Distinct ADAM metalloproteinases regulate G protein coupled receptor-induced cell proliferation and survival

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SUMMARY

Cross-talk between G protein-coupled receptor (GPCR) and epidermal growth factor receptor (EGFR) signalling systems is widely established in a variety of normal and transformed cell types. Here, we demonstrate that the EGFR transactivation signal requires metalloproteinase cleavage of EGF-like growth factor precursors in fibroblasts, ACHN kidney and TccSup bladder carcinoma cells. Furthermore, we present evidence that blockade of the metalloproteinase-disintegrin tumour necrosis factor-alpha-converting enzyme (TACE/ADAM17) by a dominant-negative ADAM17 mutant prevents angiotensin II stimulated proHB-EGF cleavage, EGFR activation and cell proliferation in ACHN tumour cells. Moreover, we found that in TccSup cancer cells the LPA-induced transactivation signal is mediated by ADAM15 demonstrating that distinct combinations of growth factor precursors and ADAMs regulate GPCR-EGFR cross talk pathways in cell lines derived from urogenital cancer. Our data show further that activation of ADAMs results in discrete cellular responses: While GPCR agonists promote activation of the Ras/MAPK pathway and cell proliferation via the EGFR in fibroblasts and ACHN cells, EGFR transactivation pathways regulate activation of the survival mediator Akt/PKB and the susceptibility of fibroblasts and TccSup bladder carcinoma cells to pro-apoptotic signals such as serum-deprivation, death receptor stimulation and the chemotherapeutic drug doxorubicin. Thus, ADAM15 and 17 function as effectors of GPCR-mediated signalling and define critical characteristics of cancer cells.
INTRODUCTION

Receptor tyrosine kinases (RTKs) play critical roles in the cellular signal transduction network as mediators of G protein-coupled receptor (GPCR) mitogenic signalling (1-3). In various cell types stimulation with GPCR agonists involves transactivation of the epidermal growth factor receptor (EGFR) resulting in the activation of the Ras/MAPK pathway (4-9). We and others have recently demonstrated that GPCR stimuli enhance cell proliferation and motility of cancer cells via transactivation of the EGFR (10-12). The molecular mechanisms of EGFR transactivation pathways are however poorly defined. In some cellular systems the GPCR-EGFR signal transmission appears to rely on cytoplasmic, ligand-independent mechanisms (13). On the other hand, the GPCR-EGFR cross talk involves a triple membrane passing signalling (TMPS) mechanism in COS-7 and PC-3 cells, i.e. the cell surface processing of proHB-EGF by metalloproteinases of the ADAM family in response to GPCR stimulation (14). A variety of metalloproteinases of the ADAM and MMP families have been implicated in HB-EGF precursor shedding including ADAM10 (15) and MDC9/ADAM9 (16,17). ProHB-EGF processing however was shown to be normal in ADAM9−/− fibroblasts, arguing against an essential role for ADAM9 in proHB-EGF cleavage in this cell type (18). Interestingly, TACE/ADAM17 deficient cells display impaired basal and stimulated solubilization of a variety of cell surface molecules including the EGF-like ligands amphiregulin and HB-EGF (19-21) suggesting ADAM17 as a major regulator of EGF-like precursor cleavage.

The EGFR and its relative HER2/neu have been implicated in the regulation of cell proliferation and survival in both physiological and patho-physiological processes. High
expression levels of EGFR and HER2 are associated with resistance of tumours to treatment with cytotoxic drugs (22,23), a short relapse time and a low survival rate (24,25). It has been demonstrated that the PI3K-Akt/PKB and the Ras-MAPK cascades are major downstream pathways of activated growth factor receptors mediating resistance to pro-apoptotic stimuli (26,27).

The results of this study indicate that prominent GPCR agonists promote activation of the Ras/MAPK cascade and cell proliferation via ADAM metalloproteinase-dependent transactivation of the EGFR in Rat1 fibroblasts and ACHN kidney carcinoma cells. Furthermore, we identify HB-EGF and ADAM17 as critical mediators of the transactivation signal in ACHN cells. Importantly, we provide evidence that GPCR-EGFR cross talk pathways are mediated by ADAM15 in TccSup bladder carcinoma cells and regulate the susceptibility of TccSup cells and fibroblasts towards pro-apoptotic stimuli such as serum deprivation, death receptor activation and chemotherapeutic agents.
EXPERIMENTAL PROCEDURES

