correlation between ligand size and the N-terminal

segment's length. Both the C-terminus and the intracellular loops interact with intracellular signaling partners, such as the associated heterotrimeric G proteins, but also with a wide variety of proteins containing structural interacting domains including PDZ, SH3 or PTB domains (Bockaert et al., 2003; Ji et al., 1998).

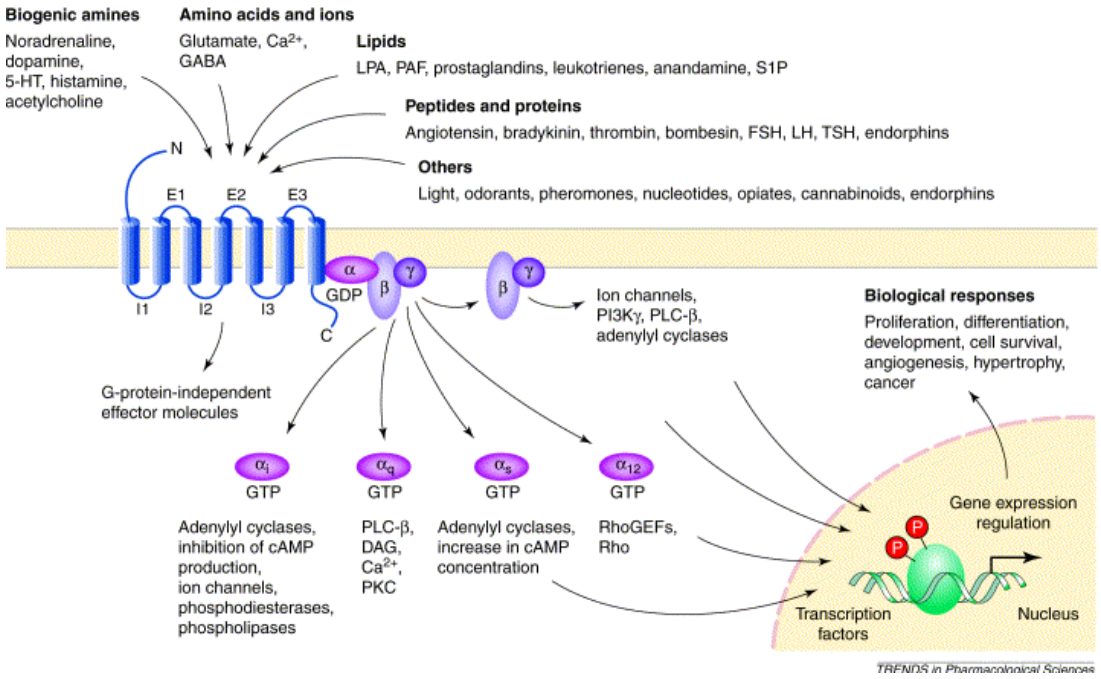

Fig. 4. Diversity of G protein coupled receptors (GPCRs). A wide variety of ligands, including biogenic amines, amino acids, ions, lipids, peptides and proteins, use GPCRs to stimulate cytoplasmic and nuclear targets through heterotrimeric G protein dependent and independent pathways. Such signaling pathways regulate key biological functions such as cell proliferation, cell survival and angiogenesis. Abbreviations: DAG, diacylglycerol; FSH, follicle-stimulating hormone; GEF, guanine nucleotide exchange factor; LH, leuteinizing hormone; LPA, lysophosphatidic acid; PAF, platelet-activating factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; S1P, sphingosine-1-phosphate; TSH, thyroid-stimulating hormone (Marinissen and Gutkind, 2001).

To add yet another layer of complexity, dimers or higher oligomers among identical GPCRs, close family members, or GPCRs from different families influence ligand binding, receptor activation, desensitization, trafficking and receptor signaling (Breitwieser, 2004)

1.4.1 Heterotrimeric G-proteins

A common biochemical characteristic of GPCRs is their interaction and activation of downstream signaling cascades by heterotrimeric G proteins, although some studies

reported G protein independent signaling through some GPCRs (Bockaert and Pin, 1999; Hall et al., 1999).

Heterotrimeric G proteins are composed of a Gα subunit interacting with a Gβγ subunit. Ligand binding to GPCRs alters the conformation of intracellular receptor domains and induces the association with heterotrimeric G proteins. This results in an exchange of GDP for GTP in the active site of the Gα subunit, followed by the dissociation of the heterotrimeric complex. Both the Gα and Gβγ subunits activate cytoplasmic or membrane bound effector molecules (Hermans, 2003).

The specific and complex signaling induced by GPCRs is due to the existence of at least 23 Gα subunits derived from 17 different genes, 6 Gβ and 12 different Gγ subunits. G proteins are generally referred to by their Gα subunits, which are subdivided on the basis of amino acid similarities in four distinct families, namely $G\alpha_s$, $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12}$ (Hermans, 2003; Pierce et al., 2001).

On the other hand, the number of downstream effectors is comparably limited and many G proteins couple to the same intracellular effectors. Effectors comprise adenylyl and guanylyl cyclase, calcium channels, GTPase-activating proteins (GAPs), guanine-nucleotide exchange-factors (GEFs), c-Src tyrosine kinase, phosphodiesterase and phospholipases. By this means, the signaling cascades induced by activated G proteins influence the level of second messengers like cyclic AMP and cyclic GMP, diacylglycerol, inositol (1,4,5)-triphosphate, phosphatidyl inositol (3,4,5)-triphosphate or calcium levels (Marinissen and Gutkind, 2001; Pierce et al., 2001)

1.4.2 Mitogenic GPCR signaling

Many reports suggest GPCRs to induce mitogenic responses and to be involved in the regulation of pathologic proliferation. Potent mitogens such as acetylcholine, angiotensin, bombesin, bradykinin, endothelin-1, isoproterenol, lysophosphatidic acid (LPA), neurotensin, prostaglandin and thrombin have been shown to induce mitogenic responses in tissue culture systems (Daaka, 2004).

The discovery of the *mas* oncogene, whose protein product displays a heptahelical structure, first revealed a connection between GPCRs and cellular transformation (Young et al., 1986). Furthermore, several known oncogenes encode mutated forms of GPCRs or their associated G proteins. Activating mutation of the α_{1B}-adrenoreceptor, thyroid-stimulating hormone receptors and luteinizing hormone receptors even in an agonist-

independent manner have been detected in adenoma of the thyroid and hyperplastic Leydig's cells, respectively (Marinissen and Gutkind, 2001; Parma et al., 1993; Shenker et al., 1993). In addition, the genomes of various transforming viruses such as Kaposi's sarcoma-associated herpesvirus contain sequences encoding constitutive active GPCRs shown to induce cancer in animal models (Montaner et al., 2003). Activating mutants of Gα subunits encoding transforming oncogenes such as *gsp*, *gip2* and *gep* are described (Dhanasekaran et al., 1998; Landis et al., 1989; Lyons et al., 1990; Marinissen and Gutkind, 2001; Radhika and Dhanasekaran, 2001; Xu et al., 1993).
Moreover, ectopically expressed GPCRs such as muscarinic acetylcholine M_1, M_2, and M_5 receptors could transform fibroblasts dependent in an agonist-dependent manner, suggesting that endogenous GPCRs can be tumorigenic when persistently simulated by agonists released from tumors in an autocrine or paracrine fashion. Inhibiting the function of GPCRs effectively prevents tumor growth in animal models leading to the possibility to develop novel therapeutics for cancer intervention which act on GPCRs.

1.4.3 The LPA receptors Edg 2, 4 and 7

LPA is an extracellular lipid mediator produced by activated platelets or from circulating lysophosphatidylcholine by autotoxin and has been implicated in the regulation of both, physiological and pathophysiological processes (Brindley, 2004; Fang et al., 2000a; Moolenaar et al., 1997). LPA represents the major mitogenic activity in serum and numerous cellular responses to LPA have been documented including rapid cytoskeletal rearrangements (Gohla et al., 1998), stimulation of cell proliferation (van Corven et al., 1989), suppression of apoptosis (Fang et al., 2000b) and induction of tumor cell migration and invasion (Fishman et al., 2001; Imamura et al., 1993). LPA levels are elevated in plasma and ascites of ovarian cancer patients (Fishman et al., 2001; Imamura et al., 1993; Xu et al., 1995; Xu et al., 1998) and LPA is likely to play a prominent role in the pathology of other types of human cancer.
Recently, cell surface receptors for LPA and the structurally related phospholipids sphingosine-1-phosphate (S1P) have been identified as members of the Edg (endothelial cell differentiation gene) subfamily of GPCRs (Kranenburg and Moolenaar, 2001) (Pyne and Pyne, 2000). Four LPA receptors, Edg2 (An et al., 1997), Edg4 (An et al., 1998a; Contos and Chun, 2000) and Edg7 (Bandoh et al., 1999) as well as five S1P receptors, Edg1, Edg3, Edg5, Edg6 and Edg 8 have been described which couple to various subtypes

of G proteins and show distinct characteristics in ligand specificity and activation of intracellular signaling pathways (Takuwa et al., 2002). Very recently, a fourth LPA receptor GPR23 has been identified which is structurally different from the Edg receptors (Noguchi et al., 2003).

The LPA receptors exhibit a high homology and are widely expressed (Figure 5). Overlapping expression patterns and the lack of subtype-specific agonists complicate the efforts to assign subtype specific physiological functions. Additionally, LPA can act independently of its receptors (Hooks et al., 2001). Recent studies on knock-out mice, however, have unveiled redundant as well as non-redundant functions for the LPA receptors that are essential for normal development and vascular maturation (Contos et al., 2002; Takuwa et al., 2002).

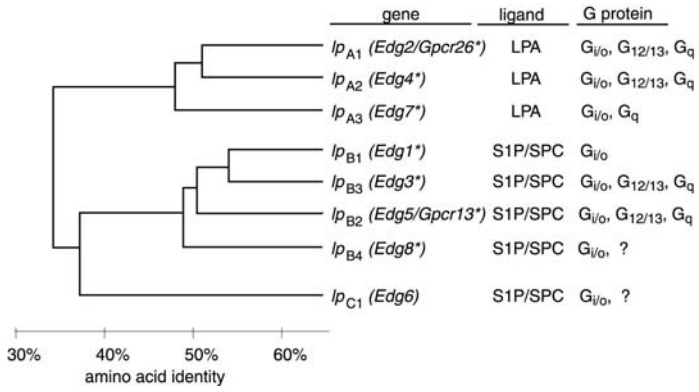

Fig. 5. Properties of lysophospholipid receptor genes. The dendrogram is based on percentage of amino acid identities among mouse LP receptor sequences (except lp_{B4}, which is from rat) and shows the predicted evolutionary divergence of the genes. Common synonyms are indicated in parentheses, and mouse gene symbols are denoted with asterisks. Probable G protein partners for each of the encoded receptors are shown, based on receptor expression studies that indicate PTX-sensitive cellular responses ($G_{i/o}$ coupling), PTX-insensitive cytoskeletal rearrangement/ Rho activation ($G_{12/13}$ coupling), or PTX-insensitive intracellular calcium mobilization and/or inositol phosphate production (G_q coupling) (Contos et al., 2000).

According to the cellular context, LPA is shown to be involved in the modulation of adenylate cyclase, stimulation of phospholipase C (PLC) and subsequent Ca^{2+} mobilization, phosphorylation of the survival mediator Akt/protein kinase B (PKB) by PI3-K and transcriptional regulation of immediate-early genes (Kranenburg and Moolenaar, 2001; Moolenaar, 1999; Moolenaar et al., 1997; Pyne and Pyne, 2000). Additionally, LPA stimulation leads to Rho induced stress fiber formation and cell rounding and activation of the Ras/MAPK pathway (Contos et al., 2000) (Figure 6). The latter pathway is connected to the induction of DNA synthesis and cellular proliferation.

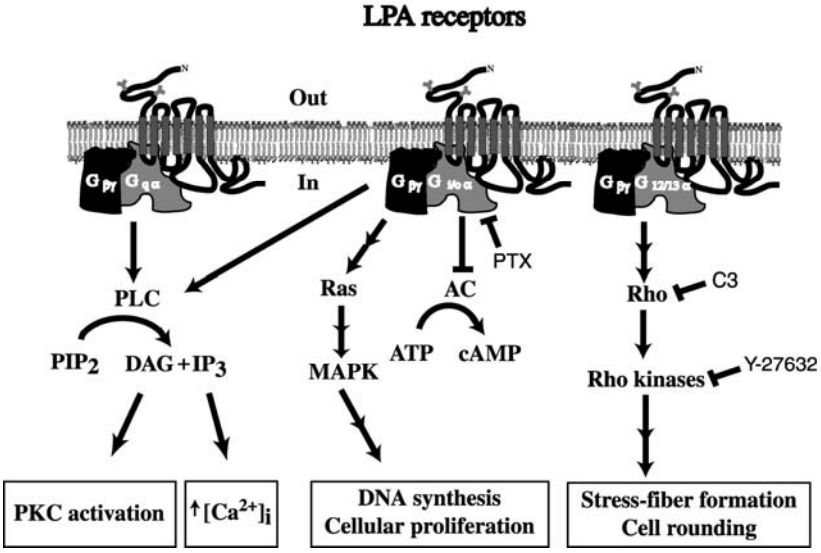

Fig. 6. G protein coupled receptor signal transduction pathways activated by LPA. Lines with arrowheads illustrate activation paths, whereas inhibition effects are illustrated by lines with crossbars. Sequential arrows indicate multiple signaling steps that are not illustrated. Activation of $G_{i/o}$ inhibits AC and thus cAMP production. This pathway also activates the Ras-MAP kinase (MAPK) cascade, which is primarily responsible for increased proliferation. In addition, $G_{i/o}$ activates PLC via its βγ subunits, which results in generation of diacylglycerol (DAG) and inositol triphosphate (IP_3) from phosphatidylinositol diphosphate (PIP_2). DAG activates protein kinase C (PKC), and IP_3 mobilizes $[Ca^{2+}]_i$. All $G_{i/o}$-mediated signaling is specifically inhibited by PTX. $G_{12/13}$ proteins are responsible for activation of the small GTPase, Rho, which can be specifically inhibited by *Botulinum* C3 exoenzyme (C3). Activated Rho stimulates Rho kinases, inducing cytoskeletal and morphological changes. Rho kinases are directly inhibited by Y-27632. The α subunits of G_q proteins are the primary effectors of PLC activation (Contos et al., 2000).

In many cellular systems, transactivation of the EGFR is reported to be mediated by LPA. Since LPA is one of the most potent mitogens known so far and implicated to be involved in the development and progression of many types of cancer, the mechanism leading to the LPA receptor induced activation of the Ras-MAPK pathway is of uppermost interest for the prevention of cancer development.

1.5 EGFR signal transactivation

In 1996, Daub et al. identified the EGFR as an essential element in GPCR-induced mitogenesis of rat fibroblasts (Daub et al., 1996). Upon treatment of cells with the G protein-coupled receptor agonists lysophosphatidic acid (LPA), endothelin-1 (ET-1) and thrombin, the EGFR and its relative HER2 were found to be rapidly tyrosine-

phosphorylated. This transactivation of a receptor tyrosine kinase couples GPCR ligands to ERK 1/2 activation and induction of *c-fos* gene expression.

Further investigations revealed that GPCR-EGFR crosstalk pathways are established in a variety of other cell types such as human keratinocytes, primary mouse astrocytes, PC12 cells and vascular smooth muscle cells and established them as widely relevant towards the activation of the MAPK signal (Fischer et al., 2003; Gschwind et al., 2001; Hart, 2003). In addition, preincubation of COS7 cells with pertussis toxin blocked LPA-induced EGFR signal transactivation, implicating the involvement of Gα subunits of the $G\alpha_{i/o}$ family of G proteins (Daub et al., 1997). Interestingly, EGFR transactivation was also observed after agonist stimulation of ectopically expressed $G\alpha_q$ coupled bombesin receptor (BombR) or $G\alpha_i$ coupled M_2 muscarinic acetylcholine receptor (M_2R) indicating that various Gα subunits of the G proteins are involved in transactivation of the EGFR depending on the stimulus and the cellular context.

Due to the rapid onset of GPCR-induced EGFR tyrosine phosphorylation and the fact that EGF-like ligands were not detectable in cell culture medium after G protein activation, the transactivation mechanism was postulated to exclusively rely on intracellular elements (Daub et al., 1996; Zwick et al., 1999). Different cytoplasmic tyrosine kinases, Ser/Thr kinases such as PKC and second messengers have been discussed as potential mediators of the transactivation signal. Src-family kinases have been suggested both as upstream and downstream mediators of GPCR induced EGFR transactivation. However, several studies indicated that the EGFR transactivation mechanism is subject to different cell type-characteristic regulatory influences. In different cellular systems EGFR activation was reported to involve intracellular calcium levels and the Ca^{2+}-regulated tyrosine kinase Pyk2 (Eguchi et al., 1998; Keely et al., 2000; Murasawa et al., 1998; Soltoff, 1998; Venkatakrishnan et al., 2000; Zwick et al., 1997).

In contradiction to a ligand-independent mechanism, it was reported that a chimeric RTK consisting of the ligand-binding domain of the EGFR and the transmembrane and intracellular portion of the PDGFR, was transactivated upon treatment of Rat1 fibroblasts with GPCR ligands resulting in a PDGFR-characteristic intracellular signal (Prenzel et al., 1999). These findings suggested a critical function of the EGFR extracellular domain in the GPCR-EGFR cross-talk mechanism, as the PDGFR was not tyrosine phosphorylated upon treatment of Rat1 cells with GPCR ligands. Furthermore, a system of co-cultured cell lines stably expressing either the M1R or the human EGFR resulted in intercellular EGFR transactivation after carbachol stimulation. In addition, LPA-, carbachol- or tetra-decanoyl-phorbol-13-acetate (TPA) induced transactivation of the EGFR and tyrosine

phosphorylation of SHC were completely abrogated by the diphtheria toxin mutant CRM197 that specifically blocks proHB-EGF function or the metalloprotease inhibitor batimastat (BB94) in COS7 and HEK 293 cells. Flow cytometric analysis directly confirmed cell-surface ectodomain shedding of proHB-EGF upon treatment with GPCR agonists or TPA.

These data supported the concept of a "Triple-Membrane-Passing-Signal" (TMPS) mechanism of EGFR signal transactivation involving a metalloprotease activity and processing of the transmembrane EGF-like growth factor precursor proHB-EGF (Prenzel et al., 1999). In this regard, growing evidence indicated transmembrane metalloproteases as the key enzymes of growth factor precursor shedding. The metalloprotease inhibitor batimastat (BB94) was shown to inhibit bombesin- and TPA-induced transactivation of the EGFR in PC-3 human prostate cancer cells and to reduce high constitutive levels of EGFR tyrosine phosphorylation in unstarved PC3 (Prenzel et al., 1999) and SCC25 squamous cell carcinoma cells (Gschwind et al., 2002). Furthermore, Dong and colleagues reported that BB94 reduced cell proliferation and cell migration of an human mammary epithelial cell line by interfering with the release of EGFR ligands (Dong et al., 1999). BB94 also inhibited proliferation of colon and breast cancer cell lines which were known to depend on autocrine signaling through the EGFR. Recently, Gschwind and colleagues showed that LPA-induced proliferation and motility of head and neck cancer cells involves metalloprotease-dependent transactivation of the EGFR (Gschwind et al., 2002). Various studies have identified members of the ADAM subclass of metalloproteases to play a critical role in GPCR mediated transactivation of the EGFR (Gschwind et al., 2003; Sahin et al., 2004). This lead to the following updated model of the triple membrane passing signal mechanism of EGFR signal transactivation (Figure 7).

Since the initial discovery of this mechanism, many studies demonstrated the broad relevance of said signaling mechanism within a variety of cellular systems and, importantly, the involvement in development and progression of pathophysiological processes. Moreover, EGFR function was reported to be critical for GPCR stimulated mitogenic signaling in several cancer cells (Castagliuolo et al., 2000; Venkatakrishnan et al., 2000).

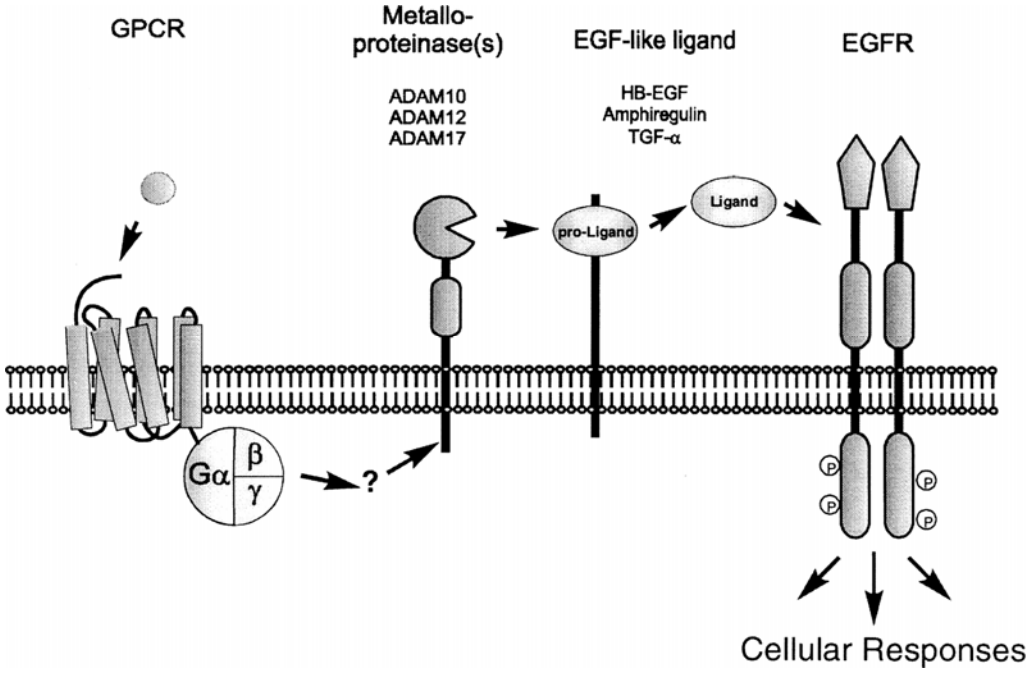

Fig. 7. The triple-membrane-passing signal (TMPS) mechanism of EGFR transactivation. GPCR stimulation induces upregulation of a metalloprotease activity, therby leading to ectodomain cleavage of EGF-like growth factor precursors. Subsequent release of the mature growth factor stimulates EGFR kinase activity and transduces the GPCR signal inside the cell to stimulate characteristic EGFR downstream signaling pathways such as MAPK, PLC-γ, STAT or PI3K activation (Fischer et al., 2003).

In addition to EGFR transactivation, other RTKs such as the insulin-like growth factor receptor (IGF-1R), hepatocyte growth factor receptor (Met-R), vascular endothelial cell growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR) and the Fibroblast growth factor receptor (FGFR)-1 have been shown to be activated by GPCR agonists (Belcheva et al., 2001; Endo et al., 2002; Fischer et al., 2004; Herrlich et al., 1998; Pai et al., 2003; Weiss et al., 1997).

1.6 Metalloproteases

Metalloproteases are important for many biological processes, ranging from cell proliferation, differentiation and remodelling of the extracellular matrix (ECM) to vascularization and cell migration. These events occur several times during organogenesis, in both normal development and during tumour progression. Mechanisms of metalloprotease action include the proteolytic cleavage of growth factors so that they become available to cells not in direct physical contact, degradation of the ECM enabling founder cells to move across tissues into nearby stroma and regulated receptor cleavage to

terminate migratory signaling. Most of these processes require a sensitive balance between the functions of matrix metalloproteases (MMPs) or metalloprotease-disintegrins (ADAMs) and natural tissue inhibitors of metalloproteases (TIMPs).

Proteases of the zinc protease superfamily are classified according to the primary structure of their catalytic sites including carboxypeptidase, DD carboxypeptidase, gluzincin, inuzincin and metzincin subgroups (Hooper, 1994). The metzincins are further subdivided in adamalysins, astacins, matrixins and serralysins (Bode et al., 1993).

For the hydrolytic processing of substrates, the active site of zinc metalloproteases contains water ions and zinc which is coordinated by three conserved histidine residues and a downstream methionine. The methionine constitutes a Met turn motif that loops around to face the consensus HEXXHXXGXXH site. The glutamic acid is believed to transfer hydrogen atoms and to polarize the zinc-bound water molecule for nucleophilic attack on the scissile peptide bond of bound substrate (Stocker and Bode, 1995).

Many metalloproteases are synthesized as inactive precursors containing a terminal prodomain which keeps the metalloprotease site inactive by a cysteine switch (Becker et al., 1995). Besides its role as an inhibitor of the protease domain, the prodomain appears to be important for maturation and intracellular transport of metalloproteases (Milla et al., 1999). The processing and activation of metalloproteases by furin, other proprotein convertases (PCs) or autocatalytic are proposed to occur at the trans-Golgi network (Anders et al., 2001; Kang et al., 2002).

1.6.1 ADAMs

Metalloprotease-disintegrins are transmembrane glycoproteins which take part in cell-cell interaction and in processing of protein ectodomains (Wolfsberg et al., 1995). They combine features of both cell surface adhesion molecules and metalloproteases and are characterized by a conserved domain structure consisting of a N-terminal signal sequence followed by a prodomain, metalloprotease and disintegrin domains, a cysteine-rich region and finally a transmembrane domain and cytoplasmic tail (Figure 8). Thus, family members are referred to as ADAM (a disintegrin and metalloprotease domain) or as MDCs (metalloprotease, disintegrin, cysteine-rich proteins).

