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Receptor Tyrosine Kinase Activation in Human Carcinoma Cells

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Erklärung

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For my Mum and Dad

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1. Introduction

The ability of mammalian cells to respond to a wide variety of extracellular signals is essential for multicellular organisms during embryonic development and adult life. These responses are coordinated through signal transduction pathways that transduce and exchange information between different cells or inside the cell between different compartments. Apart from direct cell-cell contacts signalling to neighbouring but also distant cells occurs by secreted messenger molecules, such as growth factors and hormones. These molecules bind to their cognate receptor and thereby transmit the signal inside the target cell to finally stimulate a distinct biological response including cell proliferation, migration, differentiation or apoptosis. Consequently, deregulated signal transduction events have been recognized as the underlying causes of many severe human diseases such as cancer, diabetes, immune deficiencies and cardiovascular diseases, among many others (Hanahan and Weinberg, 2000; Schlessinger, 2000; Shawver et al., 2002).

Intensive research efforts focused on the elucidation of these signalling pathways, and as increasingly larger numbers of cell signalling components and pathways are being identified and studied it has become apparent that cellular signalling pathways are not isolated from each other as linear tracts but are highly interconnected to form complex signalling networks.

Reversible protein phosphorylation has been identified as a key element in signal transduction processes (Cohen, 2002). Phosphorylation by the combined action of a protein kinase and a protein phosphatase can reversibly alter the activity of an enzyme, target proteins for degradation or increase its stability, alter its subcellular localization or change its affinity towards interacting proteins. One third of all mammalian proteins can be phosphorylated, and the sequencing of the human genome revealed that there are approximately 520 protein kinases and 130 protein phosphatases encoded by human genes (Blume-Jensen and Hunter, 2001; Manning et al., 2002). Both protein kinases and phosphatases can be subdivided into cellular and transmembrane molecules, and according to their substrate specificity into tyrosine, serine/threonine or dual specific kinases. In particular tyrosine phosphorylation has been early recognized as a major mechanism of transmembrane signalling (Cohen, 2002).

1.1. Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) represent membrane spanning cell surface receptors consisting of a glycosylated extracellular domain, a single transmembrane portion and an intracellular domain with a protein kinase core that catalyzes the transfer of the γ-phosphate of

bound ATP to the C-terminal domains and the tyrosine residues of exogenous substrates. Tyrosine kinase receptors dimerize after binding their ligands and transphosphorylate selected tyrosine residues of the cytoplasmic domain, generating docking sites for intracellular signal transducers containing phosphotyrosine interaction domains. Different transducers are recruited and activated depending on the sequence of the amino acids flanking the tyrosines in the receptor-docking sites (Blume-Jensen and Hunter, 2001; Schlessinger, 2000).

20 subfamilies of RTKs have been described, comprising more than 50 different RTKs (Figure 1). In particular, the extracellular ligand-binding domain reveals the structural diversity of RTKs as different sequence motifs have been identified within this domain. These regions comprise cysteine-rich domains, EGF-like (epidermal growth factor-like) domains, immunoglobulin-like domains, cadherin-like domains or kringle-like domains among others (Figure 1) (Blume-Jensen and Hunter, 2001).

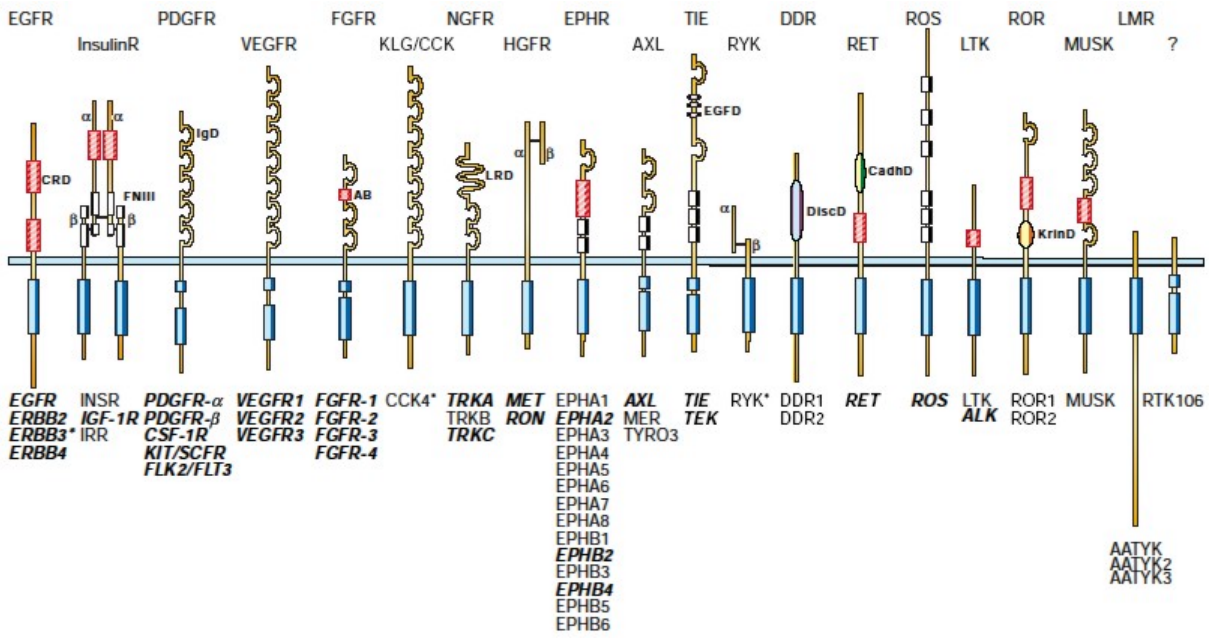

Figure 1 **Human receptor tyrosine kinases**. Abbreviations: AB, acidic box; CadhD, cadherin-like domain; CRD, cysteine-rich domain; DiscD, discoidin-like domain; EGFD, epidermal growth factor-like domain; FNIII, fibronectin type III-like domain; IgD, immunoglobulin-like domain; KrinD, kringle-like domain;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

1.1.1. The EGFR family

The epidermal growth factor receptor (EGFR) family consists of four members, besides the EGFR itself and its relatives HER2/neu, HER3 and HER4. The four family members share an overall structure of two cysteine-rich stretches in their extracellular region. While for the orphan receptor HER2 no ligand has been described so far, Her3 possesses an impaired kinase activity due to point mutations within the catalytic domain. HER2 is only activated following

its heterodimerization with another ligand-bound EGFR family receptor. However, HER2 is the preferred dimerization partner for EGFR, HER3 and HER4 (Graus-Porta et al., 1997) and HER2 containing dimers are responsible for strong and prolonged activation of downstream signalling pathways (Beerli et al., 1995; Graus-Porta et al., 1995).

The EGFR itself represents the most prominent RTK, as it was the first cell surface receptor and proto-oncogene product to be identified and molecularly cloned (Downward et al., 1984; Ullrich et al., 1984). It has been implicated in cancer development and progression 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 (Gill, 1989). In particular HER2 gene amplification has been linked to breast cancer (Slamon et al., 1989), while the EGFR is frequently co-expressed with its ligands in different forms of human cancer, such as colon (Damstrup et al., 1999), lung and prostate (Seth et al., 1999) and epithelial cells (Dong et al., 1999).

1.1.2. EGF-like ligands and receptor activation

Under normal physiological conditions, activation of the EGFR family is controlled by spatial and temporal expression of their ligands, members of the EGF-related peptide growth factor family (Peles and Yarden, 1993; Riese and Stern, 1998). These peptides are produced as transmembrane precursors, and are released as soluble growth factors after proteolytical cleavage (Figure 2) (Massague and Pandiella, 1993). The mammalian ligands that bind the EGFR include epidermal growth factor (EGF), transforming growth factor α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), epiregulin (EPR) and epigen (Riese and Stern, 1998; Salomon et al., 1999; Strachan et al., 2001). Cripto represents the most recent and a very atypical member of the EGFR ligand family, since it is not synthesized as a transmembrane protein but secreted (Rosa, 2002). The neuregulins (NRG) bind to HER3 and HER4 (NRG1 and NRG2) or only HER4 (NRG3 and NRG4). Together, stimulation of the EGFR and its family members by EGF and its relatives provides a rich and diverse signalling network (reviewed in (Yarden and Sliwkowski, 2001)).

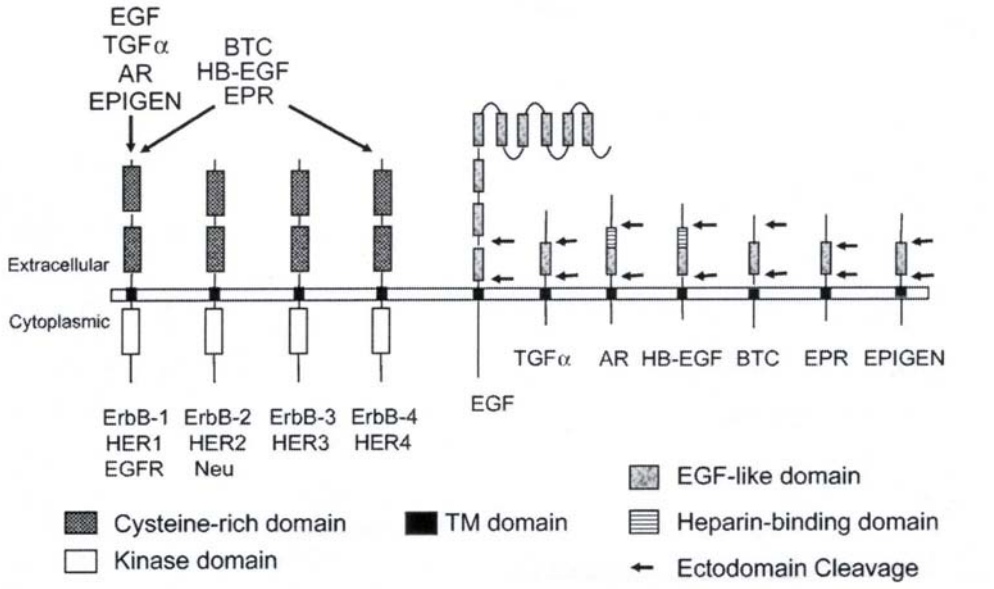

Figure 2 **The EGFR family and EGF-like ligands binding to the EGFR (Harris et al., 2003)**. The neuregulins are not shown. The EGF-like ligands that are capable of stimulating the EGFR are depicted, including their domain structure. The characteristic feature of this family of growth factors is that they are synthesized as transmembrane proteins that have to be processed to become fully active. (Jorissen et al., 2003)

Biophysical investigations revealed a 2:2 stoichiometry for ligand-receptor complexes (Lemmon et al., 1997). Recent structural studies shed new light on ligand-binding and receptor activation mechanisms (Garrett et al., 2002; Jorissen et al., 2003; Ogiso et al., 2002; Schlessinger, 2002), which support the concept of ligand-induced conformational changes leading to receptor dimerization rather than bridging of receptor monomers by ligand molecules. Receptor dimerization was shown to occur entirely receptor-mediated by a loop termed the "dimerization-loop", which is unique to EGFR family receptors. Deletions or mutations in this arm completely prevent ligand-induced EGFR activation (Garrett et al., 2002; Ogiso et al., 2002). The dimerization arm becomes exposed only after ligand-binding as the consequence of a domain rearrangement within the extracellular portion of the EGFR, thereby regulating receptor function. In contrast, the structure of HER2, which has no direct ligand, demonstrated that this receptor constitutively adopts an extended configuration with its dimerization arm exposed (Cho et al., 2003; Garrett et al., 2003). Together with biophysical studies indicating only weak homodimeric interactions of HER2 receptor molecules this observations suggest HER2 as being able to form only heterodimers with ligand-activated EGFR family receptors (Garrett et al., 2002).

1.1.3. Met - The receptor for hepatocyte growth factor

The Met receptor has been identified as the product of a human oncogene, *tpr-met* (Cooper et al., 1984; Park et al., 1987) containing a constitutively active Met kinase activity. The ligand for the Met RTK is the hepatocyte growth factor (HGF) (Giordano et al., 1989), which belongs to the scatter factor family. Since the original characterization as a proto-oncogene, the Met receptor has been shown as an important mediator of both, normal and neoplastic invasive growth. While the Met RTK is instrumental in developmental processes, deregulated Met receptor activity in many human cancers owing to genetic mutations, gene amplification, protein overexpression or production or HGF-dependent autocrine circuits has been shown (Maulik et al., 2002; Trusolino and Comoglio, 2002).

Stimulation of the Met receptor has been linked to cellular responses associated with an invasive behaviour, such as cell dissociation, migration, scattering and reduced cellular adhesion, but also stimulation of cell proliferation and survival has been demonstrated (Maulik et al., 2002). The Met RTK consists of a single membrane spanning ß-subunit, which is linked by disulfide bridges to an extracellular α-chain. The intracellular domain comprises a kinase activity and 2 tyrosine residues as autophosphorylation sites enhancing the intrinsic kinase activity upon phosphorylation, and the multi-substrate docking site containing 2 tyrosine residues that serve as signalling scaffolds for downstream effector molecules (Maulik et al., 2002). Another member of the scatter factor receptor family is the RTK RON, which is activated by its ligand macrophage-stimulating protein (MSP). Both the extracellular regions of Met and RON display structural similarities with semaphorins and plexins which together form a wide family of ligand-receptor pairs that were originally identified in the nervous system but that are now known to be widely expressed in other cell types (Giordano et al., 2002).

Recent investigations identified ß-catenin as a signalling partner of the Met receptor (Hiscox and Jiang, 1999a; Monga et al., 2002). In the cell β-catenin exerts a dual role. It functions as a member of cell adherence junctions, linking E-cadherin to the cytoskeleton allowing tight intercellular adhesion (Conacci-Sorrell et al., 2002). On the other hand, as a member of the Wnt-signalling pathway, ß-catenin can translocate into the nucleus to induce gene expression in complex with the transcription factor TCF. Independent of Wnt signalling HGF is able to induce ß-catenin phosphorylation (Hiscox and Jiang, 1999b; Shibamoto et al., 1994), leading to dissociation of the Met-ß-catenin complex, nuclear translocation of ß-catenin and concomitantly induction of TCF (T-cell factor) target genes (Monga et al., 2002; Muller et al., 2002).

1.2. RTK downstream signalling and protein interaction domains

RTK activation induces specific signal transduction pathways comprising various effector proteins inside the cell depending on the stimulus and the cellular context. Assembly and regulation of these multiprotein complexes is achieved through modular protein domains that specifically recognize other protein domains, protein modifications, lipids or DNA sequences. These protein interaction domains target proteins to a specific subcellular location, provide a means for recognition of posttranslational protein modifications or chemical second messengers, control the conformation, activity and substrate specificity of enzymes (Pawson and Nash, 2003). In general, a protein interaction domain recognizes a core determinant, with flanking or non-contiguous residues providing additional contacts and an element of selectivity. Phosphotyrosine residues are recognized by Src-Homology 2 (SH2) and phosphotyrosine-binding (PTB) domains, while SH2 domains represent the most prevalent type of phosphotyrosine binding domains. PTB domains are not restricted to bind to phosphotyrosine residues as they are also capable of binding to non-phosphorylated sequences. Apart from phosphotyrosine residues phosphoserine residues are recognized by WW and 14-3-3 domains, while FHA and WD40 domains bind to phosphothreonine residues. In addition to phosphorylated moieties, pleckstrin homology (PH), phox homology (PHOX), FERM and FYVE domains bind to phosphoinositides, and SH3 and WW domains recognize prolin-rich motifs. Figure 3 gives an overview of different protein interaction modules and their binding specificities.

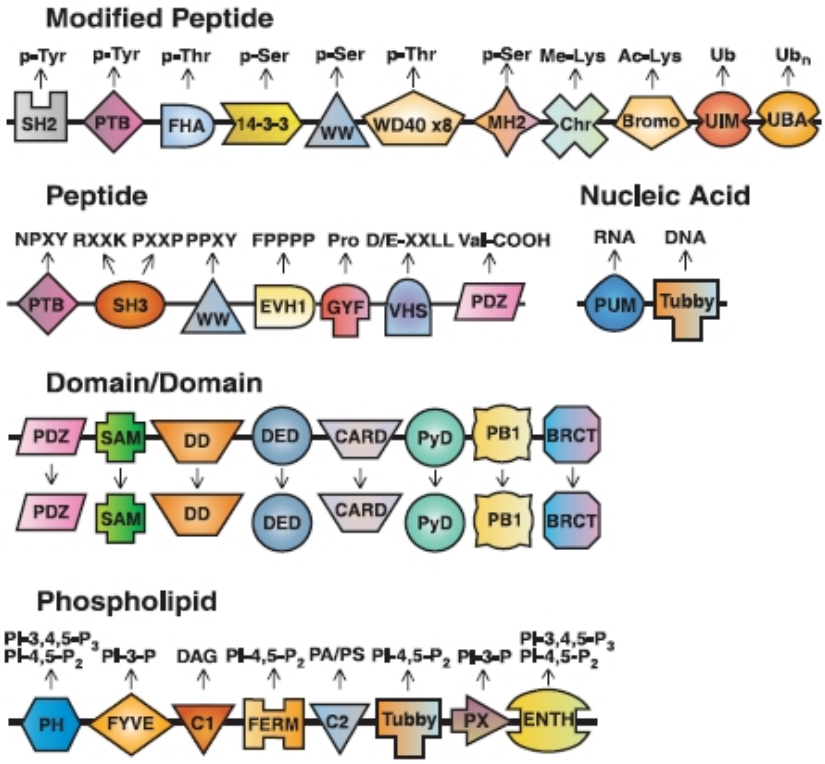

Figure 3 **Modular interaction domains as building blocks in signal transduction**. Interaction domains bind proteins, phospholipids, or nucleic acids. A subset of such domains is illustrated and their general binding functions are indicated. (Pawson and Nash, 2003)

A wide variety of proteins possesses both interaction domains and an enzymatic activity, such as the cytoplasmic kinase Src, containing SH2 domains and a kinase activity, or PLC-γ, containing both a SH2 domain and an phospholipase activity. In addition, signalling proteins consisting entirely of interaction domains have been described. Examples for these adaptor proteins comprise Grb2, Crk or Shc containing SH2 and SH3 domains to link activated RTKs to downstream signalling pathways such as the mitogen-activated protein kinases (MAPKs). In addition, docking proteins such as Gab1 become recruited to the cell membrane in response to activation of phosphatidylinositol-3 kinase (PI3K) by its PH domain (Schlessinger, 2000). Since RTKs are capable of recruiting a wide variety of adaptor proteins and downstream effectors, they have been considered as signalling platforms where specific subsets of signalling proteins are recruited.

1.3. G protein-coupled receptors

The cell surface receptor superfamily of G protein-coupled receptors (GPCRs) forms one of the largest protein families found in nature. A diverse array of external stimuli including neurotransmitters, hormones, lipids, photons, odorants, taste ligands, nucleotides and calcium

ions which can act through GPCRs give rise to remarkably diverse physiological functions. The characteristic structural feature of GPCRs, which possess no intrinsic enzymatic activity, are seven transmembrane helices of about 20-27 amino acids each. While the C terminus, the three extracellular and the three intracellular loops and the N-terminal extracellular portion vary in length, a weak correlation between ligand size and the length of the N-terminal portion has been observed, suggesting a role of this extracellular domain in ligand binding (Marinissen and Gutkind, 2001).

1.3.1. Heterotrimeric G proteins

GPCR stimulation leads to the activation of heterotrimeric G proteins by conformational changes that lead to exchange of GDP for GTP bound to the Gα-subunit and dissociation into Gα- and Gβγ-subunits. Both the Gα and Gβγ subunits activate effector molecules including second messenger generating systems (Hur and Kim, 2002; Marinissen and Gutkind, 2001). To date 16 α, 5 β and 12 γ proteins have been cloned. G proteins are generally referred to by their α subunits. On the basis of amino acid similarities, four distinct families of Gα subunits have been recognized, namely $G\alpha_s$, $G\alpha_q$, $G\alpha_i$ and G_{12} (Dhanasekaran et al., 1998; Hur and Kim, 2002; Pierce et al., 2001).

The signalling cascades induced by activated G proteins comprise adenylyl and guanylyl cyclase, calcium channels, c-Src tyrosine kinase, phosphodiesterases, phospholipases, GTPase-activating proteins (GAPs) such as Rap1GAP, guanine-nucleotide exchange-factors (GEFs) such as RhoGEF, PI3K and PKC, thereby influencing the levels of second messengers such as cyclic AMP and cyclic GMP, diacylglycerol, inositol (1,4,5)-triphosphate, phophatidyl inositol (3,4,5)-triphosphate or calcium levels or affecting e.g. stress fibre formation (Marinissen and Gutkind, 2001; Pierce et al., 2001).

1.3.2. The oncogenic potential of GPCRs and G proteins

Many potent mitogens such as thrombin, lysophosphatidic acid (LPA), bradykinin, acetylcholine receptor agonists such as carbachol, angiotensin II and others stimulate cell proliferation by acting on their cognate GPCRs in a variety of cell types. The discovery of the *mas* oncogene, whose protein product exhibits a typical heptahelical structure, provided a link between cellular transformation and GPCRs (Young et al., 1986). Moreover ectopic expression of GPCRs such as muscarinic acetylcholine M_1, M_3 and M_5 receptors could transform murine fibroblasts in an agonist-dependent fashion providing evidence that wild-type GPCRs can be tumourigenic when exposed to an excess of locally produced or

circulating agonists. Mutated GPCRs which are constitutively activated in an agonist-independent fashion have transforming abilities, such as the thyroid-stimulating hormone receptors, which occur naturally in 30% of hyperfunctioning thyroid adenomas, suggesting a direct link between GPCRs and human cancer (Marinissen and Gutkind, 2001; Parma et al., 1993). Although activating mutations are infrequent in GPCRs, these receptors can often contribute to neoplasia when persistently stimulated by agonists released from tumours, such as Gastrin-releasing peptide (GRP), neuromedin B, bradykinin, or cholecystokinin (CCK), which are secreted from small cell lung carcinoma which also express the cognate receptors and therefore exhibit autocrine or paracrine activation loops. Other GPCRs and their agonists have been implicated in colon adenocarcinomas, gastric hyperplasia and cancer (Marinissen and Gutkind, 2001). Consistent with the role for GPCRs in normal and aberrant cell growth, at least ten Gα subunits have been shown to harbour transforming potential, such as the *gsp* oncogene which encodes a GTPase-deficient $G\alpha_s$ (Marinissen and Gutkind, 2001).

1.4. Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) are important signal transducing enzymes connecting cell surface receptors to critical regulatory targets within the cell. They phosphorylate specific serine and threonine residues of target protein substrates thereby controlling cell proliferation, differentiation and transformation (Chang and Karin, 2001; Garrington and Johnson, 1999; Johnson and Lapadat, 2002). MAPKs also respond to chemical and physical stress, thereby controlling cell survival and adaptation. At least four distinctly regulated groups of MAPKs are expressed in mammals, extracellular-regulated kinase 1/2 (ERK1/2), Jun amino-terminal kinases (JNK 1/2/3), p38 proteins (p38 α/β/γ/δ) and ERK5 (Chang and Karin, 2001). Activation of ERK1/2 by RTKs represents the prototypic example for a MAPK cascade activation pathway consisting of a characteristic phosphorelay system in which a series of protein kinases phosphorylates and activates one another (Figure 4).

RTK stimulation leads to the recruitment of the adaptor protein Grb2 and association and activation of the RasGEF Sos, which in turn activates membrane-associated Ras. Ras in turn induces the serine/threonine kinase activity of the MAPK kinase kinase (MAPKKK) Raf-1 which phosphorylates and activates the MAPK kinases 1/2 (MAPKK, MEK 1/2). MEK1/2 finally activate ERK1/2 by phosphorylation of threonine and tyrosine residues in the regulatory TEY-motif (Robinson and Cobb, 1997). Subsequently ERK1/2 translocate into the nucleus to phosphorylate and activate transcription factors but also affect cytoplasmic or

transmembrane effector proteins, such as regulating transmembrane protein processing (Fan and Derynck, 1999) by phosphorylating the intracellular domain of the metalloprotease ADAM17 (Diaz-Rodriguez et al., 2002; Fan et al., 2003). JNK-family MAPKs are also known as stress-activated protein kinases as they are activated in respone to environmental stress but also by radiation and growth factors. p38 becomes activated in immune cells by inflammatory cytokines as a crucial part of the immune response, but is also stimulated by hormones, GPCR agonists and cellular stress such as osmotic and heat shock or chemotherapeutic agents (Benhar et al., 2002b; Chen et al., 2001b; Garrington and Johnson, 1999). Each MAPKK can be activated by more than one MAPKKK, increasing the complexity and diversity of MAPK signalling (Figure 4).

The mechanisms of MAPK activation by osmotic and oxidative stress have been intensely studied (Kyriakis and Avruch, 2001). Previous reports implicated the downregulation of antagonistic phosphatases as well as the regulation of scaffolding protein function in MAPK activation induced by stress agents (Benhar et al., 2002b; de Nadal et al., 2002). Moreover, small G-proteins have been demonstrated to play a role in the activation of MAPK by osmotic stress (de Nadal et al., 2002).

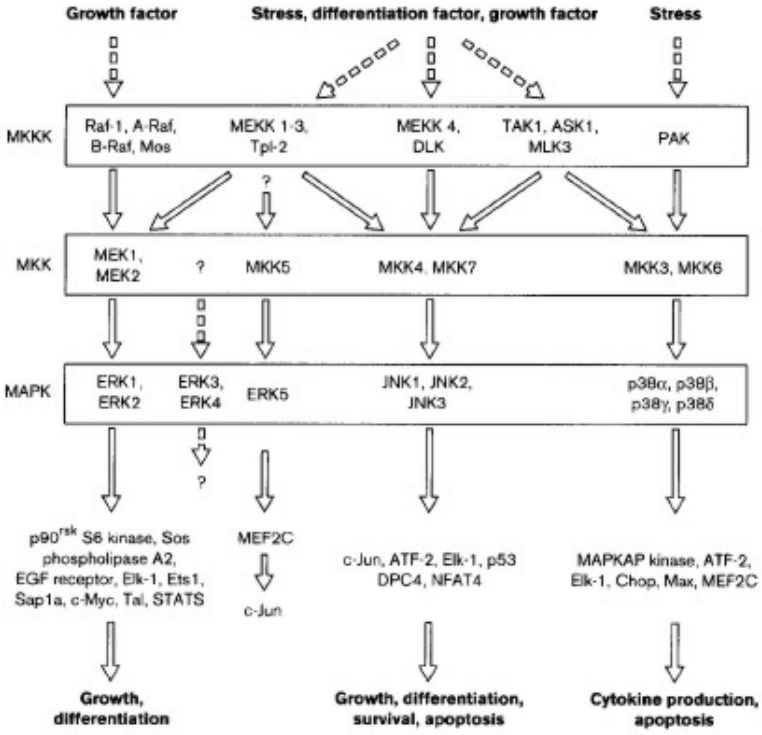

Figure 4 **Mitogen activated protein kinase modules**. Each module consists of a mitogen-activated proteine kinase kinase kinase (MKKK), a mitogen-activated proteine kinase kinase (MKK) and a MAPK. While the MKKs are relatively specific for their target MAPKs, MKKKs can activate one or more MKK. Activation of

MAPKS induces activation of different targets, comprising transcription factors but also for instance other kinases such as the EGFR. (Johnson and Lapadat, 2002)

1.5. RTK transactivation

Signal transduction pathways are organized as highly complex networks rather than linear signalling traits allowing communication and coordination of cellular signalling. Therefore increasing interest focuses on interreceptor communication mechanisms, since cross-communication between different classes of cell surface receptors allows the integration of diverse extracellular stimuli to a limited number of intracellular signal transduction pathways. Early on, the EGFR has been shown to be activated by a variety of different stimuli in addition to direct stimulation with its cognate ligands, such as stress stimuli, GPCR agonists, integrin activation, cytokine receptors or ion channels (Fischer et al., 2003; Gschwind et al., 2001; Prenzel et al., 2001). In addition, association of the EGFR with non-related RTKs such as the PDGFR has been proposed (Saito and Berk, 2001).