Reagents and Antibodies- AG1478 was obtained from Alexis Biochemicals, bradykinin was purchased from Calbiochem. EGF as well as all other GPCR ligands and chemicals were purchased from Sigma. Concanavalin A-Sepharose®4B was purchased from Sigma-Aldrich. Antibodies used were sheep polyclonal anti-EGFR antibody and mouse monoclonal antiphosphotyrosine antibody 4G10 (Upstate Biotechnology, UBI), rabbit polyclonal anti-ERK2 antibody and rabbit polyclonal anti-Akt antibody (Santa Cruz), rabbit polyclonal antiphospho-p44/42 MAPK antibody and rabbit polyclonal anti-phospho Akt (Ser473) antibody (New England Biolabs, NEB), mouse monoclonal anti-Shc antibody, mouse monoclonal anticyclin D1 antibody and mouse monoclonal Fas ligand CD95L antibody (BD Transduction Laboratories) and mouse monoclonal anti-Tubulin antibody (Sigma). Secondary HRP-conjugated antibodies were goat anti-mouse antibody (Sigma), donkey anti-sheep antibody (Dianova) and goat anti-rabbit antibody (BioRad). Goat anti-human amphiregulin, goat anti-human HB-EGF and goat anti-human TGFα blocking antibodies were obtained from R&D systems. Mouse monoclonal ICR-3R antibody (28) and rabbit polyclonal anti-HER2/neu antibody have been described before (29). The rabbit polyclonal anti-TACE antibody was purchased from Chemicon (Harrow, UK). Polyclonal anti-ADAM 15 antibodies were generated by immunizing rabbits with a peptide consisting of amino acids 757-773 of the protease.

RNA interference and RT-PCR analysis

Transfection of 21 nucleotide siRNA duplexes (Dharmacon Research, Lafayette, CO) for targeting endogenous genes was carried out using Oligofectamine (Invitrogen) and 1.9 µg of
siRNA duplex per 6-well plate with 150000 TccSup cells per well seeded one day ahead in serum-free medium. The expression level of ADAM metalloproteinases was monitored 48 h after transfection by enrichment of glycoproteins with Concanavalin A-Sepharose and detection with ADAM antibodies as well as RT-PCR analysis. Sequences of siRNA used were CGUACGC CGGAUACUCGAdTdT (control, GL2); CUCCAUCUGUUCUCCUGACdTdT, AUUGCCAGCGC GCGCCGCUGdTdT (ADAM15); GGUUU GCUUGCAGCACACCt dTdT, GUAAGGCCCAGAGUGGUdTdT (ADAM17). RNA isolated using the RNeasy MiniKit (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 CAGCACA GCTGCAAGTCATT; CCAGCATCTGCTAAGTCATT (ADAM17), GCCTCTGAGCCAGGCAGCTGCTAAGTCATT (ADAM15). PCR products were subjected to electrophoresis on a 1.5% agarose gel and DNA was visualized by ethidium bromide staining.

Cell Culture and Retroviral Infections - Rat1 fibroblasts (Genentech, Inc.), the bladder and kidney carcinoma cell lines TccSup (DSMZ) and ACHN (ATCC) were cultured as recommended by the supplier. The pLXSN constructs encoding dominant negative ADAMs lacking the pro- and metalloproteinase domain have been described before (21). All protease constructs included a C-terminal HA tag, detectable with an anti-HA monoclonal antibody (Babco). For retroviral infections the amphotropic packaging cell line Phoenix was transfected with expression plasmids by calcium phosphate/chloroquine as described previously (30). 36 h after transfection the viral supernatant was collected and used to infect subconfluent target cells (1x10⁵ cells/ 6cm dish). Polyclonal ACHN kidney cancer cell lines
stably expressing dominant-negative ADAM 17 or wildtype ADAM 17 were generated by selecting retrovirally infected cells in medium containing G418 (1g/mL) for 2 weeks.

**Stimulation of Cells, Immunoprecipitation and Immunoblotting**- Prior to lysis, cells grown to 80% confluency were serum starved and subjected to 20 min pre-incubation with AG1478 (250 nM), BB94 (5 µM) or DMSO. Next, cells were stimulated with LPA (10 µM), thrombin (2 U/mL), bradykinin (5 µM), endothelin I (100 nM), angiotensin II (1 µM) or EGF (3 ng/mL) for 3 min unless otherwise stated and lysed for 10 min on ice in HNTG buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride and 10 µg/mL aprotinin. Lysates were pre-cleared by centrifugation at 13.000 rpm for 10 min at 4°C. Supernatants were diluted with an equal volume of HNTG buffer and subsequently immunoprecipitated using the respective antibodies and 30 µL Protein A-Sepharose for 4 h at 4°C. Precipitates were washed three times with 0.5 mL HNTG buffer, suspended in SDS sample buffer and subjected to gel electrophoresis on 7.5% or 10% gels. Following SDS PAGE, proteins were transferred to a nitrocellulose membrane and immunoblotted with the respective antibodies. Signals were developed by an enhanced chemiluminescence detection system (ECL, Amersham). Filters were stripped and re-probed to confirm equal protein loading.