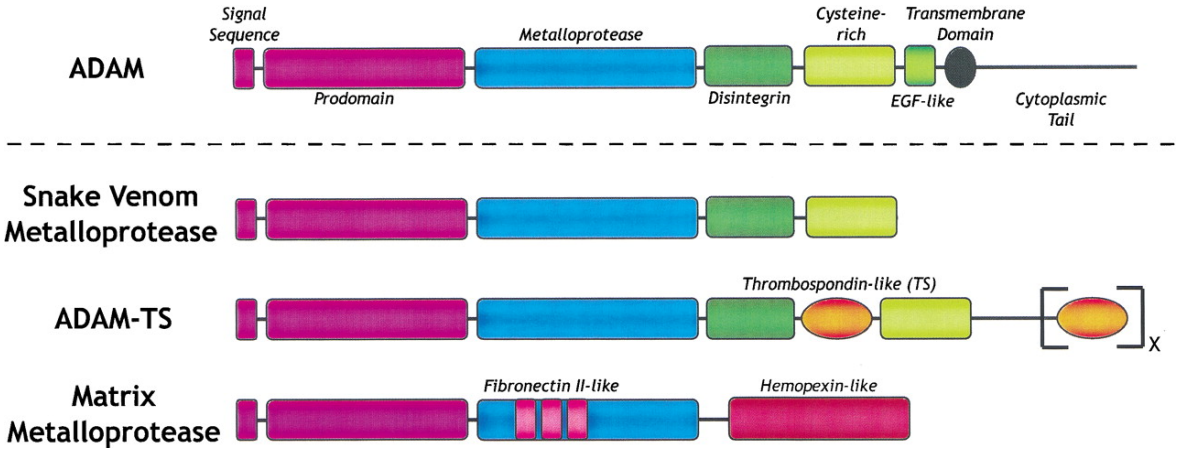

Fig. 8. The topography of the ADAMs and related metalloproteases. Generalized domain structures of the ADAMs, SVMPs, ADAM-TS, and MMP families are shown. Note that ADAM-TS family members have a variable number of thrombospondin-like (TS) motifs. The MMP shown is of the gelatinase class. Other subclasses of MMPs lack hemopexin-like sequences and/or fibronectin type II-like sequences. The subclass of MT-MMPs has transmembrane domains and cytoplasmic tails in addition to the domains shown (Seals and Courtneidge, 2003).

More than 30 ADAM cDNA sequences have been identified in organisms ranging from *S. pombe* to humans up to date (Primakoff and Myles, 2000). Although all ADAMs have a relatively well-conserved metalloprotease domain, only 15 contain the zinc-binding catalytic-site consensus sequence (HEXXH). Therefore, only 50 percent of the known ADAMs are predicted to be catalytically active. ADAMs have been implicated in diverse processes, including sperm-egg binding and fusion, membrane fusion, protein-ectodomain processing or shedding of cytokines, cytokine receptors, adhesion proteins and RTKs and growth factor precursors (Kheradmand and Werb, 2002; Schlondorff and Blobel, 1999).

The disintegrin domain of ADAMs allows the interaction with integrin receptors, but not all ADAM proteases have the RGD sequence in their disintegrin loop (Blobel and White, 1992). In contrast, the cysteine-rich and EGF-like domains are not well characterized, but it is believed that the cysteine rich domain supplements the binding capacity of the disintegrin domain. Cancer cells are shown to interact with cell surface heparin-sulfate proteoglycans in an *in vitro* binding assay with the cysteine-rich domain of ADAM12 serving as a ligand for the cell-adhesion molecule syndecan (Iba et al., 2000; Iba et al., 1999).

The cytoplasmic tails of proteases of the ADAM family vary both in length and in the protein sequence and contain motifs like PXXP binding sites for SH3 domain-containing proteins or potential phosphorylation sites for serine-threonine and/or tyrosine kinases.

Many studies postulate that the cytoplasmic tail is involved in maturation and regulation of metalloprotease activity.

The regulation of ADAM metalloprotease activity after prodomain removal is only poorly understood. Processing of membrane proteins by ADAMs requires both the membrane-anchored enzyme and its substrate to be present in cis on the same cell, probably anchored in distinct domains of the plasma membrane through cytoskeletal interactions. Upon cell activation, for example by PKC agonists, increases in cytoplasmic Ca^{2+} levels or tyrosine kinase stimulation, the attachments change and the proteases and substrates become co-clustered and can interact (Werb and Yan, 1998). Alternatively, the signaling cascade could modify the cytoplasmic domains of the proteases or substrate, producing a conformational change that either activates the enzyme or makes the cleavage site available (Schlondorff and Blobel, 1999). For most processing reactions there appears to be a constitutive level of ectodomain shedding. Processing is necessary to make paracrine growth and survival factors available including EGF-like ligands allowing for the consistent supply of EGFR agonists.

Tumor necrosis factor-α convertase (TACE/ADAM17) is the best characterized metalloprotease and has been identified as the protease responsible for the release of the inflammatory cytokine tumor necrosis factor (TNF)-α from its membrane-bound precursor proTNFα (Black et al., 1997; Moss et al., 1997). Besides TNFα, ADAM17 mediates cleavage of diverse integral membrane proteins like L-selectin, p75 TNF receptor (Peschon et al., 1998), fractalkine (Garton et al., 2001), MUC1 (Thathiah et al., 2003), β-amyliod precursor protein (βAPP) (Buxbaum et al., 1998), p55 TNFR, interleukin-1 receptor II (IL-1R II) (Reddy et al., 2000), erbB4/HER4 (Rio et al., 2000), the Notch1 receptor (Brou et al., 2000), IL-6R (Althoff et al., 2000), growth hormone-binding protein (Zhang et al., 2000) and cellular prion protein (Vincent et al., 2001). Studies using fibroblasts derived from ADAM17 knock-out mice implicated ADAM17 in the release of TGFα and other EGF-like ligands as well as the constitutive availability of these growth factors (Peschon et al., 1998; Sunnarborg et al., 2002). Mice with *tace*$^{\Delta Zn/\Delta Zn}$ null mutation die at birth with phenotypic defects, including failure of eyelid fusion, hair and skin defects, and abnormalities of lung development (Shi et al., 2003). The epithelial defects observed in *tace*$^{\Delta Zn/\Delta Zn}$ fetuses are similar to those reported in mice lacking the epidermal growth factor receptor (EGFR) (Peschon et al., 1998). Abrogation of ADAM17 function in mice leads to perinatal lethality which is not seen in EGFR or TGFα deficient mice, indicating that ADAM17 has additional substrates required for the development of important organs which are necessary for survival (Shi et al., 2003).

1.6.2 The matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are closely related to the ADAM family of metalloproteases and play a central role in breakdown and remodeling of the extracellular matrix (ECM) (Shapiro, 1998). Therefore, they are essential for development, wound healing and are involved in the pathology of hyperproliferative diseases such as cancer and arthritis (Chang and Werb, 2001; Seals and Courtneidge, 2003).

MMPs were historically divided into collagenases, gelatinases, stromelysins and matrilysins on the basis of their specificity for ECM components. However, a sequential numbering system for the more than 20 known human MMPs has been adapted and the MMPs are now classified according to their structure (Nagase and Woessner, 1999). There are eight distinct classes of MMPs: five are secreted and three are membrane-type MMPs (MT-MMPs). All MMPs are synthesized as prepro-enzymes and secreted as inactive pro-MMPs in most cases. The prodomain has a conserved unique PRCG(V/N)PD sequence. The cysteine within this sequence coordinates the catalytic zinc to maintain the latency of pro-MMPs. The catalytic domains of MMPs have an additional structural zinc ion and 2-3 calcium ions, which are required for the stability and the expression of enzymatic activity. The gelatinases MMP-2 and MMP-9 have three repeats of fibronectin-type II domain inserted in the catalytic domain.

These repeats interact with collagens and gelatins. Most of the MMPs are activated outside the cell by other activated MMPs or furin-like serine proteases. Endogenous inhibitors such as α-macroglobulins and tissue inhibitors of metalloproteinases (TIMPs) tightly control the proteolytic activities of MMPs. Many reports identified TIMPs to inhibit cell invasion *in vitro*, tumorigenesis, metastasis *in vivo* and angiogenesis (Gomez et al., 1997). The expression of many MMPs is transcriptionally regulated by growth factors, hormones, cytokines and cellular transformation (Brinckerhoff and Matrisian, 2002). Furthermore, substrates of MMPs as well as of ADAMs are EGF-like growth factors like HB-EGF which leads to the assumption that MMPs are also involved in EGFR signal transactivation (Roelle et al., 2003; Suzuki et al., 1997; Yu et al., 2002).

1.7 Molecular oncology

In normal cells cell division, survival and death are in balance promoting homeostasis. It is a general phenomenon in normal as well as transformed cells that signaling pathways are not freestanding entities but parts of larger signaling networks. Therefore, products of proto-oncogenes and tumor suppressor genes interact in overlapping pathways and dysfunction of the tightly controlled equilibrium leads to cancer.

Cancer development is a multistage process involving genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives (Hanahan and Weinberg, 2000). The genomes of tumor cells can be altered at multiple sites from subtle changes such as point mutations to severe changes in chromosome complement (Blume-Jensen and Hunter, 2001). Furthermore, cancer is the most common genetic disease: one in three people in the western world will develop cancer and one in five will die from it. Therefore, it is essential to elucidate the mechanisms behind the transformation of normal cells to cancer cells and the conversion of normal tissue into malignant tumors.

Observations of human cancers and animal models suggest that tumor development proceeds via a process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells. The vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth and which are now recognized as the six hallmarks of cancer: self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. It is assumed that these six properties are shared by most, if not all types of human tumors (Hanahan and Weinberg, 2000).

Breast cancer belongs to the most frequent tumor types in women. HER2 overexpression or increased activity has been shown to be a genetic marker for a severe type of breast cancer with a poor diagnosis (Pegram et al., 1998; Slamon and Clark, 1988; Slamon et al., 1987; Slamon et al., 1989). In some carcinomas of the breast, overexpression of the EGFR was detected (Nicholson et al., 1991). Kidney cancer on the other hand, is more common in men and one of the most frequent tumors of the urogenital tract. Until today, the most effective treatment remains the complete removal of the affected organ and thus a severely impaired quality of life for the patient (Vogelzang and Stadler, 1998). Hence, there is a

strong need to identify novel intervention targets and to design patient-tailored therapies for prevention and treatment of these tumors. In order to reverse or at least contain tumor spreading it is of utmost importance to understand the molecular mechanisms underlying cancer development and progression.

Benign tumors grow locally restricted, but the normal organization of the affected tissue gradually becomes disrupted by the increasing number of dividing cells. As the dividing cancer cells extend through the basement membrane into the surrounding stroma, malignancy begins. Invasion stimulatory pathways depend on the activation of G proteins, PI3-K and the Rac and Rho family of small GTPases. Proteolysis plays a role for the breakdown of extracellular matrix as well as cleavage of pro-invasive fragments from cell surface glycoproteins. As the dividing cancer cells extend into blood vessels, tumors undergo metastasis by spreading from the primary site to more distant sites in the body making cancer much more difficult to cure.

1.8 Aim of the study

Deregulation of both GPCR and EGFR signaling systems has been recognized as a major cause of hyperproliferative diseases. In addition, a pathway connecting these two classes of receptors has recently been discovered which entailed an arising research interest on this mechanism.

The aim of this study was to elucidate the mechanisms underlying the EGF receptor transactivation signal induced by GPCR ligands as well as the pathophysiological role of this mechanism in breast and kidney cancer cells.

A special interest of this study was the involvement of a certain class of GPCRs, the LPA receptors. LPA is one of the most potent mitogenic stimuli and therefore it is of great interest for tumor therapy to enlighten by which means LPA acts as a mitogen. It was necessary to establish appropriate model systems to study the direct influence of a single receptor on the transactivation signal as well as the biological responses.

Another part of the study focused on the involvement of a certain metalloprotease, ADAM10, in the transactivation mechanism. This was performed in a model system which is known to involve ADAM10 in Thrombin induced EGFR transactivation.

Since the signaling pathway connecting the GPCR to the metalloprotease was still unknown, multiple efforts have been taken to shed light on the molecular mechanism connecting these two parts of the pathway. GPCRs couple to heterotrimeric G proteins

which consist of a Gα and a Gβγ subunit. The involvement of these subunits was examined in different cellular systems.

2 Materials and Methods

2.1 Materials

2.1.1 Laboratory chemicals and biochemicals

Acrylamide	Serva, Heidelberg
Agar	Difco, Detroit, USA
Agarose	BRL, Eggenstein
AG1478	Alexis, Grünberg
Ampicillin	Roche, Mannheim
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, München
ATP (Adenosine 3'-triphosphate)	Pharmacia, Freiburg
Batimastat (BB94)	British Biotech, Oxford, UK
Bisacrylamide	Roth, Karlsruhe
Bromphenol blue	Sigma, Taufkirchen
BSA (Bovine serum albumin)	Sigma, Taufkirchen
CHAPS	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
Dideoxynucleotides (ddG/A/T/CTP)	Pharmacia, Freiburg
Diphtheria toxin CRM mutant	List Biological Lab., CA, USA
DTT (Dithiothreitol)	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
Fibronectin	Calbiochem, Bad Soden
Heparin	Sigma, Taufkirchen
HEPES (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid))	Serva, Heidelberg
IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Biomol, Hamburg
Ki16425	Kirin Laboratories, Tokyo
L-Glutamine	Gibco, Eggenstein
Lipofectamine®	Invitrogen, USA
Lipofectamine 2000	Invitrogen, USA
Lysozym	Sigma, Taufkirchen
LY 294002	Alexis, Grünberg
MBP (Myelin basic protein)	Sigma, Taufkirchen
Mineral oil	Sigma, Taufkirchen
Na-DOC (Sodium-desoxycholat)	Sigma, Taufkirchen
Oligofectamine®	Invitrogen, USA
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
Polybrene (Hexadimethrine bromide)	Sigma, Taufkirchen
Ponceau S	Sigma, Taufkirchen
PP1	Calbiochem, Bad Soden

PP2	Calbiochem, Bad Soden
PTX (Pertussis toxin)	List Biological Lab., CA, USA
Scintillation cocktail (Rotiszint®ecoplus)	Roth, Karlsruhe
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sodium azide	Serva, Heidelberg
Sodium fluoride	Sigma, Taufkirchen
Sodium orthovanadate	Aldrich, Steinheim
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Serva, Heidelberg
Triton X-100	Serva, Heidelberg
Tween 20, 40	Sigma, Taufkirchen
Tyrphostin AG1478	Alexis, Grünberg
Wortmannin	Sigma, Taufkirchen
Xylencyanol	

All other chemicals were purchased from Merck (Darmstadt).

2.1.2 Enzymes

AMV reverse transcriptase	Roche, Mannheim
Alkaline Phosphatase (CIAP)	Roche, Mannheim
LA-Taq-DNA Polymerase	Takara, Japan
Pfu-DNA Polymerase	Roche, Mannheim
Restriction Endonucleases	Pharmacia, Freiburg
	Roche, Mannheim
	NEB, Frankfurt/ Main
	MBI Fermentas, St. Leon-Rot
T4-DNA Ligase	Roche, Mannheim
T7-DNA Polymerase	Pharmacia, Freiburg
Taq-DNA Polymerase	Roche, Mannheim
	Takara, Japan
Trypsin	Gibco, Eggenstein

2.1.3 Radiochemicals

[γ-^{32}P] ATP	>5000 Ci/mmol
[α-^{33}P] dATP	2500 Ci/mmol

All radiochemicals were obtained from PerkinElmer Life Sciences, Köln.

2.1.4 "Kits" and other materials

Cell culture materials	Greiner, Solingen Nunclon, Dänemark Falcon, U.K.
Cellulose nitrate 0.45 µm	Schleicher & Schüll, Dassel
Concanavalin A-Sepharose® 4B	Sigma, Taufkirchen
ECL Kit	PerkinElmer, Köln
Glutathione-Sepharose	Pharmacia, Freiburg
Hyperfilm MP	Amersham, USA
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
Polyprep® Chromatography columns	Biorad, München
Protein A-Sepharose	Pharmacia, Freiburg
Protein G-Sepharose	Pharmacia, Freiburg
PuReTaq Ready-To-Go PCR Beads	Amersham Biosciences, Piscataway, NJ
QIAquick Gel Extraction Kit (50)	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
Random-Primed DNA Labeling Kit	Pharmacia, Freiburg
RNeasy Mini Kit	Qiagen, Hilden
Sephadex G-50 (DNA Quality)	Pharmacia, Freiburg
Sterile filter 0.22 µm, cellulose acetate	Nalge Company, USA
Sterile filter 0.45 µm, cellulose acetate	Nalge Company, USA
TOP10/P3 One Shot™	Invitrogen, USA
Transwells	Schubert & Weiss, Munich
Whatman 3MM	Whatman, USA

2.1.5 Growth factors and ligands

EGF (murine)	Toyoba, Japan
LPA	Sigma, Karlsruhe
Sphingosine 1-phosphate	UBI, Lake Placid
Thrombin	Sigma, Karlsruhe

All other growth factors and ligands were purchased from Sigma.

2.1.6 Media and buffers

2.1.6.1 Media for E. coli bacteria

LB-Medium	1.0 % Tryptone 0.5 % Yeast extract 1.0 % NaCl pH 7.2
2xYT-Medium	1.6 % Tryptone 1.0 % Yeast extract 1.0 % NaCl pH 7.2

When necessary the following antibiotics were added to the media after autoclavation:

Ampicillin	100 µg/ml or 50µg/ml for 1/2 Amp/Tet selection
Kanamycin	100 µg/ml
Chloramphenicol	30 µg/ml
Tetracyclin	10 µg/ml

LB-plates additionally contained 1.5 % agar.

2.1.6.2 Cell culture media

All cell culture media and additives are from Gibco (Eggenstein), fetal calf serum (FCS) was purchased from Sigma and Gibco.
Dulbecco's modified eagle medium (DMEM) supplemented with 4.5 mg/ml glucose, 2 mM L-glutamine, 1 mM sodium pyruvate.
Eagle's minimum essential medium (MEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate.
RPMI 1640 medium supplemented with 2 mM L-glutamine and 1 mM sodium pyruvayte.
Nutrient mixture F12 (HAM) supplemented with 2 mM L-glutamine and DMEM supplemented with 4.5 mg/ml glucose, 2 mM L-glutamine, 1 mM sodium pyruvate mixed 1:1.
Freeze medium containing 90 % heat-inactivated FCS and 10 % DMSO.

2.1.7 Stock solutions and buffers

Anode buffer	0.2 M Tris/ HCl, pH 8.9
BBS (2x)	50 mM BES 280 mM NaCl 1.5 mM Na_2HPO_4 pH 6.96 (NaOH)
Cathode buffer	0.1 M Tris/ HCl, pH 8.25 0.1 M Tricine 0.1 % (w/v) SDS
CHAPS lysis buffer	50 mM HEPES, pH 7.5 150 mM NaCl 1 mM EDTA 10 % Glycerol 10 mM $Na_4P_2O_7$ 10 mM CHAPS 2 mM VaO_5 10 mM NaF 1 mM PMSF 100 µg/l Aprotinin
DNA loading buffer (6x)	0.25 % Bromphenol blue 0.25 % Xylencyanol 30.0 % Glycerol 100.0 mM EDTA, pH 8.0
HBS (2x)	46 mM HEPES, pH 7.5 274 mM NaCl 1.5 mM Na_2HPO_4 pH 7.0
HNTG (1x)	20 mM HEPES, pH 7.5 150 mM NaCl 0.1 % Triton X-100 10 % Glycerin 10 mM $Na_4P_2O_7$
Gel buffer	3.0 M Tris/ HCl, pH 8.45 0.3 % (w/v) SDS
Gluthation Elution buffer	50 mM Tris/ HCl, pH 8.0 10 mM DTT 15 Mm Glutathion, reduced

Laemmli buffer (2x)	187.5 mM Tris/ HCl, pH 6.8 6.0 % SDS 30.0 % Glycerol 0.01 % Bromphenol blue 5.0 % ß-Mercaptoethanol
NET (1x)	150.0 mM NaCl 5 mM EDTA 50 mM Tris/HCl, pH 7.4 0.05 % Triton X-100
PBS (1x)	13.7 mM NaCl 2.7 mM KCl 80.9 mM Na_2HPO_4 1.5 mM KH_2PO_4, pH 7.4 (HCl)
RIPA lysis buffer	1 % NP40 1 % Na-DOC 0.1 % SDS 150 mM NaCl 10 mM $NaPO_4$, pH 7.2 2 mM EDTA 5 mM β-Glycerophosphat 4 mM VaO_5 10 mM NaF 1 mM PMSF 100 µg/l Aprotinin 1mM DTT
RIPA rescue buffer	10 mM $NaPO_4$, pH 7.2 1 mM NaF 20 mM NaCl 5 mM β-Glycerophosphat 2 mM VaO_5 1 mM PMSF 100 µg/l Aprotinin 1mM DTT
RSP-buffer	1x PBS 0.5 mM EDTA 100 µg/l Aprotinin 1 mM PMSF 0.5 mM DTT
SD-Transblot	50.0 mM Tris/ HCl, pH 7.5 40.0 mM Glycine 20.0 % Methanol 0.004 % SDS

Schagger sample buffer	4.0 % (w/v) SDS 12.0 % (w/v) Glycerol 50 mM Tris/ HCl, pH 6.8 2.0 % (w/v) β-Mercaptoethanol 0.01 % (w/v) Serva Blue G
"Strip" buffer	62.5 mM Tris/ HCl, pH 6.8 2.0 % SDS 100 mM ß-Mercaptoethanol
TAE (10x)	400 mM Tris/Acetate 10 mM EDTA pH 8.0 (Acetic acid)
TE10/0.1	10.0 mM Tris/ HCl, pH 8.0 0.1 mM EDTA pH 8.0
Tris-Glycine-SDS (10x)	248.0 mM Tris/ HCl, pH 7.5 1918.0 mM Glycine 1.0 % SDS
Triton X-100 lysis buffer	50 mM HEPES, pH 7.5 150 mM NaCl 1 mM EDTA 10 % Glycerin 1 % Triton X-100 10 mM $Na_4P_2O_7$ 2 mM VaO_5 10 mM NaF 1 mM PMSF 100 µg/l Aprotinin

2.1.8 Bacteria strains (E. coli)

E. coli	**Description**	**Origin/ Reference**
DH5aF'	F'/*end*A1 *hsd*17 (*rk-mk-*) *sup*E44 *rec*A1 *gyr*A (Nal) *thi*-1 (*lac*ZYA-*arg*F)	Genentech, San Francisco, USA
TOP10/P3	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 deoR *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G P3: Kan^R Amp^R (am) Tet^R (am)	Invitrogen, USA

2.1.9 Cell lines

Cell Line	Description	Origin/ Reference
A498	Human kidney carcinoma cell line	Sugen Inc., CA, USA
BT20	Human mammary adenocarcinoma	ATCC HTB-19
BT549	Human mammary ductal carcinoma	ATCC HTB-122
COS7	African green monkey, SV 40 transformed kidney Fibroblasts	Genentech, San Francisco, USA
HEK293	Human embryonic kidney fibroblasts, transformed with adenovirus Typ V DNA	ATCC CRL-1573
HEK293EBNA	Human embryonic kidney fibroblasts expressing EBNA1	Invitrogen, USA
HS 578T	Human mammary carcinoma	ECACC HTB-126
MDA-MB 231	Human mammary carcinoma	ATCC HTB-26
NIH 3T3	Mouse fibroblasts, Clone 7	C. Sherr
McA-RH7777	Rat hepatoma cell line	ATCC CRL-1601
Phoenix E, A	Retrovirus producer cell lines for the generation of helper free ecotropic and amphotropic retroviruses, based on HEK-293	Nolan, Stanford, USA
Rat1	Rat fibroblasts	Genentech, San Francisco, USA
SCC9	Human squamous cell carcinoma of the tongue	ATCC CRL-1629

All other cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA) and grown as recommended by the supplier.

2.1.10 Antibodies

The following antibodies were used in immunoprecipitation experiments or as primary antibodies in immunoblot analysis.

Antibody	Description/ Immunogen	Origin/ Reference
ADAM10/Kuz	Rabbit, polyclonal/ AA732-748 of human ADAM10	Chemicon, Hofheim
ADAM10 MP	Rabbit, polyclonal/ metalloprotease domain of human ADAM10	This study
ADAM12 Extra	Rabbit, polyclonal/ extracellular domain of human ADAM12	This study
ADAM15 Peptide	Rabbit, polyclonal / AA189-208 of human ADAM15	S. Hart

ADAM17 (19027)	Rabbit, polyclonal / AA807-823 of human ADAM17	Chemicon, Hofheim
Akt1/2	Rabbit, polyclonal/ AA 345-480 of human Akt1	Santa Cruz, USA
P-Akt/PKB	Rabbit, polyclonal/ phospho-Akt (Ser-473); Recognizes p-Akt of human, rabbit and rat origin	NEB
Edg2	Rabbit polyclonal/ AA recognizes the cytoplasmic domain of human and rat Edg2	Kamya Biomedical, USA
EGFR	Sheep, polyclonal/ part of cytoplasmic domain of the human EGFR	UBI, Lake Placid
EGFR (108.1)	Mouse, monoclonal/ ectodomain of the human EGFR	(Daub et al., 1997)
EGFR Cuba	Mouse monoclonal/ ectodomain of the human EGFR, IOR-R3	Habana, Cuba
ERK2 (C-14)	Rabbit, polyclonal/ peptide at C-terminus of rat ERK2	Santa Cruz, USA
ERK2 (K-23)	Rabbit, polyclonal/ peptide from sub-domain XI of rat ERK2	Santa Cruz, USA
P-ERK	Rabbit, polyclonal; recognizes phospho-p44/p42 (Thr-202/ Tyr-204) MAPK	NEB, Frankurt/M.
$G\alpha_{i/o/t/z}$ (C-20)	Rabbit, polyclonal/ peptide at carboxy terminus of $G\alpha_z$ of rat origin; recognizes mouse, rat and human $G\alpha_{i/o/t/z}$	Santa Cruz, USA
$G\alpha_{i2}$ (T-19))	Rabbit, polyclonal/ divergent domain of human $G\alpha_{i2}$; recognizes mouse, rat and human $G\alpha_{i2}$	Santa Cruz, USA
$G\alpha_q$ (E-17)	Rabbit, polyclonal/ peptide of AA 13-29 of mouse $G\alpha_q$; recognizes mouse, rat and human $G\alpha_q$	Santa Cruz, USA
$G\alpha_{12}$ (S-20)	Rabbit, polyclonal/ peptide of AA 2-21 of mouse $G\alpha_{12}$; recognizes mouse, rat and human $G\alpha_{12}$	Santa Cruz, USA
$G\alpha_{13}$ (A-20)	Rabbit, polyclonal/ peptide of AA 2-21of mouse $G\alpha_{q13}$; recognizes mouse, rat and human $G\alpha_{13}$	Santa Cruz, USA
$G\beta_1$ (C-16)	Rabbit, polyclonal/ divergent domain of human $G\beta_1$; recognizes mouse, rat and human $G\beta_1$	Santa Cruz, USA
$G\gamma_2$ (A-16)	Rabbit, polyclonal/ amino terminus of bovine $G\gamma_2$; Broad mammalian reactivity	Santa Cruz, USA
GFP	mouse, monoclonal; recognizes the green Fluorescent protein (GFP)	G. Gerisch
HA	Mouse, monoclonal; recognizes the influenza hemagglutinin epitope (HA)	Babco, CA, USA
SHC	Mouse, monoclonal	Santa Cruz
SHC	Rabbit, polyclonal/ 220 AA at C-terminus of human SHC	(Daub et al., 1997)
P-Tyr (4G10)	Mouse, monoclonal; recognizes phospho-(3)-tyrosine residues	UBI, Lake Placid

For western blot secondary antibodies conjugated with horseradish peroxidase (HRP) were utilized.