1.5.1. EGFR activation by cellular stress

Non-physiologic stimuli exerting cellular stress are capable of activating the EGFR, which has been frequently recognized as a signal transducer of stress stimuli to different downstream pathways (Carpenter, 1999; Prenzel et al., 2001). These stimuli include UV- or γ-irradiation, hyperosmolarity, reactive oxygen species or membrane depolarization (Coffer et al., 1995; King et al., 1989; Knebel et al., 1996; Rao, 1996; Rosette and Karin, 1996; Sachsenmaier et al., 1994), but also cellular stress induced by chemotherapeutic agents (Benhar et al., 2002a; Benhar et al., 2002b). In particular chemotherapeutic-induced stress signalling pathways are of special clinical interest since these pathways might provide molecular explanations for tumours resistance towards chemotherapy. The mechanism of stress-induced EGFR and also MAPK activation has entirely been attributed to EGF-like ligand-independent mechanism. Previous reportes implicated the inactivation of phosphatases by reactive oxygen species (ROS), which oxidize a critical cysteine residues in the catalytic pocket of the respective phosphatase, in the activation of stress signalling cascades (Knebel et al., 1996). Inhibition of negative regulation then results in an equilibrium shift from the non-phosphorylated to the phosphorylated state of the RTK (Meng et al., 2002; Salmeen et al., 2003). In addition, ligand-independent receptor activation has been suggested to involve non-specific clustering and internalization of the EGFR (Rosette and Karin, 1996).

1.5.2. EGFR signal transactivation

EGFR signal transactivation by GPCRs has been originally described by Daub and colleagues (Daub et al., 1996). Treatment of rat fibroblasts with lysophophatidic acid, endothelin-1 (ET-1) or thrombin leads to rapid and transient EGFR phosphorylation and subsequent activation of downstream signalling events such as mitogen-activated protein kinase (MAPK) phosphorylation or *c-fos* gene expression, which critically depend on EGFR function. Thereafter, various studies demonstrated that GPCR-induced EGFR signal transactivation occurs in a variety of cell types (Fischer et al., 2003; Gschwind et al., 2001).

Since no EGF-like ligands could be detected in conditioned cell culture media of GPCR ligand-stimulated cells, the transactivation mechanism was attributed to a ligand-independent and therefore intracellular mechanism. Different cytosolic signal transduction proteins have been implicated in the transactivation process.

Src-family tyrosine kinases have been suggested as both upstream and downstream mediators of the EGFR in the GPCR-induced transactivation. Besides Src kinases, the serine/threonine kinase PKC has been frequently suggested to be involved in EGFR signal transactivation. Moreover in different cellular systems the intracellular Ca^{2+}-concentration and the Ca^{2+}-regulated tyrosine kinase Pyk2 have been discussed as a mediator of EGFR signal transactivation (Eguchi et al., 1998; Keely et al., 2000; Soltoff, 1998; Venkatakrishnan et al., 2000; Zwick et al., 1997). Together, these reports did not clearly depict the role of cytosolic signalling proteins but rather suggest several cytoplasmic signalling molecules as mediators of the EGFR signal transactivation pathway depending on the cellular system and the signalling context.

Although different investigations suggested a ligand-independent intracellular signalling mechanism (Daub et al., 1997; Eguchi et al., 1998; Tsai et al., 1997), Prenzel and colleagues were the first to demonstrate the metalloprotease-mediated processing of the EGF-like ligand HB-EGF and therefore a ligand-dependent mechanism in EGFR signal transactivation (Prenzel et al., 1999), providing a convergence point for intracellular signalling proteins. Blocking both proHB-EGF and metalloprotease function abrogated GPCR stimulated EGFR, Shc and MAPK phosphorylation, revealing the involvement of zinc-dependent metalloproteases and the EGF-like ligand HB-EGF in the transactivation pathway. As processed HB-EGF is retained in the heparan sulphate proteoglycan matrix, the released ligand could not be detected in the cell culture supernatant. This signalling pathway was termed triple-membrane-passing signal (TMPS) as the stimulus is travelling three times through the membrane before it finally activates intracellular effector pathways. Since this

initial discovery, many reports revealed the broad relevance of this signalling mechanism within a variety of cellular systems and, importantly, pathophysiological signalling situations. As transmembrane ligand precursor molecules are a characteristic feature of the EGFR and its relatives, this transactivation mechanism is likely to be a common activation mechanism of the EGFR and the ErbB family members.

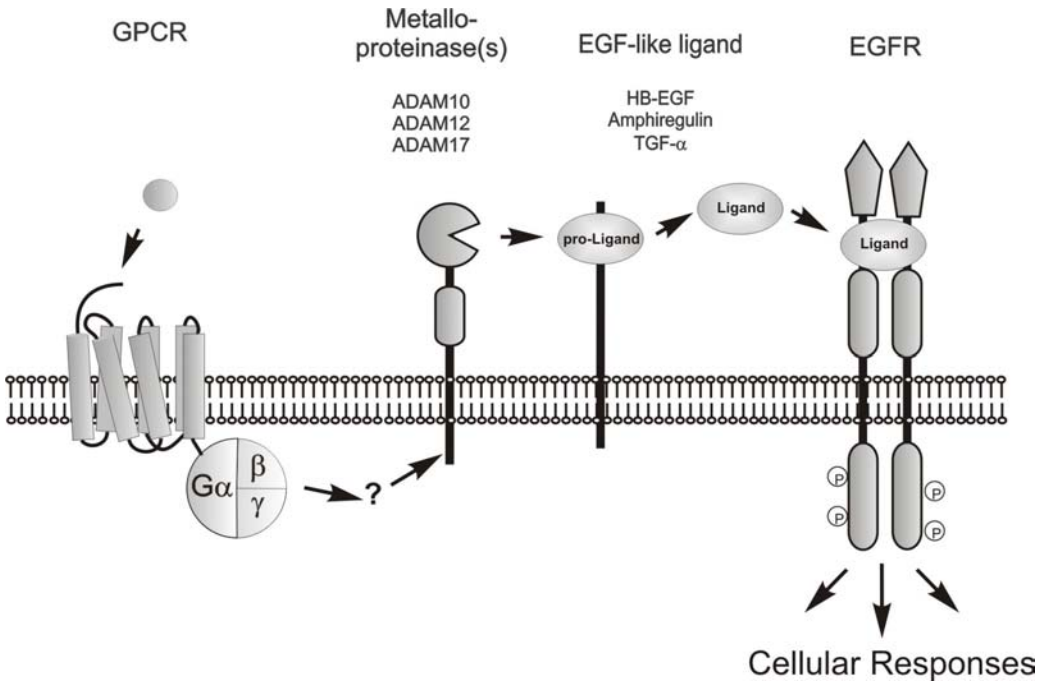

Figure 5 **Model of EGFR signal transactivation according to the triple-membrane-passing signal (TMPS)**. GPCR stimulation induces upregulation of a metalloprotease activity leading to ectodomain-cleavage of EGF-like growth factor precursor molecules. Subsequent release of the mature growth factor stimulates EGFR kinase activity transducing the GPCR signal finally inside the cell to stimulate EGFR characteristic downstream signalling pathways such as MAPK, PLC-γ, STAT or PI3K activation (Fischer et al., 2003).

1.5.3. ADAM family metalloproteases

Metalloproteases of the ADAM (a disintegrin and a metalloprotease domain) family consist of a cytoplasmic domain of variable length, a single transmembrane domain, and on the extracellular side of a catalytic, a cysteine rich and a disintegrin domain (Werb and Yan, 1998). The latter have adhesive properties by allowing interactions with integrins, heparan sulphate proteoglycans and extracellular matrix (ECM) proteins, while the metalloprotease domain can induce ectodomain shedding and cleavage of ECM. In addition the inactive pro-form contains a pro-domain which renders the protease inactive by blocking the essential zinc atom in the catalytic site by binding with a cysteine residue (White, 2003).

Members of the ADAM family of zinc-dependent metalloproteases have been frequently implicated in the processing of proEGF-like ligands and various other transmembrane

proteins (Werb and Yan, 1998; White, 2003). Studies using fibroblasts derived from TACE knock-out mice implicated ADAM17 in the release of TGF-α and other EGF-like ligands as well as the constitutive availability of these growth factors (Peschon et al., 1998; Sunnarborg et al., 2002). Furthermore, ADAM9 has been shown to release proHB-EGF after treatment of VeroH cells with tetra-doceanoyl-phorbol-13-acetate (TPA) (Izumi et al., 1998), while in the same cellular system LPA-stimulated processing of proHB-EGF is independent of ADAM9 (Umata et al., 2001), suggesting that proHB-EGF sheddases are defined by both the cellular context and the stimulus. The critical role of ADAM proteases in EGFR signal transactivation has been shown by Gschwind *et al.* who demonstrated LPA-induced processing of amphiregulin by ADAM17 in head and neck squamous cell carcinoma cells (Gschwind et al., 2003). Lemjabbar *et al.* reported that in the same cellular system different protease/ligand combinations can act as transducers of GPCR-induced EGFR stimulation (Lemjabbar and Basbaum, 2002; Lemjabbar et al., 2003). ADAM10 processing proHB-EGF has been implicated in EGFR signal transactivation in Cos7 cells (Yan et al., 2002). These findings provide evidence for the high diversity of signalling components employed in EGFR signal transactivation.

So far the identity of the signalling elements in between the GPCRs and the ADAM proteases remain elusive. Mechanistical studies on the regulation of cell surface shedding events implicated the MAPKs extracellular-regulated kinase 1/2 in the processing of TGF-α upon treatment with PGDF, FGF or EGF, while p38 was shown to control basal TGF-α shedding (Fan and Derynck, 1999). Moreover, different reports demonstrated phosphorylation of the intracellular domain of ADAM17 in response to TPA and growth factors (Diaz-Rodriguez et al., 2002; Fan et al., 2003). In contrast, Black and coworkers reported that the cytoplasmic tail of ADAM17 is dispensable for TPA-stimulated release of TNF-α and other substrates (Reddy et al., 2000).

1.5.4. Receptor cross-talk beyond EGFR signal transactivation

Cross-talk between cell surface receptors can occur in several different ways. The most direct mechanism is receptor heterodimerization which is well described for members of the EGFR family (Yarden, 2001) or Met and its relative RON (Follenzi et al., 2000). Also less related receptors have been shown to be able to associate such as EGFR and PDGFR (Saito et al., 2001) or Met and semaphorin receptors (Giordano et al., 2002). In the case of EGFR and the Met receptor, cross-communication has been reported to occur on different levels: EGFR signal transactivation induces metalloprotease-mediated ectodomain shedding of Met (Nath et

al., 2001) and aberrant EGFR activation has been shown to elevate Met expression and phosphorylation in thyroid carcinoma cells (Bergstrom et al., 2000). EGFR function has been implicated in HGF-induced hepatocyte proliferation (Scheving et al., 2002), and a possible heterodimer formation of both receptors has been suggested (Jo et al., 2000; Pai et al., 2003).
While EGFR signal transactivation represents the most prominent GPCR-RTK cross-talk mechanism, GPCR-induced RTK activation is not restricted to the EGFR. Other RTKs have been shown to be activated in response to GPCR stimulation, comprising the IGF-1 receptor (Rao et al., 1995), Trk receptor (Lee and Chao, 2001), platelet-derived growth factor receptor (PDGFR) (Herrlich et al., 1998; Linseman et al., 1995) and the vascular endothelial growth factor receptor (VEGFR) (Thuringer et al., 2002). In contrast to metalloprotease-mediated EGFR signal transactivation involving the release of EGF-like ligands, the transactivation mechanism described so far for other RTKs does not involve proteolysis of ligand precursor molecules, as their cognate growth factors are in most cases secreted rather than transmembrane molecules. Different mechanistic concepts have been proposed for ligand-independent RTK transactivation. Similar to early reports on EGFR signal transactivation cytoplasmic tyrosine kinases in particular of the Src family have been implicated in RTK transactivation, such as GPCR-induced activation of the Trk or FLK-1 receptor (Lee and Chao, 2001; Tanimoto et al., 2002). Moreover, increasing evidence implicates reactive oxygen species (ROS) as signalling intermediates in RTK activation (Bae et al., 1997; Rhee et al., 2000; Saito and Berk, 2001; Sundaresan et al., 1995; Ushio-Fukai et al., 2001).

1.5.5. Reactive oxygen species in signal transduction

An increase in ROS levels has been reported not only after RTK but also in response to GPCR stimulation in vascular smooth muscle cells or endothelial cells (Holland et al., 1998; Patterson et al., 1999). Angiotensin II-induced EGFR transactivation was shown to be blocked by reducing agents and therefore proposed to involve ROS in vascular smooth muscle cells (Ushio-Fukai et al., 2001). Interestingly, activation of ERK1/2 in HeLa cells and thrombin-induced cell proliferation in vascular smooth muscle cells was H_2O_2-dependent (Chen et al., 1995; Patterson et al., 1999). Similar to cellular stress exerted by exogenous reactive oxygen species such as stimulation with hydrogen peroxide, intracellular produced ROS are able to inactivate phosphatases thereby leading to an alteration of the tyrosine kinase/phosphatase balance towards the phosphorylated state. Several recent reports have demonstrated that exogenous oxidants or oxidants generated by peptide growth factor binding can reversibly oxidize and hence inactivate protein tyrosine phosphatases (PTPs) (Blanchetot et al., 2002; Meng et al., 2002; Rao and Clayton, 2002). In contrast to ROS as a cellular stress factor, the

production of radicals in cell signalling requires a spatial and temporal tightly controlled mechanism, allowing both a rapid and transient production but also within a restricted area and within a limited period of time to ensure specificity. Besides PTPs, G proteins of the $G\alpha_{i/o}$ subtype have been reported to be redox-sensitive (Nishida et al., 2000; Nishida et al., 2002). ROS-induced G protein activation is restricted to these pertussis toxin-sensitive Gα subunits as they contain a cysteine residue which is susceptible to oxidation and induces in the oxidized state activation of the G protein subunit (Nishida et al., 2002). Sequence alignments demonstrated that this particular cysteine residue is not present in other Gα subunits. Interestingly, elevated ROS levels have been associated with a malignant phenotype (Szatrowski and Nathan, 1991).

1.5.6. Growth factor-stimulated ROS production : NADPH oxidases

Receptor-mediated production of hydrogen peroxide (H_2O_2) has been studied mostly in phagocytic leukocytes. In these cells, the one-electron reduction of molecular oxygen by a multicomponent reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system (Nox) generates superoxide ($O_2^{\cdot-}$), which is spontaneously or enzymatically converted to H_2O_2. While almost all classical oxidases generate superoxide anions, most aspects of signalling have been linked to the more stable derivative, hydrogen peroxide. H_2O_2 meets all the criteria for an intracellular messenger, as its is a small, diffusible and ubiquitous molecule that can be synthesized and destroyed rapidly in response to external stimuli. Since the initial observation that insulin induces the generation of H_2O_2 in adipose cells (May and de Haen, 1979), the intracellular generation of this molecule has been detected in various nonphagocytic cells in association with receptor stimulation (Finkel, 2003; Lambeth, 2002; Rhee et al., 2000). The structure of Nox enzyme complexes in non-phagocytic cells is not well defined. However, homologues of the catalytic subunit $gp91^{phox}$ have been identified, while the role of other components of the complex is ill defined. The small G protein Rac is involved in the regulation of the Nox complex in phagocytic cells and has also been implicated in its regulation in nonphagocytic cells (Bae et al., 2000; Sundaresan et al., 1996; Yeh et al., 1999). Importantly, overexpression of Nox1 has been demonstrated to induce transformation of NIH-3T3 cells (Suh et al., 1999). Moreover, overexpression of Nox1 induced tumour formation in mice (Arnold et al., 2001), and recently Arbiser and colleagues reported that tumours induced by Nox1 expression enhanced angiogenesis in a ROS-dependent manner (Arbiser et al., 2002).

1.6. Aim of the study

Aim of this study is to investigate cell surface receptor cross-talk mechanisms in cellular signalling and their potential role in human cancer. To address this question, three different objectives are of special interest.

1. Recent advances in understanding the underlying molecular mechanisms of EGFR signal transactivation by the discovery of metalloprotease-induced EGF-like ligand precursor processing raised the question whether this mechanistic concept is involved in human cancer cell lines. Different cell lines derived from both non-small cell lung carcinoma (NSCLC) and pancreatic carcinoma are investigated as model systems for the ligand-dependent EGFR signal transactivation mechanism and for cellular responses dependent on this pathway which are associated with a malignant phenotype such as cell proliferation.

2. Moreover, apart from GPCR agonists other heterogeneous stimuli are known to induce EGFR phosphorylation, including stress stimuli such as osmotic or oxidative stress, mechanical stretch or UV- or γ-irradiation. Given the increasing significance of ligand-induced EGFR activation mechanisms this study addresses the relevance of EGF-like ligand processing for EGFR activation in response to cellular stress stimuli. Since chemotherapeutic agents are known to induce stress signalling cascades within the cell, the potential involvement of such a signalling pathway in cellular responses of tumour cells to chemotherapy is examined.

3. Apart from EGFR signal transactivation cross-talk between GPCRs and other RTKs has been reported but mainly in endothelial or vascular smooth muscle cells or cells of neuronal origin. Therefore during the investigations of EGFR signal transactivation in NSCLC and pancreatic carcinoma cell lines the potential cross-communication of GPCRs and other RTKs is investigated in these epithelial cell types with respect to the cross-talk mechanism and downstream signalling.

2. Materials and Methods

2.1. Materials

2.1.1. Laboratory chemicals and biochemicals

Acrylamide	Serva, Heidelberg
Agar	Difco, Detroit, USA
Agarose	BRL, Eggenstein
Ampicillin	Roche, Mannheim
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, München
ATP (Adenosine 3´-triphosphate)	Pharmacia, Freiburg
Batimastat	British Biotech, Oxford, UK
Bisacrylamide	Roth, Karlsruhe
Bromphenol blue	Sigma, Taufkirchen
BSA (Bovine serum albumin)	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
Dideoxynucleotides (ddG/A/T/CTP)	Pharmacia, Freiburg
DTT (Dithiothreitol)	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
Heparin	Sigma, Taufkirchen
HEPES (N-(2-Hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid))	Serva, Heidelberg
IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Biomol, Hamburg
L-Glutamine	Gibco, Eggenstein
Leupeptin	Sigma, Taufkirchen
Lipofectamine®	Gibco, Eggenstein
Lysozyme	Sigma, Taufkirchen
Marimastat	Sugen Inc., CA, USA
MBP (Myelin basic protein)	Sigma, Taufkirchen
Mineral oil	Sigma, Taufkirchen
MOPS (3-Morpholinopropanesulfonic acid)	Biomol, Haub
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
pNPP (p-Nitrophenyl phosphate)	Sigma, Taufkirchen
Polybrene (Hexadimethrine bromide)	Sigma, Taufkirchen
PD98059	Alexis, Grünberg
PEG (Polyethylene glycol) 4000, 6000	Serva, Heidelberg
Ponceau S	Sigma, Taufkirchen
PP2	Calbiochem, Bad Soden
PTX (Pertussis toxin)	List, Campbell, USA
Salmon sperm DNA	Sigma, Taufkirchen
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sodium azide	Serva, Heidelberg
Sodium fluoride	Sigma, Taufkirchen
Sodium orthovanadate	Aldrich, Steinheim
Scintillation cocktail (Rotiszint®ecoplus)	Roth, Karlsruhe

TEMED (N,N,N',N'-Tetramethylethylenediamine)	Serva, Heidelberg
TPA (Tetradecanoyl-phorbol-13-acetate)	Sigma, Taufkirchen
Triton X-100	Serva, Heidelberg
Tween 20, 40	Sigma, Taufkirchen
Tyrphostin AG1478	Alexis, Grünberg

All other chemicals were purchased from Merck (Darmstadt).

2.1.2. Enzymes

Alkaline Phosphatase	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
[a-^{33}P] dATP 2500 Ci/mmol
L-[^{35}S] Methionine >1000 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
Dowex AG1-X8	Bio-Rad, München
ECL Kit	PerkinElmer, Köln
Glutathione-Sepharose	Pharmacia, Freiburg
Hyperfilm MP	Amersham, USA
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm Dynatech,	Denkendorf
Poly Prep® Chromatography columns	Bio-Rad, München
Protein A-Sepharose	Pharmacia, Freiburg
Protein G-Sepharose	Pharmacia, Freiburg
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
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
Transwells	Corning, New York, USA
Whatman 3MM	Whatman, USA

2.1.5. Growth factors and ligands

Anisomycin	Calbiochem
Amphiregulin	R&D Systems
Bradykinin	Calbiochem
EGF (murine)	Toyoba, Japan
HGF (human)	Calbiochem

All other growth factors and ligands were purchased from Sigma.

2.1.6. Media and buffers

Medium for *E.coli*

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
Kanamycin 100 µg/mL
Chloramphenicol 30 µg/mL

LB-plates additionally contained 1.5% Agar.

2.1.7. Cell culture media

All cell culture media and additives were from Gibco (Eggenstein), fetal calf serum (FCS) as purchased from Sigma.

Dulbecco's modified eagle medium (DMEM) with 4.5 mg/mL glucose, 2 mM L-glutamine, 1 mM sodium pyruvate.

Eagle´s minimum essential medium (EMEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate.

Freeze medium: 90% heat-inactivated FCS, 10% DMSO.

2.1.8. Stock solutions for buffers

BBS (2x)	50 mM BES 280 mM NaCl 1.5 mM Na_2HPO_4 pH 6.96 (NaOH)
HBS (2x)	46 mM HEPES pH 7.5

	274 mM NaCl 1.5 mM Na_2HPO_4 pH 7.0
Denhardt (100x)	2.0 % Polyvinylpyrollidon 2.0 % Ficoll 2.0 % BSA
DNA loading buffer (6x)	0.25 % Bromphenol blue 0.25 % Xylencyanol 30.0 % Glycerol 100.0 mM EDTA pH 8.0
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 0.05 % Triton X-100 pH 7.4 (HCl)
PBS	13.7 mM NaCl 2.7 mM KCl 80.9 mM Na_2HPO_4 1.5 mM KH_2PO_4, pH 7.4 (HCl)
SD-Transblot	50.0 mM Tris/HCl pH 7.5 40.0 mM Glycine 20.0 % Methanol 0.004 % SDS
“Strip” buffer	62.5 mM Tris/HCl pH 6.8 2.0 % SDS 100 mM ß-Mercaptoethanol SSC (20x) 3.0 M NaCl 0.3 M Sodium citrate
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

2.1.9. Bacterial strains, cell lines and antibodies

2.1.9.1. E.coli strains

E. coli	Description	Origin/ Reference
DH5aF'	F'/endA1 hsd17 (rk-mk-),supE44, recA1, gyrA (Nal), thi-1, (lacZYA-argF	Genentech, San Francisco, USA
CJ236	dut-, ung-, thi-, relA-	(Kunkel, 1985)

2.1.9.2. Cell lines

Cell Line	Description	Origin/ Reference
COS-7	African green monkey, SV40-transformed kidney fibroblasts	Genentech
HEK-293	T Human embryonic kidney fibroblasts, transformed with adenovirus Typ V DNA	ATCC CRL-1573
DAN-G	Human pancreatic carcinoma	RZPD

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

2.1.9.3. Antibodies

The following antibodies were used in immunoprecipitation experiments, as primary antibodies in immunoblot analysis or for staining of cell surface proteins in FACS analysis.

Antibody	Description/ Immunogen	Origin/ Reference
P-Tyr (4G10)	Mouse, monoclonal; recognizes phospho-(3)-tyrosine residues	UBI, Lake Placid
EGFR	Sheep, polyclonal/ part of cytoplasmic domain of the human EGFR	UBI
EGFR (108.1)	Mouse, monoclonal/ ectodomain of the human EGFR	(Daub *et al*., 1997)
HER2/neu	Rabbit, polyclonal/ C-terminal peptide of human HER2/neu	(Daub *et al*., 1996)
Akt1/2	Rabbit, polyclonal/ AA 345-480 of human Akt1	Santa Cruz, USA
SHC	Mouse, monoclonal	Santa Cruz
SHC	Rabbit, polyclonal/ 220 AA at C-terminus of human SHC	(Daub *et al*., 1997)
Gab1	Rabbit, polyclonal/ AA 23-189 of human Gab1	(Daub *et al*., 1997)
P-ERK	Rabbit, polyclonal; recognizes phospho-p44/p42 (Thr-202/ Tyr-204) MAPK	NEB, Frankurt/M.

P-p38	Rabbit, polyclonal; recognizes phospho-p38 (Thr-180/Tyr-182) MAPK	NEB
ADAM17/TACE	Rabbit, polyclonal/ AA 807-823 of human TACE	Chemicon, Hofheim
HB-EGF	Goat, polyclonal/ recombinant, human HB-EGF	R&D Systems, Wiesbaden
ERK2	(C-14) Rabbit, polyclonal/ peptide at C-terminus of rat ERK2	Santa Cruz ERK2
(K-23)	Rabbit, polyclonal/ peptide from sub-domain XI of rat ERK2	Santa Cruz
ERK2 HA	Mouse, monoclonal; recognizes the influenza hemagglutinin epitope	Babco, California, USA
VSV (P5D4)	Mouse, monoclonal; recognizes an epitope of eleven AA derived from the vesicular stomatits virus glycoprotein VSV-G	Roche, Mannheim,
p38 (C-20)	Rabbit, polyclonal/ peptide at C-terminus of murine p38	Santa Cruz
h-Met (C-28)	Rabbit, polyclonal/ peptide at C-terminus of human c-Met	Santa Cruz

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

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

The FITC-conjugated rabbit anti-goat and FITC-conjugated goat anti-mouse secondary antibodies for flow cytometry were obtained from Sigma.

2.1.10. Plasmids and oligonucleotides

2.1.10.1. Primary vectors

Vector	Description	Origin/ Reference
pcDNA3	Mammalian expression vector, Amp_r, CMV promotor, BGH poly A, high copy number plasmid	Invitrogen, USA
pLXSN	Expression vector for retroviral gene transfer, Amp_r, Neo_r, ori 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	Genentech

	Promoter, SV 40 poly A, high copy number plasmid	

2.1.10.2. Constructs

Vector	Description	Reference
pcDNA3-HA-ERK2	cDNA of ERK2 in pcDNA3, HA-Tag	(Daub *et al*., 1997)
pcDNA3-M1R	cDNA of human M1R in pcDNA3	(Daub *et al*., 1997)
pLXSN-M1R	cDNA of human M1R in pLXSN	(Prenzel *et al*., 1999)
pcDNA3-proHB-EGF-VSV	cDNA of human proHB-EGF in pcDNA3	(Prenzel *et al*., 1999)
pGEX-cJun	cDNA of human c-Jun in pGEX	

2.1.10.3. siRNA-Oligonucleotides

siRNA	Description/Sequence	Reference
gl2	directed against firefly luciferase CGUACGCGGAAUACUUCGAdTdT	S. Hart, this group
ADAM9	AAUCACUGUGGAGACAUUUGCdTdT, AAACUUCCAGUGUGUAGAUGCdTdT	S. Hart, this group
ADAM10	AAUGAAGAGGGACACUUCCCUdTdT, AAGUUGCCUCCUCCUAAACCAdTdT	S. Hart, this group
ADAM12	AACCUCGCUGCAAAGAAUGUGdTdT, AAGACCUUGATACGACUGCUGdTdT	S. Hart, this group
ADAM15	AACUCCAUCUGUUCUCCUGACdTdT AAAUUGCCAGCUGCGCCCGUCdTdT	S. Hart, this group
ADAM17	AAGUUUGCUUGGCACACCUUdTdT AAGUAAGGCCCAGGAGUGUUdTdT AACAUAGAGCCACUUUGGAGAdTdT	S. Hart, this group

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 manufacturers´ recommendations.