**Distribution of Cell Cycle Phases and Apoptosis Assay**- Serum-starved cells (1x10^5/ 6-Well) were treated with inhibitors and growth factors as indicated. Apoptosis was induced by adding doxorubicin (1 µg/mL) or anti-CD95/FasL (50 µg/mL). After the indicated time periods cells were collected and incubated in hypotonic buffer containing 0.1 % sodium acetate, 0.1 % Triton X-100 and 20 µg/ml propidiumiodide for 2 h at 4°C. Samples were analysed on a Becton Dickinson FACScalibur flow cytometer.
Incorporation of $^3$H-Thymidine into DNA- Rat1 fibroblasts (1x10^5/12-Well) and ACHN cells (1x10^4/12-Well) were serum starved for 18 h and 48 h, respectively, and treated with inhibitors and growth factors as indicated. After 18 h cells were pulse-labelled with $^3$H-thymidine (1 µCi/ml) for 4 h. Thymidine incorporation was measured by trichloroacetic acid precipitation and subsequent liquid-scintillation counting.

ProHB-EGF ectodomain cleavage- ProHB-EGF shedding was analysed as described before (14). In brief, cells were seeded at 7x10^4 cells/6-well, grown for 24 h and serum starved for 24 h. After treatment with growth factors, cells were collected and stained with ectodomain-specific antibodies against HB-EGF (R&D Systems) for 1 h. After washing with phosphate-buffered saline (PBS), cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 15 min, washed again with PBS and analysed by FACS.

Statistical analysis- Student’s t-test was used to compare data between two groups. Values are expressed as mean +/- s.d. of at least triplicate samples. P< 0.05 was considered statistically significant.
RESULTS

GPCR agonists stimulate cell cycle progression via an EGFR- and metalloproteinase-dependent pathway in Rat1 fibroblasts- It has previously been demonstrated that GPCR signals lead to activation of the ERK/MAPK cascade as well as cell proliferation via the EGFR in Rat1 fibroblasts (1, 31) and that EGFR transactivation pathways involve an EGF-like ligand-dependent mechanism in COS-7 cells (14). To investigate the potential role of metalloproteinases in GPCR-induced mitogenesis and cell proliferation we treated Rat1 fibroblasts with the metalloproteinase inhibitor batimastat (BB94) or the EGFR-specific tyrphostin AG1478 prior to stimulation with GPCR ligands. The results showed that tyrosine phosphorylation of the EGFR and of the downstream adapter protein SHC as well as activation of the MAPK ERK1/2 in response to LPA, thrombin, bradykinin and ET-1 was abolished by BB94 and AG1478 (Fig. 1A). Next, we investigated the effect of these inhibitors on GPCR-induced cell cycle progression of quiescent Rat1 cells by flow cytometric analysis. We found that BB94 and AG1478 completely abolished S-phase progression in response to LPA, thrombin and ET-1 (Fig. 1B). S-phase entry by direct EGF stimulation, however, remained unaffected by BB94 treatment. For further quantification of mitogenic signalling in response to GPCR ligands, we measured the rate of DNA synthesis by a $^3$H-thymidine incorporation assay. Both, BB94 and AG1478 completely inhibited DNA synthesis induced by GPCR agonists (Fig. 1C). Together, these data demonstrate the critical involvement of a metalloproteinase activity in transactivation of the EGFR, downstream SHC adapter protein recruitment, activation of the ERK/MAPK pathway as well as cell proliferation induced by the GPCR agonists LPA, thrombin, bradykinin and ET-1 in Rat1 fibroblasts.

EGFR signal transactivation involves distinct combinations of ADAM metalloproteinases and EGF-like ligands in kidney and bladder carcinoma cell lines- Given that metalloproteinase-
dependent EGFR transactivation pathways are broadly established in Rat 1 fibroblasts (Fig. 1) and COS-7 cells (14) we hypothesized that metalloproteinases play functional roles in GPCR-induced transactivation of the EGFR in human cancer cells. Recently, the EGFR signalling network has been implicated in mediating critical cancer cell characteristics such as sustained cell proliferation and anti-apoptosis in tumours of the urogenital tract (32). Therefore, we investigated the responsiveness of ACHN kidney cancer and TccSup bladder cancer cells to GPCR agonists. As shown in Figure 2A, a variety of prominent GPCR ligands including angiotensin II and LPA rapidly induced EGFR tyrosine phosphorylation in these tumour cell lines. In analogy to the data obtained in Rat1 fibroblasts (Fig. 1) BB94 abrogated the EGFR transactivation signal in ACHN and TccSup cells whereas EGFR stimulation with the tyrosine phosphatase inhibitor pervanadate or with EGF was not affected (Fig. 2B, upper panel). Moreover, pre-treatment of ACHN kidney cancer and TccSup bladder cancer cells with the monoclonal anti-EGFR antibody ICR-3R which blocks binding of EGF-like ligands to the EGFR ectodomain (28), specifically abolished GPCR- and EGF-induced tyrosine phosphorylation of the EGFR (Fig. 2B, lower panel). Together, these results demonstrate that both, a metalloproteinase activity and the extracellular ligand-binding domain of the EGFR are involved in EGFR activation by GPCR stimuli and suggest the GPCR-EGFR signal transmission to occur through an EGF-like ligand-dependent mechanism in ACHN kidney and TccSup bladder carcinoma cells.