Antibody	**Dilution**	**Origin**
Goat anti-mouse	1 : 10,000	Sigma, Karlsruhe
Goat anti-sheep	1 : 25,000	Dianova, Hamburg
Goat anti-rabbit	1 : 25,000	BioRad, München

2.1.11 Plasmids and oligonucleotides

2.1.11.1 Primary vectors

Vector	**Description**	**Origin/ Reference**
pcDNA1	Mammalian expression vector, SupF gene, ColE1/M13 origin, CMV/T7 promoter, SV40 origin	Invitrogen, USA
pcDNA3	Mammalian expression vector, Amp_r, CMV promotor, BGH pA, high copy number plasmid	Invitrogen, USA
pCEFL	modified pcDNA3, CMV promoter replaced by EF-1	S. Gutkind
pLXSN	Expression vector for retroviral gene transfer, Amp_r, Neo_r, origin from pBR322, 5'-LTR and 3'-LTR from MoMuLV, SV40 promotor	Clontech, Palo Alto, USA
pLXSN-ESK	Modified pLXSN vector with multipe cloning site from pBluescript	J. Ruhe
pRK5	Expression vector, Amp_r, CMV promoter, SV 40 poly A, high copy number plasmid	Genentech, San Francisco, USA
pCEP4	Mammalian expression vector, CMV promoter, SV40 pA, OriP, EBNA-1 gene (complementary strand), Amp_r, pUC origin, TK promoter, $Hygromycin_r$, TK pA	Invitrogen, USA
pCDM8	Mammalian high copy expression Vector, SupF gene, pMBI origin from pBR322, M13 origin, CMV/T7 promoter, SV40 origin	Invitrogen, USA
pGEX5x-3	Bacterial expression vector for GST-fusion proteins, pBR322 origin, tac promo ter, Amp_r, *lac* I^q gene, protease recognition sites	Amersham, USA
pBABEpuro	Expression vector for retroviral gene transfer, Amp_r, $Puro_r$, pBS origin, SV40 promoter	C. Marshall

pRetroSuper	Mammalian expression vector, also suitable for retroviral gene transfer, H1-RNA promoter for RNA transcription under control of PolIII, PGK promoter, $Puro_r$, Amp_r	(Brummelkamp et al., 2002)
pcdef3	Mammalian expression vector, Amp_r, Neo_r, EF1a promoter, BGH pA, SV40 promoter	E. Goetzl
pRC/CMV2	CMV/T7 promoter, BGH pA, f1 origin, SV40 promoter, , Amp_r, Neo_r, ColE1 origin	Invitrogen, USA

2.1.11.2 Constructs

Vector	**Description**	**Reference**
pGEX-5x3-ADAM10,MP	cDNA of human ADAM10, metalloprotease domain, AA 214-455 in pGEX5x-3	This study
pCEP4-ADAM12E-GST	cDNA of human ADAM12, extracellular domain, AA 1-708 in pCEP4; C-terminal GST tag	This study, S. Hart
pcDNA3-hADAM10-HA	cDNA of human ADAM10 in pcDNA3; C-terminal HA-tag	A. Gschwind
pRetroSuper- ADAM10si	Expression of a RNA oligomer targeting the 3' UTR of human ADAM10 in pSuper.retro	This study
pcDNA1-$G\alpha_{i2}$ wt	cDNA of $G\alpha_{i2}$ wt in pcDNA1	M. Fauré
pcDNA1-$G\alpha_{i2}$ Q→L	cDNA of $G\alpha_{i2}$ (Q205→L) in pcDNA1	M. Fauré
pcDNA3 –$G\alpha_{13}$ wt-HA	cDNA of $G\alpha_{13}$ wt in pcDNA3; C-terminal HA-tag	S. Gutkind
pcDNA3- $G\alpha_{13}$ Q→L-HA	cDNA of $G\alpha_{13}$ (Q226→L) in pcDNA3; C-terminal HA-tag	S. Gutkind
pCEFL $G\alpha_{12}$ wt	cDNA of $G\alpha_{12}$ wt in pcDNA3	S. Gutkind
pCEFL $G\alpha_{12}$ Q→L	cDNA of $G\alpha_{12}$ (Q229→L) in pCEFL	S. Gutkind
pCEFL $G\alpha_q$ wt-HA	cDNA of $G\alpha_q$ wt in pCEFL; C-terminal HA-tag	S. Gutkind
pCEFL $G\alpha_q$ Q→L-HA	cDNA of $G\alpha_q$ (Q →L) in pCEFL; C-terminal HA-tag	S. Gutkind
pcDNA3-Edg2	cDNA of human Edg2 in pcDNA3	This study
pRC-CMV2-Edg2	cDNA of Edg2 in pRC-CMV2	E. Goetzl
pcDNA3-Edg4	cDNA of human Edg4 in pcDNA3	This study
pdef3-Edg4	cDNA of Edg4 in pcdef3	E. Goetzl
pcDNA3-Edg7	cDNA of human Edg7 in pcDNA3	This study

2.1.11.3 Important oligonucleotides

Name	Sequence
Cloning of ADAM10MP into pGEX5x-3	
ADAM10MP 3’	GCGCTCGAGTCCTCCGCCAGATCCAACAAAACAGTTG
ADAM10MP 5’	CGCCTCGAGGCCACCATGAATGGTCCAGAACTTCTGAGG
Cloning of ADAM12E + GST into pCEP4	
ADAM12ex1	AGAGATATCGCCACCGCCGGCGACGATGGCAGTG
ADAM12ex2	AGATCTAGAGGAGGATTGGTTATCTGCTTGCCGGATGG
GST 3’	GCCAGATCTTTACAGATCCGATTTTGGAGGATG
GST 5’	GCGGATCCTCCCCTATACTAGGTTATTGG
Cloning of ADAM10siRNA into pRetroSuper	
AD10(2)a	GATCCCCACTTGGCTCTCAATAAACTTTCAAGAGAAGTTTATT GAGAGCCAAGTTTTTTGGAAA
AD10(2)b	AGCTTTTCCAAAAAACTTGGCTCTCAATAAACTTCTCTTGAAA GTTTATTGAGAGCCAAGTGGG
Subcloning of G*α* subunits into pLXSN/pBABEpuro	
Alphai2forwEcoRI	GCGAATTCGCCACCATGGGCTGCACCGTGAGCGC
Alphai2revEcoRI	CGGAATTCTCAGAAGAGGCCACAGTCCTT
AlphaqforwBamHI	GCGGATCCGCCACCATGACTCTGGAGTCCATCATGGC
AlphaqrevBamHI	CGGGATCCTTAGACCAGATTGTACTCCTTCAG
Alpha13forwBamHI	GCGGATCCGCCACCATGGCGGACTTCCTGCCGTCG
Alpha13revBamHI	CGGGATCCTCACTGCAGCATGAGCTGCTT
Cloning of Edg2, 4 and 7 into pcDNA3	
Edg2 BamHI 3’	GCGGGATCCGCCACCATGGCTGCCATCTCTACTT
Edg2 ApaI 5’	GCGGGGCCCCTAAACCACAGAGTGGTCATT
Edg4 BamHI 3’	GCGGGATCCGCCACCATGGTCATCATGGGCCAGT
Edg4 ApaI 5’	GCGGGGCCCCTAAAGGGTGGAGTCCATC
Edg7 BamHI 3’	GCGGGATCCGCCACCATGAATGAGTGTCACTATGACA
Edg7 ApaI 5’	GCGGGGCCCTTAGGAAGTGCTTTTATTGCAG
RT-PCR primers for Edg2, 4 and 7 and rat actin	
hEdg2aRT	TCTTCTGGGCCATTTTCAAC
hEdg2bRT	TGCCTAAAGGTGGCGCTCAT
hEdg4aRT	CCTACCTCTTCCTCATGTTC
hEdg4bRT	TAAAGGGTGGAGTCCATCAG
hEdg7aRT	GGAATTGCCTCTGCAACATCT
hEdg7bRT	GAGTAGATGATGGGGTCCA

rat-beta-actina	TACAACCTCCTTGCAGCTCC
rat-beta-actinb	GGATCTTCATGAGGTAGTCTGTC

2.1.11.4 siRNA nucleotides

siRNA	**Sequence**
gl2	CGUACGCGGAAUACUUCGAdTdT
ADAM10	UGAAGAGGGACACUUCCCUdTdT GUUGCCUCCUCCUAAACCAdTdT
ADAM15	CUCCAUCUGUUCUCCUGACdTdT AUUGCCAGCUGCGCCCGUCdTdT
ADAM17	GUUUGCUUGGCACACCUUdTdT GUAAGGCCCAGGAGUGUUdTdT

siRNAs (Dharmacon Research, Lafayette, CO, USA) were described earlier (Fischer, 2004).

2.2 Methods in molecular biology

2.2.1 Plasmid preparation for analytical purpose

Small amounts of plasmid DNA were prepared as described previously (Lee and Rasheed, 1990).

2.2.2 Plasmid preparation in preparative scale

For transfection experiments of mammalian cells DNA of high quality was prepared using Qiagen Maxi-Kits (Qiagen, Hilden) according to the manufacturer's recommendations.

2.2.3 Enzymatic manipulation of DNA

2.2.3.1 Digestion of DNA samples with restriction endonucleases

Restriction endonuclease cleavage was accomplished by incubating the enzyme(s) with the DNA in appropriate reaction conditions. The amounts of enzyme and DNA, the buffer and ionic concentrations, and the temperature and duration of the reaction were adjusted to the specific application according to the manufacturer's recommendations.

2.2.3.2 Dephosphorylation of 5'-termini with calf intestine alkaline phosphatase (CIAP)

Dephosphorylation of 5'-termini of vector DNA in order to prevent self-ligation of vector termini. CIAP catalyzes the hydrolysis of 5'-phosphate residues from DNA, RNA, and ribo- and deoxyribonucleoside triphosphates. The dephosphorylated products possess 5'-hydroxyl termini.

For dephosphorylation 1-20 pmol DNA termini were dissolved in 44 µL deionized water, 5 µL 10x CIAP buffer (500 mM Tris/HCl pH 8.0, 1 mM EDTA pH 8.5) and 1 µL CIAP (1 U/µL). The reaction was incubated 30 min at 37° C and stopped by heating at 85° C for 15 minutes.

2.2.3.3 DNA insert ligation into vector DNA

T4 DNA Ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA. T4 DNA Ligase thereby joins doublestranded DNA with cohesive or blunt termini.

The digested, dephosphorylated and purified vector DNA (200 ng), the foreign DNA insert, 1 µL 10x T4 DNA Ligase buffer (0.66 M Tris/HCl pH 7.5, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP) and 1 µL T4 DNA Ligase (2 U for sticky ends and 4 U for blunt ends) were added to a total volume of 10 µL. The reaction was incubated at 15° C overnight. T4 DNA Ligase was inactivated by heating the reaction mixture at 65° C for 10 min. The resulting ligation reaction mixture was directly used for bacterial transformation.

2.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying, and purifying 0.5- to 25 kb DNA fragments. 1-2 % horizontal agarose gels with 1x TAE electrophoresis buffer were used for separation. The voltage was typically set to 1-10 V/cm gel length. Gels were stained by covering the gel in a dilute solution of ethidium bromide (0.5 µg/ml in water) and gently agitating for 30 min and destained by shaking in water for an additional 30 min.

2.2.4.1 Isolation of DNA fragments using low melting temperature agarose gels

Following preparative gel electrophoresis using low melting temperature agarose, the gel slice containing the band of interest was removed from the gel. This agarose slice was then melted and subjected to isolation using the QIAquick Gel Extraction Kit (Qiagen, Hilden).

2.2.5 Introduction of plasmid DNA into E.coli cells

2.2.5.1 Preparation of competent E. coli bacteria

Competent cells were made according to the procedure described before (Chung and Miller, 1988). For long-term storage competent cells were directly frozen at -70° C. Transformation frequency ranged between 10^6 and 10^7 colonies/µg DNA.

2.2.5.2 Transformation of competent E. coli bacteria

100 µL competent cells were added to 10 µL ligation mix and 20 µL 5x KCM (500 mM KCl, 150 mM $CaCl_2$, 250 mM $MgCl_2$) in 70 µL H_2O and incubated on ice for 20 min. Upon incubation at room temperature for 10 min, 1 ml LB medium was added and incubated for 1 h at 37° C with mild shaking to allow expression of the antibiotic resistance gene. Transformants were selected on appropriate LB-plates.

2.2.6 Enzymatic amplification of DNA by polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA (Mullis and Faloona, 1987). A multitude of applications have been developed including direct cloning from cDNA, *in vitro* mutagenesis and engineering of DNA, genetic fingerprinting of forensic samples, assays for the presence of infectious agents and analysis of allelic sequence variations. For long and accurate cDNA amplification LATaq™ polymerase (TaKaRa) and *Pfu* DNA polymerase (Fermentas) were used according to the manufacturer's recommendations:

0.5 µL template cDNA
2.0 µL "sense" oligonucleotide, 10 pmol/µL
2.0 µL "antisense" oligonucleotide, 10 pmol/µL
5.0 µL 10x LA PCR buffer II (without $MgCl_2$)
5.0 µL 25 mM $MgCl_2$
8.0 µL dNTP-Mix, 2.5 mM each
0.5 µL LA-Taq™ (5 U/µL)

add to 50 µL H_2O

or

0.5 µL template cDNA
2.0 µL "sense" oligonucleotide, 10 pmol/µL
2.0 µL "antisense" oligonucleotide, 10 pmol/µL
5.0 µL 10x *Pfu*-buffer with $MgSO_4$
4.0 µL dNTP-Mix, 2.5 mM each
0.5 µL *Pfu* DNA Polymerase (2.5 U/µl)

add to 50 µL H_2O

PCR reactions were performed in an automated thermal cycler („Progene", Techne). The following standard protocol was adjusted to the specific application:

initial denaturation:	3 min	94° C
amplification 20-35 cycles:	1 min	94° C (denaturation)
	1 min	50-65° C (hybridization)
	1 min respective	
	2 min/ kb product	72° C (extension)
final extension:	7 min	72° C

10 µL from each reaction were electrophoresed on an agarose gel appropriate for the PCR product size expected. PCR products were subjected to isolation using the PCR purification kit (Qiagen).

2.2.7 RT-PCR analysis

Expression of Edg2, 4 and 7 was confirmed by RT-PCR analysis. RNA isolated using RNeasy Mini Kit (Qiagen, Hilden) was reverse transcribed using AMV Reverse Transcriptase (Roche, Mannheim). 2-10 µg RNA and 1 µl random primer in a volume of 10 µl were incubated for2 min at 68°C, followed by 10 min RT. After addition of 0.5 µl RNase inhibitor, 4 µl 5x AMV RT buffer and 4 µl dNTPs (2.5 mM each) and 1µl AMV RT the volume was adjusted to 20 µl. The reaction mix was incubated at 42°C for 1h.
PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ) and 1µl RT-PCR products were used for PCR amplification according to the manufacturer's recommendations. PCR products were subjected to electrophoresis on 1.5-2% agarose gels and DNA was visualized by ethidium bromide staining.

2.2.8 DNA sequencing

DNA sequencing was performed according to the "Big Dye Terminator Cycle Sequencing Protocol" (ABI). The following mix was subjected to a sequencing-PCR run:

	0.5 µg	DNA of interest
	10.0 pmol	oligonucleotide
	4.0 µL	Terminator Ready Reaction Mix
	add to 20 µL	H_2O
25 cycles:	30 sec	94° C
	15 sec	45-60° C (annealing temperature)
	4 min	60° C

The sequencing products were purified by sodium acetate/ EtOH precipitation, dissolved in 20 µL template suppression reagent, denatured for 2 min at 90° C and analyzed on a 310-Genetic Analyzer (ABI Prism).

2.2.9 cDNA array hybridization

Filters spotted with genes of interest (cloned into pBluescript $SKII^+$) were a generous gift from A. Roidl. cDNA probes of the cell lines under varying conditions were generated by

T. Knyazeva according to standard molecular biology methods. Labeling of 3–5 µL of cDNA was performed by P. Knyazev with the Megaprime kit (Amersham) in the presence of 50 µCi [α-^{32}P]dATP. The prehybridization solution was replaced from filters by the hybridization solution containing 5x SSC, 0.5% (v/v) SDS, 100 µg/mL baker's yeast tRNA (Roche), and the labelled cDNA probe (2–5 x 10^6 cpm/mL) and incubated at 68° C for 16 h. Filters were washed under stringent conditions. A phosphorimager system (Fuji BAS 1000; Fuji) was used to quantify the hybridization signals. Average values for each slot were calculated using the formula: $A = (AB - B) \times 100/B$; [*A*, final volume; *AB*, intensity of each slot signal (pixel/mm^2); *B*, background (pixel/mm^2)].

2.3 Methods in mammalian cell culture

2.3.1 General cell culture techniques

Cell lines were grown in a humidified 93 % air, 7 % CO_2 incubator (Heraeus, B5060 Ek/CO_2) at 37° C and routinely assayed for mycoplasma contamination using a bisbenzimide staining kit (Sigma, Karlsruhe). Before seeding, cells were counted with a Coulter Counter (Coulter Electronics). Cells were cultured in the medium recommended by the manufacturer. The following cell lines required special media additives:

SCC9 Dulbecco's modified Eagle's medium (DMEM)/ Ham's F12 medium 1:1 containing 0.5 mM sodium pyruvate, 2 mM L-glutamine, 400 mg/L Hydrocortisone and 10 % FCS.

MCF10A Dulbecco's modified Eagle's medium (DMEM)/ Ham's F12 1:1 containing 0.5 mM sodium pyruvate, 5 % horse serum, 80 U/L Insulin, 1 mg/ml Hydrocortisone, 500 µg/ml Choleratoxine, 100 mg/ml EGF.

BT20, A498 Eagle's minimum essential medium (MEM) containing 10 % FCS, 1 mM sodium pyruvate, 2 mM L-glutamine and 0.1 mM non-essential amino acids.

BT549 RPMI 1640 containing 10 % FCS, 2 mM L-glutamine and 2.67 U/L Insulin.

HS578T Dulbecco's modified Eagle medium (DMEM) containing 10 % FCS, 1 mM sodium pyruvate, 2 mM L-glutamine and 10µg/ml Insulin.

2.3.2 Transfection of cultured cell lines

2.3.2.1 Transfection of cells with calcium phosphate

HEK-293T and EBNA cells in six-well dishes were transfected transiently at about 70% confluency with a total of 2 µg DNA by using a modified calcium phosphate precipitation method as described previously (Chen and Okayama, 1987). In this protocol, a calcium phosphate-DNA complex is formed gradually in the medium during incubation with cells.
The transfection mix of DNA and $CaCl_2$ in water was prepared as follows:

Dish	**6-well**	**6 cm**	**10 cm**
Area	10 cm^2	21 cm^2	57 cm^2
Volume of medium	1 ml	2 ml	4 ml
DNA in H_2O_{bidest}	2 µg in 90 µL	5 µg in 180 µL	10 µg in 360 µL
2.5 M $CaCl_2$	10 µL	20 µL	40 µL
2 x BBS (pH 6.96)	100 µL	200 µL	400 µL
Total volume	200 µL	400 µL	800 µL

To initiate the precipitation reaction the indicated volume of 2x BBS was added and mixed by vortexing. The reaction was incubated for 10 min at room temperature before being added to each well. Plates were placed in a humidified container at 3% CO_2 overnight. One day after transfection, cells were serum-starved for 24 hours in standard cell culture medium without FCS. Transfection efficiency was determined by LacZ staining after transfection of a LacZ-containing expression plasmid. For transfection of Phoenix cells HBS was used instead of BBS.

2.3.2.2 Transfection of COS7 cells with Lipofectamine®

COS7 cells were transiently transfected using Lipofectamine® (Gibco-BRL) essentially as described (Daub et al., 1997). For transfections in 6-well dishes, 90 µl of serum-free medium containing 10 µL of Lipofectamine and 1.5 µg of total plasmid DNA in 100 µl serum-free medium were mixed. After 20 min the transfection mixture was added to 800 µl serum-free medium per well.
After 4 h the transfection mixture was replaced by normal growth medium and 20 h later, cells were washed and cultured for a further 24 h in serum-free medium until lysis.

2.3.2.3 Transfection of A498 cells with Lipofectamine 2000®

A498 cells were transiently transfected using Lipofectamine 2000® (Gibco-BRL) essentially according to the manufacturer's recommendations. For transfections in 6-well dishes, 2 µg of total plasmid DNA were diluted into 250 µl of serum-free medium. 5 µl Lipofectamine 2000® (Gibco-BRL) were also diluted into 250µl of serum-free medium and allowed to incubate at room temperature for 5-10 min. After mixing of DNA and transfection reagent, the mixture was added to 2 ml of antibiotic-free, but serum containing medium per well. After 4 h the transfection mixture was removed and fresh media containing serum was added. After 20 h, cells were washed and cultured for a further 48 h in serum-free medium until lysis.

2.3.2.4 RNA interference

Transfection of pRetroSuper-ADAM10si encoding for siRNA duplexes (Dharmacon Research, Lafayette, CO) for targeting endogenous ADAM10 or siRNA dublexes (Dharmacon Research, Lafayette, CO) targeting ADAM10, 15 and 17 was carried out using Lipofectamine® 2000 (Invitrogen, USA) and 2 µg DNA or 5-10 µg siRNA dublexes, respectively, per 6-well plate as previously described (Brummelkamp et al., 2002; Tuschl et al., 1999). Transfected A498 cells were serum starved and assayed 3 days after transfection. Specific silencing of targeted genes was confirmed by western blot (ADAM10 Kuz/ADAM10 MP). Alternatively, cells were infected as described below.

2.3.3 Retroviral gene transfer in cell lines

The ecotropic packaging cell line Phoenix (Nolan, Stanford, USA) was transfected with pLXSN retroviral expression plasmids (Clontech, Palo Alto, CA) encoding wildtype and Q→L mutants of Gα subunits as well as pRetroSuper-ADAM10si by the calcium phosphate/ chloroquine method as described previously (Kinsella and Nolan, 1996). 24 h after transfection the viral supernatant was collected and used to infect NIH3T3 and Rat1 as well as A498 cells (5x 10^4 cells/6-well plate). 4 to 12 h later, retroviral supernatant was replaced with fresh medium. Selection for stable expression was started 48 h post infection with the respective antibiotic.

2.4 Protein analytical methods

2.4.1 Lysis of eucaryotic cells

Prior to lysis, cells grown to 80% confluence were treated with inhibitors and agonists as indicated in the Figure legends. Cells were washed with cold PBS and then lysed for 10 min on ice in Triton X-100 lysis buffer. Lysates were precleared by centrifugation at 13000 rpm for 10 min at 4° C. For the solubilisation of Gα subunits RIPA lysis buffer was used. The solubilisation of Edg-receptors required CHAPS lysis buffer.

2.4.2 Determination of protein concentration in cell lysates

The „Micro BCA Protein Assay Kit" (Pierce, Sankt Augustin) was used according to the manufacturer's recommendations. For samples containing glycerol the BioRad Protein Assay (BioRad Laboratories GMBH, Munich) was used according to the manufacturer's recommendations.

2.4.3 Immunprecipitation and Isolation of Glycoproteins

An equal volume of HNTG buffer was added to the precleared cell lysates that have been adjusted for equal protein concentration. Proteins of interest were immunoprecipitated using the respective antibodies and 30 µL of protein A-Sepharose for 4 h at 4° C. Glycoproteins werde isolated using 20 µl of Concanavalin A-Sepharose. Proteins bound to the respective Separose were washed three times and subsequently removed from the Sepharose by the addition of 35 µl Laemmli and heat denaturation.
GST-tagged proteins werde immunoprecipitated using Gluthation-Sepharose beads with a binding capacity of 8 mg Protein per ml. For preparative quantities one ml of Glutathion-Sepharose was used and incubated for 12 h overnight. The protein was eluted using Glutathion elution buffer.

2.4.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted as described previously (Sambrook, 1990). The following proteins were used as molecular weight standards:

Protein	**MW (kD)**	**Protein**	**MW (kD)**
Myosin	205.0	Ovalbumin	42.7
ß-Galactosidase	116.25	Carboanhydrase	29.0
Phosphorylase b	97.4	Trypsin-Inhibitor	21.5
BSA	66.2	Lysozym	14.4

2.4.4.1 Detection of low-molecular weight proteins

The detection of low-molecular weight proteins by a Tricine-SDS Gel electrophoresis was essentially performed as previously described (Schagger and von Jagow, 1987). This method has a good resolution for small protein although utilizing 10 % agarose gels.

2.4.4.2 Colloidal Coomassie staining

Protein samples intended for further analysis by mass spectrometry methods were in-gel stained with a Roti®-Blue (Roth, Karlsruhe) colloidal Coomassie staining as recommended by the manufacturer.

2.4.5 Transfer of proteins on nitrocellulose membranes

For immunoblot analysis proteins were transferred to nitrocellulose membranes (Gershoni and Palade, 1982) for 2 h at 0.8 mA/cm^2 using a "Semidry"-Blot device in the presence of Transblot-SD buffer. Following transfer proteins were stained with Ponceau S (2 g/l in 2% TCA) in order to visualize and mark standard protein bands. The membrane was destained in water.