2.3. Enzymatic manipulation of DNA

2.3.1.1. 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 was adjusted to the specific application according to the manufacturers´recommendations.

2.3.1.2. Dephosphorylation of DNA 5'-termini with calf intestine alkaline phosphatase

(CIAP)

Dephosphorylation of 5´-termini of vector DNA in order to prevent self-ligation of vector termini. CIP 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 picomoles of DNA termini were dissolved in 44 µL deionized water, 5 µL 10x reaction buffer (500 mM Tris/HCl pH 8.0, 1 mM EDTA pH 8.5) and 1 µL CIP (1 U/µL). The reaction was incubated 30 min at 37°C and stopped by heating at 85°C for 15 minutes.

2.3.1.3. 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.
In a total volume of 10 µL the digested, dephosphorylated and purified vector DNA (200 ng), the foreign DNA to be inserted, 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 mixed. The reaction was incubated at 15°C overnight. T4 DNA Ligase was inactivated by heating the reaction mixture at 65°C for 10 minutes. The resulting ligation reaction mixture was directly used for bacterial transformation.

2.3.1.4. 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. 0.6-2%, horizontal agarose gels with 1x TAE electrophoresis buffer were used for separation. The voltage was set typically to 1-10 V/cm of gel. 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.

2.3.1.5. 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).

2.3.2. Introduction of plasmid DNA into *E.coli* cells

2.3.2.1. Preparation of competent cells

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.

2.3.2.2. Transformation of competent cells

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 45 min at 37°C with mild shaking to allow expression of the antibiotic resistance gene. Transformants were selected on appropriate plates.

2.3.3. Oligonucleotide-directed mutagenesis

A DNA sequence can be specifically altered by synthesizing the desired sequence change within an oligonucleotide, and then converting this into a biologically active circular DNA strand by using the oligonucleotide to prime *in vitro* synthesis on a single-stranded circular template. This protocol (Kunkel, 1985; Messing, 1983) uses a DNA template containing a small number of uracil residues in place of thymine. Use of the uracil-containing template allows rapid and efficient recovery of mutants.

2.3.3.1. Preparation of uracil-containing, single-stranded DNA template

CJ236 bacteria were transformed with the DNA of interest (typically pcDNA3 constructs). 2 mL 2xYT-medium were inoculated with several colonies of transformed CJ236 at 37°C until the early log-phase was reached. Cultures were infected with $2x10^7$ M13K07 phages/mL (Amersham) and incubated for further 1.5 h. Next, kanamycin was added (70 µg/mL final concentration) and the culture was incubated with vigorous shaking at 37 °C overnight. Cells were pelletet twice by centrifugation (13000 rpm, 5 min) to clear the supernatant. Phage was then precipitated by adding 200 µL 2.5 M NaCl/ 20% PEG 6000 and incubation for 15 min at room temperature. Precipitated phage was collected by centrifugation. The phage sediment was resuspended in 100 µL TE10/0.1 buffer and subjected to phenol extraction/ ethanol precipitation in order to purify the single-stranded phage DNA. Quality and concentration of DNA was determined spectrophotometrically at 260 nm. For visual examination and documentation an aliquot of the single-stranded DNA was run on a 1% agarose gel.

2.3.3.2. Primer extension

The uracil-containing DNA was used as a template in oligonucleotide-directed mutagenesis experiments: 200 ng single-stranded template DNA, 2-3 pmol phosphorylated oligonucleotide, 1 µL 10x hybridization buffer (20 mM Tris/HCl pH 7,4, 2 mM $MgCl_2$, 50 mM NaCl) in a total volume of 10 µL were incubated for 2 min at 90°C and allowed to cool to room temperature. To the hybridization mixture 1 µL 10x synthesis buffer (5 mM dNTPmix, 100 mM Tris/HCl pH 7.5, 50 mM $MgCl_2$, 20 mM DTT), 5 U T4-DNA Ligase (1 µL), 1 µg T4-Gen 32 Protein (0.5 µL) and 3 U T4-DNA Polymerase (1 µL) were added. The reaction was incubated for 5 min on ice, 5 min at 25 °C and finally for 90 min at 37°C. The reaction was stopped by adding 66 µL TE. 100 ng of double-stranded DNA product were used for transformation of E. coli. Resulting clones were chosen randomLy for isolation of plasmid DNA which was analysed by sequencing.

2.3.4. 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) was used:

0.5 µL template cDNA
2 µL "sense" oligonucleotide, 10 pmol/µL
2 µL "antisense" oligonucleotide, 10 pmol/µL
5 µL 10x LA PCR buffer II (w/o $MgCl_2$)
5 µL $MgCl_2$, 25 mM
8 µL dNTP-Mix, 2.5 mM each
0.5 µL LA-Taq™ (5 U/µL)
ad 50 µL H_2O

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

first denaturation:	3 min 94°C
amplification 25-30 cycles:	1 min 94°C (denaturation)
	1 min 58°C (hybridization)
	1 min/ kb product 72°C (extension)
last 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).

2.3.5. 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 pmol oligonucleotide
4 µL Terminator Ready Reaction Mix
ad 20 µL H_2O
25 cycles: 30 sec 94°C
15 sec 45-60°C
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).

2.3.6. cDNA array hybridization

Filters spottet with genes of interest (cloned into pBluescript SKII+) were a gernerous gift from J. Ruhe, cDNA probes of the cell lines Cos-7 and NCI-H292 were generated according to standard molecular biology methods. Labeling of 3– 5 µL of cDNA was performed with the Megaprime kit (Amersham) in the presence of 50 µCi of [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 yeast tRNA (Roche), and the labeled 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)].

2.3.7. RT-PCR analysis

Specific silencing of targeted genes was confirmed by RT-PCR analysis. RNA isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) was reverse transcribed using AMV Reverse Transcriptase (Roche, Mannheim, Germany). PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ) were used for PCR amplification. Primers (Sigma Ark, Steinheim, Germany) were ADAM9, 5'-AGT GCA GAG GAC TTT GAG AA-3´ and 5'-TGC CGT TGT AGC AAT AGG CT-3´, ADAM10, 5'-TTG CTC ACG AAG TTG GAC AT-3' and 5'-TTT CCC AGG TTT CAG TTT GC-3', ADAM15, 5'-GGC TGG CAG TGT CGT CCT ACC AGA GGG-3' and 5'-GGT GCA CCC AGC TGC AGT TCA GCT CAG TCC-3'. PCR products were subjected to electrophoresis on a 2.5% agarose gel and DNA was visualized by ethidium bromide staining.

2.4. Methods in mammalian cell culture

2.4.1. General cell culture techniques

All 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 mykoplasma contamination using a bisbenzimidestaining kit (Sigma). Before seeding cells were counted with a Coulter Counter (Coulter Electronics). HEK-293, Cos7 and HuH7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate and 10% FCS. TCC-Sup were cultured in DMEM supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate and 15% FCS. NCI-H292 and DAN-G cells were maintained in RPMI medium supplemented with 2 mM L-glutamine and 10% FCS.

2.4.2. Transfection of cultured cell lines

2.4.2.1. Transfection of cells with calcium phosphate

HEK-293 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 adequate 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 following transfection, cells were serum-starved for 24 hours in standard cell culture medium without FCS.

2.4.2.2. Transfection of COS-7 cells using lipofectamine®

COS-7 cells were transiently transfected using Lipofectamine® (Gibco-BRL) essentially as described (Daub *et al*., 1997). For transfections in 6-well dishes, 1.0 mL of serum-free medium containing 10 µL of Lipofectamine and 1.5 µg of total plasmid DNA per well were used. After 4 h the transfection mixture was supplemented with an equal volume of medium containing 20% FCS and, 20 h later, cells were washed and cultured for a further 24 h in serum-free medium until lysis.

2.4.2.3. RNA interference

Transfection of 21-nucleotide siRNA duplexes (Dharmacon Research, Lafayette, CO, USA) for targeting endogenous genes was carried out using Oligofectamine (Invitrogen) for NCI-H292 cells and 4.2 µg siRNA duplex per 6-well plate as previously described (Elbashir et al., 2001). Cos-7 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, 8.4 µg siRNA duplex per 6 cm dish were incubated with 10 µL Lipofectamine 2000 in 1 mL serum-free medium for 20 minutes. The transfection mixture was added to the cell culture medium containing serum and, after 6 h, cells were washed and incubated in medium containing serum overnight.
NCI-H292 and Cos-7 cells were serum-starved and assayed 2 d after transfection. Highest efficiencies in silencing target genes were obtained by using mixtures of siRNA duplexes targeting different regions of the gene of interest.

2.4.2.4. Apoptosis Assay

TCC-Sup bladder carcinoma cells were seeded in 6-well plates, grown for 20 h and treated with 10 µM doxorubicin and CRM197 as indicated for 72 h. Cells were collected by trypsinization, resuspended in assay buffer containing propidium iodide (PI), and incubated at 4° C for 3 h. Nuclear DNA staining was analysed on a Becton Dickinson FACScalibur flow cytometer.

2.5. Protein analytical methods

2.5.1. Lysis of cells with triton X-100

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 buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/mL aprotinin. Lysates were precleared by centrifugation at 13000 rpm for 10 min at 4°C.

2.5.2. Determination of protein concentration in cell lysates

The „Micro BCA Protein Assay Kit" (Pierce, Sankt Augustin) was used according to the manufacturers´ recommendations.

2.5.3. Immunoprecipitation and *in vitro* association with fusion proteins

An equal volume of HNTG buffer was added to the precleared cell lysates that had been adjusted for equal protein concentration. Proteins of interest were immunoprecipitated using the respective antibodies and 20 µL of protein A-Sepharose for 4 h at 4°C. Alternatively, lysates were subjected to *in vitro* associations with either 3 µg of GST-Grb2 (Daub *et al*., 1997) or 2 µg of GST as control pre-bound to 30 µL of gluthathione-agarose beads. Precipitates were washed three times with 0.5 mL of HNTG buffer, suspended in 2× SDS sample buffer, boiled for 3 min, and subjected to SDS-PAGE.

2.5.4. TCA precipitation of proteins in conditioned medium

Phenylmethylsulfonyl fluoride (1 mM final concentration) was added to cell culture medium of stimulated and control treated cells and precleared by centrifugation at 13000 rpm for 10 min at 4°C. For TCA precipitation, proteins were incubated in 0.1 mg/mL sodium deoxycholate, 0.6 M TCA for 30 min on ice.

2.5.5. SDS-polyacrylamide-gelelectrophoresis (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-Galaktosidase	116.25	Carboanhydrase	29.0
Phosphorylase b	97.4	Trypsin-Inhibitor	21.5
BSA	66.2	Lysozym 14.4	

Because of the small size of pro-HB-EGF and the processed form of HB-EGF, the tricine SDS-PAGE system was used as described (Schagger and von Jagow, 1987).

2.5.6. 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/cm2 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.

2.5.7. 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% gelatin for at least 4 h. The membrane was then probed with primary antibody (typically overnight). 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 reproped with different primary antibody to confirm equal protein loading.

2.5.8. Differential detergent fractionation

Differential detergent fractionation was carried out as described before (Ramsby et al., 1994). Cells were pre-treated with inhibitors as indicated in the figure legends. Following stimulation, cells were washed in PBS, and a cytosolic-enriched fraction was obtained using a digitonin buffer (0.01% digitonin, 10 mM PIPES (pH 6.8), 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 5 mM EDTA) and centrifugation at 480x g. The supernatant was carefully removed as the cytosolic-enriched fraction. The membrane-enriched fraction was obtained by resuspending the pellet in TritonX 100 buffer (0,5% Triton X100, 10 mM PIPES (pH 7.4), 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 3 mM EDTA), centrifugation at 5000x g for 10 min and careful removal of the supernatant. The pellet obtained was were resuspended in Tween40/DOC buffer (1% Tween 40, 0.5% deoxycholate, 10 mM PIPES (pH7.4), 10 mM NaCl, 1mM $MgCl_2$). To break up nuclei, 20 strokes in a Dounce homogenizer were applied followed by ultrasonication and centrifugation at 6780x g. The supernatant was collected as the nuclei-enriched fraction. Protein concentration was determined using the BCA protein assay kit (Pierce), and equal amounts of protein were subjected to gel electrophoresis and western blotting.

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 48 h HNSCC 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. In some cases cells were transfected 24 h after seeding and serum-starved one day following transfection before being stimulated as indicated above.

2.6.2. ERK1/2 phosphorylation

For determination of ERK1/2 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. Quantitation of ERK1/2 was performed using the Luminescent Image Analyis System (Fuji). After quantitation of ERK1/2 phosphorylation, membranes were stripped of immunoglobulin and reprobed using rabbit polyclonal anti-ERK1/2 or rabbit polyclonal anti-Akt antibody to confirm equal protein loading.

2.6.3. ERK/MAPK activity

HA-ERK2 or endogenous ERK2 were immunoprecipitated from lysates obtained from sixwell dishes using 0.5 µg of anti-HA antibody or 0.4 µg of anti-ERK2 antibody, respectively. 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. Labeled MBP was quantitated using a Phosphoimager (Fuji).

2.6.4. JNK activity assay

JNK activity was assayed as described previously (Sudo and Karin, 2000). Cultured cells were lysed in lysis buffer containing 20 mM Tris (pH7.6), 0.5% Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 1 mM dithiotreitol, 0.5 mM phenylmethylsulfonylfluoride, 20 mM β-gylcerophosphate, 1 mM sodium orthovanadate and 1µg/mL leupeptin. JNK was immunoprecipitated from lysates obtained from 6-well dishes using polyclonal anti-JNK antibody. Immunoprecipitates were washed twice using lysis buffer and twice using kinase assay buffer (25 mM HEPES (pH 7.5), 20 mM β-gylcerophosphate, 20 mM PNPP, 20 mM $MgCl_2$, 2 mM dithiotreitol and 0.1 mM sodium orthovanadate). Kinase reactions were performed in 30 µL of kinase buffer supplemented with 1 µg GST-c-Jun (aa1-79), 20 µM cold ATP and 5 µCi of [γ-^{32}P]ATP at 30° C for 30 minutes. Reactions were stopped by addition of 30 µL of Laemmli buffer and subjected to gel electrophoresis on 12.5% gels. Labeled GST-c-Jun was quantitated using a Phosphoimager (Fuji).

2.6.5. Flow cytometric analysis of cell surface proteins

Was performed as described before (Prenzel *et al*., 1999). In brief, cells were seeded, grown for 20 h and in some cases retrovirally infected as indicated. Upon serum-starvation for 24 h cells were treated with inhibitors and growth factors as indicated. After collection, cells were stained with ectodomain-specific antibodies against HB-EGF, TGFα or AR for 45 min. After washing with PBS, cells were incubated with FITC-conjugated secondary antibodies for 15 min and washed again with PBS. Cells were analysed on a Becton Dickinson FACScalibur flow cytometer.

2.6.6. Detection of ROS by flow cytometric analysis

Cells were seeded in 6-well dishes and grown for 48 hrs. Prior to stimulation cells were preincubated with 20 μM DCF-DA for 30 min and subsequently stimulated as indicated for 15 min. After trypsinization, cells were centrifuged at 800x g and resuspended in PBS with propidium iodide. Cells were immediately analyzed on a Becton Dickinson FACScalibur flow cytometer.

2.6.7. Incorporation of ^{3}H-thymidine into DNA

SCC-9 or SCC-25 cells were seeded into 12-well plates (2.5 x 10^4 or 6 x 10^4 cells per well, respectively). Upon serum deprivation for 48 h, cells were subjected to preincubation with inhibitors before ligand treatment. After 18 h incubation, cells were pulse-labelled with ^{3}H thymidine (1 μCi/mL) for 4 h, and thymidine incorporation was measured by trichloroacetic acid precipitation and subsequent liquid-scintillation counting.

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

3.1. EGFR Signal Transactivation in pancreatic and non-small cell lung carcinoma

3.1.1. GPCR-induced EGFR phosphorylation in NSCLC and pancreatic carcinoma

Dysregulated EGFR signalling has been associated with both non-small cell lung carcinoma and pancreatic carcinoma (Bardeesy and DePinho, 2002; Sridhar et al., 2003). Moreover both types of cancer have been linked to autocrine signalling loops involving GPCR ligands (Moody et al., 2003). To address the question whether the EGFR provides a convergence point for GPCR-induced signalling in NSCLC and pancreatic carcinoma within a metalloprotease-dependent EGFR signal transactivation pathway, we stimulated different cell lines with GPCR agonists and investigated EGFR phosphorylation depending on metalloprotease activity by preincubation with the metalloprotease inhibitor batimastat (BB94). As shown in Figure 6 and Figure 7, GPCR stimulation induces metalloprotease-dependent phosphorylation of the EGFR in both NSCLC and pancreatic carcinoma cell lines.

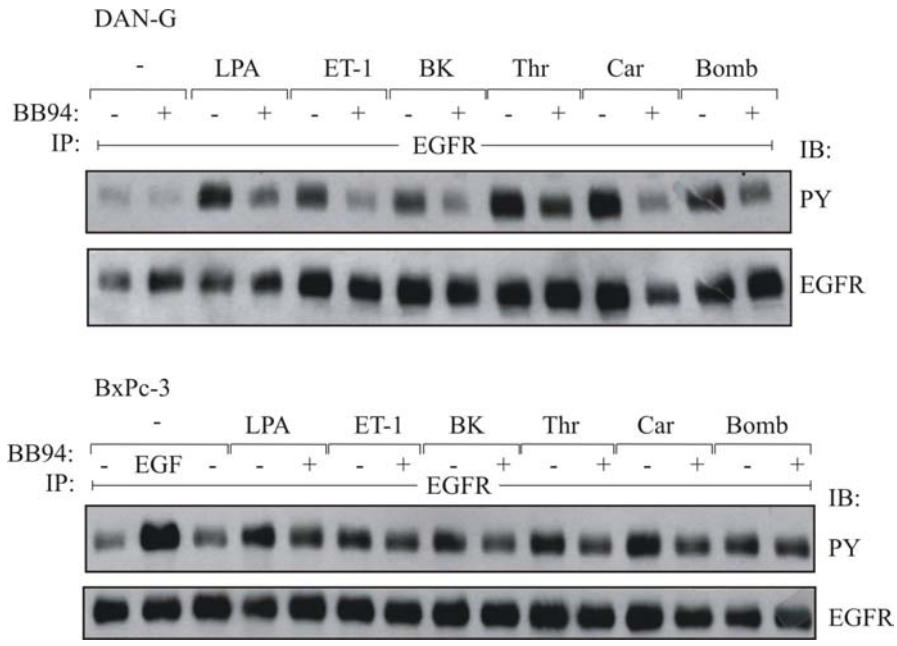

Figure 6 **EGFR signal transactivation in pancreatic carcinoma cell lines**. Following serum starvation for 24 hrs, DAN-G and BxPc-3 cells were incubated with 5 µM BB94 for 20 min and subsequently stimulated with GPCR agonists as indicated. EGFR was immunoprecipitated from cell lysates, transferred to nitrocellulose and immunoblot against phosphotyrosine was performed. The same membranes were stripped and reprobed for total EGFR content.

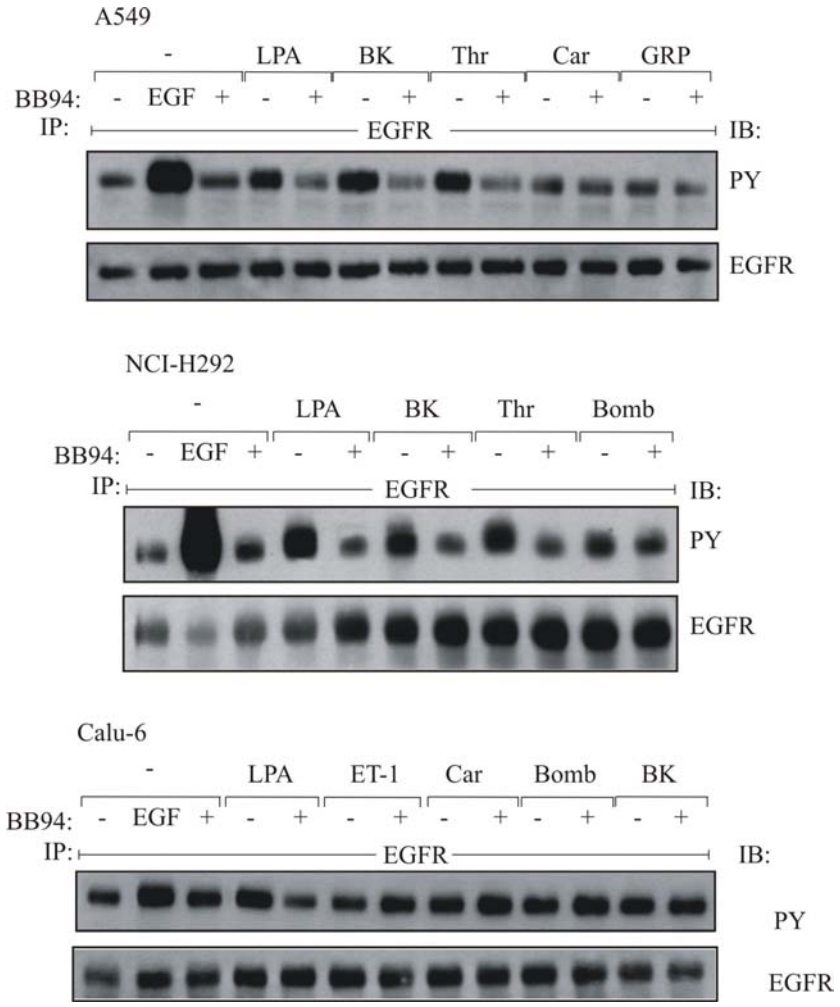

Figure 7 **Different GPCR ligands induce metalloprotease-dependent EGFR phosphorylation in NSCLC cell lines**. A549, Calu-6 and NCI-H292 cells were serum-starved for 24 hrs. Prior to stimulation, cells were preincubated with the metalloprotease inhibitor batimastat (BB94, 5 μM) for 20 min and stimulated as indicated. EGFR phosphorylation was assessed by immunoprecipitation followed by immunoblot analysis.

3.1.2. EGFR signal transactivation-induced downstream signalling

The finding that GPCR ligands induce metalloprotease-dependent EGFR signal transactivation in these cell lines raised the question whether EGFR characteristic downstream signalling dependent on this signalling mechanism could be observed. The adaptor protein Shc represents a prominent downstream signalling partner of the EGFR which becomes tyrosine phosphorylated in response to EGFR stimulation.

Figure 8 demonstrates that stimulation of A549 NSCLC cells with LPA and bradykinin (BK) induces metalloprotease-dependent Shc phosphorylation, while Thrombin (Thr) or Carbachol (Car) phosphorylate Shc only weakly. In contrast, Gastrin-releasing peptide induced Shc phosphorylation is independent of metalloprotease activity.

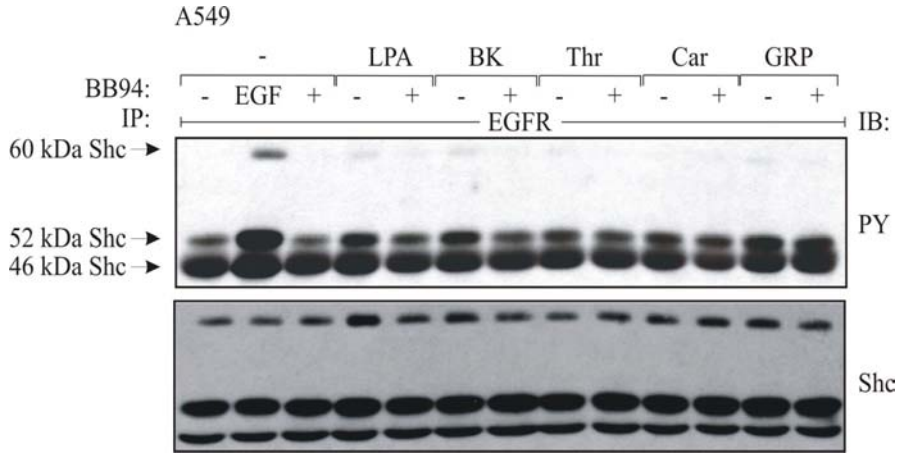

Figure 8 **GPCR-induced Shc phosphorylation depends on metalloprotease activity in A549 cells**. A549 were serum-starved for 24 hrs, preincubated with BB94 (5 µM, 20 min) and stimulated as indicated in the figure. Shc was immunoprecipitated from total cell lysates and samples were run on SDS-PAGE. Following transfer to nitrocellulose membrane the filter was probed for total Shc protein, stripped and reprobed for phosphotyrosine content.

The MAPKs ERK1/2 comprise the so far best characterized EGFR downstream signalling pathway. Moreover, they have been linked to important cellular responses such as cell proliferation, differentiation or anti-apoptosis (Chang and Karin, 2001; Johnson and Lapadat, 2002). Therefore we used phosphospecific antibodies to assess the phosphorylation state of the MAPKs ERK1 and ERK2 in A549, DAN-G and BxPc-3 cells. Interestingly, in the NSCLC cell line A549 GPCR agonist treatment induces MAPK phosphorylation but in a metalloprotease- and therefore EGFR signal transactivation-independent manner (Figure 9). A similar result was obtained in the pancreatic carcinoma cell line DAN-G (Figure 9). In contrast, in the pancreatic carcinoma cell line BxPc-3 endothelin-1 and bradykinin stimulate ERK1/2 phosphorylation in a metalloprotease-dependent fashion.