To identify EGF-like growth factors that are involved in the EGFR transactivation pathway we pre-incubated TccSup and ACHN cells with blocking antibodies against amphiregulin (20 µg/mL), TGFα (1 µg/mL) or HB-EGF (20 µg/mL). Pre-incubation with amphiregulin or TGFα neutralizing antibodies attenuated the LPA-induced transactivation signal in TccSup cells whereas direct stimulation of the EGFR by EGF was not affected (Fig. 2C). In ACHN cells EGFR stimulation by angiotensin II was completely inhibited by the HB-EGF
neutralizing antibody, pre-treatment with amphiregulin or TGFα blocking antibodies however showed no effect. These findings demonstrate that EGFR activation in response to GPCR agonists involves both TGFα and amphiregulin in TccSup bladder cancer and HB-EGF in ACHN kidney cancer cells.

To identify metalloproteinases which are involved in angiotensin II and LPA-induced cleavage of EGF-like ligand precursors in ACHN and TccSup cells, respectively, we investigated the effect of dominant-negative mutants of ADAM 10, 12, 15 and 17 (ΔMP10, 12, 15, 17) (21) on GPCR stimulated EGFR transactivation. As shown in Figure 2D upper panel, the transactivation signal was specifically blocked by ΔMP17 in ACHN cells upon stimulation with angiotensin II, and by ΔMP15 in TccSup cells upon stimulation with LPA.

To determine the relative expression of ADAM15 in urogenital cancer cell lines we raised polyclonal antibodies against the ectodomain of ADAM15 by immunizing rabbits with a peptide consisting of amino acids 757-773 of the ADAM15 ectodomain. In HEK293 cells ectopically expressing ADAM15, the anti-ADAM15 antiserum recognized a band of about 100 kD under non-reduced and of about 105 kD under reduced conditions (Fig. 2D, middle panel). The anti-ADAM15 antibodies however did not cross-react with over-expressed ADAM17 in HEK293 cells as well as endogenous ADAM17 in TccSup cells. The specificity of the anti-ADAM15 antiserum was further confirmed by the finding that ADAM15 siRNAs that specifically downregulated the ADAM15 mRNA levels as determined by RT-PCR (Fig. 2D, middle panel) reduced the ADAM15 Western blot signal in TccSup cells (Fig. 2D, middle panel). By immunoblotting Concanavalin A-enriched glycoproteins with the anti-ADAM15 antiserum we observed that infection of TccSup cells with the ΔMP15 construct reduced the endogenous expression level of ADAM 15 while the ΔMP17 construct showed no effect (Fig. 2D, lower panel). The ΔMP17 mutant however reduced the levels of ADAM17 in TccSup cells. These findings combined with our recent observation that ΔMP17 down-
regulated endogenous ADAM17 in SCC-9 cells (21) suggest that the ΔMP mutants of ADAM15 and ADAM17 inhibit metalloproteinase signalling by reducing the expression level of the endogenous protease. Moreover, we found that the protein expression levels of endogenous ADAM15 were similar in A498, TccSup and ACHN cancer cells whereas ADAM15 levels were higher in 5637 cells (Fig. 2D, lower panel), further supporting the hypothesis that in spite of a similar ADAM expression profile, different GPCR stimuli activate different ADAMs in TccSup and ACHN cancer cell lines.

To further substantiate the requirement for ADAM17 in GPCR-induced ectodomain cleavage of proHB-EGF, we investigated the effect of angiotensin II on the cell surface content of endogenous proHB-EGF by flow cytometry. Treatment of ACHN cells with angiotensin II resulted in a decrease in proHB-EGF on the cell surface (Fig. 2E). In contrast, angiotensin II-induced proHB-EGF shedding was markedly inhibited in ACHN cells stably expressing dominant negative ADAM17 (ΔMP17). ΔMP15 however showed no effect. Together, these findings demonstrate a role of ADAM17 in angiotensin II-triggered cleavage of proHB-EGF and EGFR transactivation in ACHN cells as well as identify ADAM15 as a critical sheddase in LPA-induced EGFR activation in TccSup bladder carcinoma cells.