2.4.6 Immunoblot detection

After electroblotting the transferred proteins are bound to the surface of the nitrocellulose membrane, providing access for reaction with immunodetection reagents. Remaining binding sites were blocked by immersing the membrane in 1x NET, 0.25 % gelatine for at least 4 h. The membrane was then probed with primary antibody (typically overnight at 4° C). Antibodies were diluted 1:500 to 1:2000 in NET, 0.25 % gelatin. The membrane was washed 3x 20 min in 1x NET, 0.25 % gelatin, incubated for 1 h with secondary antibody and washed again as before. Antibody-antigen complexes were identified using horseradish peroxidase coupled to the secondary anti-IgG antibody. Luminescent substrates were used to visualize peroxidase activity. Signals were detected with X-ray films or a digital camera unit. Membranes were stripped of bound antibody by shaking in strip-buffer for 1 h at 50° C. Stripped membranes were blocked and reprobed with different primary antibody to confirm equal protein loading.

2.5 Generation of polyclonal antibodies

2.5.1 Large scale expression of GST-fusion proteins

Bacteria were transformed with the pGEX-5x3-ADAM10MP. Two or three colonies were inoculated into 100 ml LB-media with 50 µg/ml ampicillin and grown overnight at 37° C. This culture was diluted into 1 L LB-media containing antibiotic and allowed to grow at 37° C to an OD_{600} of 0.4 to 0.5. The expression of the target gene was then induced by the addition of 1 ml 0.2 M IPTG to a final concentration of 0.2 mM. After 4 to 5 hours bacteria were harvested and centrifuged at 6000 rpm and the pellet was resuspended in 18 ml ice-cold RSP-buffer. Bacteria were lysed by sonication at 70 %, 3 times for 1 min on ice. Triton X-100 was added to a final concentration of 1%. The mixture was the incubated on ice for 5 min followed by centrifugation for 5 min at 10000 g at 4° C. The supernatant was incubated with 1 ml Glutathione-Sepharose beads o/n at 4° C. The beads were washed three times with ice-cold PBS and then transferred to a BioRad column (BioRad, Munich). The protein was eluted with 10 ml elution buffer. Fractions of 1 ml were collected and tested for the presence of the protein by SDS-PAGE analysis. Fractions containing the protein were dialyzed against PBS

containing 10 % glycerol and protein concentration was measured using BioRad Protein Assay (BioRad Laboratories GmbH, Munich).

For insoluble proteins the pellet from the last step was resuspended in 10 ml 1.5 % N-Lauroysarcosine containing 25 mM Triethanoloamine and 1 mM EDTA and incubated for 1 h at 4° C. After centrifugation the supernatant was also incubated with 1 ml Glutathione-Sepharose-beads as described above.

HEK293-EBNA cells were transfected with pCEP4-ADAM12E-GST using the Calcium-phosphate method as described. After 48 h cells were selected using 1 µm/ml Hygromycin. The supernatant was harvested every 2 days for a period of 3 weeks. After centrifugation at maximum speed the supernatant was incubated with 1 ml Glutathione-Sepharose beads overnight at 4° C. Then, the beads were washed three times with ice-cold PBS and transferred to a BioRad column. Elution and dialysis was performed as indicated above. Addititonally, cells were lysed using Triton X-100 lysis buffer and then also incubated with 1 ml Glutathione-Sepharose beads overnight at 4° C and purification of the protein was performed as previously described.

2.5.2 Immunisation of rabbits

Chinchilla and New Zealand rabbits were immunized with 0.5 mg of protein in a 1:1 emulsion with Freund's Adjuvans complete (Sigma, Karlsruhe) to a final volume of 2 ml. At day 0, 2 ml of preimmune serum were drawn from the ear vein of the rabbit. Two weeks later, a second injection of 0.5 mg protein in a 1:1 emulsion with Freund's Adjuvans incomplete (Sigma, Karlsruhe) was performed. After two weeks a first test bleed was drawn from the ear vein and checked for antibody production. Four to six weeks after the second injection a third injection in Freund' Adjuvans incomplete followed. The rabbit was bleed out 14 days after the last injection.

The serum was kept at room temperature for 1 h and then at 4° C overnight. Afterwards, the serum was centrifuged at 2000 rpm for 10 min and the supernatant was collected. A second centrifugation step at 9000 rpm for 10 min followed and again the supernatant was removed. Both supernatants were combined and sodium azide was added to a final concentration of 0.02 %. The serum was aliquoted and stored at -20° C.

2.6 Biochemical and cell biological assays

2.6.1 Stimulation of cells

Cells were seeded in cell culture dishes of appropriate size and grown overnight to about 80% confluence. After serum-starvation for 24 or 48 h cells were treated with inhibitors and agonists as indicated in the Figure legends, washed with cold PBS and then lysed for 10 min on ice.

2.6.2 Erk 1/2 and Akt/PKB phosphorylation

For determination of Erk 1/2 and Akt phosphorylation, approximately 20 µg of whole cell lysate protein/lane was resolved by SDS-PAGE and immunoblotted using rabbit polyclonal phospho-specific Erk/MAPK antibody. Akt phosphorylation was detected by protein immunoblotting using rabbit polyclonal anti-phospho-Akt antibody. Quantification of Erk 1/2 was performed using the Luminescent Image Analyis System (Fuji). After quantification of Erk 1/2 phosphorylation, membranes were stripped of immunoglobulin and reprobed using rabbit polyclonal anti-Erk 1/2 or rabbit polyclonal anti-Akt antibody to confirm equal protein loading.

2.6.3 Erk/MAPK activity

Endogenous Erk 2 was immunoprecipitated from lysates obtained from six-well dishes using 0.4 µg of anti-Erk 2 antibody. Precipitates were washed three times with HNTG buffer, and washed once with kinase buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 200 µM sodium orthovanadate). Kinase reactions were performed in 30 µL of kinase buffer supplemented with 0.5 mg/ml myelin basic protein, 50 µM ATP and 1 µCi of [γ-^{32}P]ATP for 10 min at room temperature. Reactions were stopped by addition of 30 µL of Laemmli buffer and subjected to gel electrophoresis on 15 % gels. Labelled MBP was quantificated using a Phosphoimager (Fuji).

2.6.4 Focus formation assay

NIH3T3 and Rat1 cells were infected with different Gα-subunits and their oncogenic variant using the packaging cell line Phoenix E (Kinsella and Nolan, 1996). Ras-infected cells were utilized as a positive control. The cells were stimulated with 10 µg/ml LPA every other day. Cells were cultured for 2-3 weeks until foci were visible and subsequently fixed and stained with crystal violet. (0.5 % crystal violet, 20 % methanol).

2.6.5 Proliferation assay

In a 96-well flat bottom plate (Nunc, Naperville, Ill.) approximately 2,000 cells/100 µl of cell suspension were seeded. Upon serum-starvation for 24 h cells were incubated with inhibitors and growth factors for the indicated times. MTT, a tetrazolium dye (3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide; thiazolyl blue, Sigma, St. Louis, MO) was added to each well to a final concentration of 1 mg/ml MTT. Plates were incubated in the presence of MTT for 4 h. Mitochondrial dehydrogenase activity reduces the yellow MTT dye to a purple formazan, which is solubilized (DMSO, acidic acid, SDS) and absorbance was read at 570 nm on an micro-plate reader.
A non-toxic alternative to MTT is Alamar Blue™ (Biosource, Camarilla, CA, USA). According to the manufacturer's recommendations, 10 µl of Alamar Blue™ are added to each well and absorbance at 590 nm can be read out after different times, because the cells are not affected.

2.6.6 *In vitro* wound closure

The assay was performed as previously described (Fishman et al., 2001) with some modifications. Confluent monolayers of kidney and breast cancer cells were wounded with a uniform scratch, the medium was removed and cells were washed twice with PBS. Medium without FCS was added and cells were subjected to 20 min preincubation with DMSO (control), 250 nM AG1478, 10 µM batimastat or 10 nM Ki16425 before ligand treatment. Cells were permitted to migrate into the area of clearing for 8-24 h. Wound closure was monitored by visual examination using a Zeiss microscope.

2.6.7 Migration of cancer cells

Cell migration assays of A498 kidney cancer and MDA-MB 231 breast cancer cells were performed using Transwells (Sieuwerts et al., 1997). Serum free medium containing LPA as a chemoattractant was added to the lower well of a chamber. $1x10^5$ cells in exponential growth were harvested and then preincubated with the respective inhibitor for 20 min and added to the upper well of the chamber in serum free medium. The chambers were incubated for 6-24 h in a humidified 7 % CO_2, 37° C incubator. Finally, the cells that have migrated to the lower surface of the membrane were stained with crystal violet and counted under the microscope. Alternatively, cells migrated to the lower surface were fixed with methanol and stained with crystal violet. The stained cells were solubilized in 10 % acetic acid, and the absorbance at 570 nm was measured in a micro-plate reader.

2.7 Statistical analysis

Student's *t*-test was used to compare data between two groups. Values are expressed as mean ± standard deviation (s. d.) of at least triplicate samples. $P<0.05$ was considered statistically significant.

3 Results

The EGFR signaling pathway is known to be involved in the regulation of various cellular processes and is a key player in hyperproliferative diseases such as cancer (Zwick et al., 2001; Zwick et al., 1999).
Furthermore, a novel pathway linking GPCRs to the EGFR signal pathway has been discovered involving the shedding of EGF-like ligands by a metalloprotease (Daub et al., 1996; Prenzel et al., 1999).
The aim of the study was to elucidate the relevance of the EGFR signal transactivation by GPCR agonists in the development and progession of cancer. The focus of the investigation was to analyze the pathway activating the metalloprotease by GPCR ligands.

3.1 ADAM-specific antibodies

The development of new tools for the investigation of the EGFR transactivation signal was of primary interest. Antibodies specifically blocking the metalloprotease function of ADAM proteins can be helpful in the investigation of the role of the metalloprotease in the EGFR transactivation pathway and may lead to the design of novel therapeutics.
The metalloprotease domains of different ADAM proteins were expressed in the mammalian cell line HEK293, but it was soon recognized that the metalloprotease domain itself is neither correctly processed nor stably secreted. These findings were confirmed by the study of Schlondorff et al. who found that the prodomain of ADAM proteins is necessary for correct processing and intracellular maturation of ADAM proteins (Schlondorff et al., 2000).
Therefore, the metalloprotease domain of ADAMs which is not heavily glycosylated was bacterially expressed. The complete extracellular fragment of ADAM proteins including the prodomain was expressed in the mammalian cell line HEK293EBNA.

3.1.1 ADAM10,MP antibody

The metalloprotease domain of ADAM10 fused to an N-terminal GST-tag was successfully expressed in the bacterial expression system DH5α and purified using GSH-Sepharose beads.

The beads with the bound protein were then used to immunize a Chinchilla rabbit. The resulting antibody serum was tested for its specificity (Figure 9).

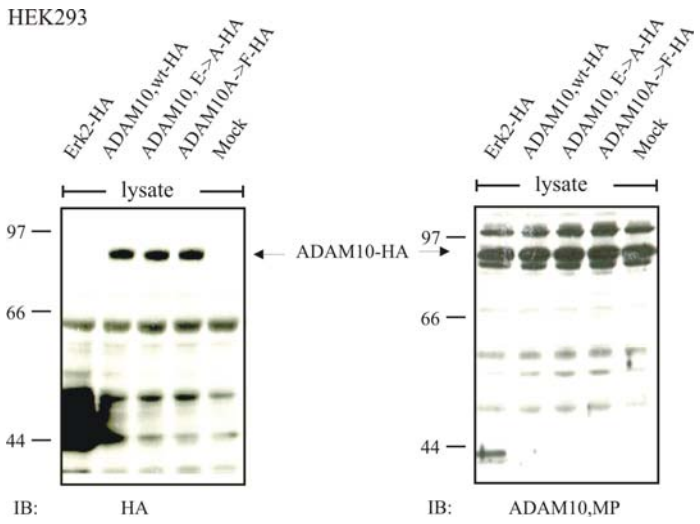

Fig. 9. Specificity test of ADAM10,MP polyclonal serum. Equal amounts of lysate from HEK293 cells overexpressing Mock control, Erk2-HA control, ADAM10wt-HA, the mutants ADAM10,E→A-HA and ADAM10A→F-HA were immunoblotted (IB) with HA-tag antibody and ADAM10,MP polyclonal serum, respectively.

The antibody revealed not to be specific in immunoprecipitation experiments as N-terminal sequencing and MALDI-TOF analysis of the respective bands proved (data not shown), but it was a useful tool for immunoblot analysis. However, this antibody was not suitable for the detection of point mutations in the metalloprotease region of ADAM10 as all mutants are equally well recognized.

3.1.2 ADAM12 antibody

The complete extracellular fragment of ADAM12 fused to an N-terminal GST-tag was expressed in HEK293EBNA cells. As the secretion of the soluble fragment was insufficient, both the secreted protein and the protein from the cell lysate were purified using GSH-Sepharose beads and used for immunization of a New Zealand rabbit. Subsequently, the serum was tested for its specificity (Figure 10).

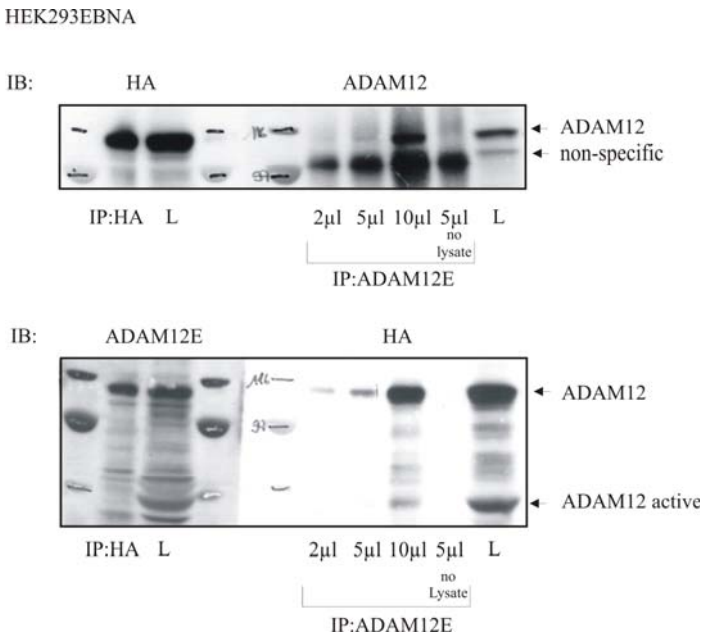

Fig. 10. Specificity test of ADAM12E polyclonal serum. Equal amounts of lysate from HEK293 cells overexpressing ADAM12-HA were submitted to immunoblot (IB) analysis with HA-tag antibody and ADAM12E polyclonal serum as indicated. Additionally, immunoprecipitation (IP) with equal amounts of lysates was performed using 3 µl HA antibody and the indicated amounts of ADAM12E polyclonal serum and subsequently immunoblotted (IB) with HA-tag antibody and ADAM12E polyclonal serum, respectively.

ADAM12E antibody serum was able to detect overexpressed ADAM12 both in immunoblot analysis and in immunoprecipitation experiments. In immunoprecipitation analysis, the active form of ADAM12 could also be visualized. Futrther experiments demonstrated that endogenous expression of ADAM12 can only be demonstrated if the protein level is comparably high (data not shown).

3.2 Reconstituted LPA receptor expression in McA-RH7777 cells

LPA is the strongest mitogenic factor present in serum and is believed to be involved in the development and progession of different cancer types including breast, ovarian, colon and prostate cancer (Fang et al., 2000a; Fishman et al., 2001; Goetzl et al., 1999; Kue et al., 2002; Shida et al., 2003). Therefore, it was of utmost interest to study the functions of the LPA receptors and their involvement in the transactivation of the EGFR signaling pathway.

3.2.1 Stable expression of LPA receptors in McA-RH7777 cells

The McA-RH7777 cell line is known to be deficient of any expression of the LPA receptors Edg2, 4 and 7 (Im et al., 2000). Thus, it represents an ideal model system for analyzing differential functions of the three LPA receptors of the Edg receptor family. In this study, polyclonal cell lines stably expressing the LPA receptors Edg2, 4 and 7 were established. Expression of the LPA receptors was determined by RT-PCR (Figure 11).

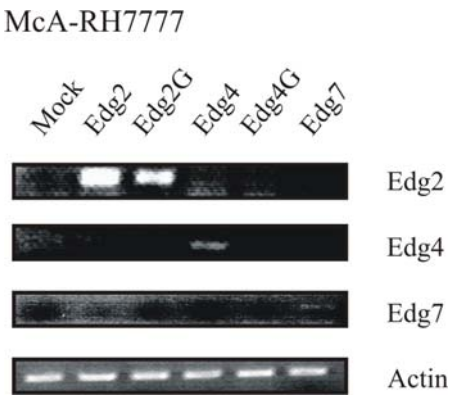

Fig. 11. RT-PCR analysis of the polyclonal McA-RH7777 cell lines stably expressing Edg 2, 4 and 7. Equal amounts of total RNA isolated from McA-RH7777 cells stably expressing the respective receptor or Mock-control were subjected to RT-PCR analysis. PCR products were amplified using PuReTaq Ready-to-Go PCR beads with the respective primers for 35 cycles. Actin mRNA levels demonstrate equal loading.

While the expression level of Edg2 was comparably high in both polyclonal cell lines (Edg2 and Edg 2G), only the Egd4 but not the Edg4G cell line expressed the Edg4 gene product. The expression of Edg7 was comparably weak. Additionally, Figure 11 clearly illustrates that the McA-RH7777 cell line was deficient of Edg2, Edg4 and Edg7 mRNA expression.

3.2.2 Expression profile of different ADAM proteins in McA-RH7777 cells

Previous studies revelaed the involvement of ADAM proteins in EGFR signal transactivation in various cell lines (Gschwind et al., 2003; Schafer et al., 2004b). Hence, it was important to demonstrate the expression of members of this protein family in McA-RH7777 cells.

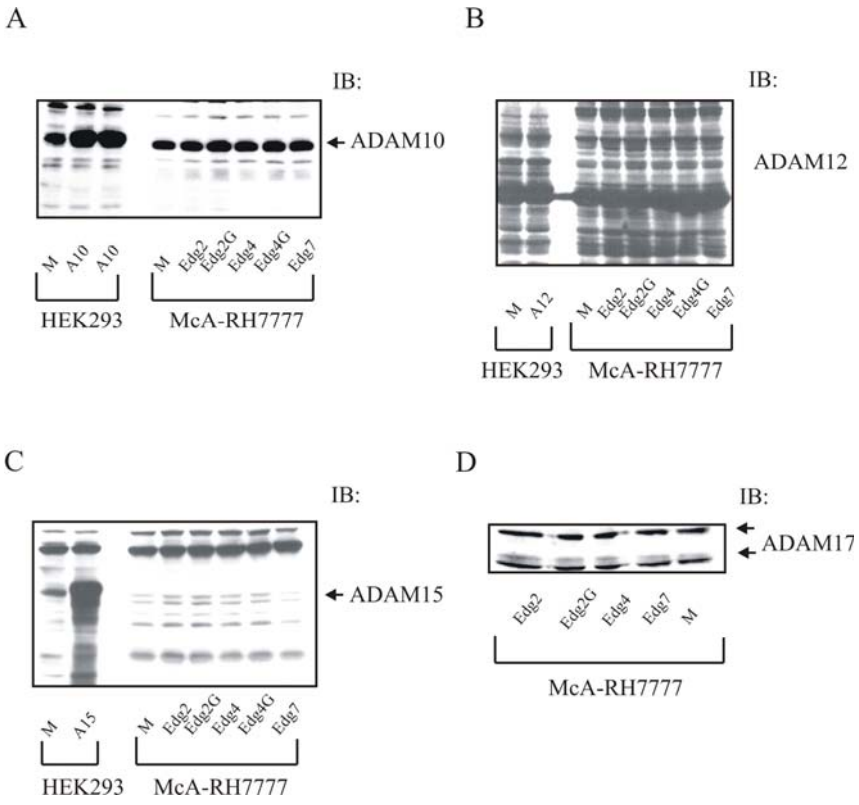

Fig. 12. Expression of ADAM proteins in McA-RH7777 cells. Equal amounts of lysates from HEK293 cells overexpressing Mock control or the respective ADAM construct as well as lysates from the different polyclonal McA-RH7777 cell lines were subjected to immunoblot (IB) analysis and probed with the respective antibody as indicated.

As shown in Figure 12, expression of ADAM10 and ADAM17 in McA-RH7777 cells was not detected in immunoblot analysis. Expression of ADAM12 and ADAM15 could not be verified in McA-RH7777 cell lines. ADAM15 and ADAM12 antibodies were specifically raised against human ADAMs but due to sequence homology they were suspected to be cross-reactive with the respective rat protein.

3.2.3 EGFR transactivation can not be restored in cells stably expressing Edg 2, 4 or 7

Reconstituted expression of LPA receptors in McA-RH7777 cells leads to functional signaling of the LPA receptors (An et al., 1998a; An et al., 1997; Bandoh et al., 1999). In the context of this study, one important question was if expression of the LPA receptors was able to establish a pathway linking LPA stimulation of the LPA receptors to the activation of the

EGFR signaling pathway. Hence, McA-RH7777 cells stably expressing the LPA receptors Edg2, 4 or 7 were stimulated with LPA and assayed for EGFR activation. In addition, the cells were preincubated with known inhibitors of the TMPS pathway as well as the PI3K pathway.

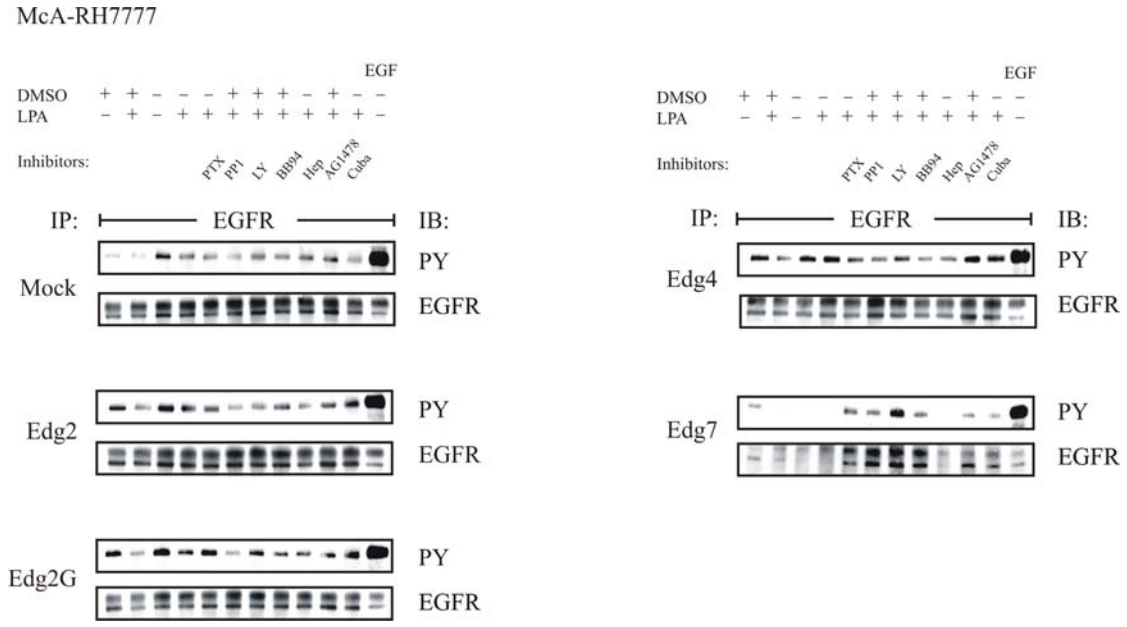

Fig. 13. EGFR phosphorylation induced by LPA stimulation and its inhibition. McARH7777 cells stably expressing Mock control, Edg2 or Edg2G, Edg4 or Edg7 were starved for 24 h and pretreated with either DMSO (20 min) as a control or the following inhibitors: PTX (125 ng/ml, 18h), PP1 (20µM, 20 min), LY294,002 (100 µM, 20 min), BB94 (10 µM, 20 min), Heparin (100 ng/ml, 20 min), AG1478 (250 nM, 20 min) or the EGFR blocking antibody Cuba (20µg/ml, 30 min). Afterwards, cells were stimulated with 10 µM LPA or 3 ng/ml EGF for 3min. Equal amounts of lysate were immunoprecipitated (IP) with EGFR 108 monoclonal antibody and immunoblotted (IB) with anti-phosphotyrosine (PY) antibody. Reprobing with EGFR antibody ensured equal loading.

The basal phosphorylation of the EGFR was elevated in the McA-RH7777 cell lines stably expressing Edg2 and 4, but LPA stimulation did not lead to an increase in EGFR activation. Therefore, no inhibition of the EGFR activation was detectable, either. Blocking PI3K functionwith the inhibitor LY294,002, however, resulted in increased EGFR phosphorylation suggesting that PI3K activity blocks EGFR activation.

3.2.4 Restored Erk phosphorylation upon Stimulation with LPA

It was further analyzed if the restored expression of the LPA receptors was able to mediate signaling to known downstream targets of the LPA receptors. Among them, the MAPK pathway is of primary interest since it activates DNA synthesis and induces cell proliferation (Contos et al., 2000). The influence of LPA stimulation on the activation state of the MAPK Erk 1/2 was determined by immunoblot analysis (Figure 14).