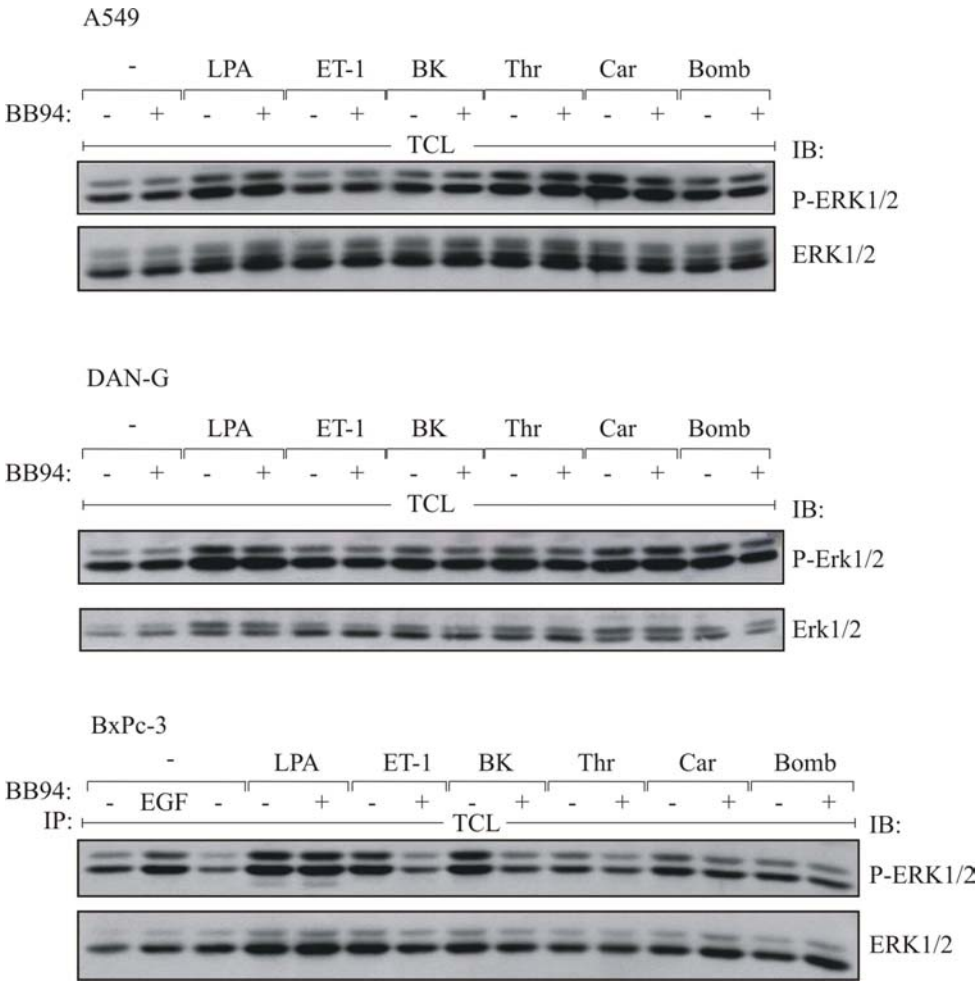

Figure 9 **ERK1/2 phosphorylation in response to GPCR agonist treatment dependent on metalloprotease activity**. A549, DAN-G and BxPc-3 cells were serum-starved for 24 hrs, incubated with BB94 (5 µM, 20min) and stimulated as indicated for 3 min. Total cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose and immunoblot analysis against phospho-ERK1/2 was performed. The same filters were reprobed for total ERK1/2 content.

Moreover, as shown in Figure 10, metalloprotease- and EGFR-dependent ERK2 activation in response to both LPA and bradykinin treatment could be observed in the NSCLC carcinoma cell line NCI-H292 using an ERK2 kinase assay. While bradykinin stimulation was completely abolished by both the metalloprotease inhibitor BB94 and the selective EGFR kinase inhibitor AG1478, LPA-induced ERK2 activation was only blocked by 50% by metalloprotease inhibition (Figure 10).

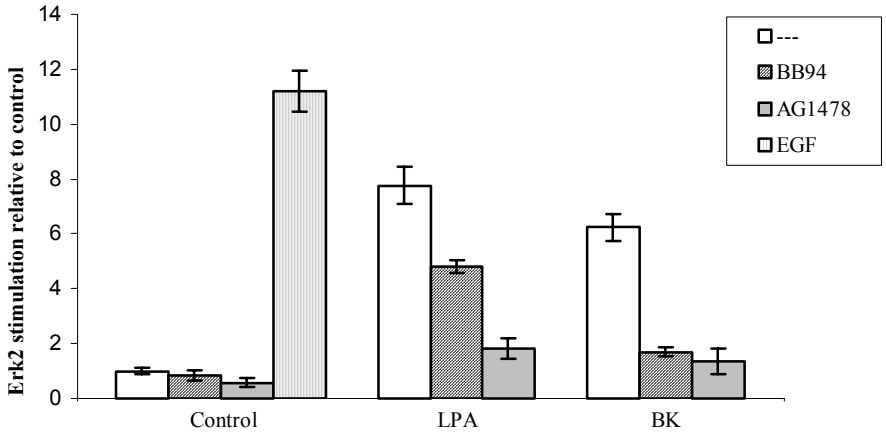

Figure 10 **MAPK activity assay of ERK2 in NCI-H292 cells in response to LPA stimulation**. NCI-H292 cells were serum-starved for 24 hrs, incubated with AG1478 (250 nM, 20 min), BB94 (5 µM, 20 min) or an equal volume DMSO (20 min) and stimulated with LPA (10 µM, 10 min), BK (2 µM, 10 min) or EGF (2 ng/mL, 10 min). ERK2 was immunoprecipitated and a radioactive kinase assay performed as described under materials and methods. Quantitative analysis of ERK phosphorylation from three independent experiments (mean ± s.d.) using the FUJI LAS1000 imaging system.

3.1.3. LPA stimulation induces DNA synthesis in NCI-H292 cells

Having established a role for metalloprotease-dependent EGFR signal transactivation in ERK1/2 activation depending on the cellular system and the stimulus, we addressed the question whether this GPCR-stimulated signal is able to induce cell proliferation. We used the thymidine incorporation assay to assess the DNA synthesis rate in NCI-H292 cells in response to LPA treatment, depending on both metalloprotease and EGFR function, since this cell line showed the strongest response to GPCR agonist treatment on both the EGFR and ERK level (Figure 7,Figure 10). As shown in Figure 11, LPA stimulation induces a 2,3 fold induction of DNA-synthesis, which is comparable to treatment with 2 ng/mL EGF. While metalloprotease inhibition blocked MAPK activity only to 50%, BB94 is able to reduce the LPA stimulated thymidine incorporation to basal levels. The same result was obtained using the EGFR inhibitor AG1478 (Figure 11).

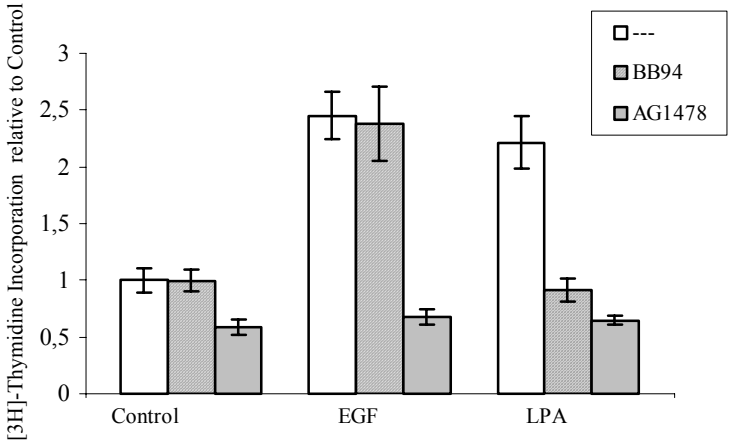

Figure 11 **Metalloprotease-dependent EGFR signal transactivation mediates LPA-induced DNA-synthesis in NCI-H292 cells**. NCI-H292 were seeded in 12-well plates ($2*10^4$ per well) and serum-starved for 24 hrs. Following preincubation with AG1478 (250 nM, 20 min) or BB94 (5 µM, 20 min) cells were stimulated over night with LPA (10 µM) or EGF (2 ng/mL). The following day thymidine incorporation assays was performed as described under materials and methods. Data shown are representative for three independent experiments (mean ± S.D.).

3.2. Stress signalling in human tumour cells is mediated by HB-EGF and ADAM proteases

3.2.1. Distinct kinetics of EGFR and MAPK activation in Cos-7 and human carcinoma cell lines

Previous investigations attributed heterogeneous EGFR activation mechanisms, i.e. activation of the EGFR by stimuli other than EGF-like ligands such as GPCR agonists or non-physiologic stress stimuli, to completely ligand-independent and therefore intracellular pathways (Daub et al., 1996; Knebel et al., 1996). However, recent results demonstrated the involvement of EGF-like ligands in EGFR activation by GPCR agonists (Prenzel et al., 1999). This finding lead us to investigate whether cellular stress stimuli such as osmotic and oxidative stress, which both induce tyrosine phosphorylation of the EGFR and activation of MAPKs in a wide variety of cell systems (Carpenter, 2000; de Nadal et al., 2002; King et al., 1989), utilize a ligand-dependent mechanism to activate the EGFR. To elucidate the underlying mechanisms, we performed time course experiments in Cos-7 and TCC-Sup bladder carcinoma cell lines and analysed EGFR phosphorylation with phosphotyrosine-specific and MAPK phosphorylation with activation-specific antibodies by immunoblot analysis. As shown in the upper panels of Figure 12, tyrosine phosphorylation of the EGFR in response to the stress agents sorbitol (0.3 M) and hydrogen peroxide (200 µM) occurred in

both cell lines within 5 to 10 min and thus preceded the activation of ERK and JNK MAPKs, which became phosphorylated after 10 and 15 min, respectively (Figure 12 A/B, lower panel). In contrast, phosphorylation of p38 occurred as an immediate response to stress agents. The results of these time course experiments were consistent with a role of p38 as upstream mediator of EGFR activation and further pointed to a function of ERK1/2 and JNK as downstream effectors of the EGFR in response to stress stimuli.

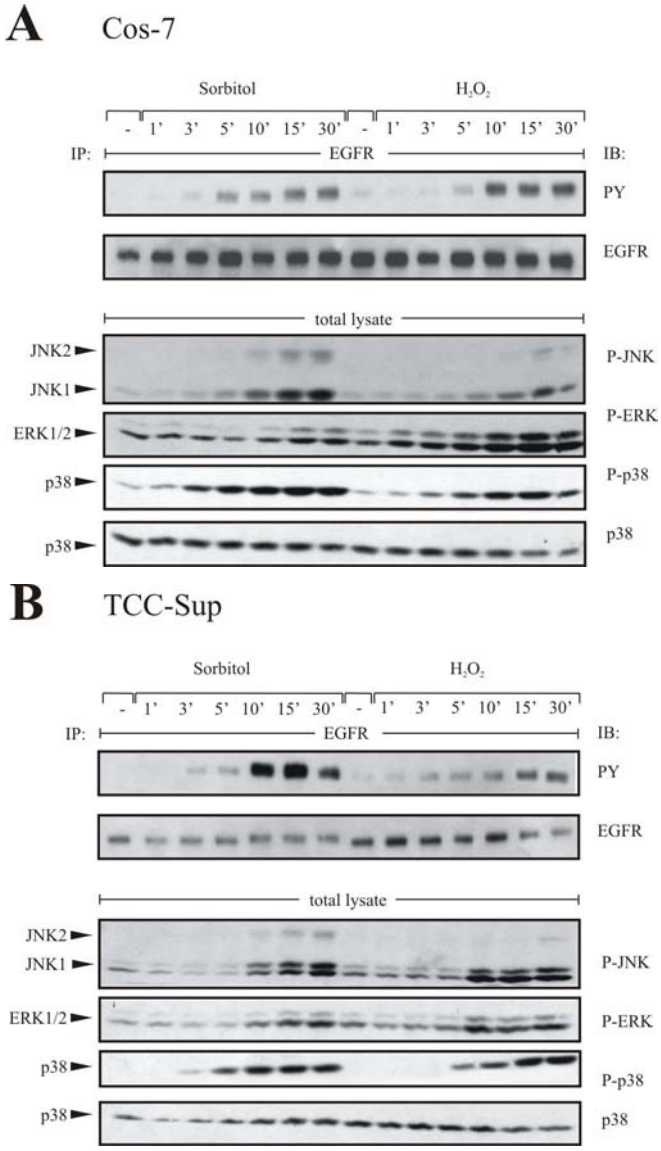

Figure 12 **Time-course of stress-induced EGFR and MAPK phosphorylation in different cell lines. (A) EGFR and MAPK phosphorylation in response to osmotic and oxidative stress**. Cos-7 cells were treated with sorbitol (0.3 M) and hydrogen peroxide (200 µM) for the indicated time periods. Following immunoprecipitation (IP) of cell extracts with anti-EGFR antibody proteins were immunoblotted (IB) with anti-phosphotyrosine antibody and re-probed with anti-EGFR antibody. Phosphorylated MAPKs were detected by immunoblotting total lysates with anti-phospho-ERK, anti-phospho-JNK and anti-phospho-p38 antibody. The same filters were re-probed with anti-p38 antibody. (B) Stress-induced EGFR phosphorylation in human bladder carcinoma cell lines. TCC-Sup cells were treated as indicated in (A).

3.2.2. Stress-induced EGFR activation is independent of Src activity

Chen *et al.* reported hydrogen peroxide-induced activation of the EGFR in a ligand-independent manner in Cos-7 cells by activation of the non-receptor tyrosine kinase c-Src, which in turn phosphorylates the EGFR (Chen et al., 2001a). To test whether this ligand-independent mechanism accounts for both oxidative and osmotic stress-induced EGFR phosphorylation in Cos-7 cells we employed the same experimental setup using the Src specific inhibitor PP2 as described by Chen *et al*. Figure 13 shows a concentration course of the Src inhibitor and its effect on EGFR phosphorylation induced by EGF. A PP2 concentration of 10 µM as used by Chen *et al.* inhibited EGFR kinase function unspecifically (Figure 13, upper panel). When PP2 was used at a concentration that did not affect EGFR kinase activity no inhibition of stress-induced EGFR phosphorylation could be observed, suggesting that Src is not involved in EGFR activation in response to osmotic or oxidative stress in Cos-7 cells.

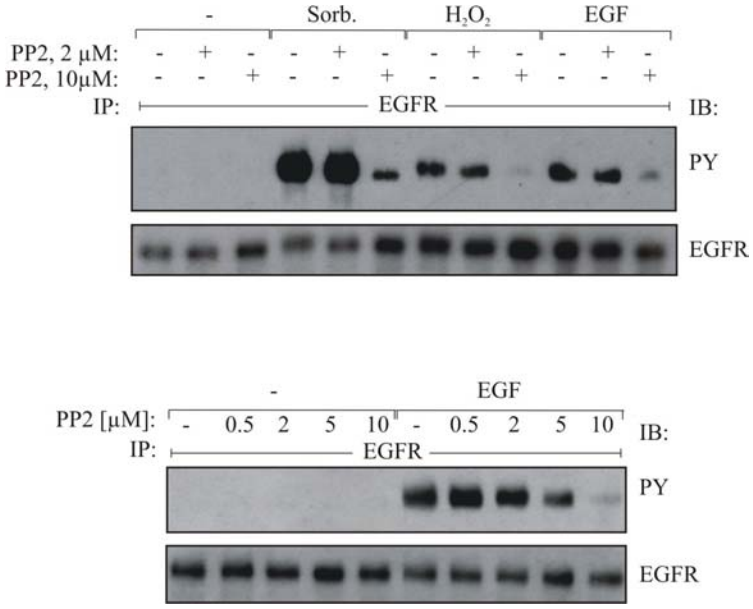

Figure 13 **Stress-induced EGFR activation is independent of Src activity**. Serum-starved Cos-7 cells were preincubated with PP2 for 30 min at the indicated concentrations. After stimulation with sorbitol (0.3 M, 10 min), H_2O_2 (200 µM, 10 min) or EGF (2 ng/mL, 3 min) EGFR was immunoprecipitated and immunoblot analysis was performed to assess its phosphorylation state. The same filters were stripped and reprobed for EGFR content.

3.2.3. Redox-dependent activation of $G\alpha_{i/o}$ is not involved in stress-induced EGFR activation

Nishida and colleagues reported the activation of Gα subunits of the $G\alpha_{i/o}$ family in response to treatment with hydrogen peroxide by oxidation of a cysteine residue characteristic for these Gα protein subtype (Nishida et al., 2000). Since these G protein subunits have been

implicated in EGFR signal transactivation (Daub et al., 1997), we investigated whether this direct G protein activation mechanism independent of GPCR stimulation is involved in oxidative and osmotic stress-induced EGFR activation. As shown in Figure 14, preincubation of Cos-7 cells with the bacterial pertussis toxin (PTX), which modifies and inactivates $G\alpha_{i/o}$ subunits by ADP-ribosylation (Schiavo and van der Goot, 2001), had no effect on sorbitol- or hydrogen peroxide-induced EGFR activation. In contrast, LPA-stimulated EGFR signal transactivation serving as a positive control was blocked to 50%, as reported by Daub and colleagues (Daub et al., 1997).

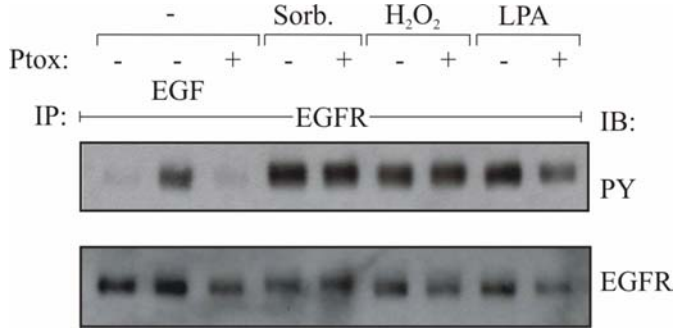

Figure 14 **Pertussis toxin-sensitive Gα subunits are not involved in stress-induced EGFR activation**. Cos-7 cells were seeded, serum-starved for 24 hrs and at the same time incubated with pertussis toxin 18 hrs prior to stimulation. After 24 hours of serum starvation cells were stimulated with 0.5 M sorbitol and 200 µM hydrogen peroxide for 10 min and 10 µM LPA for 3 min. Cells were lysed and assessed for EGFR phosphorylation content as described under Figure 12.

3.2.4. p38 controls EGFR activation by osmotic and oxidative stress

The finding that p38 activation preceded EGFR tyrosine phosphorylation (Figure 12) raised the question whether p38 acts as an upstream regulator of EGFR stimulation in Cos-7 and human carcinoma cell lines. Preincubation of Cos-7 cells with the selective EGFR-kinase inhibitor AG1478 did not affect p38 phosphorylation in response to stress agents (Figure 15), demonstrating that p38 activation was independent of EGFR activity.

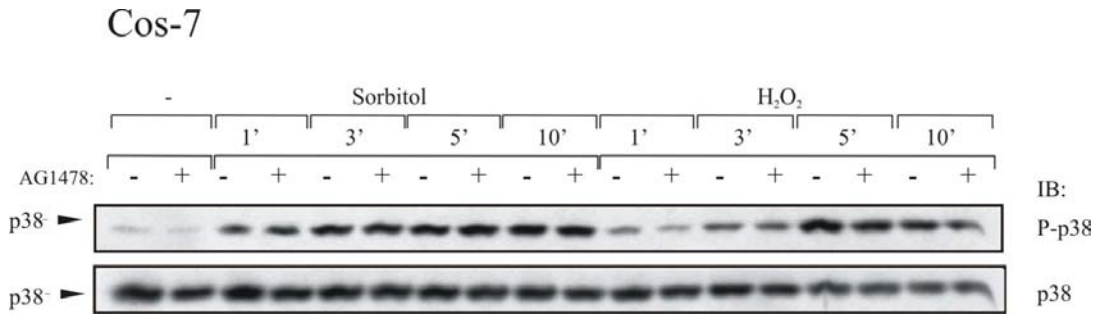

Figure 15 **Time-course of p38 phosphorylation dependent on EGFR kinase function**. Cos-7 cells were pretreated with AG1478 (250 nM) or an equal volume of empty vehicle (DMSO) for 20 min and stimulated with 0.3 M sorbitol or 200 µM hydrogen peroxide for the indicated periods. Cell extracts were immunoblotted with anti-phospho-p38 antibody and reprobing of the same filters with polyclonal anti-p38 antibody.

To address the question whether p38 is located upstream of the EGFR, we used the p38-specific inhibitor SB202190 to investigate the effect of blocking p38 activity on stress-induced EGFR activation. As shown in Figure 16A, preincubation of Cos-7 cells with SB202190 completely abrogated stress-induced EGFR activation while leaving lysophosphatidic acid (LPA) and EGF-induced tyrosine phosphorylation unaffected. In contrast, the MEK1/2 inhibitor PD98059 did not affect EGFR phosphorylation. Analogous results were obtained with the bladder carcinoma cell line TCC-Sup (Figure 16B). Taken together, these results implicate p38 as an upstream mediator of EGFR activation in the stress-induced signalling response of Cos-7 and TCC-Sup bladder carcinoma cells.

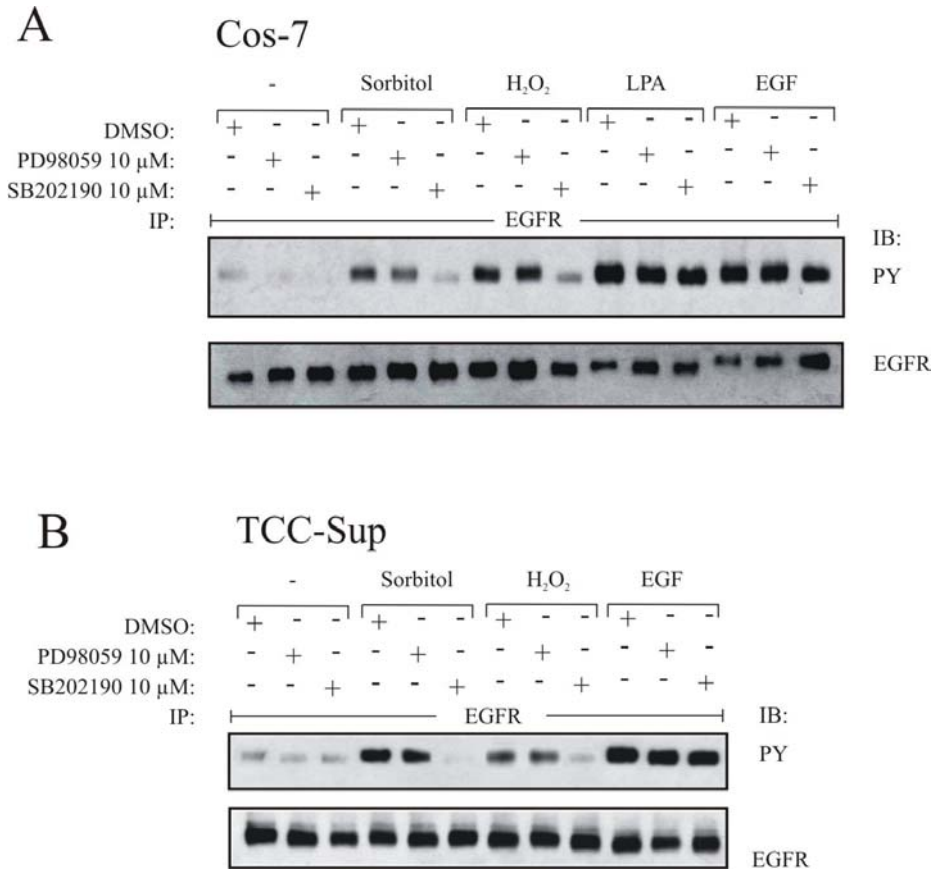

Figure 16 **Stress-induced EGFR activation depends on p38 but not on ERK activity in Cos-7 and TCC-Sup bladder carcinoma cells**. (A) Cos-7 cells were pre-treated with PD98059 (50 µM), SB202190 (10 µM) or an equal volume of empty vehicle (DMSO) for 30 min and stimulated with 0.3 M sorbitol and 200 µM hydrogen peroxide for 10 min and the GPCR agonist LPA (10 µM) or EGF (2 ng/mL) for 3 min as positive controls. Cell extracts were assayed for EGFR tyrosine phosphorylation content. **(B)** Stress-induced EGFR activation depends on p38 activity in TCC-Sup carcinoma cells. TCC-Sup cells were pre-treated as described under (A) and stimulated with 0.3 M sorbitol and 200 µM hydrogen peroxide for 10 min and EGF (2 ng/mL) for 3 min as a positive control. After lysis cell extracts were assayed for EGFR tyrosine phosphorylation content.

3.2.5. Stress-induced EGFR phosphorylation depends on metalloprotease activity and HB-EGF function

Recent investigations underscored the importance of EGF-like ligand precursor processing in EGFR phosphorylation upon stimuli such as GPCR signals (Zwick et al., 1999). Based on these findings, we asked whether EGFR activation by stress stimuli may also involve a ligand-dependent mechanism. We therefore preincubated cells with the metalloprotease inhibitor batimastat (BB94), which has been shown to inhibit EGF-like ligand processing and subsequent EGFR transactivation (Zwick et al., 1999). Upon stimulation with stress agents, EGFR tyrosine phosphorylation was monitored by immunoblot analysis with phosphotyrosine-specific antibody. As shown in Figure 17, BB94 almost completely blocked sorbitol or hydrogen peroxide-induced EGFR phosphorylation in Cos-7 and TCC-Sup bladder carcinoma cells (upper and middle panel). In the lung carcinoma cell line NCI-H292, BB94 reduced EGFR activation by about 50%, suggesting an alternative parallel activation mechanism (Figure 17, lower panel). Next, we investigated the effect of the diphtheria toxin mutant CRM197 which specifically abolishes HB-EGF function, on stress-activated EGFR tyrosine phosphorylation. Indeed, CRM197 inhibited EGFR phosphorylation to the same extent as BB94, suggesting that HB-EGF is critically involved in stress-induced EGFR activation in the three cell lines tested. Control stimulations showed that EGFR tyrosine phosphorylation by LPA was completely prevented by both inhibitors while neither CRM197 nor BB94 affected direct receptor stimulation upon EGF treatment.

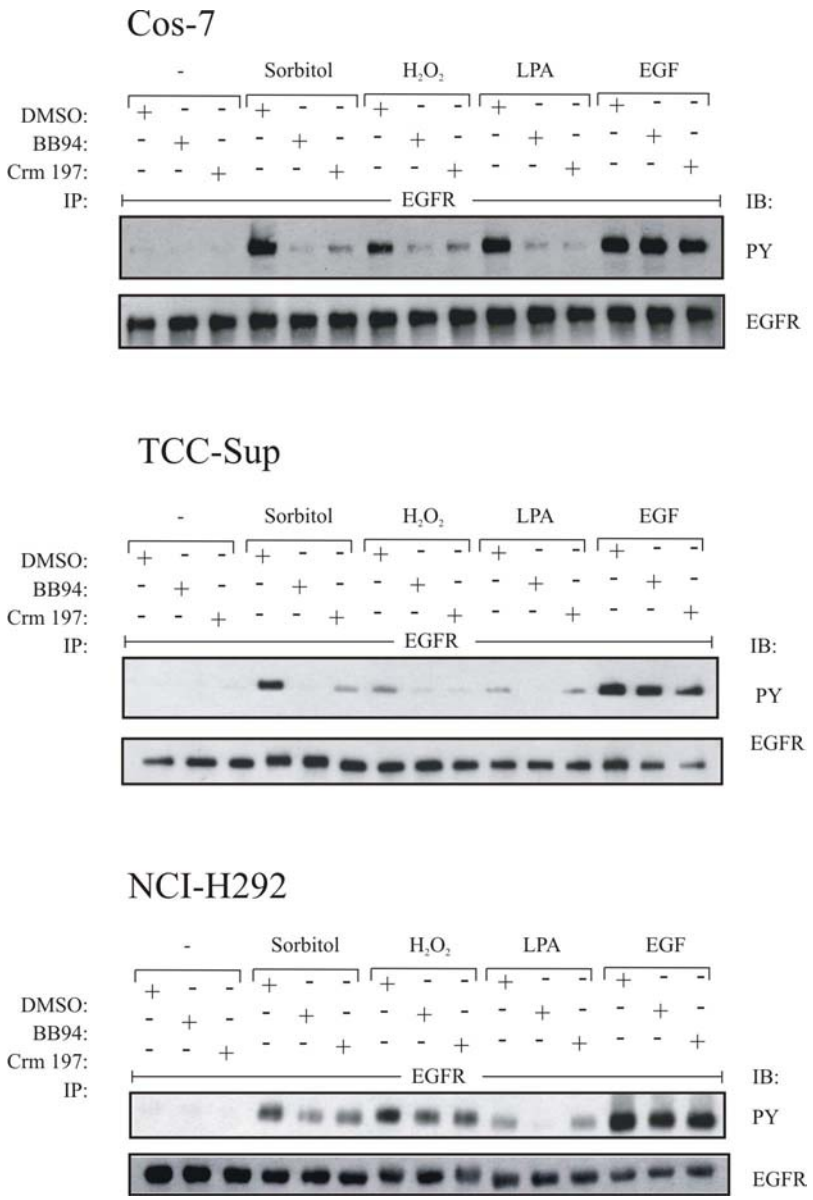

Figure 17 **EGFR activation in response to osmotic and oxidative stress depends on metalloprotease-mediated HB-EGF processing.** (A) Effect of metalloprotease and HB-EGF inhibition on EGFR phosphorylation. Cos-7, NCI-H292 and TCC-Sup cells were serum-starved for 24 hrs, pre-treated with BB94 (10 µM), the diphtheria toxin mutant CRM197 (10 µg/mL) or an equal volume of empty vehicle (DMSO) for 20 min, and stimulated for 10 min with 0.3 M sorbitol, 200 µM hydrogen peroxide, 10 µM LPA or 2 ng/mL EGF. Following immunoprecipitation of cell extracts with anti-EGFR antibody proteins were immunoblotted with anti-phosphotyrosine antibody and re-probed with anti-EGFR antibody.