**ADAM17 and the EGFR regulate proliferation of ACHN kidney cancer cells in response to GPCR stimuli**- Since the EGFR plays a central role in the regulation of the MAPK signal we investigated the effect of EGFR and metalloproteinase inhibition on GPCR-induced ERK1/2 activation in ACHN cells. BB94 and AG1478-treatment completely abrogated ERK1/2 activation in response to angiotensin II and LPA as determined by immunoblotting total cell lysates with activation state-specific ERK antibodies (Fig. 3A, upper panel). Recently, EGFR activity was shown to be required for GPCR-induced cell cycle progression by promoting accumulation of cyclin D1 in mid-late G1 phase in non-transformed cells (31). Therefore, we
assessed whether the EGFR is involved in GPCR-induced cyclin D1 expression in ACHN cells. Our results show that stimulation with angiotensin II and LPA lead to accumulation of cyclin D1 which was sensitive to AG1478 and the metalloproteinase inhibitor BB94 (Fig. 3A, lower panel). Next, we investigated ACHN cell proliferation in response to angiotensin II. We found that GPCR-induced DNA synthesis was specifically blocked by BB94 and AG1478 (Fig. 3B). Moreover, expression of the dominant-negative ADAM 17 mutant resulted in a decrease in the rate of DNA synthesis upon stimulation with angiotensin II. Expression of the ADAM 17 mutant inhibited DNA synthesis by EGF to a minor extend suggesting the involvement of ADAM17 in the regulation of basal and/or EGF-induced EGFR ligand availability (Fig. 3C) as described before (21). In conclusion, our results substantiate the importance of ADAM17-dependent EGFR transactivation for the accumulation of cyclin D1 and stimulation of cell proliferation by angiotensin II in ACHN kidney cancer cells.

LPA prevents apoptosis via transactivation of the EGFR in Rat1 fibroblasts and TccSup bladder carcinoma cells- In addition to cell proliferation, the EGFR and the oncoprotein HER2/neu regulate the responsiveness of a variety of cell types to pro-apoptotic stimuli such as serum-deprivation, death receptor activation and cytotoxic drugs. Previously, HER2/neu has been reported to be transactivated by GPCRs in Rat1 fibroblasts (1) and to be expressed at high levels in aggressive bladder tumours (33). Therefore we raised the question if HER2/neu is activated in response to LPA in TccSup bladder cancer cells. Our results demonstrate that LPA stimulation resulted in tyrosine phosphorylation of HER2/neu in TccSup bladder cancer cells and that HER2/neu transactivation was sensitive to BB94 (Fig. 4A). In addition, tyrosine phosphorylation of HER2/neu following LPA or EGF treatment was abolished by the EGFR inhibitor AG1478. Together, a metalloproteinase activity as well as the EGFR are critical for LPA-induced HER2/neu signal transactivation in TccSup bladder cancer cells.
PI3K and Akt/PKB are critical elements of survival pathways activated by growth factors, cytokines and integrins (34). We therefore determined the effect of GPCR stimulation in Akt/PKB activity by immunoblotting Rat1 and TccSup cell lysates with phospho-specific Akt/PKB antibodies. Indeed, Akt/PKB was strongly activated upon stimulation with GPCR ligands in an EGFR and metalloproteinase-dependent manner in both cell systems (Fig. 4B). Moreover, apoptosis of Rat1 fibroblasts induced by serum deprivation was blocked in the presence of LPA or EGF (Fig. 4C). The anti-apoptotic effect of LPA was specifically abrogated by AG1478 or BB94, again demonstrating the critical involvement of the EGFR and metalloproteinas. It was previously shown that activation of growth factor pathways enhances mechanisms of drug resistance in mammary carcinoma cells (35). Therefore, we investigated the involvement of EGFR transactivation in survival of Rat1 fibroblasts treated with doxorubicin, a prominent drug used in chemotherapy. We found that LPA prevented doxorubicin-induced apoptosis of Rat1 fibroblasts in a BB94 and AG1478-sensitive fashion (Fig. 4D). Interestingly, a recent report provided evidence that EGF stimulation protects breast adenocarcinoma cells from Fas-induced apoptosis through an Akt/PKB-mediated pathway (36). Hence, we investigated the involvement of EGFR signal transactivation in the regulation of survival of TccSup bladder carcinoma cells. We used a monoclonal anti-Fas CD95 antibody to induce apoptosis in TccSup bladder cancer cells. As shown in Figure 4E, LPA effectively prevented CD 95-induced apoptosis of TccSup cells in a BB94 and AG1478-sensitive manner. Collectively, these results demonstrate that metalloproteinase-dependent EGFR transactivation pathways are critically involved in activation of the survival mediator Akt/PKB as well as in the prevention of apoptosis induced by serum deprivation and doxorubicin in Rat1 fibroblasts and anti-Fas antibody in TccSup bladder carcinoma cells.
DISCUSSION