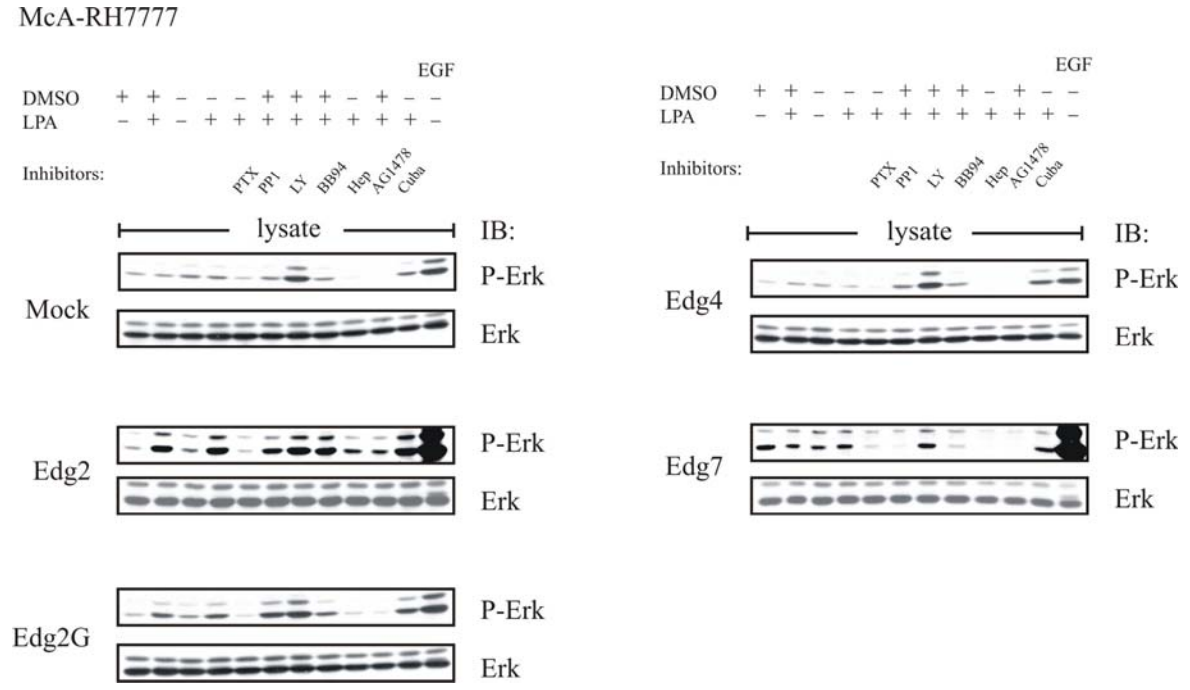

Fig. 14. Erk 1/2 phosphorylation induced by LPA stimulation and its inhibition. McARH7777 cells stably expressing Mock control, Edg2 or Edg2G, Edg4 or Edg7 were starved for 24 h and pretreated with either DMSO (20 min) as a control or the following inhibitors: PTX (125 ng/ml, 18h), PP1 (20µM, 20 min), LY294,002 (100 µM, 20 min), BB94 (10 µM, 20 min), Heparin (100 ng/ml, 20 min), AG1478 (250 nM, 20 min) or the EGFR blocking antibody Cuba (20µg/ml, 30 min). Subsequently, cells were stimulated with 10 µM LPA or 3 ng/ml EGF for 7 min. Equal amounts of total lysate were immunoblotted (IB) with activation state specific Erk antibody followed by reprobing with Erk 1/2 antibody to ensure equal loading.

In Edg2 overexpressing McA-RH7777 cells, LPA stimulation strongly activated Erk 1/2. This effect showed to be weaker in Edg4 and Edg7 polyclonal cells, but expression of these receptors was comparatively low with respect to the Edg2 overexpressing McA-RH7777 cell lines.

The activation of Erk 1/2 was sensitive to treatment by Pertussis toxin (PTX) which specifically inhibits $G\alpha_{i/o}$ subunits, confirming previous studies that demonstrate LPA

mediated signaling to be typically coupled to $G\alpha_{i/o}$ subunits (Takuwa et al., 2002). Neither the src kinase inhibitor PP1 nor the PI3K inhibitor LY294,002 affected the LPA induced Erk 1/2 activation proving that both kinases were not involved in the activation of Erk 1/2 in this cellular context. Interestingly, the metalloprotease inhibitor Batimastat (BB94) and the EGFR inhibitor AG1478 as well as the HB-EGF and Pro-Amphiregulin inhibitor Heparin lead to abrogation of the LPA induced Erk 1/2 activation, suggesting the involvement of a TMPS pathway in activation of the MAPK Erk 1/2, although activation of the EFGR itself could not be demonstrated (Figure 13). Inhibition of the EGFR with the blocking antibody Cuba did also not influence EGFR phosphorylation, which could have proven the direct involvement of the EGFr binding domain. This antibody, however, was specifically designed against the human EGFR and might not be functional for rat EGFR.

3.2.5 Akt activation upon stimulation with LPA

In order to analyze whether a separate pathway bypassing the EGFR was activated upon LPA stimulation, the activation of the protein kinase PKB/Akt upon stimulation with LPA was examined (Figure 15).

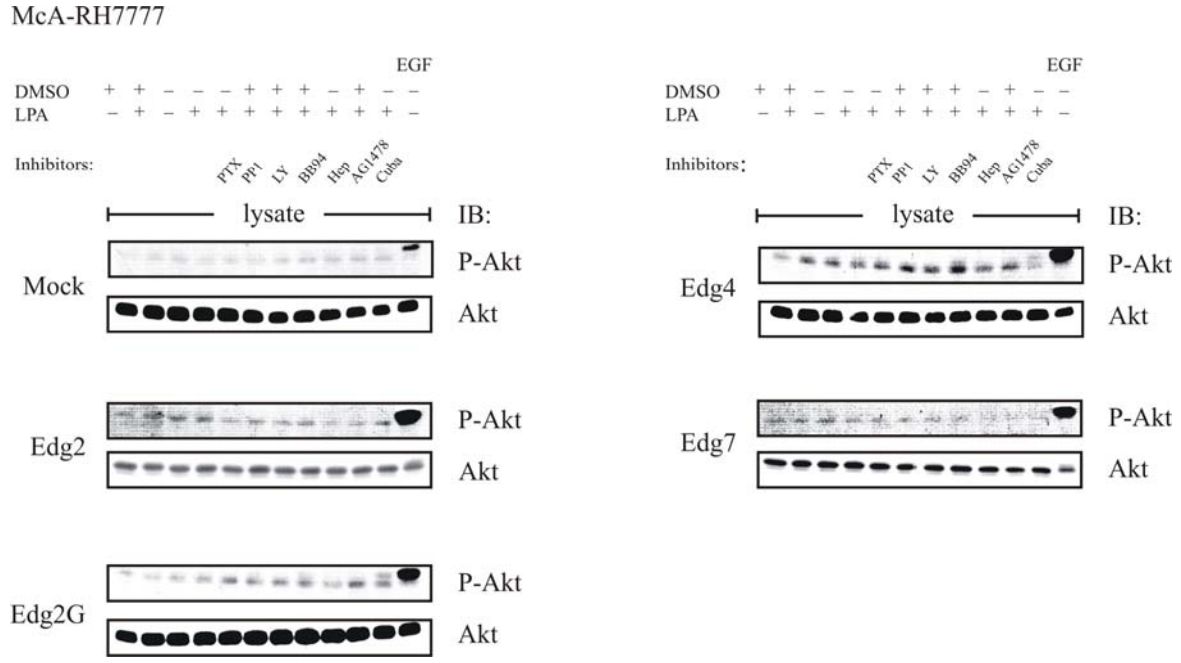

Fig. 15. Akt-activation induced by LPA stimulation and its inhibition. McARH7777 cells stably expressing Mock control, Edg2 or Edg2G, Edg4 or Edg7 were starved for 24 h and pretreated with either DMSO (20 min) as a control or the following inhibitors: PTX (100 ng/ml, 18h), PP1 (20µM, 20 min), LY294002 (100 µM, 20

min), BB94 (10 µM, 20 min), Heparin (100 ng/ml, 20 min), AG1478 (250 nM, 20 min) or the EGFR blocking antibody Cuba (20µg/ml, 30 min). Afterwards, cells were stimulated with 10µM LPA or 5ng/ml EGF as a control for 7 min. Equal amounts of total lysate were immunoblotted (IB) with activation state specific Akt antibody followed by reprobing with an Akt antibody to ensure equal loading.

The basal level of active Akt 1/2 was slightly enhanced, but no specific Akt activation due to stimulation with LPA was detected.

Summarizing the results obtained in McA-RH7777 cells, the expression of LPA receptors, especially of Edg2, lead to LPA mediated activation of the MAPK pathway by an Akt 1/2, src kinase family and PI3K independendent. However, activation of Erk 1/2 was metalloprotease and EGF-like ligand dependent. Additionally, blocking EGFR function with the specific inhibtor AG1478 abrogated Erk 1/2 activation suggesting the involvement of the TMPS pathway, although the activation of the EGFR itself could not be demonstrated.

3.3 EGFR transactivation in A498

Kidney cancer is one of the most frequent tumors of the urogenital tract. Until today, the most effective treatment remains the complete removal of the affected organ (Vogelzang and Stadler, 1998). Hence, there is a strong need to identify novel intervention targets for prevention and treatment of these tumors.

Both LPA and Thrombin stimulation have been demonstrated to induce EGFR signal transactivation in the kidney carcinoma cell line A498 (Figure 16, (Hart, 2004; Schafer et al., 2004a). While the EFGR transactivation induced by LPA is mediated predominantly by ADAM17, Thrombin induced EGFR transactivation is known to be exclusively mediated by ADAM10 (Hart, 2004; Schafer et al., 2004a).

Therefore, this cell line was selected to study the combined action of two different stimuli as well as the specific influence of the metalloprotease ADAM10 on the EGFR signal transactivation pathway.

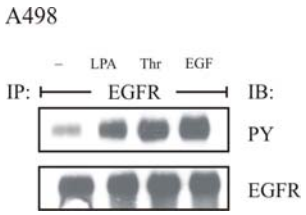

Fig. 16. EGFR transactivation induced by LPA and Thrombin stimulation. A498 cells were starved for 48 h and subsequently stimulated with 10 µM LPA, 2 U/ml Thrombin or 3 ng/ml EGF for 3 min. Equal amounts of lysates were immunoprecipitated with EGFR108 antibody and immunoblotted with phosphotyrosine specific antibody. Subsequent reprobing with EGFR antibody verified equal loading.

3.3.1 LPA and Thombin induced EGFR transactivation in A498

Further analysis of the two TMPS pathways activated by LPA and Thrombin demonstrated that the two stimuli utilize different pathway which converge at the EGFR.

LPA as well as Thrombin mediated EGFR transactivation was abrogated with BB94, a broad spectrum metalloprotease inhibitor, as well as the EGFR inhibitor AG1478 (Figures 17 and 18). In A498 cells, the LPA receptor involved in LPA mediated signaling did not couple to $G\alpha_{i/o}$ subunits, because the $G\alpha_{i/o}$ specific inhibitor Pertussis toxin (PTX) did not inhibit the EGFR signal transactivation by LPA.

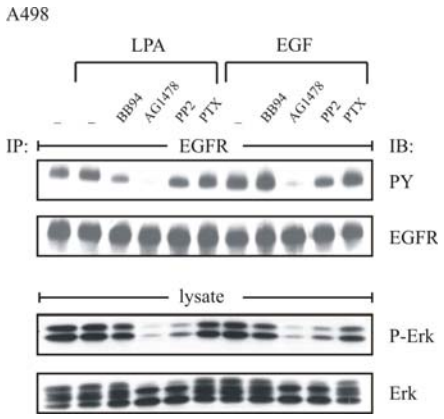

Fig. 17. Inhibition of LPA induced EGFR transactivation. A498 cells were starved for 48 h and preincubated with BB94 (5µM, 20 min), AG1478 (250 nM, 20 min), PP2 (20 µM, 20min) or PTX (125 ng/ml, 18 h). Subsequently, cells were stimulated with 10 µM LPA or 3 ng/ml EGF for 3 min. Equal amounts of lysated were immunoprecipitated with EGFR108 antibody and immunoblotted with phosphotyrosine specific antibody. Subsequent reprobing with EGFR antibody verified equal loading. Equal amounts of the same lysate were immunoblotted with activation state specific antibodies against Erk 1/2 and subsequently reprobed with Erk antibody to ensure equal loading.

Additionally, the src kinase inhibitor PP2 was able to reduce the LPA and EGF induced EGFR activation by approximately 50 % implying a role for src kinase in the TMPS pathway upstream of the EGFR (Figure 17).

Diphtheria toxin mutant Crm and Heparin, inhibitors of the EGF-like ligand HB-EGF and pro-Amphiregulin, did not inhibit Thrombin mediated EGFR signal transactivation (Figure 18). Therefore, neither pro-Amphiregulin nor HB-EGF were involved in this pathway. Since it has been demonstrated that LPA mediated GPCR-EGFR crosstalk involves HB-EGF (Hart, 2004; Schafer et al., 2004a), the two stimuli activated different metalloproteases and induced the shedding of different EGF-like ligands.

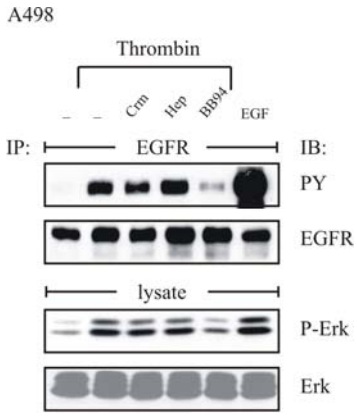

Fig. 18. Inhibition of Thrombin induced EGFR transactivation. A498 cells were starved for 48 h and preincubated with Diphtheria toxin mutant Crm (10 µg/ml, 20 min), Heparin (100 ng/ml, 20min) or BB94 (5µM, 20 min). Subsequently, cells were stimulated with 2 U/ml Thrombin or 3 ng/ml EGF for 3 min. Equal amounts of lysates were immunoprecipitated (IP) with EGFR108 antibody and immunoblotted (IB) with PY specific antibody. Subsequent reprobing with EGFR antibody verified equal loading. Equal amounts of the same lysate were immunoblotted with activation state specific antibodies against Erk 1/2 and subsequently reprobed with Erk antibody to ensure equal loading.

3.3.2 Effects of double stimulation of EGFR transactivation in A498 cells

Obviously, two essentially independent pathways resulting in EGFR Phosphorylation were activated in A498 cells. Hence, combined stimulation with the two different stimuli LPA and Thrombin was suggested to proportionally increase the EGFR transactivation signal.

Figure 19 demonstrates that LPA induced EGFR signal transactivation was $G\alpha_{i/o}$ independent, but HB-EGF dependent, while Thrombin induced TMPS pathway was $G\alpha_{i/o}$ dependent, but HB-EGF independent as inhibition with PTX, Heparin and Diphtheria toxin mutant Crm

proved. Therefore, both pathways only converged at the EGFR. Hence, the EGFR surface expression might be the only limiting factor in double stimulation of EGFR signal transactivation, but the signal intensity was proportionally increased when both stimuli were applied at once, verifiying that the amount of EGFR on the cell surface was not a limiting factor.

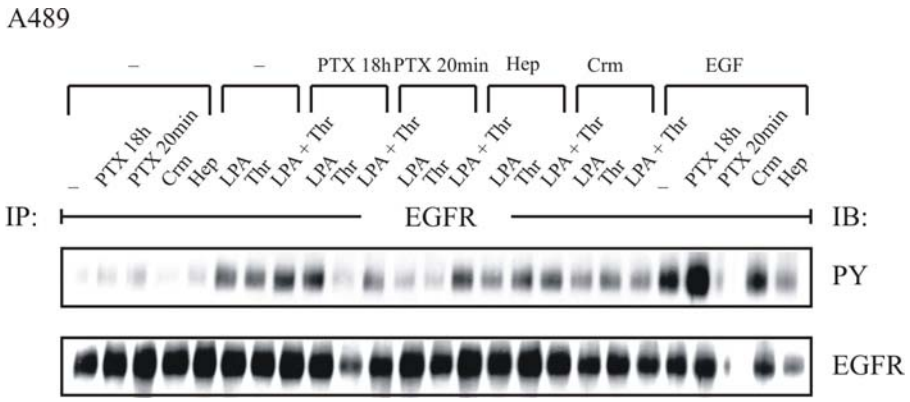

Fig. 19. Double stimulation of EGFR transactivation by LPA and Thrombin stimulation. A498 cells were starved for 48 h and preincubated with PTX (125 ng/ml, 18 h or 20 min, respectively), Crm (10 µg/ml, 20 min), or Heparin (100 ng/ml, 20min). Subsequently, cells were stimulated with 10 µM LPA, 2 U/ml Thrombin, a combination of both or 3 ng/ml EGF for 3 min. Equal amounts of lysates were immunoprecipitated (IP) with EGFR 108 antibody and immunoblotted (IB) with phosphotyrosine (PY) specific antibody. Subsequent reprobing with EGFR antibody verified equal loading.

3.3.3 The effect of ADAM10,MP polyclonal antibody on Thrombin induced EGFR transactivation in A498 cells

It is known that Thrombin induced EGFR signal transactivation is mediated exclusively by ADAM10, but the role of the metalloprotease activity of ADAM10 in the shedding of EGF-like ligands had to be further analyzed. Thus, it was studied whether the polyclonal antibody serum ADAM10,MP, binding to the metalloprotease domain of ADAM10, inhibited the Thrombin induced EGFR activation.

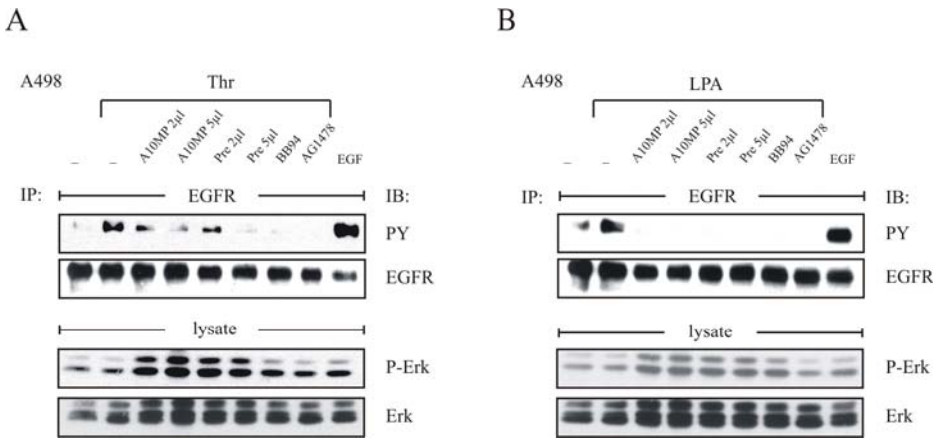

Fig. 20. Blocking EGFR transactivation by preincubation with ADAM10MP polyclonal serum. A498 cells were starved for 48h and then preincubated with the indicated amounts of ADAM10,MP polyclonal serum. Afterwards, cells were stimulated with 3 ng/ml EGF and 2 U/ml Thrombin (A) or 10 µM LPA (B) for 3 min. Equal amounts of lysates were immunoprecipitated (IP) with monoclonal EGFR 108 antibody and submitted to immunoblot (IB) analysis with Py specific antibodies. Sebsequent reprobing with EGFR antibody ensured equal loading. Additionally, equal amounts of total lysates were immunoblotted with p-Erk antibody and reprobed with Erk antibody to verify equal loading.

Figure 20 demonstrates that the preincubation of A498 cells with ADAM10,MP polyclonal serum lead to abrogation of the ADAM10 mediated EGFR signal transactivation induced by Thrombin stimulation, but it did not abrogate the activation of the downstream mediator Erk 1/2. In addition, the preimmune serum used as a control also diminished EGFR activation, proving that this inhibition was an unspecific effect of the serum. The LPA induced EGFR transactivation was also inhibited by ADAM10,MP and preimmune serum revealing that this inhibition was not a specific property of ADAM10,MP antibodies.

3.3.4 Transactivation is reduced by transient transfection of ADAM10 Si-RNA

A recently discovered method to confirm the involvement of ADAM10 in the Thrombin mediated EGFR transactivation in A498 cells is the downregulation of ADAM10 expression by small interfering RNAs (siRNAs) (Brummelkamp et al., 2002). Hence, a retroviral construct expressing siRNAs against the 3'UTR of ADAM10 was designed and tested in transient expression in A498 cells (Figure 21). Targeting a region outside the coding sequence of ADAM10 allowed ectopic expression of different ADAM10 constructs.

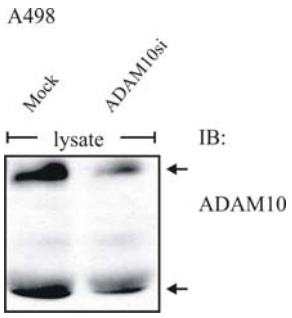

Fig. 21. The expression of ADAM10 is reduced by expression of ADAM10siRNAs. A498 cells were transfected with pRetroSuper-ADAM10siRNA. 48 h post-transfection cells were lysed and equal amounts of lysate were immunoblotted with ADAM10 antibody.

The expression of ADAM10 in the transfected A498 cells was strongly reduced.

Subsequently, the effect of ADAM10siRNA on the Thrombin induced EGFR signal transactivation was evaluated in order to confirm previous results with synthetic siRNAs (Hart, 2004). As shown in Figure 22, the reduction of ADAM10 expression by the expression of the pRetroSuper-ADAM10siRNA construct lead to abrogation of the Thrombin induced EGFR signal as well as inactivation of the downstream target Erk 1/2.

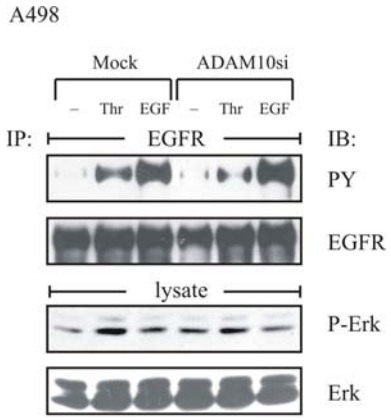

Fig. 22. Expression of ADAM10siRNA leads to abrogation of EGFR signal transactivation. A498 cells were transfected with pRetroSuper-ADAM10siRNA. Transfected cells were starved for 48 h and then stimulated with 2 U/ml Thrombin or 3 ng/ml EGF for 3 min. Equal amounts of lysates were immunoprecipitated (IP) with EGFR 108 antibody and immunoblotted (IB) with phosphotyrosine (PY) specific antibody. Subsequent reprobing with EGFR antibody verified equal loading. Equal amounts of the same lysate were immunoblotted (IB) with activation state specific antibodies against Erk 1/2 and subsequently reprobed with Erk antibody to ensure equal loading.

3.3.5 Generation of stably expressing ADAM10siRNA A498 cells

In order to study the influence of different mutants of ADAM10 lacking either the metalloprotease domain or the cytoplasmic tail on the EGFR signal transactivation, A498 cells were infected either with the pRetroSuper-ADAM10siRNA construct or control vector and selected for stable expression with 1 µg/ml Puromycin. After two weeks, colonies were picked and monoclonal cell lines were established. These cell lines were tested for the expression of ADAM10. Most of the monoclonal cell lines expressing ADAM10 siRNAs exhibited a significantly reduced level of ADAM10 expression (Figure 23).

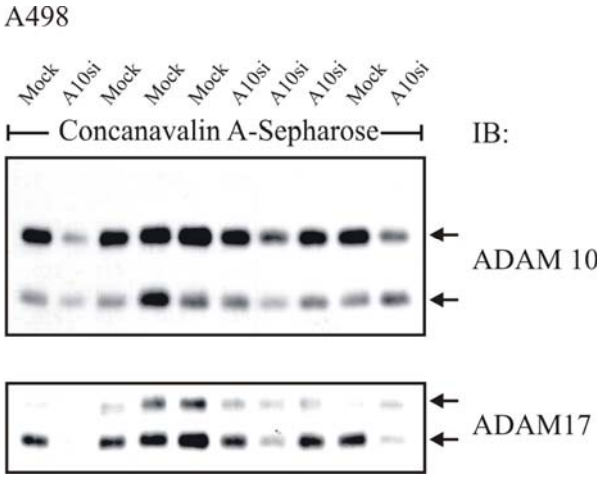

Fig. 23. The expression levels of ADAM 10 and ADAM17 in A498 cells stably expressing pRetroSuper-ADAM10siRNA. A498 cells were infected three times every four hours with retroviral supernatant from PhoenixA cells transfected with pRetroSuper-ADAM10siRNA. 24 h post-infection, cells were split and selected for expression of ADAM10siRNA with 1µg/ml Puromycin. Two weeks later, single cell colonies were picked. Equal amounts of lysate from the monoclonal cell lines were purified using ConcanavalinA-Spepharose and immunoblotted (IB) with ADAM 10 and ADAM17 antibodies, respectively.

However, the downregulation of ADAM10 was not specific as the expression of ADAM17 was deregulated in a similar fashion (Figure 23). Therefore, these cell lines could not be used for studying the specific influence of ADAM10 downregulation.

3.3.6 Ki16425 inhibits LPA induced EGFR transactivation and *in vitro* wound closure in A498 cells

Recently, a functional antagonist for the LPA receptors, Ki16425, with a preference for the Edg 2 and Edg7 receptor was discovered. Short term action of LPA such as Ca^{2+}-influx and cAMP production, but also long term action including migration and DNA synthesis could be abrogated by Ki16425 (Ohta et al., 2003; Yamada et al., 2004)
Preincubation of A498 cells with the LPA receptor antagonist Ki16425 completely abrogated LPA induced EGFR signal transactivation (Figure 24).

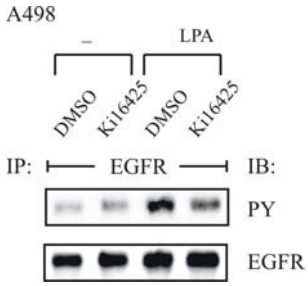

Fig. 24. LPA receptor inhibitor Ki16425 abrogates Thrombin induced EGFR signal transactivation in A498 cells. A498 cells were starved for 48 h. Cells were preincubated with DMSO as control or 10 nM Ki16425 for 20 min. Subsequently, the cells were stimulated with 10 µM LPA for 3 min. Equal amounts of lysate were immunoprecipitates with EGFR 108 antibody and immunoblotted (IB) with phosphotyrosine (PY) specific antibody. Subsequent reprobing with EGFR antibody verified equal loading.