Furthermore, we investigated whether this ligand-dependency is also observed at the EGFR substrate level. Shc adaptor proteins are well-characterised adaptor proteins linking the EGFR to activation of the Ras/Raf/ERK-MAPK signalling cascade. As shown in Figure 18, both CRM197 and BB94 pre-treatment of Cos-7 cells strongly suppressed SHC tyrosine phosphorylation in response to stress stimuli resembling that of the EGFR itself. Therefore, phosphorylation of Shc by stress stimuli critically depends on a ligand-dependent EGFR phosphorylation mechanism in Cos-7 cells.

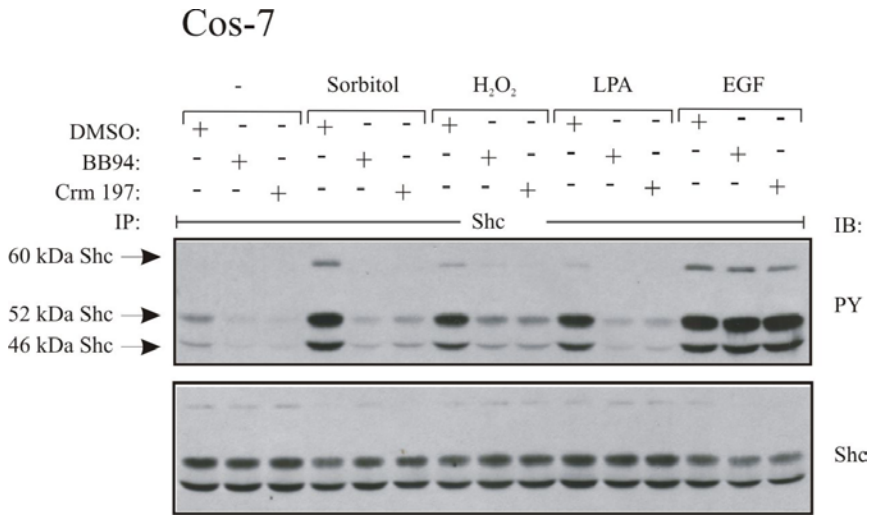

Figure 18 **Analysis of Shc phosphorylation in response to stress agents**. Cos-7 cells were treated as described under Figure 17. After immunoprecipitation of Shc from cell extracts with a polyclonal anti-Shc antibody proteins were immunoblotted with anti-phosphotyrosine antibody and re-probed with anti-Shc antibody.

3.2.6. Ectodomain shedding of proHB-EGF is induced in response to osmotic and oxidative stress in Cos-7 cells

To further substantiate the role of HB-EGF in this ligand-dependent EGFR stimulation mechanism, we directly investigated proHB-EGF processing by flow cytometric analysis of the total amount of ligand precursor present on the cell surface of Cos-7 cells prior to and after stimulation with sorbitol or hydrogen peroxide. As shown in Figure 19, both stimuli lead to a significant decrease of HB-EGF precursor detectable on the cell surface. Moreover, in accordance with the results presented in Figure 17, pre-treatment with the metalloprotease inhibitor BB94 abolished proHB-EGF processing.

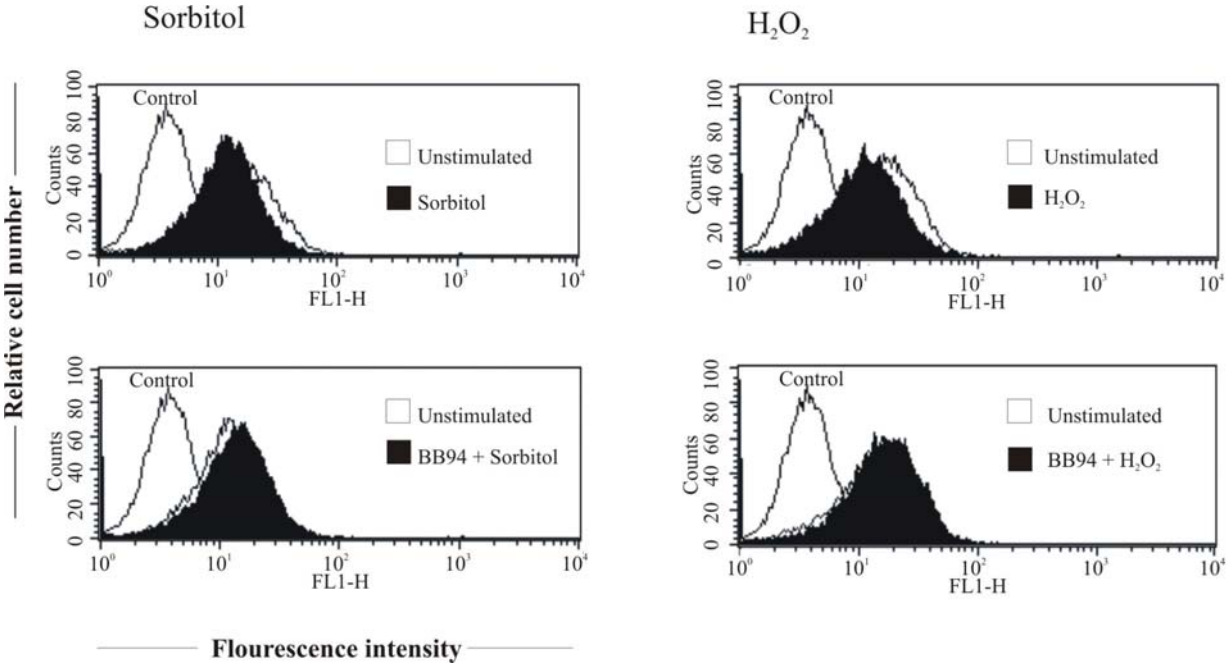

Figure 19 **Flow cytometric analysis of proHB-EGF processing in response to stress agents**. Cos-7 cells were pre-treated with BB94 (10 µM) or an equal volume of empty vehicle (DMSO) for 20 min, and stimulated with 0.3 M sorbitol or 200 µM hydrogen peroxide for 30 min. Cells were collected and stained for surface proHB-EGF and analyzed by flow cytometry.

In addition to the reduction of HB-EGF precursor, we used Cos-7 cells ectopically expressing proHB-EGF to determine the amount of mature soluble HB-EGF released from the cell surface into the supernatant. As shown in Figure 20, both treatment with sorbitol or hydrogen peroxide induced an increase of mature HB-EGF in the cell culture supernatant as determined by immunoblot analysis. Again, HB-EGF release was blocked by preincubation with the metalloprotease inhibitor BB94 confirming the involvement of a metalloprotease.

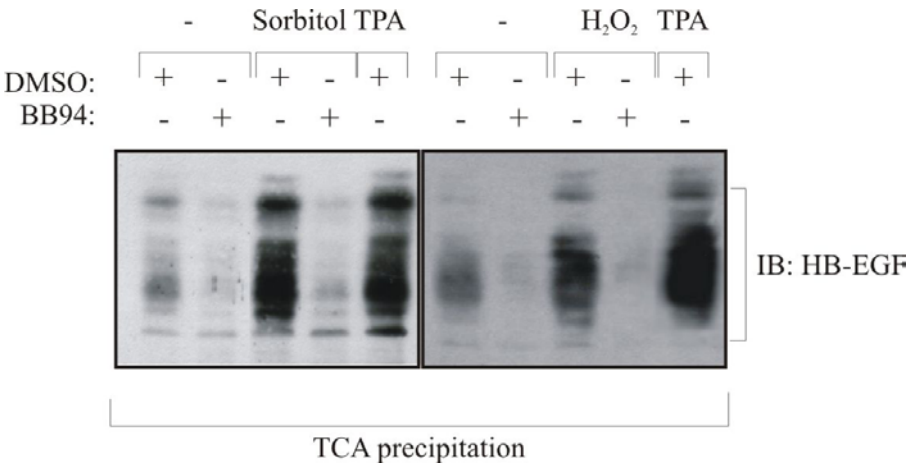

Figure 20 **Immunoblot analysis of conditioned media**. Cos-7 cells were transiently transfected with proHB-EGF cDNA. After serum starvation for 24 hours cells were stimulated for 20 minutes with sorbitol (0.3 M) or hydrogen peroxide (200 µM), and proteins within the supernatant were precipitated using trichloroacetic acid (TCA) precipitation. Precipitated proteins were subjected to tricin-sodiumdodecylsulfate gel electrophoresis

following the protocol of Schägger-Jagow and subsequent immunoblot analysis with anti-HB-EGF antibody. TPA stimulation has been included as a positive control.

3.2.7. **Metalloproteases of the ADAM family mediate EGFR activation by osmotic and oxidative stress**

The finding that metalloprotease-dependent mechanisms significantly contribute to stress-induced EGFR and Shc activation raised the question which metalloprotease(s) are involved in this signalling cascade. cDNA microarray analysis of NCI-H292 cells revealed that those ADAM family proteases which have already been implicated in EGF-like ligand cleavage are expressed in this cell line (Asakura et al., 2002; Izumi et al., 1998; Peschon et al., 1998; Sunnarborg et al., 2002; Yan et al., 2002) (Figure 12). In Cos-7 cells cDNA microarray analysis revealed expression of ADAM9, 10, 15 and 17 and of the EGF-like ligands HB-EGF, EGF and TGF-α (Stefan Hart, this group, personal communication).

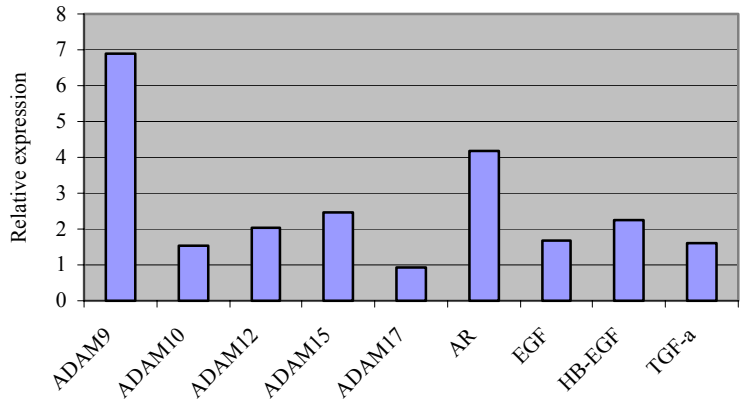

Figure 21 **cDNA micro-array analysis of NCI-H292 cells for ADAM expression and EGF-like ligand** . Expression analysis was performed as described in the section materials and methods.

We used the RNA interference technique to inhibit the endogenous expression of the proteases ADAM9, -10, -12, -15 and ADAM17. Figure 22 shows the efficient and specific knockdown of target gene expression by the siRNAs against ADAM9, 10 and 15 using RT-PCR analysis. The siRNAs against ADAM12 and 17 have been described previously (Gschwind et al., 2003). Transient transfection of these siRNAs directed against the individual proteases and subsequent phosphotyrosine analysis of the EGFR upon stimulation with either sorbitol or hydrogen peroxide revealed that ADAM10 and ADAM17 are involved in EGFR activation in response to both stress stimuli in Cos-7 cells (Figure 23 A). Since we were particularly interested in the regulation of these processes in human cancer cells, we further investigated the involvement of ADAM family members in the lung carcinoma cell line NCI-

H292. Similar to the results obtained in Cos-7 cells, ADAM17 also mediates the stress-induced EGFR activation as demonstrated in Figure 23 B. But, in contrast to Cos-7 cells, ADAM9 and not ADAM10 is involved in the stress response of NCI-H292 cells. Thus, cell-type specific but overlapping sets of ADAM family members regulates HB-EGF-dependent EGFR activation in response to stress agents.

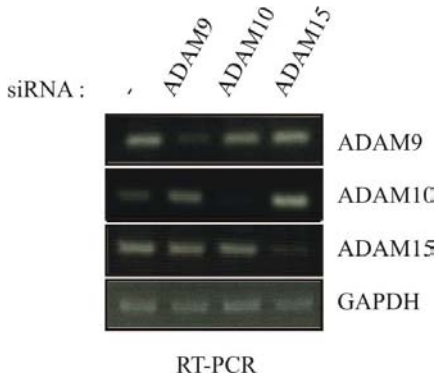

Figure 22 **Blockade of ADAM metalloprotease expression by RNA interference (RNAi)**. NCI-H292 cells were transfected with siRNA against ADAM9, ADAM10 or ADAM15, cultured for 2 days and analyzed for gene expression by RT-PCR as indicated.

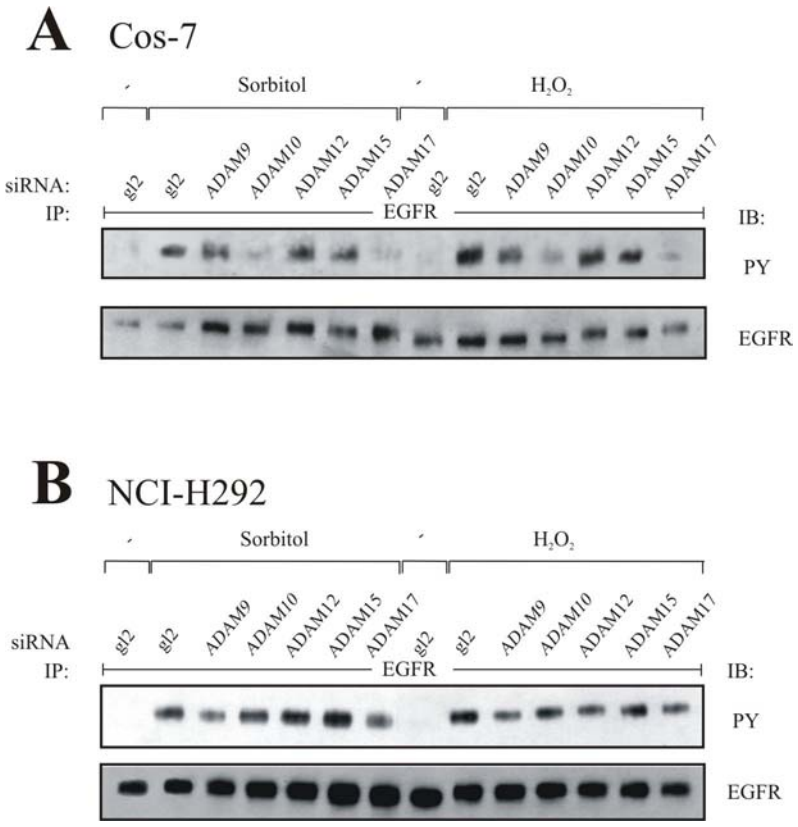

Figure 23 **RNAi-induced downregulation of ADAM metalloproteases interferes with stress-induced EGFR activation**. (A) Cos-7 cells were transfected with siRNAs against ADAM9, -10, -12, -15 and –17, serum-starved for 24 hours, stimulated with 0.3 M sorbitol or 200 µM hydrogen peroxide for 10 min and assayed for EGFR tyrosine phosphorylation content. (B) NCI-H292 cells were treated as described under (A).

3.2.8. Activation of the MAPKs ERK1/2 and JNK in response to hyperosmolarity and oxidative stress is mediated by HB-EGF-dependent EGFR activation

Since the MAPKs ERK1/2 and JNK are activated by hypertonicity and reactive oxygen species (de Nadal et al., 2002; Kyriakis and Avruch, 2001), we asked whether the ligand-dependent EGFR phosphorylation contributes to the induction of these MAPK family members by stress stimuli. To investigate the overall dependence of stress-induced MAPK activation on the EGFR kinase activity, we used the selective EGFR inhibitor AG1478. Furthermore, we compared the effect of tyrosine kinase inhibition with the suppression of ligand-dependent EGFR activation by BB94 and CRM197. As shown in Figure 24 (upper panel), both sorbitol and hydrogen peroxide-induced activation of ERK1/2 is blocked by AG1478. Moreover, BB94 and CRM197 are almost as effective in blocking ERK1/2 phosphorylation. These data suggest that stress-induced ERK1/2 activation in Cos-7 cells almost completely depends on EGFR activation, which can be largely attributed to a ligand-dependent mechanism.

The same experimental setup was used to address the question whether the same mechanistic concept is valid for stress signalling in human carcinoma cell lines. As shown in Figure 6A, we found that hyperosmolarity- and oxidative stress-induced ERK1/2 activation in TCC-Sup and NCI-H292 cells was substantially blocked by AG1478, BB94 or CRM197 (Fig. 6A, middle and lower panel). Interestingly, although oxidative stress induces EGFR phosphorylation only partially through a ligand-dependent mechanism in NCI-H292 cells, ERK activation depends mainly on proHB-EGF processing in this cell type.

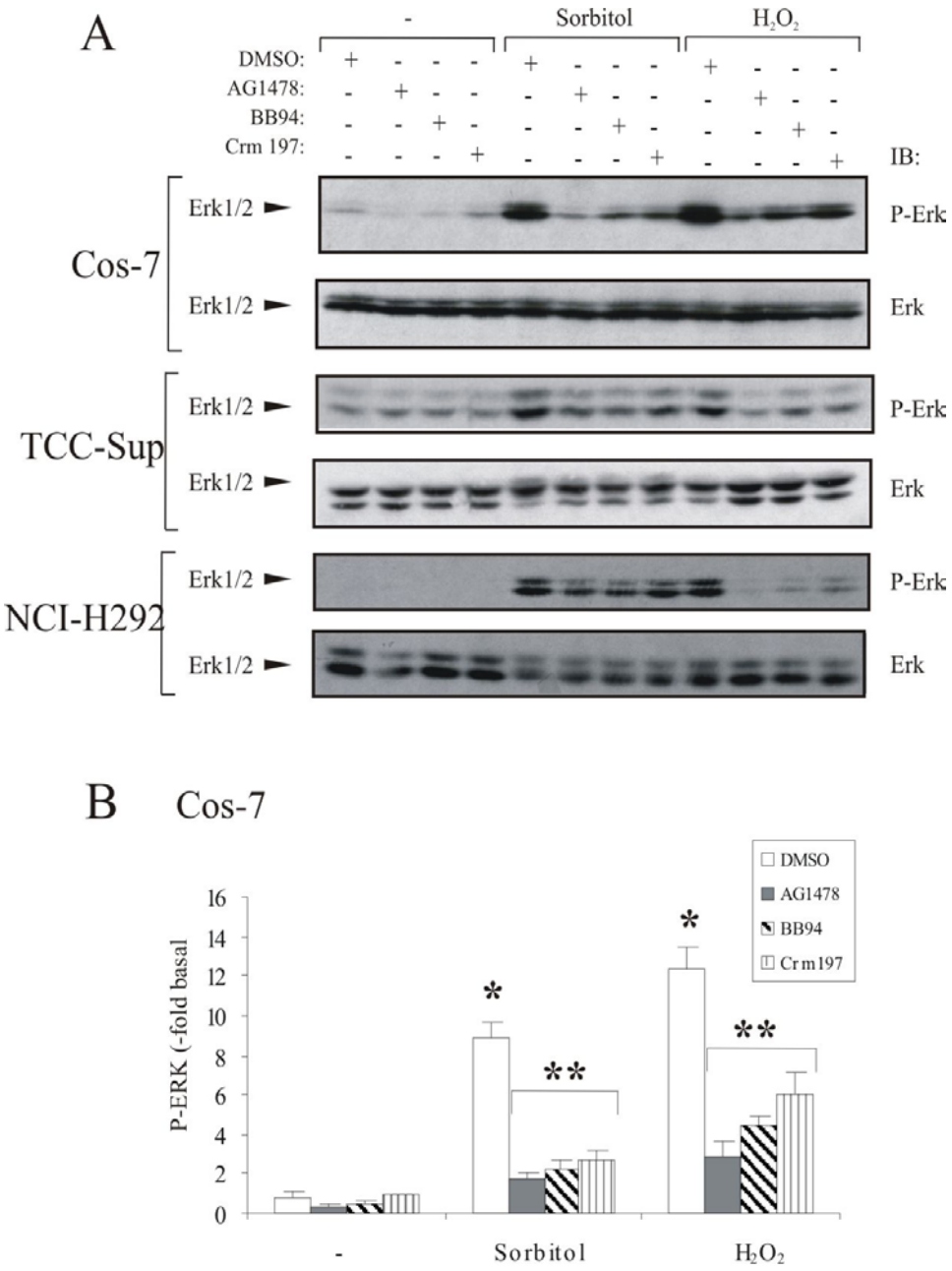

Figure 24 **ERK1/2 activation in response to stress agents and blockade of EGFR, metalloprotease and HB-EGF function**. Cos-7 cells transiently transfected with pcDNA3-HA-ERK2, TCC-Sup and NCI-H292 cells were pre-treated with AG1478 (250 nM), BB94 (10 µM), CRM197 (10 µg/mL) or an equal volume of empty vehicle (DMSO) for 20 min and stimulated with 0.3 M sorbitol or 200 µM hydrogen peroxide for 30 min. After cell lysis total lysates were immunoblotted with anti-phospho-ERK antibody, followed by reprobing of the same membranes with polyclonal anti-ERK antibody. Quantitative analysis of ERK phosphorylation from three independent experiments (mean ± s.d.) using the FUJI LAS1000 imaging system. **P* < 0.001 for control versus stimulation. ***P* < 0.006 for stimulation versus stimulation + inhibitors.

In addition to ERK1/2, we were interested in the signalling mechanisms leading to activation of JNK MAPKs. Figure 25 (upper panel) shows the JNK activity induced by osmotic and oxidative stress upon pre-treatment with AG1478, BB94 or CRM197. While sorbitol-induced JNK activity is largely independent of the EGFR in Cos-7 cells, 50% of the oxidative stress-induced JNK activity depend on the EGFR and HB-EGF. Similar results were obtained in the

bladder carcinoma cell line TCC-Sup as shown in Figure 25 (middle panel). In contrast, hydrogen peroxide stimulated JNK activation in the lung carcinoma cell line NCI-H292 appears to be independent of the EGFR, while JNK activity in response to hypertonicity partially depends on a ligand-dependent EGFR activation pathway.

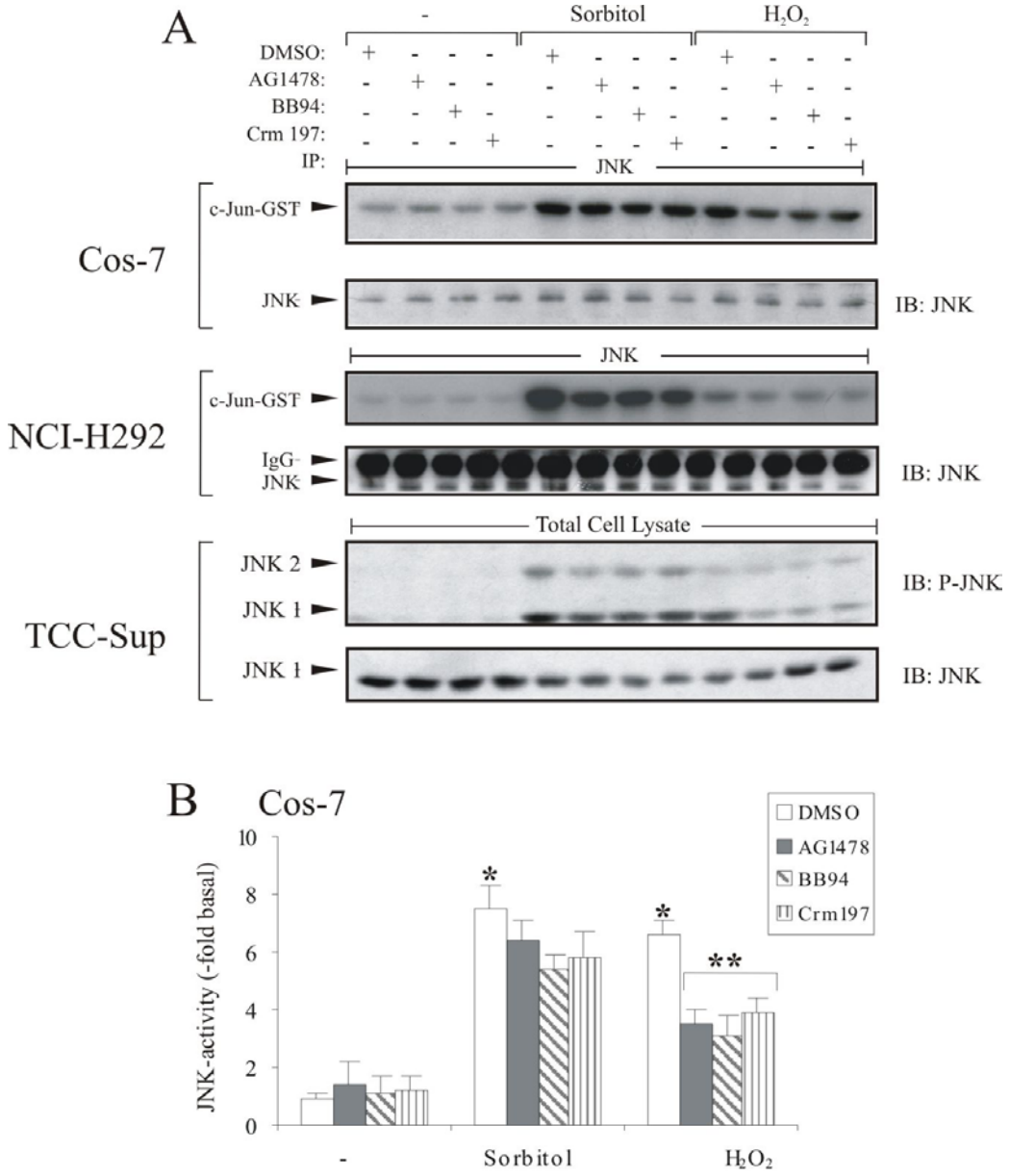

Figure 25 **JNK activation in response to stress agents and blockade of EGFR, metalloprotease and HB-EGF function**. (A) Cos-7 and NCI-H292 cell were treated as described under Figure 24. After lysis, JNK was immunoprecipitated using an anti-JNK antibody, and JNK activity was assayed using GST-c-JUN fusion protein as a substrate. Phosphorylated GST-c-JUN was visualized by autoradiography and JNK was immunoblotted in parallel using polyclonal JNK antibody. Quantitative analysis of GST-c-JUN phosphorylation from three independent experiments (mean ± s.d.) using the FUJI LAS1000 imaging system. **P* < 0.001 for control versus stimulation. ***P* < 0.01 for H_2O_2 stimulation versus H_2O_2 + inhibitors. TCC-Sup cells were treated as described under (A). After cell lysis, JNK phosphorylation was assayed by immunoblotting cell extracts with anti-phospho-JNK antibody and reprobing of the same filters with anti-JNK antibody.