Given that GPCR-EGFR cross-talk pathways are widely established in normal and transformed cell types, we investigated the mechanisms and the role of EGFR signal transactivation in cell proliferation and anti-apoptosis in fibroblasts and urogenital cancer cells. Our data provide evidence that treatment of Rat1 fibroblasts, ACHN kidney and TccSupp bladder carcinoma cell lines with a variety of physiologically abundant GPCR agonists results in rapid EGFR activation through a metalloproteinase activity (Fig. 1A, 2B). EGFR signal transactivation also required the extracellular ligand-binding domain of the EGFR (Fig. 2B). These findings are consistent with the triple membrane-passing signal (TMPS) concept of EGFR transactivation that has recently been established in COS-7 and PC-3 cells (14). Our finding that in TccSup cells LPA treatment also leads to tyrosine phosphorylation of the oncoprotein HER2/neu (Fig. 4A) confirms our previous observations in Rat-1 fibroblasts (1) and further expands the significance of the TMPS pathway. A critical role of HER2/neu in the etiology of bladder cancer has been suggested by the finding that HER2/neu is expressed at high levels in neoplastic epithelium of tumours when compared with normal tissue (37).

By using neutralizing antibodies specific for EGF-like growth factor precursors we identified HB-EGF as well as amphiregulin and TGFα as mediators of the transactivation signal in ACHN and TccSup cancer cells, respectively (Fig. 2C). The enzymes implicated in shedding of EGF-like growth factor precursors belong to the ADAM family of zinc-dependent proteinases, which are widely expressed in many tissues. TACE/ADAM17 has previously been demonstrated to mediate cleavage of EGF-like precursors in murine fibroblasts (20,38)
and squamous cell carcinoma cells (21). Indeed, a dominant-negative ADAM17 mutant specifically blocked angiotensin II-induced proHB-EGF cell surface cleavage (Fig. 2E), the EGFR transactivation signal (Fig. 2D) and cell proliferation (Fig. 3C) in ACHN kidney cancer cells. These findings establish ADAM17 as a critical angiotensin II-responsive HB-EGF sheddase in ACHN cells. Other mechanisms, in which HB-EGF-dependent transactivation of the EGFR is mediated by ADAM10 in lung epithelial cells (39) and COS-7 cells (15) or by ADAM12 in cardiomyocytes (40), have been reported. The possible involvement of these sheddases in ACHN cells, however, was excluded in this study (Fig. 2D).

Adding to the complexity and obvious cell type specificity of EGFR transactivation pathways, we found that dominant negative ADAM15, but not ADAM17 prevented LPA-induced EGFR transactivation in TccSup cells (Fig. 2D). ADAM15 has recently been shown to contain an active metalloproteinase domain and has been implicated in cell adhesion and ectodomain processing (41). The cytoplasmic domain of ADAM15 contains proline-rich sequences that mediate interactions with Src-like kinases in hematopoietic cells (42). Whether Src-ADAM15 interactions and Src-dependent tyrosine phosphorylation of ADAM15 is critical for mediating the transactivation signal in TccSup cells remains to be elucidated.

When we investigated the role of EGFR transactivation in GPCR-induced mitogenic signalling, we found that inhibition of EGFR function or metalloproteinase activity blocked tyrosine phosphorylation of SHC and ERK1/2 activation in fibroblasts (Fig. 1A) and ACHN cells (Fig. 3A). These data demonstrate that the EGFR is instrumental in transducing mitogenic signals in response to GPCR agonists in these cell systems. Our current results further indicate that DNA synthesis and S-phase cell cycle progression by LPA requires EGFR and metalloproteinase activity in Rat1 fibroblasts (Fig. 1B-C) as well as in ACHN cells in response to angiotensin II (Fig. 3B).
A further important aspect of our findings is that, in addition to the proliferative responses, EGFR signal transactivation plays a direct role in the regulation of cell survival. The growth-promoting signalling events in Rat1 fibroblasts and TccSup cancer cells are accompanied by phosphorylation of the survival mediator Akt/PKB downstream of the EGFR (Fig. 4B). Activation of Akt has recently been shown to suppress apoptosis of Rat1 fibroblasts that had been detached from the extracellular matrix and to promote progression of quiescent cells into the S phase of the cell cycle (43). Furthermore, we found that LPA treatment drastically increased survival of fibroblasts after serum deprivation or doxorubicin treatment in an EGFR- and ADAM-dependent manner (Fig. 4C-D). In analogy to our observations, LPA was shown to cause resistance of cancer cells to chemotherapy in a recent study (44).

Another report demonstrated that GPCR ligands protect human lymphoblastoma cells from apoptosis triggered by CD95 antibodies via yet unknown mechanisms (45). We observed that death receptor-mediated apoptosis in TccSup bladder carcinoma cells was effectively prevented by LPA co-stimulation (Fig. 4E). Death receptor pathways are of pathophysiological significance since 75% of transitional cell carcinoma (TCC) cells display apoptosis upon activation of the Fas/ Fas-ligand pathway (46). Again, the anti-apoptotic effect of LPA on TccSup cells was dependent on the EGFR and metalloproteinase activity substantiating the importance of TMPS pathways in the regulation of cell death-versus-survival decisions (Fig. 4E).