For the first time, it was unambiguously demonstrated that the G protein coupled receptor was directly involved in EGFR signal transactivation. Thus, the LPA mediated transactivation was not independent the LPA receptors in A498 cells, but critically relied on the function of the LPA receptors.
LPA induces *in vitro* wound closure and migration in A498 cells (Schafer, 2004). In addition, this study analyzed the direct involvement of the LPA receptors in the LPA mediated wound closure and chemotaxis in A498 cells. For this purpose, A498 cells were grown to confluency and starved for 48 h. After applying a uniform scratch, cells were preincubated with LPA receptor inhibitor Ki16425 or inhibitors of the EGFR transactivation pathway and subsequently stimulated with LPA. The cells were allowed to migrate into the wound for 14 h. While LPA induced complete wound closure within 14 h, Thrombin stimulation did not lead to wound closure in A498 cells. Inhibition with the LPA receptor inhibitor Ki16425

completely abrogated LPA induced wound closure, while the inhibitors of the TMPS pathway only lead to a reduction in wound closure. Therefore, Ki16425 is the more potent inhibitor of LPA induced wound closure, suggesting that the LPA receptors additionally activate a second pathway evoking wound closure (Figure 25). LPA was even a more potent stimulus than serum for wound closure and chemotaxis (Figure 25).

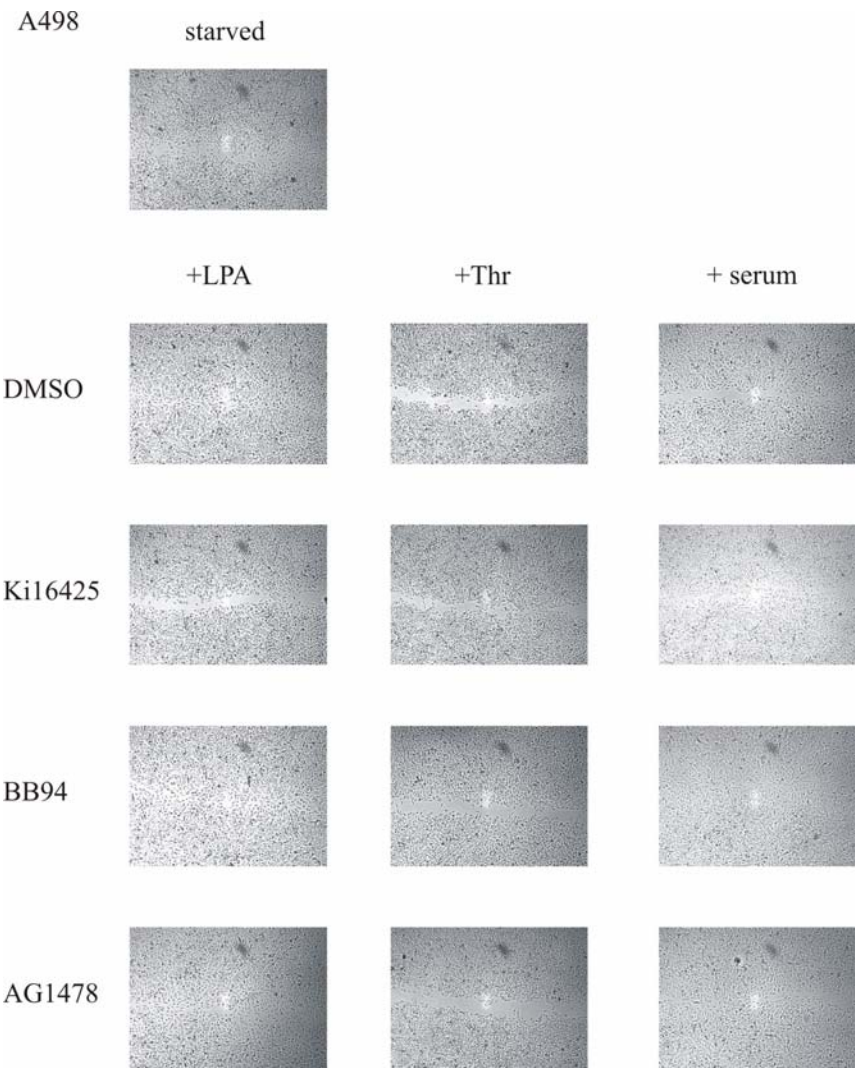

Fig. 25. The influence of Ki16425, BB94 and AG1478 on the LPA induced wound closure in A498 cells. A498 cells were grown to confluency and starved for 48 h. A uniform scratch was applied using a pipette tip. Subsequently, cells were preincubated with 100 nM Ki16425, 5 µM BB94, 250 nM AG1478 or DMSO as a control for 20 min and then stimulated with LPA (10 µM), EGF (3 ng/ml) or serum (10 %). Cells were allowed to migrate into the wound for 14 h.

3.4 EGFR signal transactivation by LPA in breast cancer cells

Breast cancer belongs to the most frequent tumor types in women. The overexpression of HER2 which is the preferred binding partner for the EGFR is a marker for the aggressiveness of breast cancer tumors. (Pegram et al., 1998; Slamon and Clark, 1988; Slamon et al., 1987; Slamon et al., 1989). In some carcinomas of the breast, overexpression of the EGFR was additionally detected (Nicholson et al., 1991). LPA is a potent mitogenic stimulus and has been previously implicated in the proliferation and migration of human breast cancer (Goetzl et al., 1999; Sliva et al., 2000)

Therefore, it was of great interest whether transactivation of the EGFR signal by the GPCR ligand LPA leads to the aggressive behavior of breast cancer.

3.4.1 Expression of LPA receptors, ADAM proteins and EGF-like ligands in breast cell lines

Analysis by cDNA arrays detected an upregulated expression of the LPA receptors in various breast cancer cells.

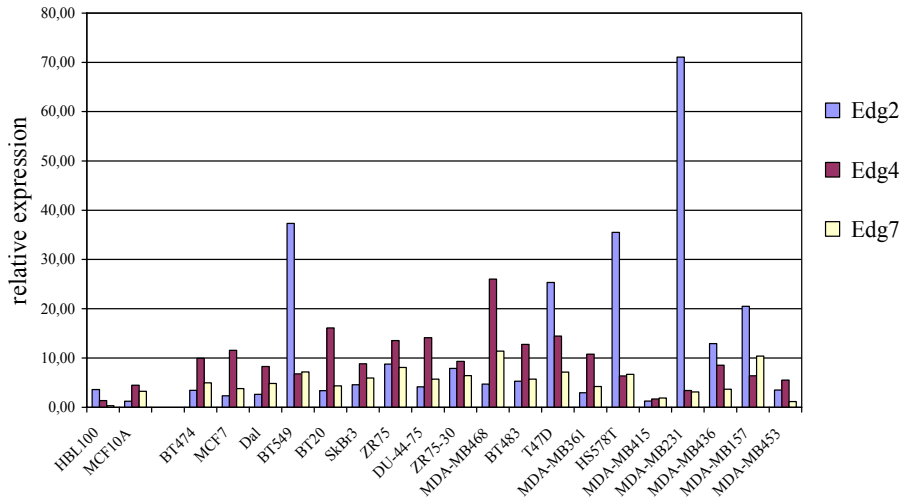

Fig. 26. Relative expression of LPA receptors in various breast cell lines as detected by cDNA array analysis. On the left are two normal breast cell lines HBL 100 and MCF10A followed by different breast cancer cell lines.

The normal breast epithelial cell line MCF10A was chosen as control cell line. None of the normal breast cell lines exhibited high expression of LPA receptors. The weakly invasive adenocarcinoma cell line BT20 expressed all LPA receptors at a low level and served as an additional control. On the contrary, the invasive carcinoma cell lines MDA-MB 231, HS578T and the invasive ductal carcinoma cell line BT549 highly expressed Edg2, but Edg4 and Edg7 transcripts were hardly detectable. Therefore, these three cell lines were suitable systems to study the influence of a single LPA receptor, namely Edg2 (Figure 26).
Expression of the gene products of different ADAM proteins were also demonstrated in cDNA array analysis (Figure 27).

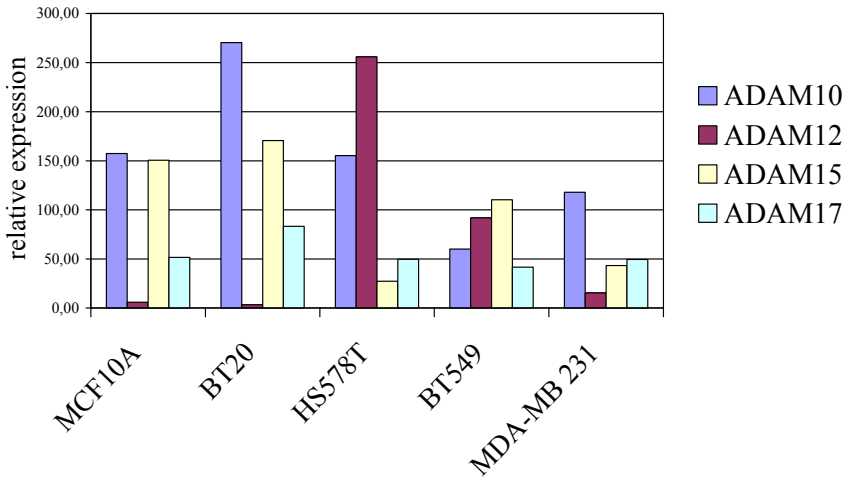

Fig. 27. Expression levels of ADAM proteins in the selected cell lines. Depicted are the relative mRNA expression levels of ADAM10, 12, 15 and 17 in selected breast cell lines.

ADAM10 and ADAM17 expression was also detected in immunoblot analysis of the selected cell lines. Except for HS578T, all cells strongly expressed ADAM10. ADAM17 was expressed by all cell lines to a similar extent (Figure 28).

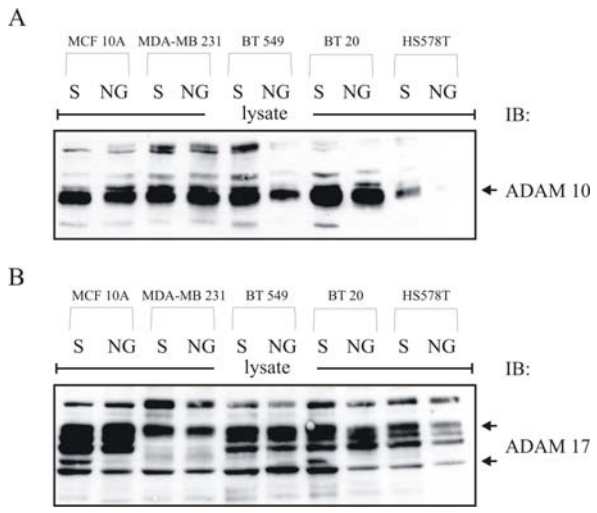

Fig. 28. Detection of ADAM10 and 17 in immunoblot analysis. Equal amounts of lysate from 24 h starved cella and cell in normal growth were immunoblotted (IB) with ADAM10 and ADAM17 antibody, respectively.

The expression of EGF-like ligands and the EGFR receptor itself revealed an exceptionally high expression of the EGFR in the BT20 cell line and high levels of Amphiregulin in MCF10A and MDA-MD 231 cells. BT 20 expressed only low levels of all EGF-like ligands (Figure 29).

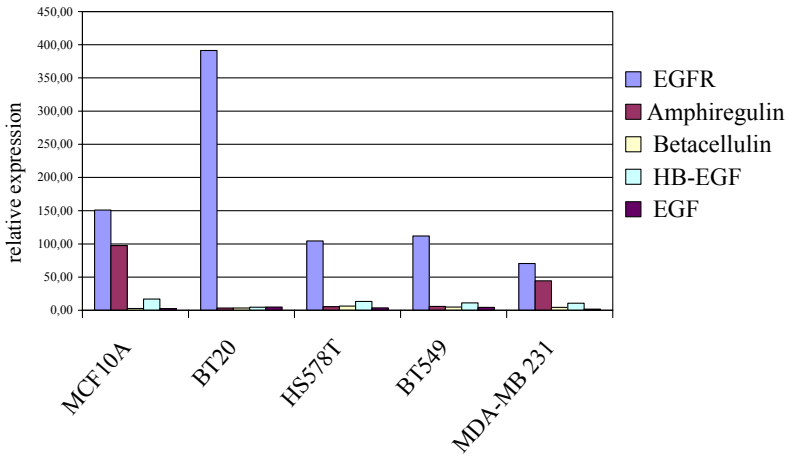

Fig. 29. Expression levels of the EGFR and its ligands in the selected breast cell lines as detected by cDNA array analysis. Depicted are relative mRNA expression levels of the EGFR, Amphiregulin, Betacellulin, HB-EGF and EGF in the indicated breast cell lines.

3.4.2 Transactivation of breast cell lines by LPA

The ability to induce EGFR signal transactivation by LPA stimulation was examined in the selected cell lines (Figure 30). In addition, the possibility to inhibit basal EGFR and Erk 1/2 activity with the known inhibitors of the TMPS pathway, Batimastat and AG1478, was investigated.

LPA activated the TMPS pathway both in the normal and the highly invasive breast cancer cell lines BT549 and MDA-MB 231. In BT20, the EGFR exhibited high basal phosphorylation and no additional induction upon stimulation with LPA was observed. The basal EGFR phosphorylation was not blocked by inhibition with Batimastat. On the contrary, the EGFR signal transactivation by LPA in MCF10A, BT549 and MDA-MB 231 cells as well as the EGFR phosphorylation in normal cells was abrogated by inhibition with Batimastat (Figure 30).

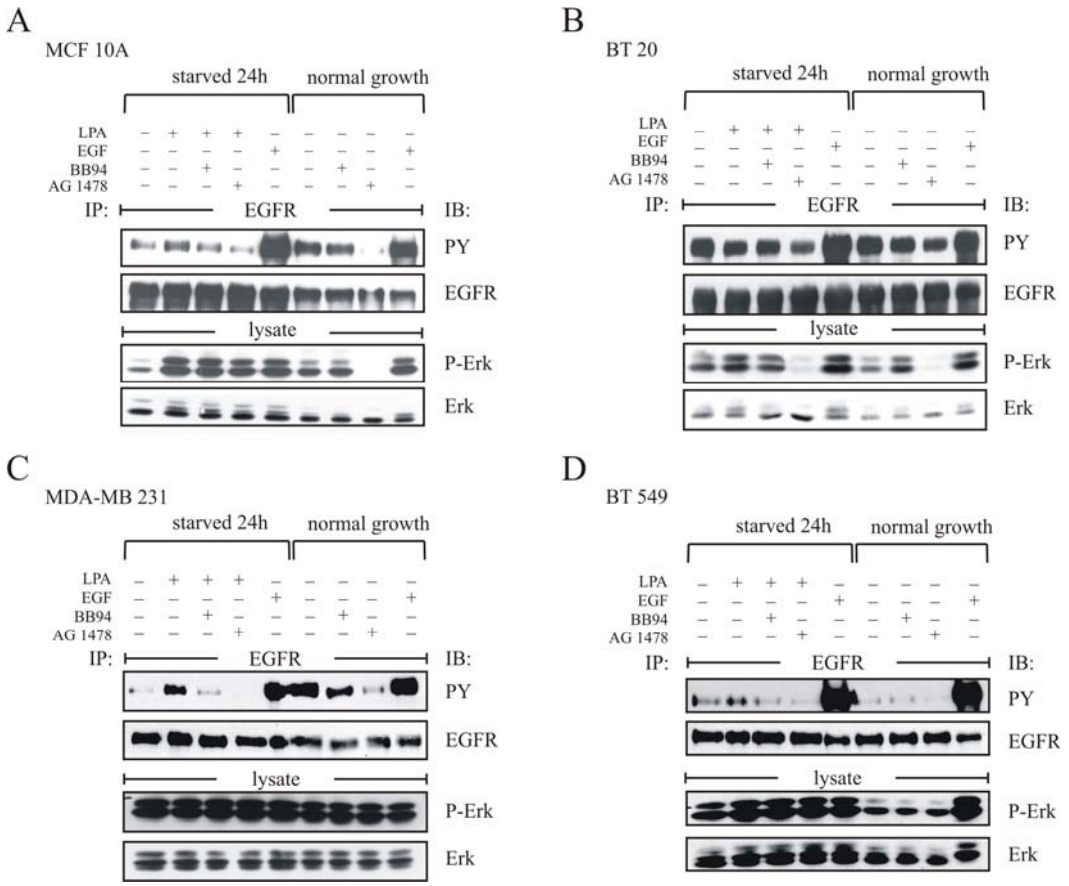

Fig. 30. LPA induces EGFR signal transactivation in MCF10A, BT549 and MDA-MB 231, but not in BT20 cells. Cells were starved for 24 h and after preincubation with DMSO as control, 5 µM BB94 or 250 nM AG1478 for 20 min and stimulated with 10 µM LPA or 3 ng/ml EGF for 3 min. Additionally, cells in normal

growth were incubated with 5 µM BB94 or 250 nM AG1478 for 20 min. Equal amounts of lysates were immunoprecipitated (IP) with EGFR 108 antibody and immunoblotted (IB) with phosphotyrosine (PY) specific antibody. Subsequent reprobing with EGFR antibody verified equal loading. Equal amounts of the same lysates were immunoblotted (IB) with activation state specific antibodies against Erk 1/2 and subsequently reprobed with Erk antibody to ensure equal loading.

3.4.3 Array data of breast cancer cells

In order to identify crucial players in the transactivation pathway and to further characterize these breast cell lines, cDNA array analysis was conducted with the selected breast cell lines after serum deprivation and stimulation with LPA as well as cells in normal growth inhibited with the broad spectrum metalloprotease inhibitor BB94 for 6 h.

Dr. Pjotr Knyazev, visiting Professor in the Department of Molecular Biology at the MPIB, had previously shown by cDNA array analysis that the LPA receptor Edg2 is overexpressed in a large variety of breast cancer cell lines and appeared to be coregulated with several metalloproteases. The data obtained from Dr. Knyazevs cDNA array experiments provided evidence for a connection of Edg2 with the invasive potential of breast cancer cells and the EGFR signal transactivation mechanism, because the LPA receptor Edg2 was observed to be coregulated with metalloproteases which are known to be involved in the TMPS pathway in breast cancer cells as shown in Figure 31. Edg2 was observed to be in one closely related cluster of genes with similar regulation of expression as the metalloproteases ADAM12 and MMP14, the metalloprotease inhibitors TIMP1, 2 and 3 and the EGF-like ligand HB-EGF (Knyazev, unpublished results).

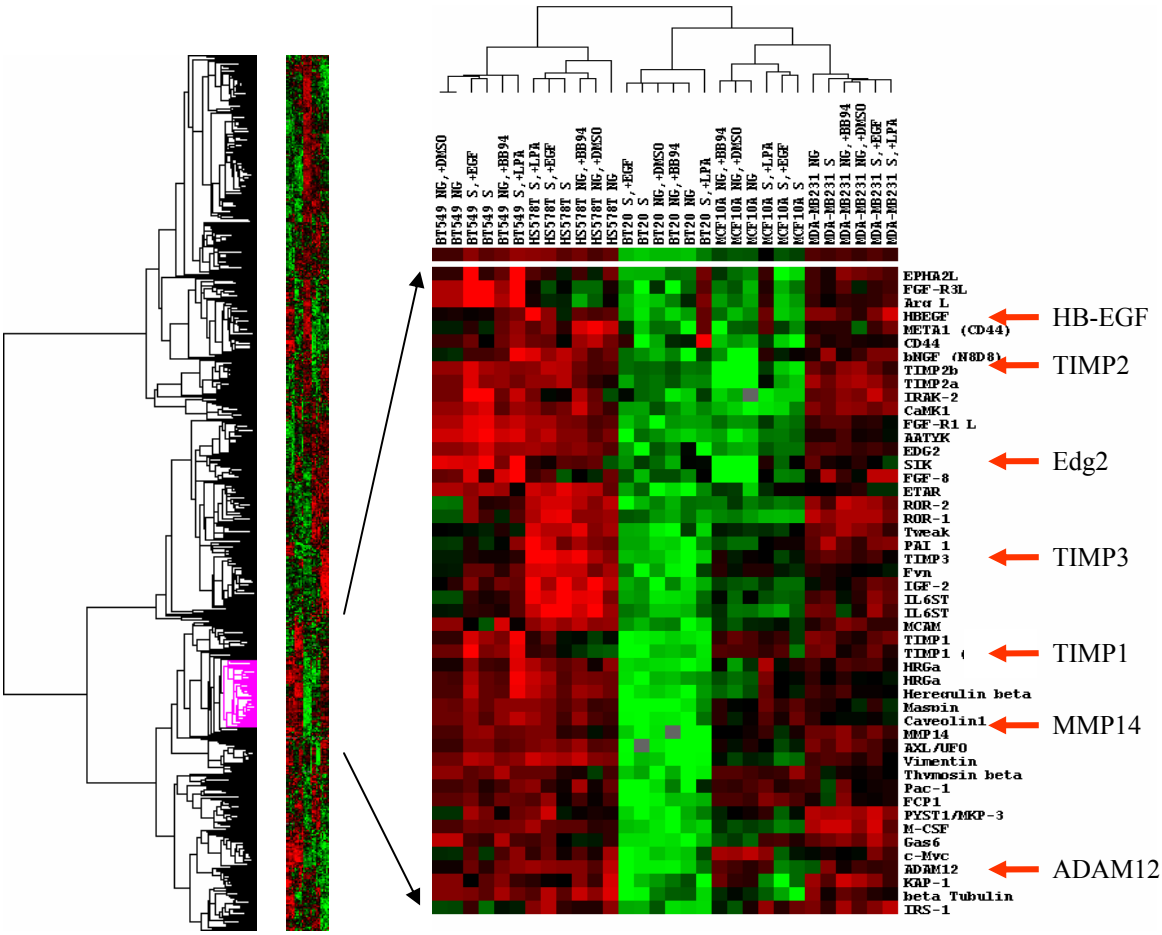

Fig. 31. cDNA array analysis: Cluster I of genes with similarly regulated genes. MCF10A, BT20, HS578T, BT549 and MDA-MB 231 cells were either starved for 24 h and simulated with 10 µM LPAor 3 ng/ml EGF for 6 h or grown for 24 h and inhibited with 10 µM BB94, control cells were treated with DMSO for 6 h. cDNA from these cells was prepared, labeled with α[^{33}P]dATP and hybridized on array filters as described. Data obtained from these filters was normalized and subjected to Cluster analysis. Genes depicted in red were highly expressed while genes shown in green exhibited an extremely low expression. The correlation factor of this cluster was 0.72.

In the highly invasive cell lines HS578T, BT549 and MDA-M 231 these genes were highly expressed, while the weakly invasive cell line BT20 exhibited lower expression of these genes. The normal breast cell lines exhibited an intermediate expression pattern.

3.4.3.1 The EGFR clusters with HER2

The EGFR itself did not cluster with the other components of the TMPS pathway. Instead, it was detected in a separate cluster together with HER2 as shown in Figure 32.

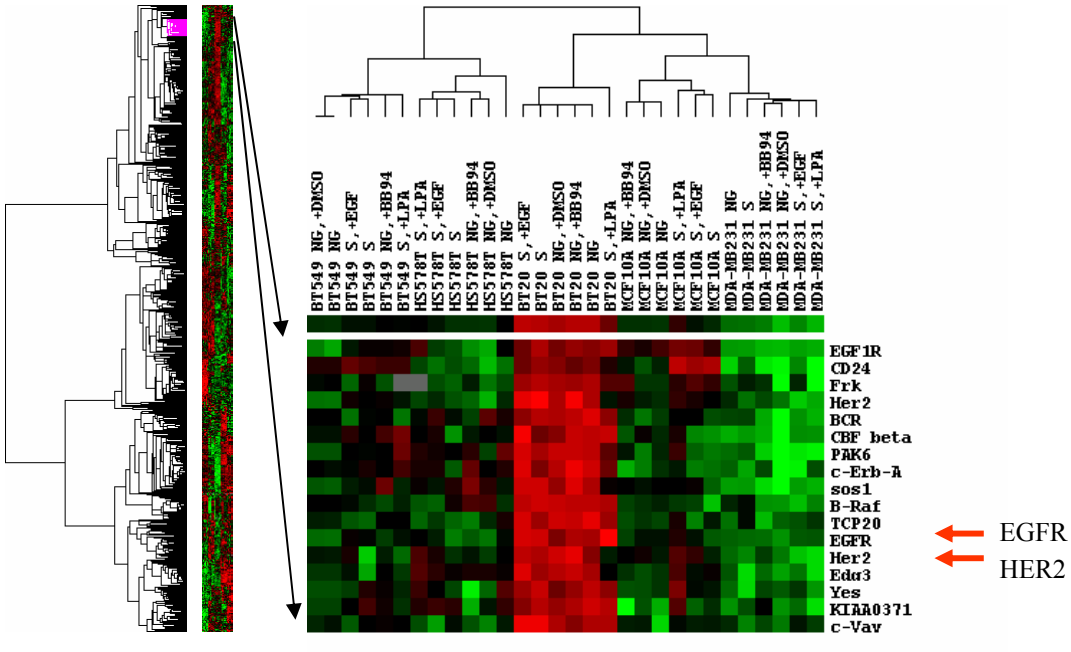

Fig. 32. cDNA array analysis: Cluster II of genes with similarly regulated genes. MCF10A, BT20, HS578T, BT549 and MDA-MB 231 cells were either starved for 24 h and simulated with 10 µM LPA or 3 ng/ml EGF for 6 h or grown for 24 h and inhibited with 10 µM BB94, control cells were treated with DMSO for 6 h. cDNA from these cells was prepared, labeled with α[^{33}P]dATP and hybridized on array filters as described. Data obtained from these filters was normalized and subjected to Cluster analysis. Genes depicted in red were highly expressed while genes shown in green exhibited an extremely low expression. The correlation factor of this cluster was 0.79.

While the expression of the EGFR and HER2 was normal to low in the normal breast epithelial cell line MCF10A and the highly invasive cell lines HS578T, BT549 and MDA-MB 231, the weakly invasive cell line BT20 revealed an exceptionally high expression of the EFGR and HER2, which explained the high basal activity of the EGFR. From these results, it was concluded that the overall expression pattern correlated with the transactivation behavior of the breast cells. LPA was able to induce EGFR signal transactivation in BT549, MDA-MB 231 and MCF10A cells with with a low basal EGFR expression and high expression of other components of the TMPS pathway, whereas in BT20 cells with an inverse gene expression pattern LPA did not induce GPCR-EGFR crosstalk.