In agreement with the finding that p38 acts as an upstream mediator of stress-induced EGFR activation Figure 16, p38 activity neither depended on the EGFR kinase activity nor on metalloprotease or HB-EGF function in Cos-7 cells (Figure 26).

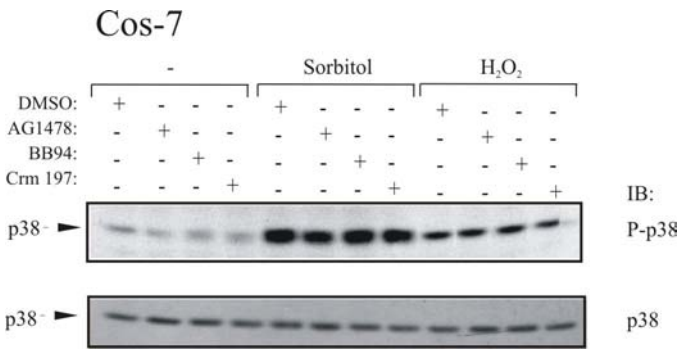

Figure 26 **p38 phosphorylation is independent of EGFR, metalloprotease and HB-EGF function**. Cos-7 cells were treated as described under Figure 24. p38 phosphorylation was assayed by immunoblotting cell extracts with anti-phospho-p38 antibody and re-probing the same filters with anti-p38 antibody.

3.2.9. Blockade of HB-EGF function strongly enhances doxorubicin-induced cell death

Treatment of tumour cells with chemotherapeutics has been shown to activate the stress kinases p38 and JNK (Benhar et al., 2002b). Similar results with respect to p38 activation were obtained with TCC-Sup bladder carcinoma cells after exposure to the chemotherapeutic doxorubicin (Figure 27).

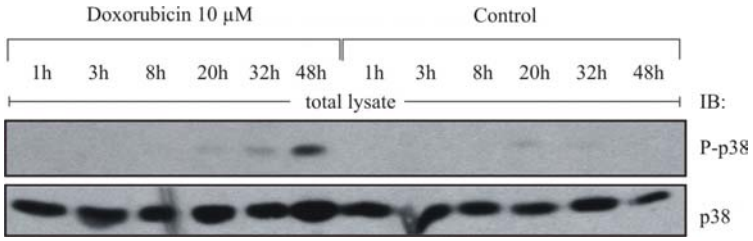

Figure 27 **p38 activation in response to doxorubicin treatment**. TCC-Sup cells were seeded and treated with doxorubicin for the indicated time points. After cell lysis, p38 activation was assessed by immunoblotting cell extracts with anti-phospho-p38 antibody and reprobing of the same filters with anti-p38 antibody.

In this physiological context, p38-triggered proHB-EGF processing and subsequent EGFR activation might provide a cellular survival signal and thereby counteract doxorubicin-induced apoptosis. To test this hypothesis, we treated TCC-Sup cells with the specific HB-EGF blocker CRM197 and measured its effect on doxorubicin-induced cell death. Remarkably, CRM197 treatment strongly enhances the apoptotic response to doxorubicin

when compared to doxorubicin alone, while CRM197 had only a minor effect on cell survival. Thus, these results point to a physiological significance of p38-dependent EGFR activation as a pathway employed by tumour cells to evade apoptosis upon chemotherapy-induced cellular stress.

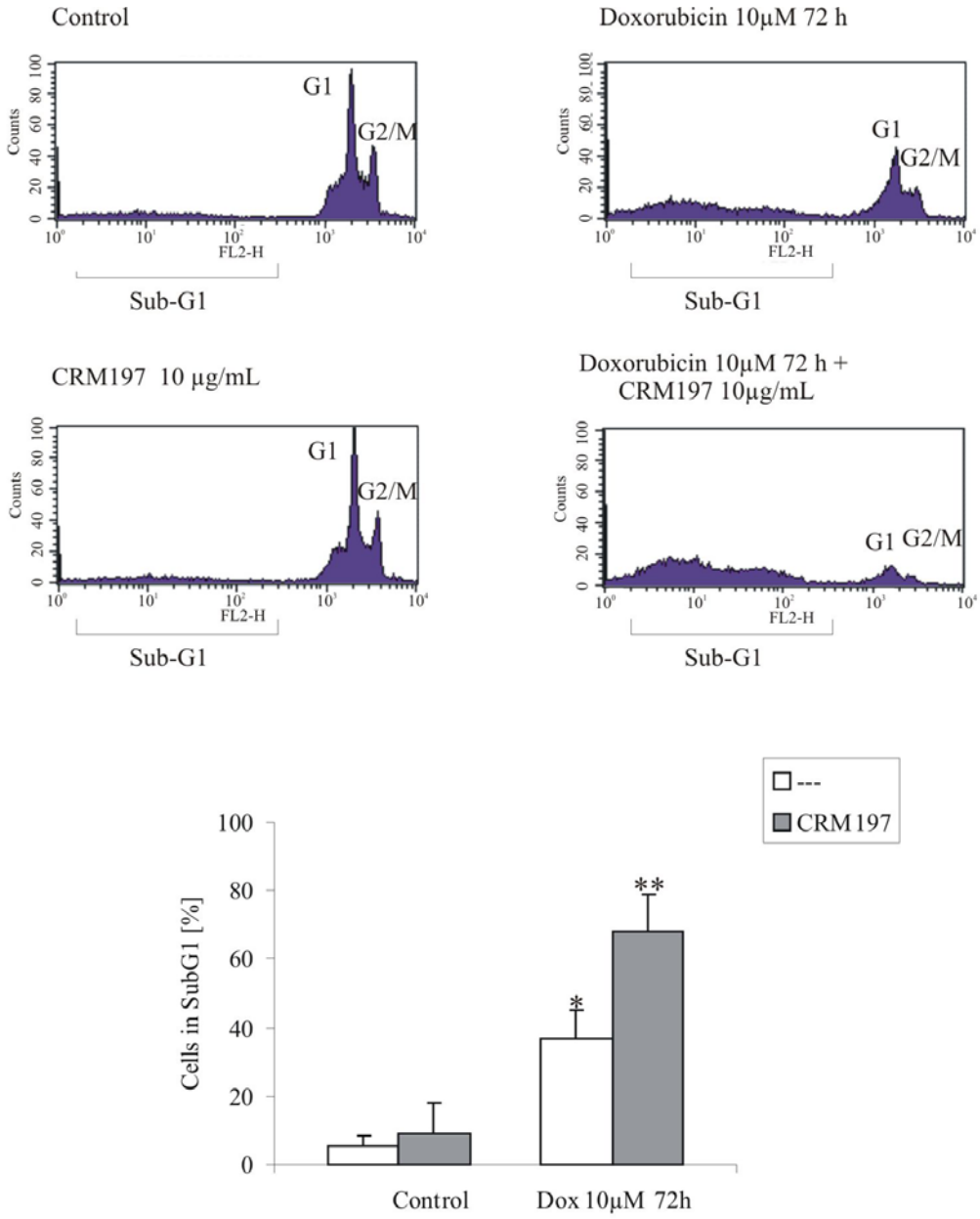

Figure 28 **Blockade of HB-EGF function enhances cell-death in response to doxorubicin**. Cells were treated for 72 h with 10 µM doxorubicin and 10 µg/mL Crm197 every 24 h as indicated. After collection of cells in assay buffer, nuclei were stained with PI and analyzed by flow cytometric analysis. Quantification of four independent experiments (mean ± SD). *$P < 0.001$ for control versus doxorubicin. **$P < 0.004$ for doxorubicin versus doxorubicin + CRM197.

3.3. Met Receptor transactivation by GPCRs and EGFR is mediated by ROS

3.3.1. Analysis of cell surface protein phosphorylation in response to GPCR stimulation in pancreatic carcinoma cells

In order to address the question whether other RTKs become activated and therefore tyrosine phosphorylated in response to GPCR agonist treatment, we stimulated DAN-G pancreatic carcinoma cells with LPA or Thrombin for 3 min. To selectively extract cell surface proteins, lectin chromatography was applied to the cell lysates using lentilsepharose and subsequently the fraction of glycosylated proteins that bind to lentilsepharose was analyzed for phosphotyrosine content.

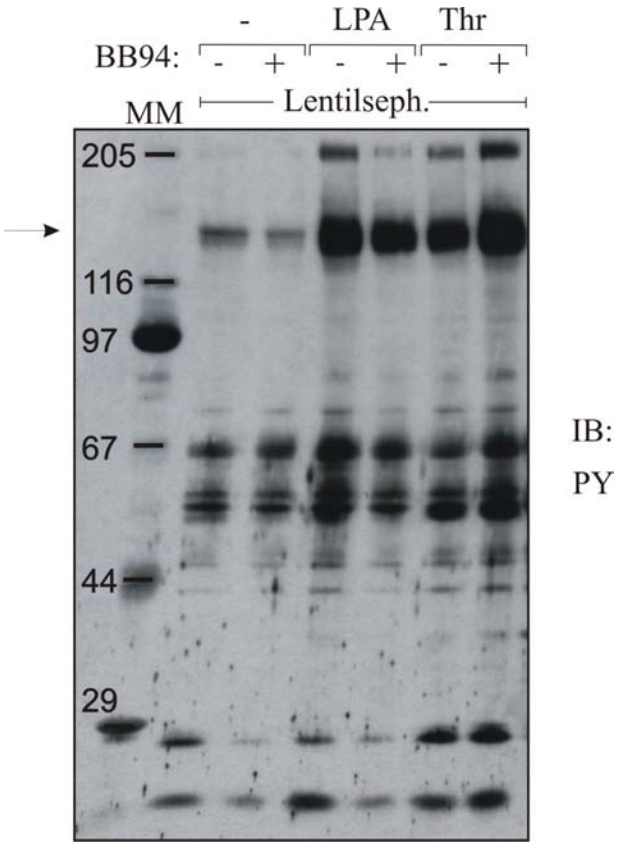

Figure 29 **Lentilsepharose extraction of DAN-G pancreatic carcinoma cells**. DAN-G pancreatic carcinoma cells were serum-starved for 24 hrs, preincubated with BB94 (10 µM) and stimulated with LPA (10 µM) or Thrombin (2U/mL) for 3 min. Cell lysates were extracted for glycosylated cell surface proteins using lentilsepharose. Extracts were applied to a 7.5-12% gradient SDS-PAGE, transferred to nitrocellulose and immunoblot analysis against phosphotyrosine was performed.

As shown in Figure 29 both LPA and Thrombin induce phosphorylation of a 140 kDa protein band independent of metalloprotease function, as revealed by preincubation with BB94. This tyrosine phosphorylated protein is distinct of the EGFR, which migrates at 170 kDa.

3.3.2. GPCR- and EGFR Stimulation induce Met receptor tyrosine phosphorylation

The result of the experiment shown in Figure 29 revealed a yet unidentified protein which becomes tyrosine phosphorylated in response to GPCR agonist treatment. Since this protein was glycosylated it was likely to be a transmembrane protein. Moreover, the tyrosine phosphorylation of this molecule suggested a RTK as the protein of interest. Indeed immunoblot experiments identified this protein as the Met receptor and in consequence as a new RTK that becomes transactivated in response to GPCR stimulation (Figure 30).

We used immunoblot analysis to assess the phosphorylation state of the Met receptor in response to treatment with different GPCR agonists. As shown in Figure 30, Met receptor tyrosine phosphorylation is rapidly induced in response to different GPCR ligands in the pancreatic carcinoma cell line DAN-G and the hepatocellular carcinoma cell lines HepG2 and HuH7 (Figure 30).

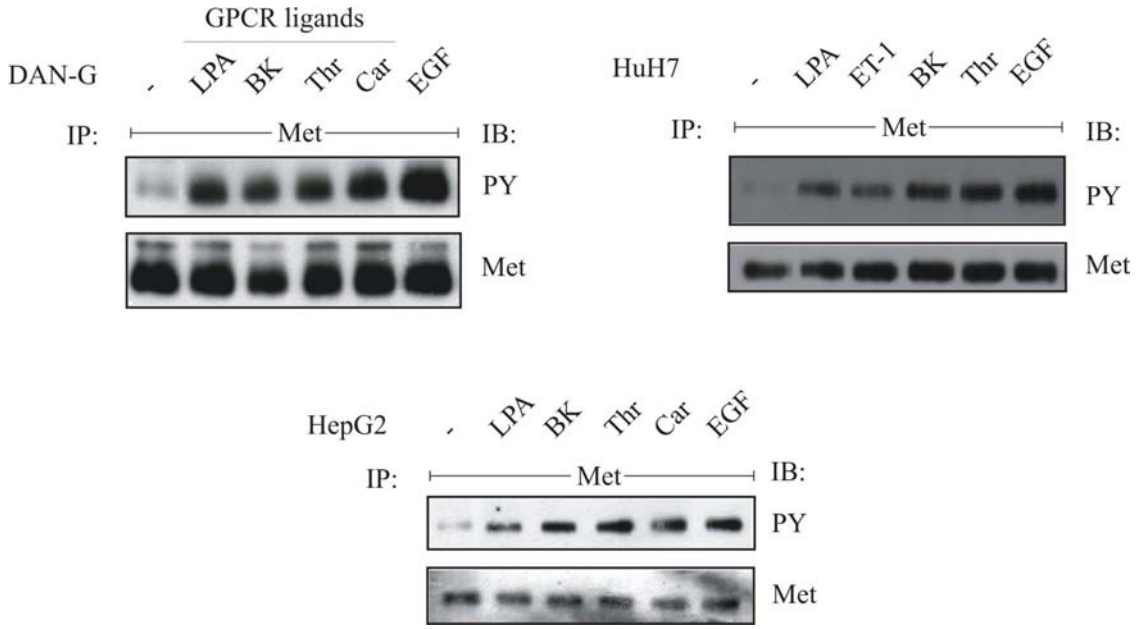

Figure 30 **GPCR agonists and EGF induce Met receptor transactivation**. Cells were serum-starved for 24 hrs, treated with lysophosphatidic acid (LPA, 10 µM), Bradykinin (BK, 2 µM), Thrombin (Thr, 2U/mL), Carbachol (Car, 10 µM) or EGF (4 ng/mL) as indicated for 3 min. Following immunoprecipitation (IP) of cell extracts with anti-Met antibody proteins were immunoblotted (IB) with anti-phosphotyrosine antibody and re-probed with anti-Met antibody. The upper band of higher molecular weight shown in the DAN-G Met receptor reblot represents the precursor form of the Met receptor, consisting of both the α- and β-chain. The lower migrating band represents the processed mature β-chain.

Time course experiments revealed that this activation occurs readily after 3 minutes and declines after 7 to 15 minutes (Figure 31), demonstrating a rapid and transient transactivation of the Met receptor. Interestingly, not only GPCR ligands but also EGF induced stimulation

of the Met receptor (Figure 30). In both cases Met receptor tyrosine phosphorylation induced by its transactivation resembles stimulation by 4 ng/mL exogenous HGF (Figure 32).

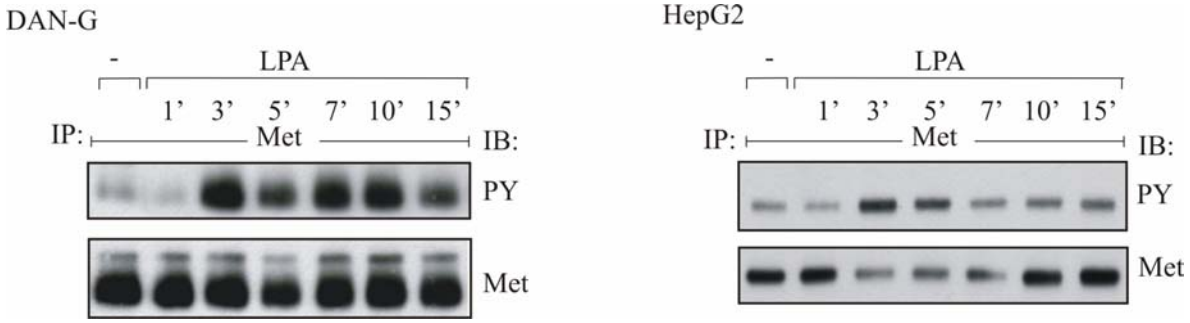

Figure 31 **Time course of GPCR- and EGF-induced Met receptor transactivation**. Cells were stimulated with LPA (10 µM) for the indicated time periods. Cell lysates were treated as described under Figure 30.

3.3.3. GPCR-induced Met Transactivation occurs independent of EGFR kinase activity

EGFR signal transactivation is the so far best characterized GPCR-RTK cross-talk mechanism. As EGFR-Met association and phosphorylation of Met by the EGFR's kinase activity have been proposed in previous reports (Jo et al., 2000; Pai et al., 2003), we wanted to investigate whether GPCR-Met transactivation occurs via this previously suggested mechanism. This putative signalling pathway renders the Met receptor as a direct signaling partner of the EGFR.

To address this question we used the EGFR kinase specific inhibitor AG1478 to block EGFR-dependent phosphorylation events. As demonstrated in Figure 32, blockade of the EGFR kinase activity did not interfere with GPCR-induced Met phosphorylation, demonstrating that the EGFR kinase is dispensable for Met transactivation in response to treatment with GPCR agonists. In contrast, GPCR-induced EGFR signal transactivation is blocked by AG1478 treatment, as transactivation of the EGFR depends on its kinase activity (Daub et al., 1996; Prenzel et al., 1999) (Figure 32).

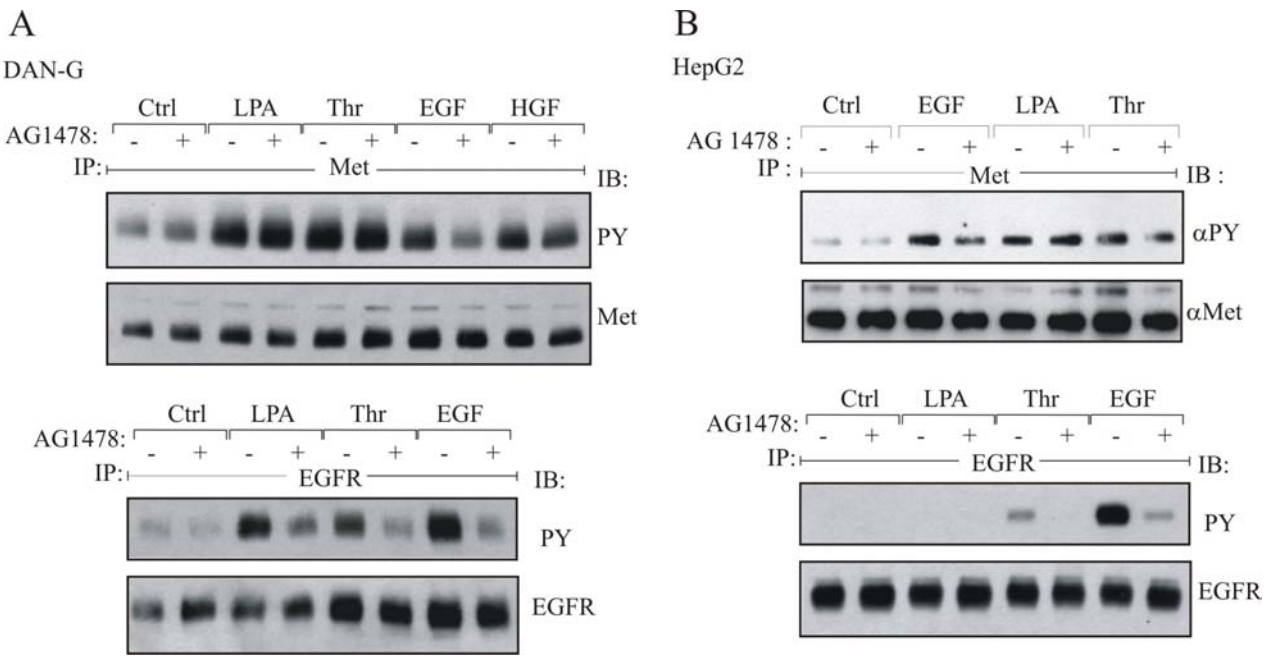

Figure 32 **GPCR-induced Met transactivation is independent of EGFR kinase activity**. DAN-G (A) and HepG2 (B) cells were pre-treated with AG1478 (250 nM) or an equal volume of empty vehicle (DMSO) for 20 min and subsequently stimulated with LPA (10 µM), Thr (2U/mL), EGF (4 ng/mL) or HGF (4 ng/mL) as positive control for 3 min. Following immunoprecipitation of cell extracts with anti-Met antibody proteins were immunoblotted with anti-phosphotyrosine antibody and reprobed with anti-Met antibody. EGFR phosphorylation content was analyzed by immunoprecipitation of cell extracts with anti-EGFR antibody and immunoblotting with anti-phosphotyrosine. The same filters were reprobed with anti-EGFR antibody.

As anticipated, Met transactivation by EGF was abrogated by preincubation with AG1478 (Figure 32 A/B, lower panel), as the EGFR kinase is crucial to transmit the EGF-stimulated signal, regardless of direct association or an indirect communication with the Met receptor. But since no co-precipitation between the EGFR and Met receptor or vice versa could be detected, neither in unstimulated nor in stimulated cells, these observations suggest a mechanism distinct of RTK association between the EGFR and the Met receptor for Met transactivation.

3.3.4. LPA-induced Met transactivation is independent of Src, PKC, PI3K and MEK1/2 activity

To further investigate the mechanism leading to Met receptor transactivation we used different inhibitors against cellular signalling molecules that have earlier been revealed as GPCR effector molecules and which have been implicated in GPCR-RTK cross-talk (Wetzker and Bohmer, 2003). Figure 33 shows that preincubation of DAN-G cells with inhibitors against Src (PP1), PKC (GF109203X), PI3K (LY204002) or MEK1/2 had no effect on Met receptor transactivation, suggesting that these signalling proteins are not involved as upstream mediators in the Met receptor transactivation mechanism.

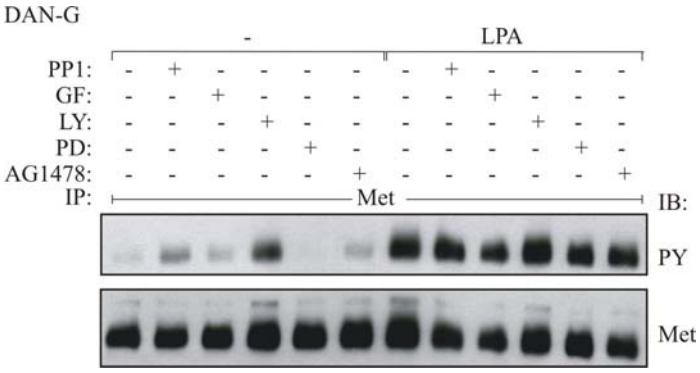

Figure 33 **Inhibition of Src, PKC, PI3K and MEK1/2 has no effect on Met receptor transactivation**. DAN-G pancreatic carcinoma cells were serum-starved for 24 hrs, preincubated with PP1 (10 µM, 30 min), GY109203X (10 µM, 30 min), LY294002 (10 µM, 30 min) or PD98059 (25 µM, 60 min) and stimulated with LPA (10 µM) for 3 min. Met receptor was immunoprecipitated and analyzed for phosphotyrosine content. The same filter was stripped and reprobed for Met receptor content.

3.3.5. Met receptor transactivation is independent of serine proteases

The ligand for the Met RTK HGF is secreted from its producing cell and has to be activated by serine proteases to become active. Although HGF is not a transmembrane protein, this proteolytic activation step could potentially resemble the metalloprotease-dependent shedding event involved in EGFR signal transactivation. To test this hypothesis we used different serine protease inhibitors to interfere with the putatively GPCR-induced proteolytic activation step of HGF. The serine protease inhibitor aprotinin has earlier been used to block activation of HGF (Li et al., 1998). As shown in Figure 34, preincubation of DAN-G cells with aprotinin had no effect on Met receptor transactivation by LPA. Interestingly, BB94 exerted also no effect on Met receptor tyrosine phosphorylation. Since aprotinin does not inhibit all serine proteases, we used the stronger serine protease inhibitor Pefabloc™ to further substantiate this result. In agreement with the result obtained in Figure 34, Figure 35 shows that inhibition of serine protease-dependent shedding events has no effect on Met receptor transactivation. Higher concentrations of 4 mM Pefabloc™ reduced the overall level of Met receptor, both the precursor and the mature β-chain form (Figure 35).

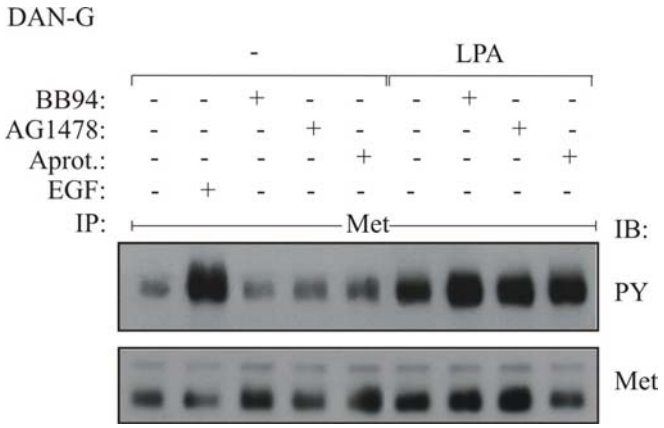

Figure 34 **Inhibition of serine proteases by aprotinin has no effect on Met receptor transactivation**. DAN-G cells were preincubated with 100 µg/mL aprotinin (Aprot.) for 60 min, AG1478 (250 nM, 20 min) or BB94 (10 µM, 20 min), subsequently stimulated with LPA (10 µM) or EGF (2 ng/mL) for 3 min and Met receptor phosphorylation was assessed as in Figure 33.

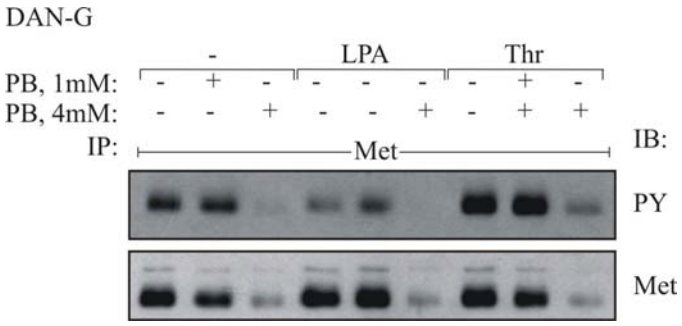

Figure 35 **Met receptor transactivation is not affected by inhibition of serine proteases with Pefabloc™** . Serum-starved DAN-G cells were pre-incubated with the indicated concentrations of Pefabloc™ for 60 min and stimulated with LPA (10 µM) or Thr (2 U/mL) for 3 min. Met receptor phosphorylation was assessed as described in Figure 33.