In summary, our findings highlight the importance of EGFR signal transactivation in cancer cell proliferation and survival and strongly support a role of ADAM metalloproteinases as determinants of cancer progression.
REFERENCES


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FOOTNOTES

The abbreviations used are: ADAM, a disintegrin and metalloproteinase; DMSO, dimethysulfoxide; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; HB-EGF, heparin-binding EGF-like growth factor; LPA, lysosphatidic acid; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; RTK, receptor tyrosine kinase

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FIGURE LEGENDS

FIG. 1. GPCR ligands induce mitogenic signalling and cell proliferation in Rat1 fibroblasts through metalloproteinase-dependent transactivation of the EGFR. A, Rat1 cells were serum-starved for 18 h, pre-incubated with DMSO, BB94 (5 µM) or AG1478 (250 nM) for 20 min and stimulated with 10 µM LPA, 2 U/mL thrombin, 5 µM bradykinin or 100 nM endothelin I for 3 min. After immunoprecipitation with anti-EGFR or anti-SHC antibodies, proteins were immunoblotted with anti-phosphotyrosine (PY) antibody. Reprobing of the same filters with SHC antibodies confirmed equal protein loading. To determine ERK activation cells were stimulated for 7 min and total cell lysates were immunoblotted with anti-phospho-ERK or anti-ERK2 antibodies. B, Effect of EGFR- and metalloproteinase-inhibition on S-phase progression. Quiescent Rat1 cells were pre-incubated with inhibitors and stimulated with growth factors for 24 h. Cells were harvested and analysed by flow cytometry (∗, p < 0.05). C, Cell proliferation as determined by 3H-thymidine incorporation. Quiescent Rat1 cells were pre-incubated with inhibitors and stimulated with growth factors for 18 h. Cells were pulse-labelled with 3H-thymidine and thymidine incorporation was measured by liquid-scintillation counting (∗, p < 0.025).

FIG. 2. GPCR-induced EGFR transactivation requires shedding of EGF-like ligands by ADAM 17 and 15. A, ACHN kidney and TccSup bladder cancer cell were serum-starved for 48 h and 24 h, respectively, and treated with various GPCR ligands (1 µM angiotensin II, 200 nM bombesin, bradykinin, 1 mM carbachol, ET-1, LPA and thrombin) or EGF for 3 min. After immunoprecipitation the EGFR was immunoblotted with anti-PY and reprobed against total EGFR. B, Quiescent ACHN and TccSup cells were pretreated with BB94 or the
monoclonal anti-EGFR antibody ICR-3R for 1 h. Then, cells were stimulated with the ligands or pervanadate (PV, 1 mM) for 3 min. Tyrosine phosphorylated EGFR was detected as described under A. C, Effect of neutralizing antibodies on the transactivation signal. Quiescent TccSup or ACHN cells were pre-incubated with 20 µg/mL amphiregulin, 20 µg/mL HB-EGF or 1 µg/mL TGFα blocking antibodies for 1 h and stimulated with LPA, angiotensin II or EGF for 3 min. The EGFR was immunoprecipitated and immunoblotted with anti-PY antibody. D, ACHN as well as TccSup cell lines were infected with pLXSN constructs encoding dominant negative mutants (∆MP) of ADAM 10, 12, 15 and 17. Quiescent cells were stimulated with LPA, Angiotensin II or EGF for 3 min and tyrosine-phosphorylated EGFR was detected by immunoblotting (upper panel). HEK293 cells were transiently transfected with expression constructs encoding ADAM15 and ADAM17, respectively. Glycoproteins from total cell lysates were enriched by precipitation with Concanavalin A-sepharose and immunoblotted against ADAM15 and ADAM17 under reduced and non-reduced SDS-PAGE conditions (middle panel). Specific downregulation of endogenous ADAMs. Total lysate of TccSup cells infected with pLXSN constructs encoding ∆MP 15 and 17 was enriched with Concanavalin A-sepharose and immunoblotted against ADAM 15 as well as ADAM 17 (*=unspecific band). To further demonstrate the specificity of the ADAM15 antiserum TccSup cells were transfected with siRNAs against ADAM 15 and 17 or control siRNA. Gene expression was analysed by RT-PCR or immunoblot with ADAM 15 and 17 antibodies. Determination of the expression level of ADAM 15 in A498 and ACHN kidney as well as 5637 and TccSup bladder cancer cell lines (lower panel). Cell lysates containing 400 µg protein were resolved by SDS–PAGE and immunoblotted against ADAM 15. To ensure loading of equal protein amounts the membrane was reprobed with anti-tubulin antibody. Signals were quantified using a Fuji 1000 CCD camera and the “Image Gauge” program. E, Flow cytometric analysis of proHB-EGF ectodomain cleavage. After stimulation with
angiotensin II for 20 min, ACHN cells stably expressing dominant negative ADAM 17 (ΔMP17), ADAM15 (ΔMP15) or control cells were stained for surface HB-EGF and analysed by flow cytometry.