3.4.3.2 The influence of LPA stimulation and BB94 inhibition on the gene expression pattern

The stimulation of starved breast cancer cells as well as inhibition of breast cancer cells in normal growth with the broad spectrum metalloprotease inhibitor Batimastat for 6 hours did not lead to changes in the gene expression pattern of more than 4 fold as determined in dot blot analysis (data not shown) which was the cut-off limit for significant up- or downregulation in the studied system. Further analysis with longer stimulation and inhibition might reveal more significant changes in the gene expression after LPA stimulation or metalloprotease inhibition.

3.4.4 Blocking LPA induced transactivation in breast cells with Ki16425

The discovery of a fairly specific inhibitor for the Edg receptors (Ohta et al., 2003; Yamada et al., 2004) allowed to study the direct involvement of the LPA receptors in the EGFR signal transactivation.

The inhibitor Ki16425 strongly decreased LPA induced EGFR signal transactivation in MCF10A and MDA-MD231 cells (Figure 33), again clearly demonstrating that the LPA receptor Edg2 is directly involved in the EGFR transactivation pathway. Interestingly, lower concentrations (10 nM) of the inhibitor yielded stronger inhibition in short term experiments. As Sphingosine-1-phosphate also binds to Edg2, the Sphingosine-1-phosphate induced transactivation of the EGFR signal was also diminished by preincubation with Ki16425. However, other families of GPCRs such as the Thrombin receptor were not inhibited by the LPA receptor antagonist Ki16425 proving the specificity of this inhibitor (Figure 33).

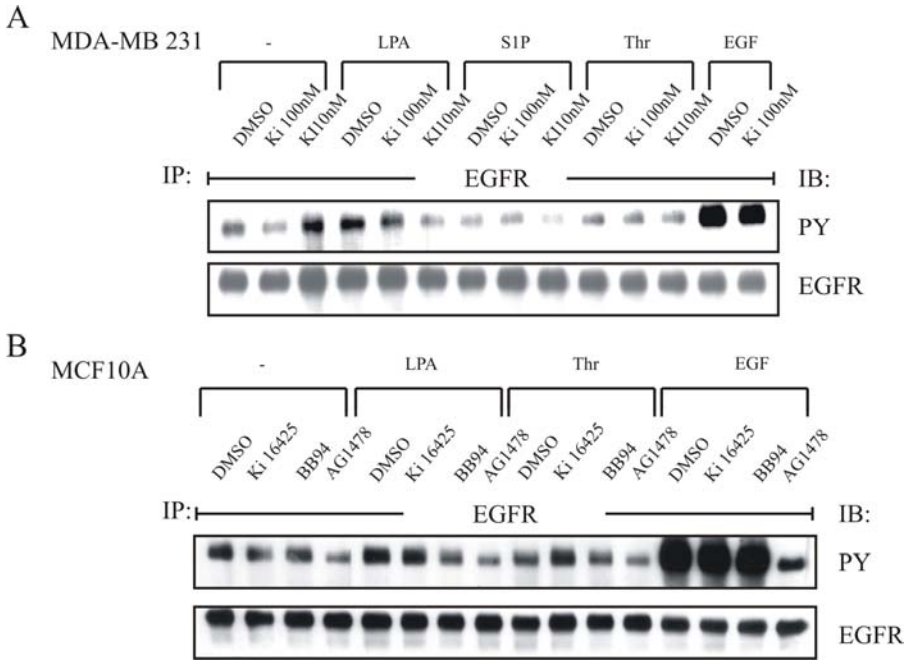

Fig. 33. Low concentrations of Ki16425 can abrogate LPA induced EGFR signal transactivation in breast cells. MDA-MB 231 (A) and MCF10A cells (B) were starved for 24 h and after 20 min preincubation with Ki16425 (10 and 100 nM as indicated) stimulated with 10 µM LPA, 10 µM Sphingosine-1-phosphate, 2 U/ml Thrombin or 3 ng/ml EGF for 3 min. Equal amounts of lysate were immunoprecipitated (IP) with EGFR 108 antibody and immunoblotted (IB) with PY-specific antibody followed by reprobing with EGFR antibody to verify equal loading.

3.4.5 LPA induced wound closure in breast cancer cells

LPA or EGF stimulation did not induce wound closure in the normal breast epithelial cell line MCF10A (data not shown), while LPA induces complete wound healing within 12-14 hours both in BT549 and MDA-MB 231 cell lines (Figures 34 and 35).

Contrarywise, EGF as well as Thrombin had hardly any effect on the wound healing behavior. The wound healing was completely abrogated by inhibition with LPA receptor inhibitor Ki16425 (Figures 34 and 35). Inhibitors of TMPS pathway BB94 as well as AG1478 could in comparison to Ki16425 only reduce wound healing (Figure 36). Thus, it had to be concluded that a second pathway leading to wound closure is activated by the LPA receptor Edg2. These results clearly show that wound closure is only partly mediated by the transactivation pathway in BT549 cells.

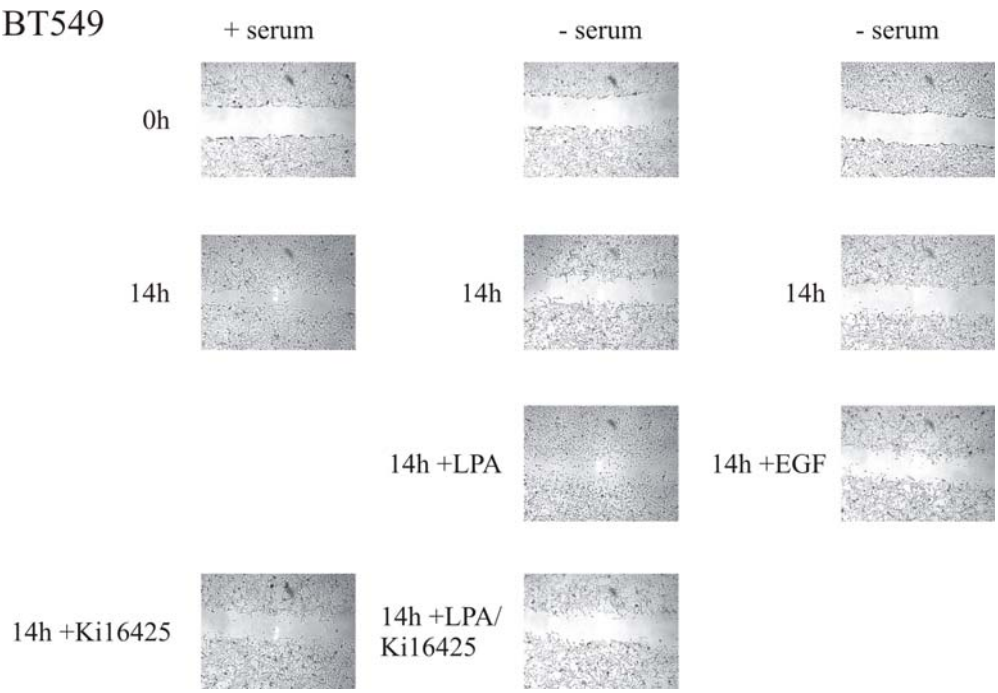

Fig. 34. Ki16425 abrogates LPA induced wound closure in BT549 cells. BT549 cells were grown to confluency and starved for 24 h. A uniform scratch was applied using a pipette tip. Subsequently, cells were preincubated with 100 nM Ki16425, 5 µM BB94, 250 nM AG1478 or DMSO as a control for 20 min and then stimulated with LPA (10 µM), EGF (3 ng/ml) or serum (10 %). Cells were allowed to migrate into the wound for 14 h.

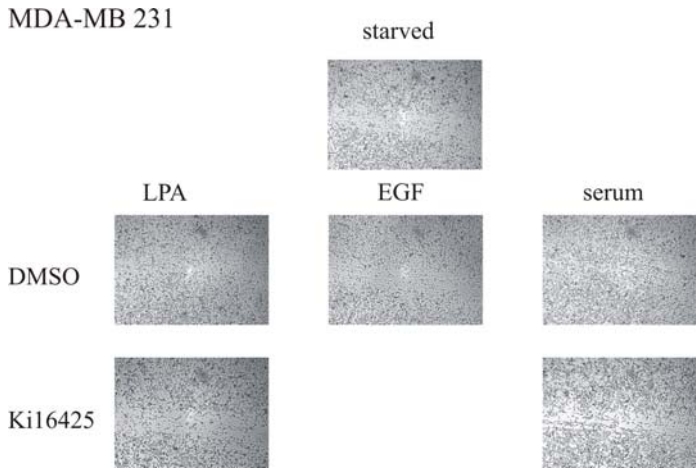

Fig. 35. Ki16425 inhibits LPA induced wound closure in MDA-MB 231 cells. MDA-MB 231 cells were grown to confluency and starved for 24 h. A uniform scratch was applied using a pipette tip. Subsequently, cells were preincubated with 100 nM Ki16425 or DMSO as a control for 20 min and then stimulated with LPA (10 µM) or EGF (3 ng/ml) or serum (10 %). Cells were allowed to migrate into the wound for 12 h.

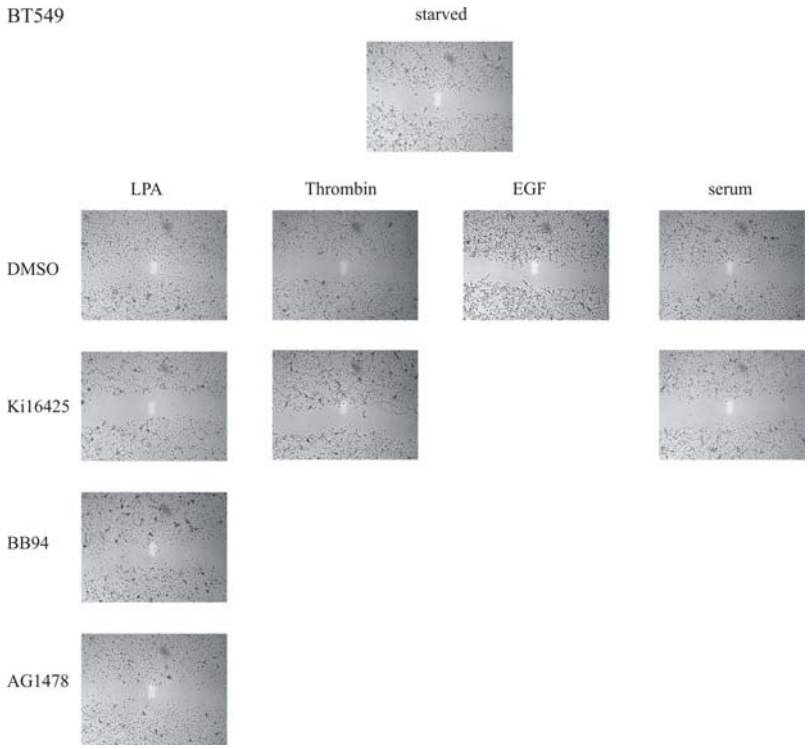

Fig. 36. The influence of Ki16425, BB94 and AG1478 on the LPA mediated wound closure in BT549 cells. BT549 cells were grown to confluency and starved for 24 h. A uniform scratch was applied using a pipette tip. Subsequently, cells were preincubated with 100 nM Ki16425, 5 µM BB94, 250 nM AG1478 or DMSO as a control for 20 min and then stimulated with LPA (10 µM), Thrombin (2 U/ml) or EGF (3 ng/ml). Cells were allowed to migrate into the wound for 12 h.

3.5 The involvement of G protein subunits

While many studies report Gα subunits to be involved in oncogenic signaling (Dhanasekaran et al., 1998; Gudermann et al., 2000; Neves et al., 2002), comparably little is known about the signaling events induced by Gβγ subunits (Jones et al., 2004).

The fact that Pertussis toxin abrogates EGFR signal transactivation in many cell lines leads to the assumption that Gα subunits, in particular $G\alpha_{i/o}$ subunits, are involved in the mediation of the EGFR signal transactivation (Gschwind et al., 2003; Hart, 2004). The finding that constitutively active mutants of Gα subunits encode transforming oncogenes such as *gsp*, *gip2* and *gep* (Dhanasekaran et al., 1998; Landis et al., 1989; Lyons et al., 1990; Marinissen and Gutkind, 2001; Radhika and Dhanasekaran, 2001; Xu et al., 1993) reinforces the suggestion that Gα subunits are key regulators of the EGFR signal transactivation.

3.5.1 Overexpression of Gα-subunits

LPA receptors are described to predominanatly couple to $G\alpha_{i/o}$ subunits, while Thrombin receptors often couple to $G\alpha_{12}$ and $G\alpha_{13}$ subunits (Neves et al., 2002). In contrast, the Carbachol receptors are mainly associated with $G\alpha_q$ or $G\alpha_{11}$ subunits (Luo et al., 2001). For these reasons the following combinations of GPCR ligands and Gα subunits were studied (Figure 37). Presumably, the constitutively active mutants of Gα subunits activate the EGFR signaling pathway independent from ligand stimulation.

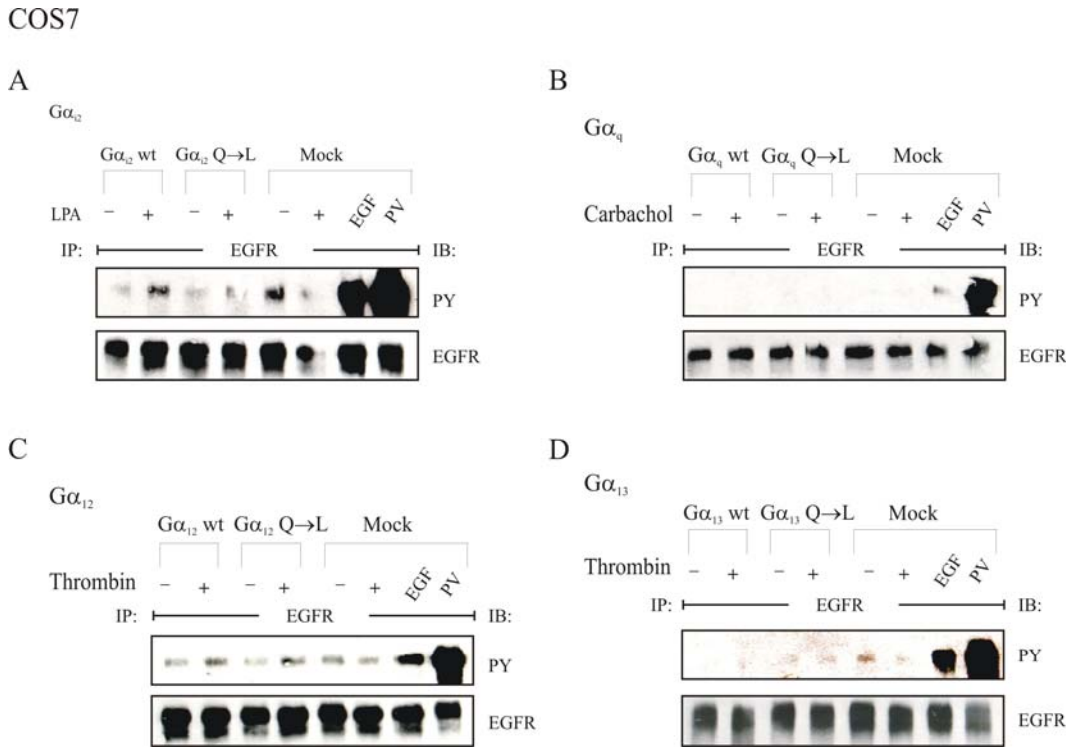

Fig. 37. Overexpression of different Gα subunits and their activating mutants in COS7 cells. COS7 cells were transfected with $G\alpha_{i2}$, $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$ subunits as well as the corresponding Gα Q→L mutants. 24 h post-transfection, cells were serum-starved for 20 h and subsequently stimulated with 10 µM LPA, 3 ng/ml EGF or 100 µM PV, respectively. Equal amounts of cell lysates were immunoprecipitated (IP) with EGFR 108 antibody and immunoblotted (IB) with PY antibody. Reprobing with EGFR antibody verifies equal loading.

A strongly enhanced EGFR activation was not induced by the expression of any of the Gα subunits or their oncogenic mutants. Stimulation with the corresponding stimuli did not result in enhanced activation of the EGFR signal (Figure 37). Therefore, the Gα subunits affected the EGFR signal transactivation pathway neither in a ligand-dependent nor independent fashion.

Further analysis of the expression of $G\alpha_{i2}$ subunit and its oncongenic variant demonstrated that the expression of both subunits lead to increased basal levels of EGFR phosphorylation, but merely a slight enhancement after LPA stimulation (Figure 38).

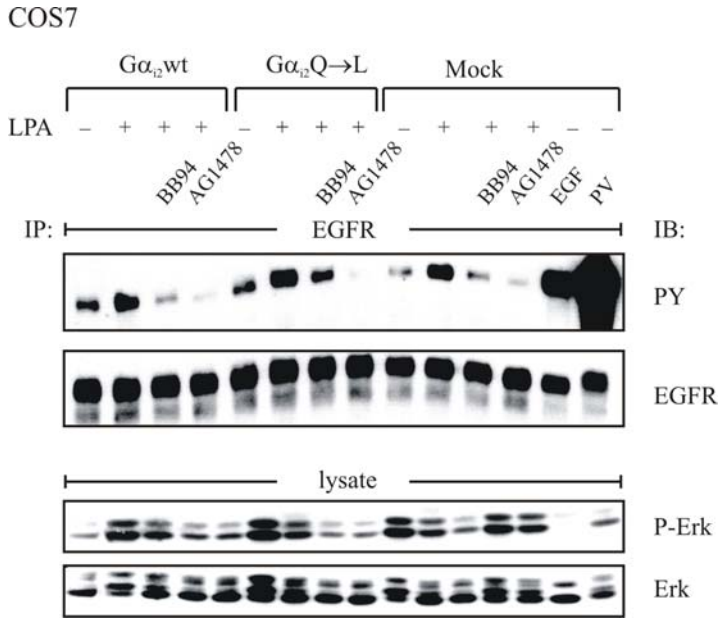

Fig. 38. Overexpression of $G\alpha_{i2}$ subunits in COS7 cells. COS7 cells were transfected with Mock control, Ga_{i2}wt or $G\alpha_{i2}Q\rightarrow L$ subunit expressing constructs. Cells were starved for 24 h and after preincubation with BB94 (5µM, 20 min) or AG1478 (250 nM, 20 min) stimulated with LPA (10µM) or EGF (3ng/ml) for 3 min. Equal amounts of lysates were immuonprecipitated (IP) with EGFR specific monoclonal antibody and immunoblotted (IB) with PY-specific antibody followed by reprobing with anti-EGFR antibody to ensure equal loading. Equal amounts of the same lysates were immunoblotted (IB) with P-Erk specific antibody followed by reprobing with anti-Erk antibody.

3.5.2 Overexpression of Gβγ subunits

A large variety of Gβ and Gγ subunits are expressed in mammals (Jones et al., 2004). Gβγ subunits are suggested to play an important role in the Gi mediated activation of the MAPK Erk 1/2 (Crespo et al., 1994; Faure et al., 1994). Additionally, it was discovered that Gβ and Gγ are only well expressed in combination, since only the Gβγ dimer is sufficiently stable (Luttrell et al., 1997).

The $G\beta_1\gamma_2$ subunit was tested for its ability to enhance EGFR signal transactivation as well as Erk 1/2 activation when overexpressed in various cell lines. Expression of different $G\beta_1$ and $G\gamma_2$ constructs was examined in COS7 cells as shown in Figure 39.

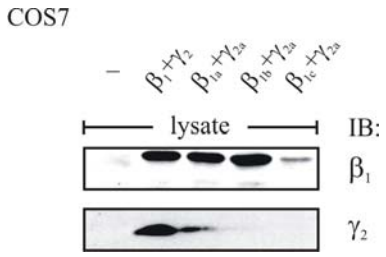

Fig. 39. $G\beta_1\gamma_2$ overexpression in COS7 cells. COS7 cells were transfected with either Mock control, $G\beta_1$, $G\gamma_2$ or $G\beta_1$ and $G\gamma_2$ subunit expressing constructs. Equal amounts of lysates were subjected to SDS-PAGE analysis followed by Western blot analysis. Expression of $G\beta_1$ and $G\gamma_2$ was detected by $G\beta_1$ and $G\gamma_2$ specific antibodies.

The expression of $G\beta_1$ and $G\gamma_2$ was well detected (Figure39). These constructs were used for further experiments to study the actions of $G\beta_1\gamma_2$ as presented in the following.

3.5.2.1 Influence of Gβγ-subunit overexpression on the EGFR transactivation in different cell lines

$G\beta_1$ and $G\gamma_2$ were coexpressed in Rat1, SCC9, COS7 and HEK239 cells. The ability of the $G\beta_1\gamma_2$ subunit to induce activation of the EGFR and the MAPK Erk 1/2 was determined by immunoblot analysis as demonstrated in Figures 40 and 41.
In HEK293 cells, $G\beta_1$ was able to induce Erk 1/2 activation to a comparable extent as stimulation with LPA. $G\gamma_2$ expression, however, was not be detected (Figure 40).

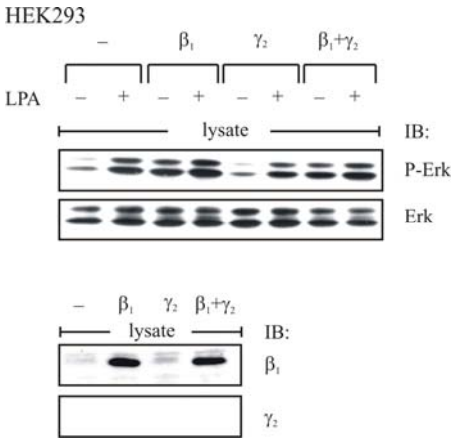

Fig. 41. $G\beta_1\gamma_2$ overexpression in HEK293 cells. HEK293 cells were transfected with either Mock control, $G\beta_1$, $G\gamma_2$ or $G\beta_1$ and $G\gamma_2$ subunit expressing constructs. Cells were starved for 24 h and equal amounts of lyates were immunoblotted (IB) with P-Erk specific antibody followed by reprobing with anti-Erk antibody. Expression of $G\beta_1$ and $G\gamma_2$ was detected by $G\beta_1$ and $G\gamma_2$ specific antibodies.

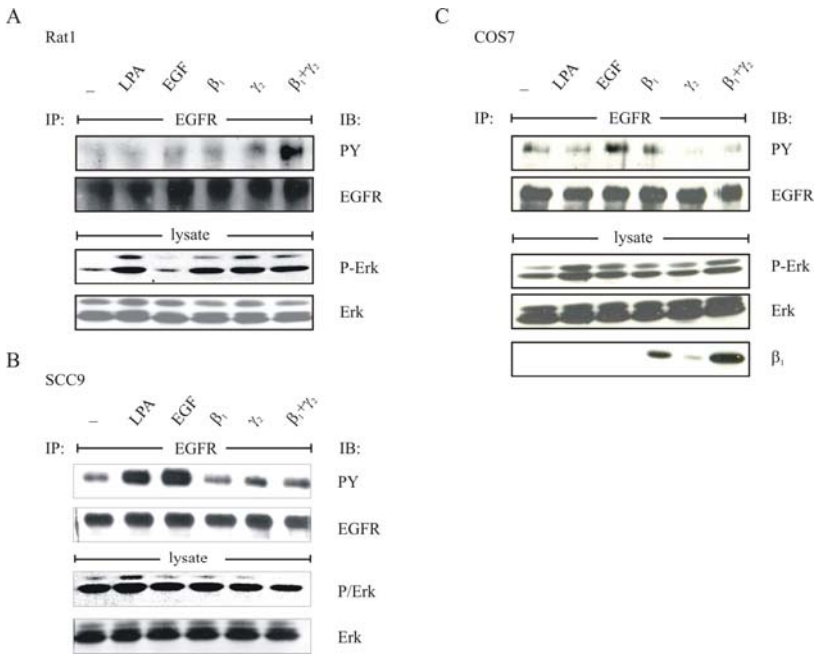

Fig. 40. Influence of Gβγ subunits on the EGFR signal transactivation in various cell lines. Rat1 (A), SCC9 (B) and COS7 (C) cells were transfected with either Mock control, $G\beta_1$, $G\gamma_2$ or $G\beta_1$ and $G\gamma_2$ subunit expressing constructs. Cells were starved for 24h and stimulated with LPA (10μM) or EGF (3ng/ml) for 3 min. Equal amounts of lysates were immuonprecipitated (IP) with EGFR specific monoclonal antibody and immunoblotted (IB) with PY-specific antibody followed by reprobing with anti-EGFR antibody to ensure equal loading. Equal amounts of the same lysates were immunoblotted (IB) with P-Erk specific antibody followed by reprobing with anti-Erk antibody.

In Rat1 cells, overexpression of $G\beta_1\gamma_2$ increased EGFR phosphorylation as well as Erk 1/2 activation (Figure 41).

Neither in SCC9 nor in COS7 cells, an elevated EGFR or Erk 1/2 activity was demonstrated (Figure 41). Therefore, the action of the $G\beta_1\gamma_2$ subunit seemed to be limited to the cellular context of Rat1 and HEK293 (Figure 40) and was predominantly mediated by the $G\beta_1$ subunit. The activation of the EGFR and Erk1/2 is due to the overexpression of $G\beta_1\gamma_2$ and is ligand independent.

4 Discussion

Crosstalk between members of different receptor families has been recognized to be a well-established concept in signal transduction. Signaling networks are essential in the control of a variety of cellular processes. GPCRs as well as RTKs belong to the most prominent cell surface proteins regulating the cell's response to environmental signals.

Aberrant signaling of RTKs is known to be crucially involved in the development and progression of hyperproliferative diseases such as cancer (Blume-Jensen and Hunter, 2001). Constitutive activation of RTKs originates from gene amplification, overexpression, activating mutations or autocrine stimulation of the receptors by growth factors (Zwick et al., 2001). On the other hand, deregulated GPCR signaling by autocrine production of GPCR agonists or activating mutations has been frequently associated with different types of human cancer (Marinissen and Gutkind, 2001; Moody et al., 2003). Considering the pathophysiological significance of both GPCR mediated and direct EGFR signaling, the discovery of a pathway connecting these receptors gave rise to extensive studies of said pathway.

This study demonstrated the direct involvement of LPA receptors in the EGFR signal transactivation in different cellular contexts. It was demonstrated that the LPA receptors activate more that one pathway leading to wound closure. Furthermore, the pathway linking GPCR stimulation to the activation of the involved metalloprotease was investigated.