3.3.6. GPCR- and EGF-stimulated ROS production in carcinoma cell lines

Inactivation of tyrosine phosphatases by growth factor-induced production of ROS has been frequently implicated in the activation of RTKs (Bae et al., 1997; Finkel, 2003; Rhee et al., 2000; Sundaresan et al., 1995; Ushio-Fukai et al., 2001). Since the rapid and transient activation mechanism revealed by time course experiments (Figure 31) is in good agreement with a rapid and transient production of ROS, we asked whether Met receptor transactivation is mediated through a growth factor-stimulated increase in the level of reactive oxygen intermediates. Thus we used the fluorescence dye dichlorofluorescein diactate (DCF-DA) to detect ROS production. DCF-DA is a membrane-permeable non-fluorescent compound, which is deesterified inside the cell. Subsequent oxidation of dichlorofluorescein generates the fluorescent dye itself. Flow cytometric analysis of cells treated with DCF-DA and

subsequent stimulation with EGF or the GPCR-ligands LPA and Thrombin clearly demonstrated an increase of reactive oxygen species within the cell (Figure 36).

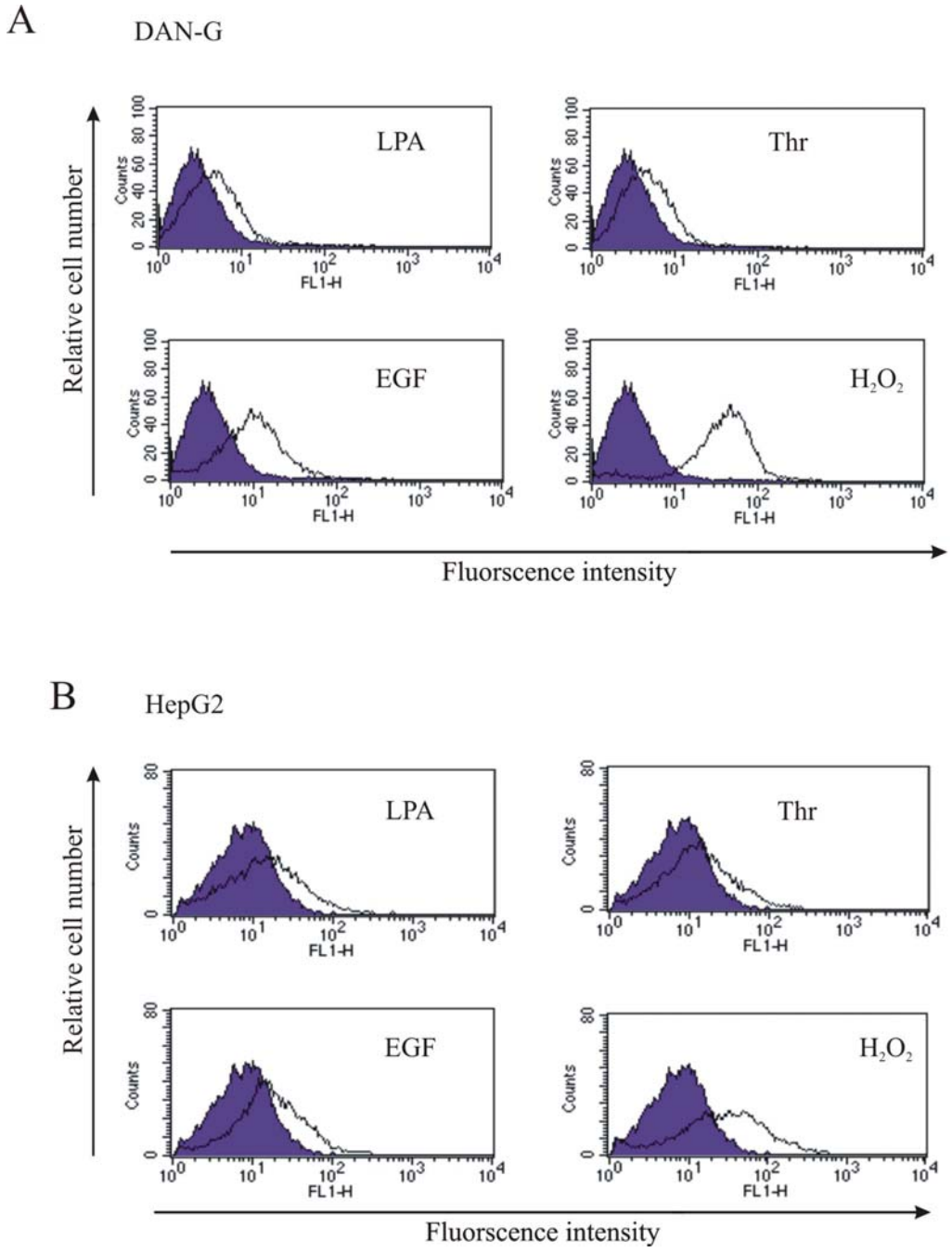

Figure 36 **GPCR agonists and EGF increase ROS production in DAN-G and HepG2 cells**. (A/B) Cells were seeded into 6-well dishes, grown for 48 hrs and incubated with 2',7'-dichlorofluorescein diacetate (DCF-DA, 20 µM) for 30 min. After stimulation with LPA (10 µM), Thr (2 U/mL), EGF (4 ng/mL) or hydrogen peroxide (H_2O_2, 500 µM) as a positive control for 15 min cells were collected, resuspended in PBS supplemented with propidium iodide and immediately analyzed by flow cytometric analysis.

To further substantiate the role of ROS in the Met receptor transactivation mechanism we preincubated the cells with the reducing agent epigallocatechin gallate (EGCG). As demonstrated in Figure 37, EGCG treatment of DAN-G cells interfered with EGF- and

GPCR-induced Met receptor tyrosine phosphorylation, underlining the involvement of ROS in this signaling pathway. On the other hand, EGCG preincubation leaves EGFR signal transactivation unaffected (Figure 37). Taken together, these results demonstrate the critical role of ROS production in the Met receptor transactivation mechanism.

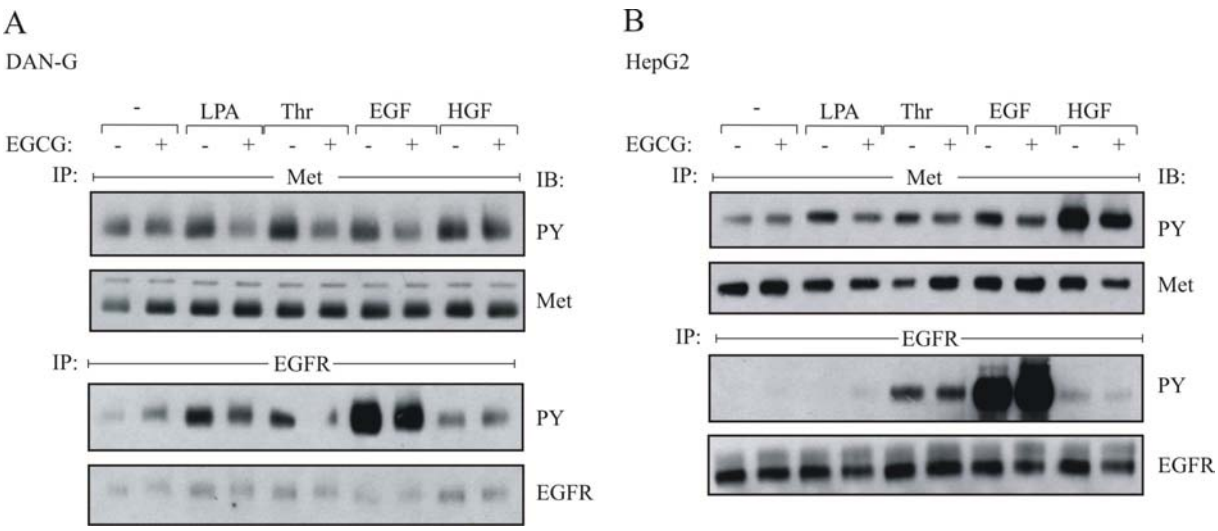

Figure 37 **EGCG abolishes Met receptor transactivation.** Cells were preincubated with epigallocatechin gallate (EGCG, 40 µM) for 60 min. Following stimulation as described under Figure 32 Met or EGFR respectively were immunoprecipitated and analyzed for phosphotyrosine content. The same filters were stripped and reprobed for Met and EGFR respectively.

3.3.7. Met receptor transactivation involves NADPH oxidase activity

Recent investigations implicated membrane-bound NADPH oxidases in the growth factor stimulated production of ROS (reviewed in (Finkel, 2000; Finkel, 2003; Rhee et al., 2000)). To investigate whether Nox enzymes are involved in Met receptor transactivation, we used the Nox inhibitor diphenyleneiodonium chloride (DPI) to interfere with Nox-dependent ROS production and analyzed Met receptor tyrosine phosphorylation by immunoblot analysis. As shown in Figure 38, blockade of Nox function interfered with both GPCR- and EGF-induced Met phosphorylation in HepG2 and DAN-G cells, suggesting the involvement of Nox in Met receptor transactivation. EGFR signal transactivation was not affected by DPI treatment (Figure 38).

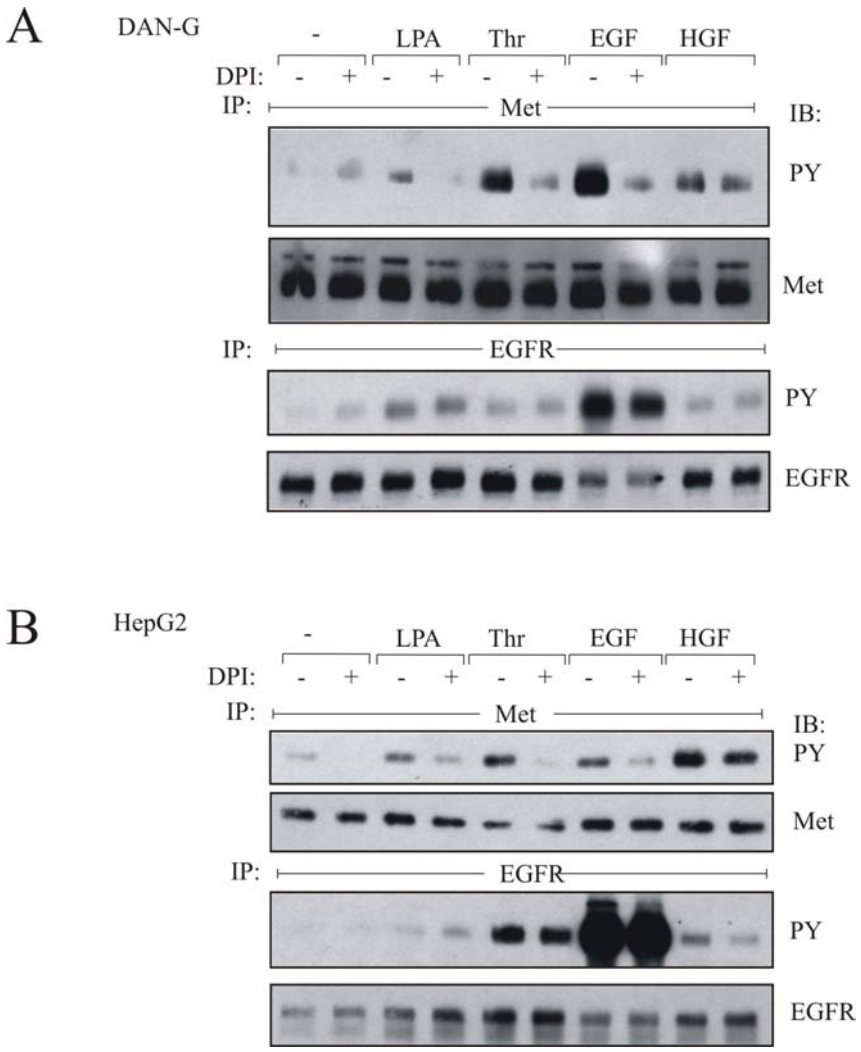

Figure 38 **ROS production depends on NADPH-oxidase activity**. (A/B) Following preincubation with diphenyleneiodonium chloride (DPI, 10 μM) for 30 min, cells were stimulated as indicated and Met (upper panel) or EGFR (lower panel) were analyzed for phosphotyrosine content by immunoblot analysis.

Investigations on regulatory mechanisms of Nox activity implicated the small GTPase Rac as one of the cytosolic components of the Nox enzyme complex (Sundaresan et al., 1996). Moreover, Rac has been shown as a downstream signalling target of both GPCRs and RTKs (LIT). Therefore we used the ToxinB, which blocks GTPases of the Rac, Rho and Cdc42 family, to interfere with Rac function (Schiavo and van der Goot, 2001). Indeed, preincubation with ToxinB abrogated both GPCR- and EGF-induced Met transactivation (Figure 39), while EGFR signal transactivation was not influenced (Figure 39). These data support the mechanistic concept that Nox enzymes are involved in the cross-talk mechanism leading to Met phosphorylation.

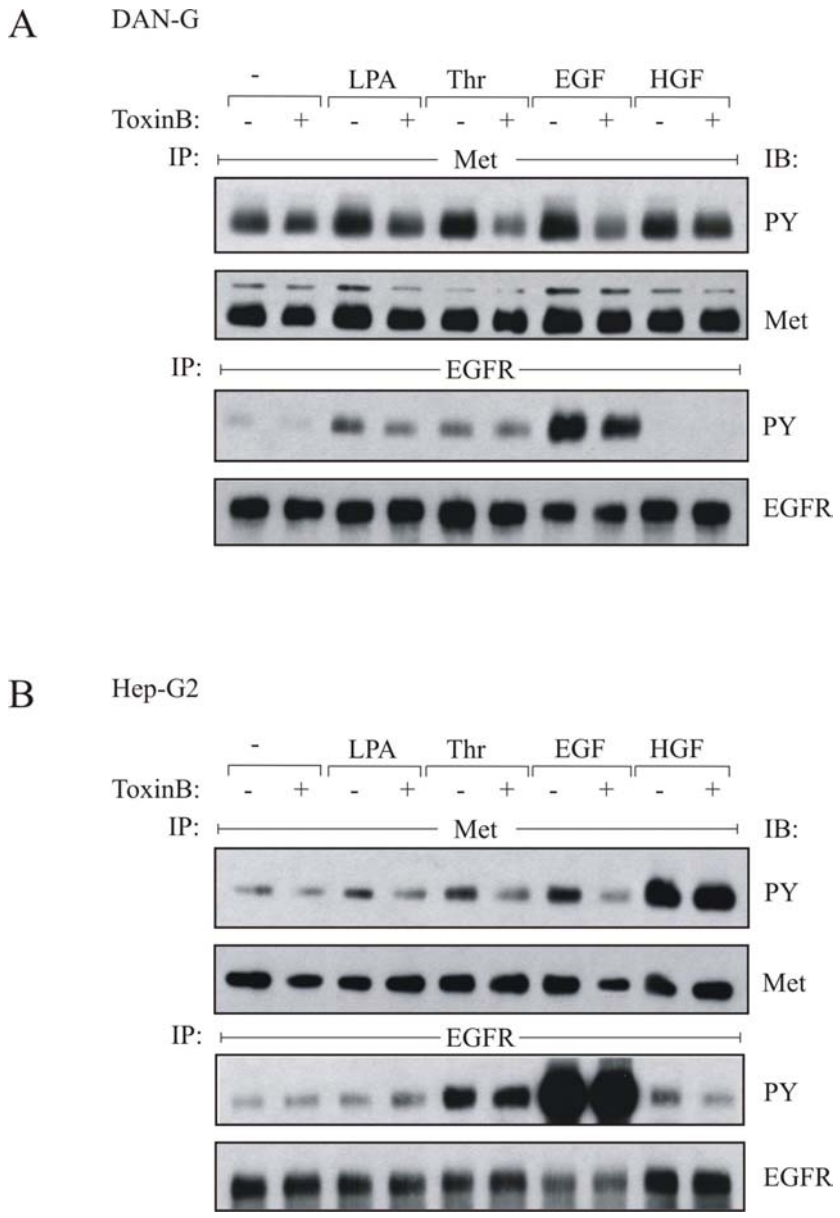

Figure 39 **ToxinB treatment blocks Met receptor transactivation**. Cells were pretreated with ToxinB (2 ng/mL) for 2 hrs, stimulated as indicated and analyzed as described under Figure 38.

3.3.8. Met receptor transactivation is independent of NO-synthase activity

Besides reactive oxygen species also reactive nitrogen species (RNS) have gained increasing attention as signalling intermediates (Schindler and Bogdan, 2001). To test whether the production of RNS plays a role in Met receptor transactivation, we used the specific NO-synthase inhibitor N^G-Monomethyl-L-arginine monoactate (L-NMMA) to interfere with RNS production. As shown in Figure 40 inhibition of DAN-G cells with L-NMMA did neither effect GPCR-induced nor EGF-stimulated Met receptor transactivation, nor was HGF stimulation used as a positive control affected.

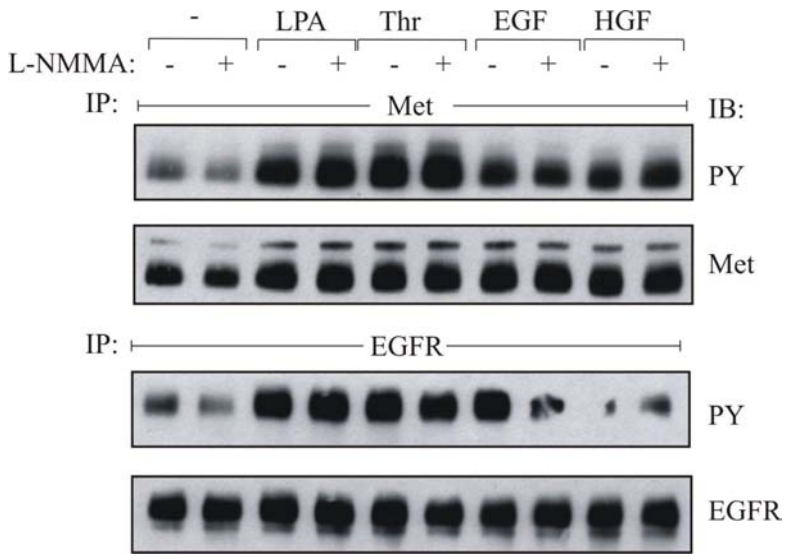

Figure 40 **Met receptor transactivation is independent of NO-synthase activity**. DAN-G cells were serum-starved and treated with L-NMMA (100 µM, 30 min). Following stimulation with LPA (10 µM), Thr (2 U/mL), EGF (4 ng/mL) or HGF (4 ng/mL), Met receptor and EGFR were immunoprecipitated from split cell lysates and immunoblot analysis for phosphotyrosine content was performed. The same blots were stripped and probed for Met or EGFR protein, respectively.

3.3.9. Met transactivation induces dissociation of the ß-Catenin-Met receptor complex in HuH7 and DAN-G cells

ß-catenin has been shown to be constitutively associated with the Met receptor in hepatoma and hepatocellular carcinoma cells (Herynk et al., 2003; Hiscox and Jiang, 1999a). Immunoprecipitation of Met in the hepatocellular carcinoma cell line HuH7 co-precipitated ß-catenin (Figure 41), which is constitutively associated and tyrosine phosphorylated in HuH7 cells (Figure 41, upper panel). Stimulation of Met transactivation by both GPCR ligands and EGF induced the dissociation of this ß-catenin-Met receptor complex (Figure 41).

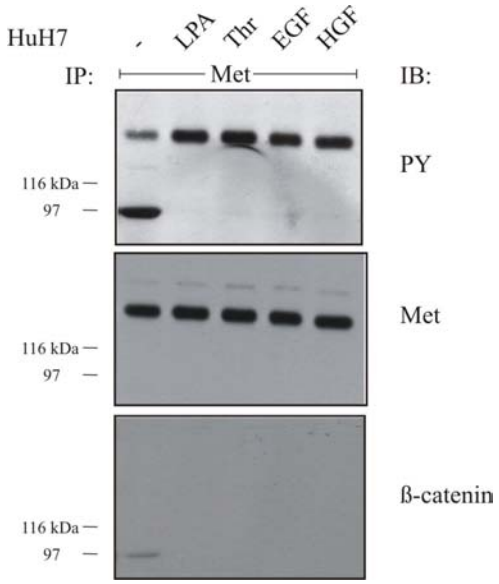

Figure 41 **Met transactivation induces dissociation of the Met-ß-catenin complex**. HuH7 cells were serum-starved for 24 hrs and stimulated as indicated. Following immunoprecipitation of the Met receptor filters were probed for phosphotyrosine content or ß-catenin.

To analyze the translocation of ß-catenin in response to Met receptor transactivation we used the differential detergent fractionation technique to prepare cytoplasmic-, membrane- and nuclei-enriched fractions. Figure 42 demonstrates that stimulation of HuH7 and DAN-G cells with LPA, Thrombin, EGF or HGF induced translocation of ß-catenin from the membrane to the cytoplasmic fraction and an increase of ß-catenin in the nuclei-enriched fraction.

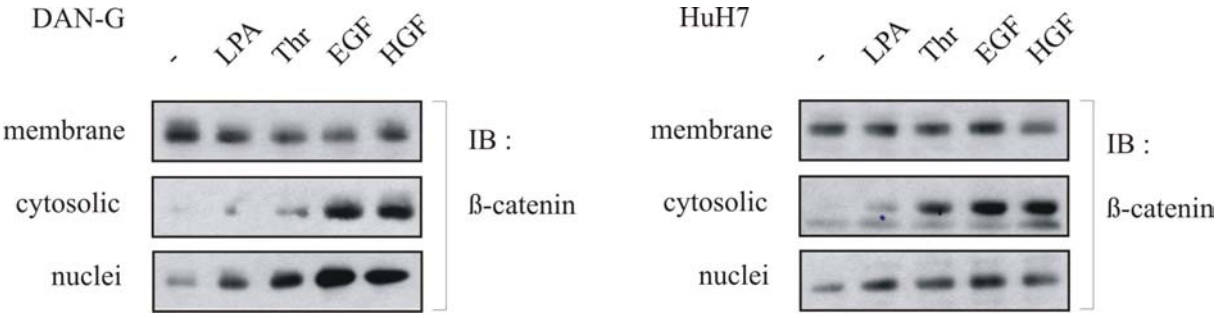

Figure 42 **Met transactivation induces nuclear translocation of ß-catenin**. DAN-G or HuH7 cells were stimulated as depicted and subsequently cytoplasmic-, membrane- and nucleic-enriched fractions were isolated by differential detergent fractionation (DDF) as described under materials and methods. Equal amounts of total lysates were analyzed for ß-catenin content by immunoblot analysis.

4. Discussion

Deregulated RTK signalling has been frequently linked to the development and progression of pathophysiological disorders such as cancer (Blume-Jensen and Hunter, 2001; Zwick et al., 2001). Besides the EGFR and its relative HER2 which have been early on recognized as signalling proteins with a high oncogenic signalling potential, RTKs of other families have been implicated in the pathogenesis of cancer, such as c-Met or the platelet-derived growth factor receptor (PDGFR). In addition to RTKs the autocrine production of GPCR agonists has been frequently associated with different types of human cancer (Marinissen and Gutkind, 2001; Moody et al., 2003). With respect to the pathophysiological significance of cell surface receptor signalling this study investigates the role of RTK activation mechanism in response to heterogeneous stimuli including GPCR agonists and cellular stress stimuli in human tumour cell lines.

4.1. Metalloprotease-mediated EGFR signal transactivation in NSCLC and pancreatic carcinoma cell lines

Previous publications implicated the EGFR in the molecular pathology of both NSCLC and pancreatic carcinoma (Bardeesy and DePinho, 2002; Sridhar et al., 2003). Moreover GPCR agonist signalling loops have been demonstrated in both types of cancer (Moody, 1996; Moody et al., 2003; Siegfried et al., 1999).

The data presented in this study provide evidence for the functional relevance of GPCR stimulation and EGFR downstream signalling pathways employing metalloprotease-dependent EGFR signal transactivation. Stimulation with GPCR ligands induces the rapid tyrosine phosphorylation of the EGFR in both tumour types (Figure 6 and Figure 7), depending on the cellular system and the stimulus investigated. The NSCLC cell lines A549 and Calu-6 reveal only a weak GPCR-induced stimulation of EGFR phosphorylation which can be attributed to an increased basal receptor tyrosine phosphorylation. In both carcinoma cell lines the small G protein Ras is constitutively activated due to mutation (Heasley et al., 1997; Rajesh et al., 1999) which has been linked to autocrine signalling loops involving the EGFR and its ligands leading to high basal tyrosine phosphorylation (Watanabe et al., 1996; Zushi et al., 1997).

Preincubation with the metalloprotease inhibitor batimastat abrogated GPCR-induced EGFR phosphorylation demonstrating the involvement of a metalloprotease activity. On the other hand, bombesin-stimulated EGFR phosphorylation was independent of metalloprotease

function in BxPc-3 cells (Figure 6), suggesting a ligand-independent pathway in parallel to the ligand-dependent EGFR signal transactivation in this cell line.

Besides the EGFR itself, GPCR agonist treatment induces EGFR characteristic downstream signalling cascades. Metalloprotease-dependent phosphorylation of the adaptor protein Shc occurs in response to GPCR stimulation (

Figure 8), which is linked to stimulation of the MAPKs Erk1/2 (Figure 9/Figure 10). The role of the EGFR as a transducer of GPCR stimulation to the MAPKs Erk1/2 has been demonstrated in a variety of other cell systems, including Rat-1 (Daub et al., 1996), Cos-7 (Daub et al., 1997; Prenzel et al., 1999), PC-12 (Kim et al., 2000), HEK-293 cells (Della Rocca et al., 1999) and head and neck squamous cell carcinoma (Gschwind et al., 2002). Interestingly, in the NSCLC cell line NCI-H292, LPA induces an increase in DNA synthesis which depends on both EGFR kinase and metalloprotease function, indicating that metalloprotease-dependent EGFR signal transactivation in response to LPA treatment is capable of inducing cell proliferation in this cell line (Figure 11). In contrast, neither A549 or Calu-6 cells nor the pancreatic carcinoma cell lines DAN-G or BxPc-3 revealed a GPCR-stimulated increase in DNA synthesis (data not shown). All of these cell lines harbour activating mutations of the Ras protein which accounts in particular for 90% of all pancreatic carcinoma (Bardeesy and DePinho, 2002; Watanabe et al., 1996; Zushi et al., 1997). In consequence the GPCR-induced EGFR and subsequent Ras activation might only provide weak enhancement compared to the constitutive activity. LPA- and other GPCR agonist-stimulated DNA synthesis has also been observed in Rat-1, Swiss 3T3 and HNSCC cells (Daub et al., 1996; Gschwind et al., 2002; Santiskulvong et al., 2001). Together with these results the data presented in this study corroborate the instrumental role of the EGFR in linking GPCRs to mitogenic signals.

4.2. EGFR activation in response to cellular stress

How mammalian cells respond to physical stress has been intensely studied, but, despite these extensive research efforts, various mechanistic aspects of stress-induced signalling have remained elusive (Benhar et al., 2002b; Finkel and Holbrook, 2000; Kamata and Hirata, 1999; Kyriakis and Avruch, 2001). The present study investigates growth factor-dependent mechanisms leading to EGFR and subsequent MAPK activation in response to osmotic and oxidative stress in human carcinoma cells.