**FIG. 3. Effect of EGFR and ADAM metalloproteinase inhibition on GPCR-induced ERK activation and cell proliferation in ACHN kidney cancer cells.** A, Quiescent ACHN cells were pre-incubated with inhibitors and then stimulated with angiotensin II, LPA or EGF for 7 min or 8 h. Crude lysates were immunoblotted with phospho-specific ERK and cyclin D1 antibodies, respectively. B, Effect of BB94 and AG1478 on GPCR-induced ACHN cell proliferation. Quiescent ACHN cells were pre-incubated with inhibitors and stimulated with angiotensin II or EGF for 18 h. Cells were pulse-labelled and ³H-thymidine incorporation was measured by liquid-scintillation counting (*, p < 0.02). C, ACHN kidney cancer cells stably expressing dominant negative (ΔMP) ADAM 10, 12, 15 and 17 were serum-starved for 48 h and stimulated with angiotensin II or EGF for 18 h. Incorporation of ³H-thymidine into the cells was measured by liquid-scintillation counting (*, p < 0.02).

**FIG. 4. LPA prevents apoptosis induced by serum-starvation, doxorubicin and anti-CD95/FasLigand via EGFR transactivation.** A, Transactivation of HER2. Quiescent TccSup cells were pre-incubated with inhibitors and stimulated with LPA or EGF. Cell lysates were immunoprecipitated with antibodies against HER2 and immunoblotted against PY. B, Activation of Akt/PKB by GPCR ligands. Following pre-incubation with inhibitors quiescent TccSup and Rat1 cells were stimulated and Akt activation was determined by immunoblotting with anti-phospho-Akt antibody. Re-probing against total Akt confirmed loading of equal protein amounts. C, Effect of LPA, EGF, AG1478 and BB94 on apoptosis induced by serum deprivation. Quiescent Rat1 cells were pre-incubated with inhibitors and
stimulated with growth factors for 48 h. Cells were harvested and analysed by flow cytometry (*, p < 0.02). D, Effect of BB94, AG1478 and growth factors on doxorubicin-induced apoptosis. Quiescent Rat1 cells were pre-incubated with inhibitors, stimulated with LPA for 1 h and then treated with Doxorubicin (1µg/mL). After 12 h the percentage of apoptotic cells was determined by FACS analysis (*, p < 0.002). E, Effect of LPA, BB94, and AG1478 on doxorubicin-induced apoptosis. Quiescent TccSup cells were pre-incubated with inhibitors, stimulated with LPA and then treated with anti-CD95/FasLigand (50 µg/mL). After 24 h the relative amount of apoptotic cells was determined by FACS analysis (*, p < 0.004).
Figure 1

A

Ratl:

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B

Ratl:

![Chart B]

C

Ratl:

![Chart C]
Figure 2

A

ACHN:

stimulation: IP: BB94 ICR-3R

IB: PY EGFR

TccSup:

stimulation: IP: BB94 ICR-3R

IB: PY EGFR

B

ACHN:

stimulation: BB94 ET-1 LPA EGF PY

IB: BB94 ICR-3R

TccSup:

stimulation: BB94 ET-1 LPA EGF PY

IB: BB94 ICR-3R

C

ACHN:

stimulation: AngII EGF

inhibition: IP: 3M PD 10 nM PD 100 nM PD 1

IB: PY EGFR

TccSup:

stimulation: LPA EGF

inhibition: IP: 3M PD 10 nM PD 100 nM PD 1

IB: PY EGFR
Figure 2
Figure 3

A

ACHN:

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IB: phosphoERK

ERK2

IB: cyclin D1

ERK2
Figure 3

B

ACHN:

![Bar graph showing cpm for control, AngII, and EGF treatments with BB94 and AG1478 treatments indicated.]

C

ACHN:

![Bar graph showing cpm for control, Angiotensin II, and EGF treatments with Mock and deltaMP treatments indicated.]
Figure 4

A

TecSup:

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B

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Rat1:

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</table>

Phospho/Akt

Akt
Figure 4

C

Rat1:

![Bar chart showing % cells in sub G0 for control, LPA, and EGF treatments. The chart includes error bars and asterisks indicating statistical significance.]

D

Rat1:

![Bar chart showing % cells in sub G0 for control, Doxo, Doxo+LPA, and Doxo+EGF treatments. The chart includes error bars and asterisks indicating statistical significance.]

35
Figure 4

E

TeeSup:

![Graphs showing cell cycle distribution and cell count](image)

TeeSup:

![Bar chart showing cell count](image)
Distinct ADAM metalloproteinases regulate G protein coupled receptor-induced cell proliferation and survival
Beatrix Schäfer, Beatrice Marg, Andreas Gschwind and Axel Ullrich

J. Biol. Chem. published online August 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400129200

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