4.1 Reconstituted LPA receptor Edg2 expression in McA-RH7777 cells activates a TMPS like mechanism

In order to study the role of LPA receptors in the EGFR transactivation pathway and to assign subtype specific action of the LPA receptors Edg2, 4 and 7, a LPA receptor deficient dell line was transfected with Edg2, Edg4 or Edg7. Previously, it was shown that ectopic expression of Edg2, 4 and 7 in the LPA receptor deficient rat hepatoma cell line MCA-RH7777 leads to functional LPA receptor signaling such as intracellular calcium mobilization (An et al., 1998b).

The basal phosphorylation of the EGFR was elevated in the McA-RH7777 cell lines stably expressing Edg2 and 4, but LPA stimulation did not lead to significant EGFR activation (Figure 13). In Edg2 overexpressing McA-RH7777 cells, LPA stimulation restored Erk 1/2 activation (Figure 14). The activation of Erk 1/2 was sensitive to treatment by Pertussis toxin (PTX), which specifically inhibits $G\alpha_{i/o}$ subunits, confirming previous studies which suggested LPA mediated signaling to be often coupled to $G\alpha_i$ subunits (Takuwa et al., 2002). Erk 1/2 activation was independent of src kinase and PI3K function. Interestingly, the metalloprotease inhibitor Batimastat and the EGFR inhibitor AG1478 as well as the HB-EGF and Pro-Amphiregulin inhibitor Heparin lead to abrogation of the LPA induced Erk 1/2 activation. Akt 1/2 was also not activated subsequent to LPA stimulation (Figure 15).
Summarizing the above, the LPA receptors, especially Edg2, activated the MAPK pathway by a src family kinase, Akt 1/2 and PI3K independendent, but metalloprotease and EGF-like ligand dependent pathway. Although EGFR activation could not be detected, Erk 1/2 activation was inhibited by the EGFR specific inhibitor AG1478. On the basis of these findings, the involvement of a TMPS pathway leading to the activation of Erk 1/2 was suggested, although the activation of the EFGR itself could not be demonstrated (Figure 13).
Two possible explanations for this phenomenon were the following. Either the AG1478 was not as specific for the EGFR in rat cells as in human cells hinting at the involvement of a closely related receptor tyrosine kinase or a comparably weak activation of the EGFR was able induce signaling to Erk 1/2 in McA-RH7777 cells. This hypothesis seemed more plausible since also inhibition of the shedding of ligands specifically activating the EGFR abrogated Erk 1/2 activation.

4.2 LPA receptor Edg2 function is essential for EGFR signal transactivation

Recently, the LPA receptor specific inhibitor Ki16425 was discovered, which is functional both in inhibiting short term functions of LPA receptors and long-term actions of LPA stimulation such as DNA synthesis and migration (Ohta et al., 2003; Yamada et al., 2004). Previous studies reported that LPA and autotaxin stimulated cell motility of neoplastic and non-neoplastic cells through Edg2 can be inhibited by Ki16425 (Hama et al., 2004). On the contrary, some studies suggested receptor-independent actions for LPA (Hooks et al., 2001).

Hence, it was necessary to confirm that the LPA receptors were directly involved in the LPA induced transactivation of the EGFR.
This study clearly demonstrated that in kidney cancer and breast cells Ki16425 abrogated LPA induced EGFR signal transactivation and therefore proved, for the first time, the direct involvement of LPA receptors, especially Edg2, in the EGFR signal transactivation pathway (Figure 24 and Figure 33).

4.3 LPA activates two distinct pathways leading to wound closure

Various studies support the assumption that the EGFR acts as a central integrator of diverse GPCR signals (Carpenter, 1999; Gschwind et al., 2001).
Wound healing is a complex process featuring cell growth and motility as well as chemotaxis. Previous reports have shown that LPA enhances *in vitro* wound closure and invasion in ovarian cancer cells (Xu et al., 1995). Additionally, it was observed that LPA stimulates closure of wounded monolayers of human endothelial cells (Lee et al., 2000). *In vivo* data in bladder showed that wound healing is mediated by the EGFR and HER2 via processing of EGF, TGFα or amphiregulin (Bindels et al., 2002). Metalloproteinase-dependent EGF-like ligand shedding has been reported to mediate EGFR transactivation and migration of vascular smooth muscle cells (Eguchi et al., 2003). In colon cancer EGFR transactivation by Prostaglandin E2 regulates cell migration and invasion (Buchanan et al., 2003). Furthermore, it was suggested that LPA is a critical factor regulating motility of pancreatic cancer cells (Yamada et al., 2004) and the invasion of ovarian cancer cells (Fishman et al., 2001). Other studies indicated the involvement of HB-EGF contributing to migration of prostate cancer cells (Madarame et al., 2003) and the modulation of invasion of metastatic breast cancer cells by amphiregulin (Kondapaka et al., 1997). The metalloprotease ADAM17/TACE was identified as a key element of GPCR-EGFR crosstalk promoting cancer cell motility (Gschwind et al., 2003). Recently, the involvement of ADAM 15 in the migration of mesangial cells was demonstrated (Martin et al., 2002). The TMPS pathway is believed to play a crucial role in the regulation in cell motility and wound closure.
Here, we present data supporting that GPCR-induced EGFR signal transactivation is one pathway leading to wound closure in kidney and breast cancer cells. The LPA receptor itself as well as metalloprotease and EGFR function were demonstrated to be involved in the migratory behavior of A498, BT549 and MDA-MB 231 cells after LPA stimulation

(Figure 25, Figures 34-36). In agreement with these results, Price et al. showed that the breast cancer cell line MDA-MB 231 reacts with a potent chemotactic response but not with a proliferative response to EGF (Price et al., 1999).

The LPA receptor inhibitor Ki15425 completely abrogate the chemotactic behavior of kidney and breast cells while the inhibition of the TMPS pathway only reduced wound closure (Figures 25 and 36). From these results, it was concluded that two different pathways are activated upon LPA stimulation, both resulting in chemotaxis. In contrast, Thrombin stimulation did not induce wound closure in A498, and BT549 cells even though it is able to activate the EGFR (Figure 25, Figure 36).

Taken together, the results of this study indicated that LPA induced chemotaxis of A498, BT549 and MDA-MB-231 cells was regulated by both EGFR independent and dependent signaling pathways. Therefore, the Triple-Membrane-Passing signal (TMPS) mechanism of EGFR signal transactivation provides only one molecular explanation how GPCR ligands regulate chemotaxis and wound closure in breast and kidney cancer cells.

4.4 Combination of LPA and Thrombin leads to enhanced EGFR signal transactivation in A498 cells

LPA was previously described to induce EGFR tyrosine phosphorylation in several model systems including Rat1 (Daub et al., 1996), HEK293 (Della Rocca et al., 1999), PC-12 (Kim et al., 2000), Swiss 3T3 (Gohla et al., 1998), HaCaT and COS7 (Daub et al., 1997). Three LPA receptors of the Edg receptor family with differences in G protein-coupling are described.

In A498 cells, LPA stimulated EGFR signal transactivation was not inhibited by PTX (Figure 16) suggesting predominantly PTX-insensitive G protein subunits to be involved. On the contrary, the Thrombin mediated EGFR signal transactivation was PTX-sensitive and therefore was assigned to the $G\alpha_{i/o}$ family (Figure 19).

While LPA induced EGFR signal transactivation was mediated by ADAM17 and HB-EGF, GPCR-EGFR crosstalk stimulated by Thrombin involved ADAM10 and an EGF-like ligand different from HB-EGF, since Heparin was not able to block the TMPS pathway (Figure 18). Additionally, this study demonstrated that LPA mediated GPCR-EGFR crosstalk involved a src kinase activity upstream of the EGFR (Figure 17). This confirmed previous findings that

Src kinase was involved in GPCR-EGFR cross communication (Guerrero et al., 2004) and regulated downstream signaling via Erk1/2 and PI3-K/Akt (Das et al., 2004).
This study proved that both transactivation pathways converge only at the EGFR. In double stimulation experiments with LPA and Thrombin, a proportionally increased EGFR signal was detected, indicating that the amount of EGFR on the cell surface was not limiting in this experiment (Figure 19). To verify the involvement of preclustering of the metalloprotease with the GPCR and/or the EGF-like ligand additional lipid raft experiments will be necessary in the future.

4.5 Thrombin mediated EGFR signal transactivation is abrogated by deregulation of ADAM10

Metalloprotease mediated ectodomain shedding of growth factor precursors *in vivo* is not yet completely understood. Growing evidence suggested the involvement of several members of the ADAM family of zinc-dependent proteases in the processing of EGF-like precursors (Werb and Yan, 1998). ADAM9 is implicated in the shedding of proHB-EGF by TPA in Vero-H cells (Izumi et al., 1998), while LPA-induced proHB-EGF cleavage in the same cell system is independent of ADAM9 (Umata et al., 2001). The severe phenotype of mice lacking ADAM17 suggests an essential role for this metalloprotease in the processing of proTGFα and possibly other EGFR ligands in normal development. Absence of functional ADAM17 results in impaired basal cleavage of proAR and proHB-EGF in murine fibroblasts (Merlos-Suarez et al., 2001; Sunnarborg et al., 2002). Recently, Lemjabbar et al. described ADAM17 to be the responsible sheddase of proAR in the lung cancer cell line NCI-H-292 after incubation of the cells with tobacco smoke (Lemjabbar et al., 2003). Recent reports implicate ADAM10 (Lemjabbar and Basbaum, 2002; Yan et al., 2002) and ADAM12 (Asakura et al., 2002) in proHB-EGF-dependent EGFR signal transactivation in MDA-MB 231 cells. The detailed regulation of the metalloprotease's specificity remains unclear. Erk has been shown to bind and phosphorylate the cytoplasmic tail of ADAM17 at threonine 735 in response to TPA stimulation (Diaz-Rodriguez et al., 2002). Experiments with chimeras of TGFα and APP propose that the secondary structure of the juxtamembrane region is the important recognition element for the sheddase (Arribas et al., 1997).
This study provided further support for ADAM10 playing a key role in Thrombin induced transactivation of the EGFR in A498 cells since the deregulation of ADAM10 by the

expression of ADAM10siRNAs lead to abrogation of Thrombin induced EGFR signal transactivation (Figures 21 and 22). In order to examine the role of different domains of ADAM10 in the activation of the catalytic domain and the shedding of EGF-like ligands, A498 cells stably expressing an ADAM10siRNA construct were established. Interestingly, long-term silencing of ADAM10 with a siRNA construct targeting the 3'UTR of ADAM10 also lead to downregulation of ADAM17 expression which made it impossible to ascribe the observed effects to the single action of ADAM10 (Figure 23). This finding was in conformity with recent findings that long-term siRNA silencing can lead to off-target effects, especially downregulation of closely related family members (Scherr and Eder, 2004).

4.6 Inhibitory antibodies against ADAM proteins

Antibodies are potent therapeutics in cancer treatment whereas low molecular weight compounds are often less specific and lead to side effects during therapy. The monoclonal antibody Trastuzumab targeting HER2 in combination with conventional chemotherapy leads to increased survival rates of breast cancer patients (Finn and Slamon, 2003; Ropero et al., 2004). Acute myeloid leukemia (AML) cell growth can be inhibited by mono-specific and bi-specific anti-CD33 x anti-CD64 antibodies (Balaian and Ball, 2004). Recently, a monoclonal antibody against the EGF-like ligand cripto was identified to react with various cancer, but not normal cells thus providing a novel target for antibody-based immunotherapy (Xing et al., 2004).

In this study, specific antibody sera against ADAM10 and ADAM12 were generated (Figures 9 and 10). In A498 cells, Thrombin induced EGFR signal transactivation is mediated exclusively by ADAM10, while ADAM17 mediated transactivation is induced by LPA (Hart, 2004; Schafer, 2004; Schafer et al., 2004a).

Hence, the ADAM10,MP polyclonal serum was tested for its ability to interfere with Thrombin mediated EGFR signal transactivation in this cellular context. The serum was able to block the GPCR-EGFR cross communication, but this effect could not be assigned to a specific binding of ADAM10,MP antibodies as the antibody also interfered with LPA induced activation of ADAM17 (Figure 20). Additionally, the preimmune serum showed a similar effect. Therefore, the inhibition of the TMPS pathways leading to EGFR signal transactivation was unspecifically blocked by serum components.

Once a cell line is discovered, in which transactivation of the EGFR stimulated by one agent is solely mediated by ADAM12, the ADAM12 serum can be tested for its inhibition properties.

4.7 cDNA array analysis of breast cancer cells

cDNA array analysis is a potent method to identify proteins involved in cancer development. Normal and cancer samples can be directly compared and abnormal gene expression can be detected. Especially for breast cancer it is studied whether gene chip analysis can help to predict treatment success (Hampton, 2004). But this method can be used on any type of cancer for which important tumor markers are identified.

LPA was able to induce GPCR-EGFR cross communication in both normal and highly invasive breast cancer cell lines examined suggesting that EGFR signal transactivation plays an important role in transformed and non-transformed cells (Figure 30). Mammary epithelial cells revealed a comparably weak GPCR-induced stimulation of EGFR phosphorylation which was attributed to the low EGFR expression level in normal and highly invasive breast cancer cell lines as detected in the cDNA array analysis (Figure 32).

Knyazev has previously shown by cDNA array analysis that the LPA receptor Edg2 is overexpressed in a large variety of breast cancer cell lines (Figure 26).

The data from this cDNA array analysis provided further evidence for the correlation of Edg2 with the invasive potential of breast cancer cells and the EGFR signal transactivation mechanism, because the LPA receptor Edg2 was observed to cluster with proteins which are suggested to be involved in the TMPS pathway in breast cancer cells. Edg2 was part of one closely related cluster of genes with similar regulation of expression together with the metalloproteases ADAM12 and MMP14, the metalloprotease inhibitors TIMP1, 2 and 3 and the EGFR ligand HB-EGF (Figure 31).

The normal breast epithelial cell line MCF10A and the highly invasive cell lines HS578T, BT549 and MDA-MB 231 expressed normal to low amounts of the EGFR and HER2, while the weakly invasive cell line BT20 exhibited extremely high expression of the EFGR and HER2, which explained the high basal activity of the EGFR in these cells.

The expression pattern correlated with the transactivation behavior: BT20 with a high basal EGFR expression and low expression of other components of the TMPS pathway did not

induce GPCR-EGFR crosstalk upon LPA stimulation. On the contrary, LPA induced EGFR signal transactivation in the other cell lines exhibiting an inverse gene expression pattern.
The comparison of the gene expression pattern of LPA-stimulated and non-stimulated starved breast cancer cells as well as breast cancer cells in normal growth inhibited with the broad spectrum metalloprotease inhibitor Batimastat or DMSO as control did not exhibit major changes in the gene expression pattern determined in dot blot analysis (data not shown). From these results, it was concluded that persistent stimulation with GPCR agonists or repression of metalloprotease activity did not alter the gene expression of TMPS pathway related components in the investigated time frame of six hours. On the other hand, normal and breast cancer cells were clearly separated on the basis of their gene expression pattern. As overexpression of components of the TMPS pathway correlates with the invasive potential of BT549, HS578T and MDA-MB 231 cells, the GPCR-EGFR crosstalk is presumably involved in the invasive behavior of breast cancer cells.

4.8 The involvement of G protein subunits in the TMPS pathway

Many studies suggest Gα subunits to be involved in oncogenic signaling (Dhanasekaran et al., 1998; Gudermann et al., 2000; Neves et al., 2002). Constitutively active mutants of Gα subunits encode transforming oncogenes such as *gsp*, *gip2* and *gep* (Dhanasekaran et al., 1998; Landis et al., 1989; Lyons et al., 1990; Marinissen and Gutkind, 2001; Radhika and Dhanasekaran, 2001; Xu et al., 1993) suggesting that Gα subunits are key regulators of the EGFR signal transactivation.
As Pertussis toxin (PTX) abrogates EGFR signal transactivation in many cell lines, it is presumed that Gα subunits, especially $G\alpha_{i/o}$ subunits, are involved in the EGFR signal transactivation (Gschwind et al., 2003; Hart, 2004).
While many studies focus on the Gα subunits, comparably little is known about the signaling events induced by Gβγ subunits (Jones et al., 2004). Gβγ subunits are implicated in intracellular calcium mobilization, stimulation of PI3K and cyclic AMP production.
This study investigated the involvement of G protein subunits in the TMPS pathway. Overexpression of $G\alpha_{i2}$, Ga_q, $G\alpha_{12}$ and $G\alpha_{13}$ subunits as well as their constitutively active mutants did not significantly enhance EGFR signal transactivation in COS7 cells. Neither ligand-dependent nor independent EGFR signal transactivation was observed for any of the

studied Gα subunits (Figure 37 and Figure 38), but as there exist at least 23 Gα subunits derived from 17 different genes the number of possibilities are extremely high.

Gβγ subunits are suggested to play an important role in the Gi mediated activation of the MAPK Erk 1/2 (Crespo et al., 1994; Faure et al., 1994). In mammals, 6 Gβ and 12 different Gγ subunits are expressed.

Among these, this study focused on the actions of the $G\beta_1\gamma_2$ subunit in the EGFR signal transactivation. Coexpression of $G\beta_1$ and $G\gamma_2$ was performed, because it was discovered that only the Gβγ dimer is sufficiently stable (Luttrell et al., 1997). Overexpression of the $G\beta_1\gamma_2$ subunit was able to activate the EGFR and the downstream target Erk 1/2 in a ligand-independent manner in Rat1 and HEK293 cells (Figures 40 and 41). In SCC9 and COS7 cells EGFR activation due to $G\beta_1\gamma_2$ overexpression could not be observed. Therefore, the $G\beta_1\gamma_2$ subunit was able to mediate EGFR signal transactivation and subsequent Erk 1/2 activation in the cellular context of Rat1 and HEK293 cells (Figure 40 and Figure 41).

4.9 Perspectives

Given the importance of EGFR signaling pathways in pathophysiological disease, the factors leading to the EGFR signal transactivation, especially the pathway resulting in the activation of the metalloprotease have to be elucidated.

The mechanism leading to the specificity of the ADAM proteins for different EGF-like ligands requires further analysis as the same ADAM has varying specificity in different cell lines. One possible strategy might be to identify intracellular binding partners of ADAM protein using a membrane-based yeast two hybrid screen or pull-down assays.

It was shown that the β2-adrenergic receptor co-immunoprecipitates with the EGFR receptor in stimulated COS7 cells suggesting the formation of a macromolecular complex containing other elements of the TMPS pathway (Maudsley et al., 2000). This preclustering of GPCRs with ADAM metalloproteases and EGF-like ligands could be an explanation for the variation in specificity of the metalloprotease depending on the stimulus, even though other EGF-like ligands are present. Lipid raft experiments as well as FRET (Fluorescence Resonance Energy Transfer) or BRET (Bioluminescence Resonance Energy Transfer) analysis are further options to verify this hypothesis. In addition, it needs to be clarified if the activity of the metalloprotease is regulated by phosphorylation events.

A broad spectrum siRNA screening can help to further elucidate the role Gα and Gβγ subunits in the EGFR signal transactivation pathway. Additionally, it would be interesting to study the involvement of other second messengers such as Ca^{2+}-influx. Further, the role of the recently described GPCR oligomerization in the EGFR signal transactivation pathway is of great interest (Breitwieser, 2004).

As stimulation of the LPA receptors does not solely lead to the transactivation of the EGFR signal pathway, the importance of both independent pathways in cancer cell development have to be further and differentially analyzed. If there is a shift mechanism leading to an increase in signal transduction via the EGFR, this mechanism needs to be identified.

Specific inhibition of single components of the TMPS using low molecular weight inhibitors or blocking antibodies can help to gain new insights into the physiological role of this pathway and will provide novel therapeutic targets for disease intervention.

Hardly any *in vivo* data exists that provides support for the relevance of the EGFR signal transactivation pathway. Further, *in vivo* studies of the EGFR transactivation pathways can substantiate the data collected in *in vitro* studies and prove the relevance of the EGFR signal transactivation pathway. EGFR knockout mice have a similar phenotype like TACE knockout mice (Hansen et al., 1997). Furthermore, it was demonstrated that TACE regulates TGFα ligand availability *in vivo* (Peschon et al., 1998). HB-EGF knockout mice which mostly die early have enlarged, dysfunctional hearts and poorly differentiated lungs (Jackson et al., 2003). An ADAM 10-deficient mouse displays multiple defects of the central nervous system, the somites and the cardiovascular system (Hartmann et al., 2002). Triple null mice lacking EGF, amphiregulin, and TGFα are growth retarded and showed intestinal defects (Troyer et al., 2001). Mice deficient for three LPA receptors show dysmorphism of the head, semilethality due to defective suckling behaviour and generation of a small fraction of pups with frontal haematoma (Contos et al., 2002). Further analysis of the EGFR pathway in these knock out mice can confirm the existing *in vitro* data and will give rise to the validation of targets of anti-tumor therapy and lead to the development of novel intervention therapies.

5 Summary

The most important results from this study were the following:

Reconstitution of LPA receptor expression in McA-RH7777 lead to Erk 1/2 activation induced by LPA stimulation depending on a metalloprotease, an EGF-like ligand and RTK activity, but was independent of PI3K and src kinase function. Therfore, the activation of a TMPS-like pathway was suggested although the direct involvement of the EGFR could not be demonstrated.

In A498 cells, the amount of EGFR on the cell surface was not a limiting factor in the EGFR transactivation pathway as double stimulation of two independent pathways converging at the EGFR was not limited. The involvement of ADAM 10 in Thrombin mediated EGFR signal transactivation could be demonstrated by expression of a siRNA construct in A98 cells.

In invasive breast cancer cells Edg2 was upregulated and for the examined breast derived cells Edg2 possessed a gene regulation which was similar to the metalloproteases MMP14 and ADAM12 as well as the metalloprotease inhibitors TIMP1, 2 and 3 and the EGF-like ligand HB-EGF. These genes were observed in one closely related cluster in cDNA array analysis. The gene regulation of the EGFR is part of the same cluster as its preferred dimerisation partner HER2. However, they were found in a cluster separate from the other components of the TMPS pathway. The EGFR expression levels were inversely correlated to the invasive potential of these breast cancer cells.

For the first time, the direct involvement of the LPA receptor Edg2 in the EGFR signal transactivation pathway was demonstrated both for breast and kidney cancer cells with the LPA receptor inhibtor Ki16425. LPA induced wound closure in these cells was not exclusively mediated by the EGFR transactivation pathway as the direct inhibition of the LPA receptor Edg2 completely abrogated wound closure, while in comparison the broad spectrum metalloprotease inhibitor BB94 and the EGFR inhibitor AG1478 merely lead to reduced wound closure.

While overexpression of the $G\beta_1\gamma_2$ subunit ligand-independently activated the EGFR signal transactivation pathway in a certain cellular context, the Gα subunits and their oncogenic mutants did not activate the TMPS pathway neither ligand dependent nor independent.

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7 Abbrevations

Ab	Antibody
ADAM	A disintegrin and metalloprotease domain
Amp	Ampicilline
Amp_r	Ampicilline resistence
APS	Ammoniumpersulfate
AR	Amphiregulin
ATP	Adenosintriphosphate
bp	Base pairs
BSA	Bovine serum albumin
°C	Degree celsius
cAMP	Cyclic adenosinmonophosphate
Ca^{2+}	Calcium Ions
CaM Kinase	Ca^{2+}-calmodulin-dependent kinase
c-fos	Cellular homologue to v-fos (FBJ murine osteosarcoma viral oncogene)
c-jun	Cellular homologue to v-jun (avian sarcoma virus 17 oncogene)
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
DN	Dominant negative
DMSO	Dimethylsulfoxide
DNA	Desoxyribonukleic acid
dsDNA	Dooble-stranded DNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethlendiamintetraacetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
Fig	Figure
g	Gramm
Gab1	Grb2-associated binder-1
Gab2	Grb2-associated binder-2
GDP	Guanosindiphosphate
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor binding protein 2
GST	Glutathion-S-transferase
GTP	Guanosintriphosphate
h	Hour
HA	Hemagglutinin
HB-EGF	Heparin-binding EGF-like growth factor

H_2O_{bidest}	Twice-destilled, deionised Water
HEPES	N-(2-Hydroxyethyl)-piperazin-N‘-2-Ethansulfonic acid
HER	Human EGFR-related
HNSCC	Head and neck squamous cell carcinoma
Ig	Immunglobulin
IP	Immunoprecipitation
IP_3	Inositol-1,4,5-trisphosphate
IPTG	Isopropyl-ß-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
kb	Kilobase
kDa	Kilodalton
l	Liter
LPA	Lysophosphatydic acid
µ	Micro
m	Milli
M	Molar
MAP	Mitogen-activated protein
MAPK	MAP kinase
MBP	Myelin basic protein
MEK	MAPK/ERK Kinase
min	Minute
MMP	Matrix metalloprotease
n	Nano
OD	Optical density
p.a.	Per analysis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	Polyethylenglycole
PI 3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-diphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl-fluoride
PNPP	p-Nitrophenyl-phosphate
PTX	Pertussis toxin
PY	Phospho-tyrosine
Raf	Homologue to v-raf (murine sarcoma viral oncogene)
Ras	Homologue to v-ras (rat sarcoma viral oncogene)
RNA	Ribonucleic acid
rpm	Rotations per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
SAPK	Stress-activated protein kinase
S. D.	Standard deviation
SDS	Natriumdodecylsulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sec	Second

SH2, 3	domain Src homology 2, 3 domain
SHP-2	SH2-containing PTP-2
Sos	Son of sevenless
src	Homologue to v-src (sarcoma viral oncogene)
TACE	TNFα-converting enzyme
TCA	Trichloroacetic acid
TGFα	Transforming growth factor alpha
TEMED	N, N, N‘, N‘-Tetramethyletylendiamine
Tet	Tetracycline
TNFα	Tumor necrosis factor alpha
TPA	12-O-Tetradecanoyl-phorbol-13-acetate
Tris	Tris(hydroxymethyl)aminomethan
Tween 20	Polyoxyethylensorbitanmonolaureate
U	Enzymatic activity unit
o/n	Overnight
UV	Ultraviolett
V	Volt
Vol	Volume
wt	Wild type

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