4.2.1. Osmotic and oxidative stress mediate metalloprotease- and HB-EGF dependent EGFR activation

The data presented here demonstrate that EGFR phosphorylation induced by both osmotic and oxidative stress requires a metalloprotease activity triggering the release of mature HB-EGF in Cos-7 cells and human carcinoma cell lines (Figure 17). These findings significantly extent results from earlier reports, which indicated a role of mechanisms such as receptor aggregation, phosphatase inactivation or the stimulation of intracellular kinases in stress-induced EGFR activation (Blanchetot et al., 2002; Knebel et al., 1996; Rosette and Karin, 1996). These previously described mechanisms might contribute to the partially ligand-independent EGFR phosphorylation in NCI-H292, whereas a ligand-dependent activation fully accounts for receptor phosphorylation by osmotic and oxidative stress in Cos-7 and TCC-Sup bladder carcinoma cells. Thus, the respective contribution of different routes leading to EGFR activation appears to depend on the cellular context. It is noteworthy that Chen *et al*. (Chen et al., 2001a) attributed the hydrogen peroxide-induced EGFR activation in Cos-7 cells to a ligand-independent mechanism involving c-Src. In contrast to these earlier observations, inhibition of EGFR activation by oxidative stress with the Src inhibitor PP2 in the same cell system could not be observed (Figure 13). Nishida *et al*. reported the activation of $G\alpha_{i/o}$ proteins by H_2O_2 due to oxidation of a cysteine residue which is characteristic for this $G\alpha$ subfamily (Nishida et al., 2000; Nishida et al., 2002). Since these $G\alpha_{i/o}$ proteins are sensitive to pertussis toxin, a contribution of ROS-induced $G\alpha_{i/o}$ activation could be excluded by preincubation with the bacterial toxin (Figure 14).

Stress-induced signalling might also trigger a positive feedback loop further enhancing ligand-dependent EGFR activation, since previous investigations revealed stress-induced expression of HB-EGF and amphiregulin (Miyazaki et al., 2001). Interestingly, Zenz *et al*. demonstrated a functional correlation between c-Jun, a downstream target of the JNK family, the EGFR and HB-EGF, as in c-Jun deficient keratinocytes both HB-EGF and EGFR expression was reduced (Zenz et al., 2003).

4.2.2. ADAM family proteases mediate stress-induced EGFR phosphorylation

Although Frank *et al*. recently implicated HB-EGF in hydrogen peroxide-induced EGFR phosphorylation in vascular smooth muscle cells (Frank et al., 2003), the various components involved in this process have not been defined. This study provides experimental evidence that ADAM proteases are responsible for shedding of proHB-EGF upon cellular stress. Interestingly, while GPCR-mediated EGFR transactivation occurs through distinct individual

ADAM proteases (Gschwind et al., 2003; Yan et al., 2002), we found that two or more ADAM proteases become active upon cellular stress. ADAM17 appears to be generally involved in stress stimulated shedding events, while also ADAM9, ADAM10 and ADAM12 can contribute depending on the cell system and type of stimulus. All of these enzymes have been previously implicated in EGF-like ligand shedding (Asakura et al., 2002; Izumi et al., 1998; Lemjabbar and Basbaum, 2002; Yan et al., 2002). The identification of stress-induced ADAM family members distinct from those regulated through GPCRs is corroborated by previous reports demonstrating that ADAM9 cleaves proHB-EGF in response to TPA stimulation in VeroH cells (Izumi et al., 1998), while in the same cellular system proHB-EGF processing after LPA stimulation is independent of ADAM9 (Umata et al., 2001). Taken together, these data suggest that proHB-EGF sheddases are defined by both the cellular context and the stimulus. Moreover, ADAM9 knock-out mice lack an obvious phenotype and ADAM9 $^{-/-}$ fibroblasts display no defects in proHB-EGF processing (Weskamp et al., 2002), which strongly argues for functional redundancy among proHB-EGF cleaving enzymes in vivo.

4.2.3. p38 controls EGFR activation upon cellular stress

How are the metalloproteases of the ADAM family activated, finally leading to EGFR phosphorylation and downstream signalling responses? Previous reports demonstrated regulation of metalloprotease-mediated ectodomain cleavage of transmembrane proteins in response to growth factors and TPA by the MAPK ERK1/2, while the basal level of ectodomain shedding has been attributed to p38 activity (Fan and Derynck, 1999; Gechtman et al., 1999; Rizoli et al., 1999). Moreover, p38 has been implicated as an upstream mediator of the EGFR in the sorbitol-induced EGFR activation in human non-transformed keratinocytes (Cheng et al., 2002). In contrast to the results presented herein, the authors of this report excluded a ligand-dependent mechanism based on medium transfer experiments. As the released EGF-like ligand may be retained in the extracellular matrix through binding to heparan sulfate proteoglycans (Prenzel et al., 1999), an involvement of ligand-dependent EGFR activation cannot be ruled out by this type of experimental approach. These reports and the finding that p38 activity in our systems is independent of the EGFR phosphorylation state prompted us to ask whether stress-activated p38 is the upstream signalling element that controls ligand-dependent EGFR activation. Indeed, we found that preincubation with a specific p38 inhibitor abrogated stress-induced EGFR activation, while blocking ERK1/2 activation leaved EGFR phosphorylation unaffected. On the contrary, p38 activation itself in

response to stress agents is independent of the EGFR as assessed by the EGFR selective inhibitor AG1478. Furthermore, time course experiments revealed that p38 activation precedes EGFR phosphorylation, which is a necessary prerequisite for p38 being located upstream of the EGFR, while ERK1/2 and JNK activation occurs even later. Together, these data suggest p38 as the upstream inducer of ligand-dependent EGFR activation and its subsequent downstream signalling.

4.2.4. The role of HB-EGF function in doxorubicin-induced apoptosis of cancer cells

Activation of ERK1/2 and JNK in response to oxidative and osmotic stress represents an important step in the cellular stress response (reviewed in (Kyriakis and Avruch, 2001)). Stress signalling via MAPKs is often increased in cancer cells, which frequently produce high levels of ROS *per se* (Burdon, 1995; Szatrowski and Nathan, 1991). Moreover, anticancer drugs or radiation therapy can further activate stress signalling cascades (Benhar et al., 2002b), which has also been attributed to the production of ROS caused by these agents. Here, this study demonstrates that stress-induced ligand-dependent EGFR activation is a prerequisite for subsequent ERK1/2 and, to a lesser extent, also JNK MAPK activation (Figure 24/Figure 25). As ligand-dependent, EGFR-mediated MAPK signalling can control cell survival through induction of apoptosis regulators such as the Bcl-2 family (Jost et al., 2001), it might also play a role in the protection of bladder carcinoma cells from doxorubicin-induced apoptosis observed in our experiments. Consistent with this hypothesis, blockade of HB-EGF function strongly enhanced doxorubicin-induced cell death (Figure 28). As chemotherapeutic agents have been previously shown to activate stress signalling cascades (Benhar et al., 2002b; Losa et al., 2003; Sanchez-Prieto et al., 2000), this signalling mechanism provides a molecular explanation for the ability of tumour cells to evade drug-induced cell death. Figure 43 schematically summarizes the results presented in this study as a model of metalloprotease- and HB-EGF-dependent EGFR activation upon cellular stress.

Increasing evidence implicates particularly ROS-induced oxidative stress in a variety of human disorders as diverse as cardiovascular, neurodegenerative or hyperproliferative diseases and cancer. Therefore, these results are of special significance for the understanding of pathophysiological disorders and the development of respective therapeutic approaches.

The findings presented herein emphasize the importance of ADAM family proteases and HB-EGF as critical mediators of the stress response in human cancer cells and suggest that cross-

communication between different groups of MAPK employs ADAM proteases and the EGFR as signalling intermediates.

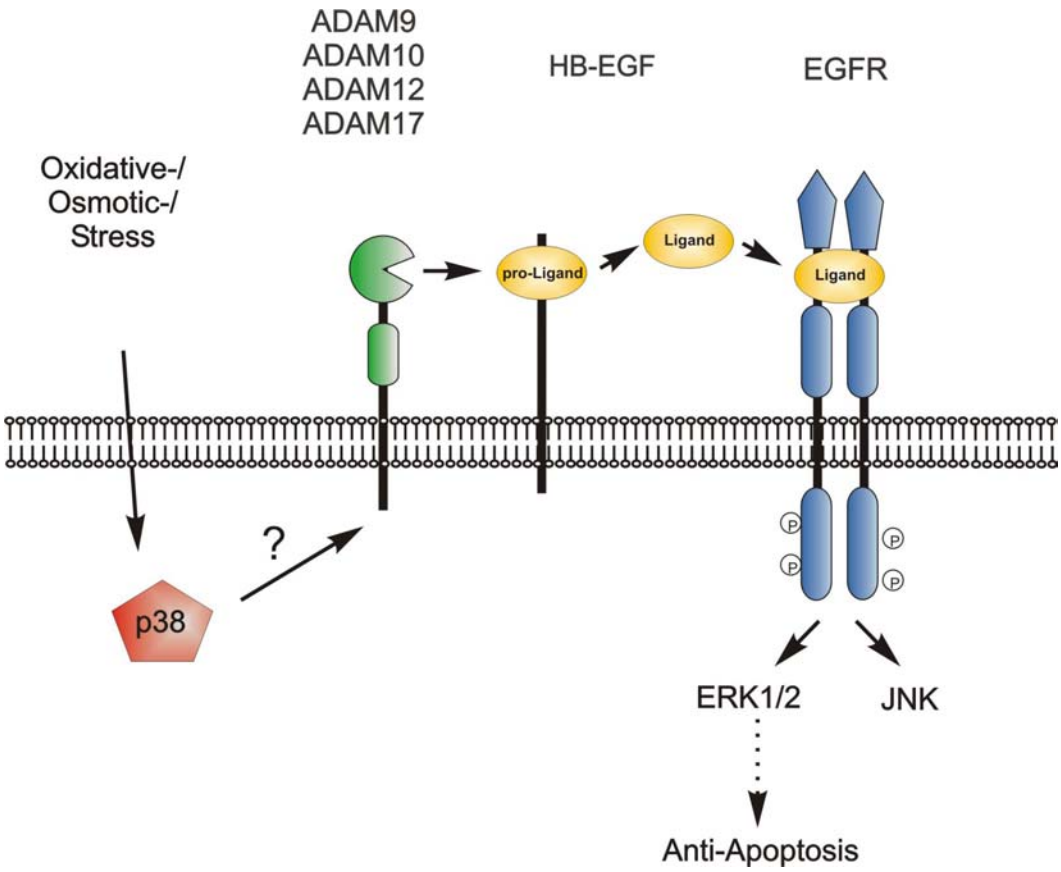

Figure 43 **Model of the cellular stress response mediated by metalloprotease-dependent release of HB-EGF**. Stress stimuli such as oxidative or osmotic stress, but also chemotherapeutics activate p38. p38 is able to induce the activity of ADAM family proteases leading to specific processing of proHB-EGF, release of the mature growth factor and subsequent EGFR stimulation. Dependent on this mechanistic concept EGFR characteristic downstream signalling cascades are induced comprising ERK1/2 and JNK activity, and cellular responses such as cell survival in response to stress are modulated.

4.3. Met receptor transactivation

Cross-talk between cell surface receptors has been early recognized as a crucial signaling mechanism to expand the cellular communication network. Related RTKs such as members of the EGFR family are capable of forming heterodimers thereby affecting downstream signaling pathways (Yarden, 2001). A further layer of complexity was added by the discovery of the heterogeneous GPCR-RTK cross-talk mechanism with EGFR signal transactivation serving as the prototypic interreceptor signaling pathway (Daub et al., 1996; Prenzel et al., 1999). In addition to above mentioned pathways integrins and cytokine receptors have also been reported to be involved in cross-communication (Moro et al., 1998; Trusolino et al.,

2001) (Yamauchi et al., 1997). Therefore, increasing interest focuses on these heterogeneous interreceptor cross-communication networks.

4.3.1. EGF-induced Met receptor phoshorylation

This study provides experimental evidence that the EGFR transactivates the Met RTK in human pancreatic and hepatocellular carcinoma cells. Previous investigations demonstrated cross-talk between both receptors on the transcriptional level and Met ectodomain shedding in response to EGFR signal transactivation (Bergstrom et al., 2000; Nath et al., 2001). Direct association of Met with and phosphorylation by the EGFR has been suggested as the underlying mechanism of this cross-communication (Jo et al., 2000). EGF- or TGF-α-induced Met phosphorylation and Met-EGFR co-precipitation were restricted to A431 cells which overexpress EGFR due to gene amplification. However, in hepatocellular carcinoma cell lines that express moderate levels of both receptors, the authors could neither detect transactivation of Met nor coprecipitation of Met and EGFR (Jo et al., 2000). Since in this study neither association of Met and EGFR was found in hepatocellular and pancreatic carcinoma cells, it was investigated whether an alternative mechanism than heterodimerization might account for Met receptor transactivation.

4.3.2. The role of ROS production in EGF-stimulated Met receptor transactivation

Using a flow cytometric analysis of cells stained with the redox-sensitive fluorescent dye DCF-DA it could be shown that reactive oxygen species are generated in response to EGF treatment (Figure 36). The critical role of ROS in this cross-talk mechanism was further corroborated by the finding that preincubation with the reducing agent EGCG interferes with Met receptor transactivation (Figure 37). While different cellular sources for ROS have been reported (Thannickal and Fanburg, 2000), the data of this study provide evidence for the involvement of plasma membrane bound NADPH oxidases (Figure 37/Figure 38/Figure 39). These enzymes have previously been implicated in the acute and rapid production of ROS in response to growth factor treatment (Finkel, 2000; Finkel, 2003; Rhee et al., 2000; Thannickal and Fanburg, 2000). Moreover, the finding that inhibition of the small GTPases Rac, Rho and Cdc42 by ToxinB interfered with Met transactivation does also point towards an involvement of Nox enzymes, as Rac has been shown to regulate the activity of the Nox enzyme complex.

4.3.3. GPCR agonist-induced Met receptor transactivation

In addition to EGF, also stimulation of cells with GPCR ligands such as LPA, Bradykinin, Thrombin or Carbachol induced the rapid and transient phosphorylation of the Met receptor (Figure 30). Analogous to EGF-induced Met transactivation it could be demonstrated that GPCR-induced Met phosphorylation critically depends on the production of ROS by NADPH oxidases (Figure 36/ Figure 37/ Figure 38/Figure 39). Flow cytometric analysis showed that stimulation with either LPA, Thrombin or EGF induced a moderate shift towards higher fluorescence compared to stimulation with the positive control H_2O_2. This observation can be anticipated as the ROS production induced by physiologic stimuli in epithelial cells should be strictly limited in its duration and localization, which are in this case distinct microdomains at the plasma membrane. In consequence, only a fraction of the fluorescent dye distributed throughout the cell can be oxidized in response to growth factor treatment. The increase in ROS production corresponds to a moderate increase of Met receptor tyrosine phosphorylation, which is equivalent to stimulation with 4 ng/mL HGF (Figure 32). Interestingly, this level of receptor tyrosine phosphorylation is comparable to the level of EGFR transactivation reached by GPCR ligands.

Very recently, Pai and colleagues reported that prostaglandine E2 is capable to induce Met receptor tyrosine phosphorylation in colorectal carcinoma cells (Pai et al., 2002). They suggested an indirect mechanism involving the EGFR, since preincubation with the EGFR kinase specific inhibitor AG1478 abolished Met activation (Pai et al., 2002). Interestingly, EGF treatment did not significantly enhance Met phosphorylation. However, treatment of hepatocellular and pancreatic carcinoma cells with AG1478 did not affect Met phosphorylation in response to GPCR agonists, suggesting that GPCR-induced Met phosphorylation does not dependent on EGFR kinase activity in these cellular systems. Since the EGFR itself is able to induce ROS production (Bae et al., 1997), in some cellular systems EGFR signal transactivation might be used by GPCRs that are unable to directly stimulate the production of reactive oxygen intermediates to induce ROS-dependent Met transactivation.

The involvement of ROS in Met transactivation is corroborated by the previous findings that PDGF induces ROS production in HepG2 cells (Bae et al., 2000). The same result has been obtained in A431 cells in response to EGF treatment (Bae et al., 1997). Since in colorectal carcinoma cells that have been used in the study by Pai and colleagues (Pai et al., 2002) functional components of Nox enzymes have been found (Kikuchi et al., 2000; Perner et al., 2003), the results presented herein can provide a molecular explanation for activation of Met in response to heterogeneous stimuli in the cellular systems reported by Stolz *et al*. and Pai *et*

al. (Jo et al., 2000; Pai et al., 2002). ROS have been previously implicated in receptor cross-talk mechanisms in particular in endothelial or vascular smooth muscle cells, such as Angiotensin II-induced EGFR transactivation (Ushio-Fukai et al., 2001). The results of this study extend this finding and demonstrate this functional correlation of GPCR and Met cross-talk in transformed epithelial cells of hepatocellular and pancreatic carcinoma.

4.3.4. Met receptor transactivation induces dissociation of the β-catenin-Met complex

Previous reports demonstrated the association between the Met receptor and ß-catenin (Hiscox and Jiang, 1999a; Monga et al., 2002; Muller et al., 2002). Interestingly, it could be shown that in the hepatocellular carcinoma cell line HuH7 and in the pancreatic carcinoma cell line DAN-G not only direct Met activation by HGF but also Met transactivation by GPCR agonists and EGF stimulates the nuclear translocation of ß-catenin. Nuclear ß-catenin has been associated with enhanced transcription of TCF target genes that are involved in epithelial-to-mesenchymal-transition and the development of an invasive phenotype (Behrens, 2000; Conacci-Sorrell et al., 2002; Mareel and Leroy, 2003). The Met receptor has been shown to be instrumental to invasive growth in both physiological and pathophysiological signaling context (Trusolino and Comoglio, 2002). Interestingly, previous reports implicated co-expression of Met and EGFR in tumor progression and development in gastrinoma and hepatocellular carcinoma. In the latter, overexpression of both Met and the EGFR is associated with increased tumor size, tumor stage, lymph node metastasis and poor prognosis and reduced survival (Peghini et al., 2002). Daveau *et al.* reported the presence of enhanced levels of mRNA for both receptors in particular in poorly differentiated tumors and in patients with early tumor recurrence (Daveau et al., 2003). Noteworthy, Miura and colleagues demonstrated that ROS potentiated the invasive activity of hepatoma cells by autocrine/paracrine loop of HGF (Miura et al., 2003).

The results of this study provide a molecular mechanism for EGFR-dependent transactivation of the Met receptor and extend this concept to cross-talk involving GPCRs and Met. With respect to the outstanding role of the Met receptor in tumor invasion, these findings are of special significance for the understanding of cancer progression and metastasis in response to heterogeneous growth factor stimulation involving the Met receptor. Figure 44 shows a model of GPCR-induced Met receptor transactivation in hepatocellular and pancreatic carcinoma cells based on the results presented in this study. In this model, GPCR stimulation induces in a Rac-dependent manner a NADPH oxidase activity resulting in an increased ROS

production. The rise in ROS levels inactivates a yet unidentified phosphatase, thereby increasing the basal phosphorylation of the Met receptor giving rise to a phosphorylation signal.

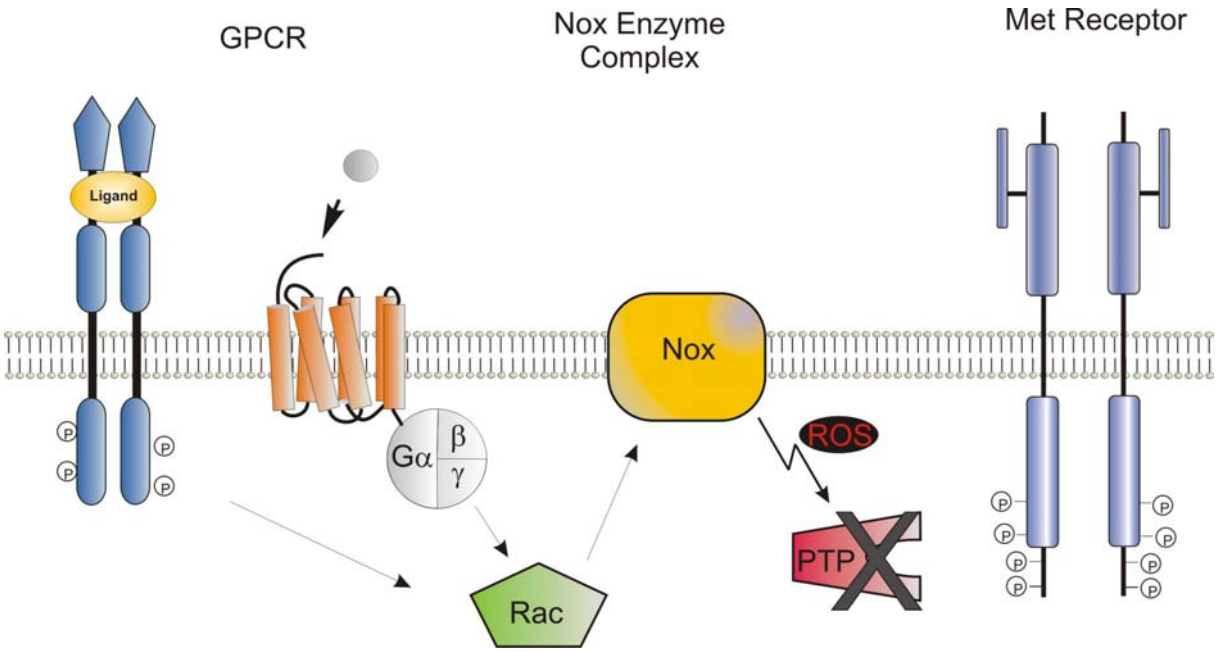

Figure 44 **Model of GPCR- and EGFR-induced Met receptor transactivation**. Stimulation of both GPCRs and EGFR induce in a Rac-dependent fashion the production of ROS by NADPH-oxidase enzyme complexes. Following inactivation of one or more Met specific phosphatase the equilibrium of phosphorylated and dephosphorylated receptor becomes shifted towards the phosphorylated state.

4.4. Perspectives

An increasing number of reports underlines the role of ligand-dependent EGFR signal transactivation in diverse human disorders, including cancer, cardiac and gastrointestinal hypertrophy, helicobacter pylori-induced pathophysiological processes or cystic fibrosis (reviewed in (Fischer et al., 2003)). In particular the role of ADAM family metalloproteases in EGFR activation has gained increasing interest and consequently different members of this family could be identified as critical mediators of EGFR signal transactivation. Since this mechanism provides a link to couple autocrine GPCR signalling loops to the EGFR, future work has to focus on the relation of deregulated GPCR signalling and the TMPS pathway. Of special significance are elements that link GPCRs to the respective metalloprotease-ligand pair since they might provide targets for specific therapeutical intervention. The developmental role of proteins which participate in the transactivation pathway such as ADAM17 and HB-EGF has been addressed by studies using knock-out mice (Jackson et al., 2003; Peschon et al., 1998). However, both the physiological and pathophysiological role of these molecules within the EGFR signal transactivation *in vivo* awaits further elucidation.

An additional layer of complexity was added by the finding that different metalloprotease-ligand combinations can exist, depending on the cellular context and stimulus (Fischer et al., 2003). In addition, the present study demonstrates within the context of cellular stress signalling situations that different ADAM proteases are able to induce cleavage of a single distinct EGF-like ligand, while other EGF-related growth factors are present. Future studies will be necessary to identify the regulatory mechanisms that determine the substrate specificity of these metalloproteases. The results presented in this study indicate a role for the MAPK p38 in the ligand-dependent EGFR activation upon cellular stress, while the mechanistic details linking p38 to activation of ADAM proteases still have to be elucidated.

The data presented here point out the role of EGF-like ligand processing as a mechanistic concept for tumour cells to evade chemotherapy. However, the broad significance of the mechanisms elucidated in this study and the potential relevance towards the development of targeted cancer therapies will need to be addressed in future studies on the basis of both cell culture models and primary tumours. Of special interest is the expression analysis of tumours based on the characterization of the signalling pathways relevant to resistance to chemotherapy. While antibodies directed against the EGFR itself or its relative HER2 are readily used in combination with chemotherapy (Zwick et al., 2001), targeting a distinct growth factor is likely to provide an enhanced selectivity.

This work provides evidence for the GPCR- and EGFR-induced transactivation of the Met receptor tyrosine kinase. The mechanistic concept involving the NADPH oxidase-dependent acute production of ROS emphasizes the role of reactive oxygen intermediates as signal transducers and supports the concept for a role of deregulated radical signalling in pathophysiological disorders. Increased ROS levels have been early linked to tumour cells (Szatrowski and Nathan, 1991). While this increased production has been associated with an high metabolic rate in these cells, recent reports suggest a role of hydrogen-peroxide producing enzymes in tumour development and progression (Arbiser et al., 2002; Arnold et al., 2001; Suh et al., 1999). The results of this study reveal the Met receptor as a target of enhanced ROS levels. However, future studies have to identify further components of this signalling mechanism, such as the phosphatase which becomes inactivated in response to ROS production.

5. Summary

The classical view of RTK activation comprises direct stimulation of the receptor by its ligand. The finding that heterogeneous stimuli distinct of the cognate ligand induce phosphorylation of RTKs such as the EGFR was attributed to intracellular signal transduction pathways such as the inactivation of phosphatases or the action of cellular tyrosine kinases. However, recent investigations demonstrated that EGFR signal transactivation in response to GPCR stimulation relies on a ligand-dependent mechanism, combining the wide diversity of GPCRs with the oncogenic EGFR signalling potential. This study investigated whether this signalling pathway occurs in pancreatic and non-small cell lung carcinoma. In both tumour types metalloprotease-dependent transactivation of the EGFR was observed and linked to activation of the mitogen-activated protein kinases ERK1/2. Interestingly, LPA treatment enhanced DNA synthesis in a NSCLC cell line, indicating a potential role for EGFR signal transactivation in tumour cell proliferation.

Cellular stress stimuli induce signalling cascades such as the EGFR and MAPKs in mammalian cells. Similar to EGFR signal transactivation receptor stimulation was thought to rely on ligand-independent mechanisms. In contrast, this study provides evidence for the involvement of an EGF-like ligand in stress-induced EGFR phosphorylation. The mechanism comprises p38-dependent activation of ADAM family proteases releasing HB-EGF and can be linked to ERK1/2 and JNK activation. Interestingly, cell death in response to doxorubicin treatment, which induces p38 activity, is strongly enhanced by blockade of HB-EGF, suggesting a role of this signalling pathway for tumour cells to evade drug-induced cell death.

Since cross-communication between different classes of cell surface receptors gains increasing interest, this study addressed the question whether other RTKs than the EGFR can be transactivated in epithelial tumour cells. This work demonstrates GPCR- and EGFR-induced Met receptor transactivation depending on the rapid and transient production of reactive oxygen species. This Met receptor transactivation induces dissociation of the Met-β-catenin complex and subsequent nuclear translocation of β-catenin, providing a link between GPCR and EGFR signalling to the Met receptor and its potent invasive signalling capacities.

Taken together, these data emphasize the significance of communication between heterogeneous receptor signalling pathways. While interreceptor cross-talk provides an mean of signal fine tuning, these data indicate a role of such signalling pathways for tumour cells to stimulate cellular proliferation, modulate their invasive behaviour or allow them to evade from drug-induced cell death.

6. References

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7. Abbreviations

AA	Amino acid
Ab	Antibody
ADAM	A disintegrin and metalloprotease domain
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
cDNA	Complementary DNA
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
ECM	Extracellular matrix
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
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
HGF	Hepatocyte Growth Factor
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
NRG	Neuregulin
Nox	NADPH oxidase
OD	Optical density
p.a.	Per analysis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	Polyethylenglycole
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-diphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl-fluoride
PNPP	p-Nitrophenyl-phosphate
PTP	Protein tyrosine phosphatase
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
ROS	Reactive oxygen species
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 elektrophoresis
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
TCF	T cell factor
TGFα	Transforming growth factor alpha
TEMED	N, N, N‘, N‘-Tetramethyletylendiamine
TMPS	Triple-membrane-passing-signal
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
VEGFR	Vascular endothelial growth factor receptor
Vol	Volume
Wt	Wild type

Curriculum Vitae

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