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CORRECTION

Shedding of c-Met is regulated by crosstalk between a G-protein coupled receptor and the EGF receptor and is mediated by a TIMP-3 sensitive metalloproteinase

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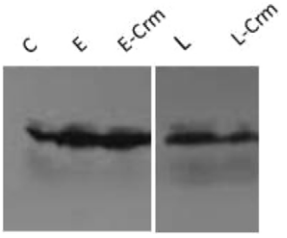
There was an error published in *J. Cell Sci.* **114**, 1213-1220.

In this manuscript, the western blots presented in Fig. 2 and Fig. 4 represent the results of a single experiment in which control samples (untreated) and those that had been treated with EGF and LPA were run on separate SDS-PAGE gels. In the final preparation of the figures for publication, the gels were rearranged to make composite figures.

We wish to correct an error in Fig. 5. The samples used in two lanes in Fig. 5, namely E-CRM and L were inadvertently duplicated. A blot from a duplicate experiment performed during the same period of the project is presented below. It demonstrates that the results are consistent with that shown in the original published figure. The figure legend remains unaltered. The raw data image of the duplicate experiment has been submitted to the Editors of *Journal of Cell Science*.



None of these changes alters the conclusions of the paper. We apologize to our readers for the inconvenience caused as a result of the error.

Shedding of c-Met is regulated by crosstalk between a G-protein coupled receptor and the EGF receptor and is mediated by a TIMP-3 sensitive metalloproteinase

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SUMMARY

A wide repertoire of transmembrane proteins are proteolytically released from the cell surface by a process known as 'ectodomain shedding', under both normal and pathophysiological conditions. Little is known about the physiological mechanisms that regulate this process. As a model system, we have investigated the metalloproteinase-mediated cleavage of the hepatocyte growth factor receptor, Met. We show that epidermal growth factor (EGF) receptor activation, either directly by EGF or indirectly via the G-protein coupled receptor (GPCR) agonist lysophosphatidic acid (LPA), induces cleavage of Met through activation of the Erk MAP kinase signalling cascade. The tyrosine kinase activity of the EGFR was a prerequisite for this stimulation, since treatment of cells with a synthetic inhibitor of this receptor, AG1478, completely abrogated shedding. The metalloproteinase mediating Met cleavage was specifically inhibited by the tissue inhibitor of metalloproteinases (TIMP)-3, but not by TIMP-1 or TIMP-2. Furthermore, the level of Met shedding could be modulated by different cell-matrix interactions. Our results indicate that ectodomain shedding is a highly regulated process that can be stimulated by EGFR signalling pathways and integrin ligation.

Key words: Ectodomain shedding, Epidermal growth factor receptor, ADAM, TIMP

INTRODUCTION

Ectodomain shedding is a process in which the extracellular domains of transmembrane proteins are proteolytically shed from the cell surface (Bosenberg and Massague, 1993; Blobel, 1997; Werb and Yan, 1998). This process allows a cell to alter its surface phenotype rapidly in response to environmental changes and to yield soluble intercellular regulators. Several different types of membrane proteins undergo protein ectodomain shedding, including cytokines such as tumour necrosis factor-α (TNF-α; Black et al., 1997; Moss et al., 1997) and the kit ligand (Yee et al., 1993), cytokine receptors such as the TNF receptors (Madge et al., 1999) and growth factor precursors such as heparin binding-epidermal growth factor (HB-EGF; Izumi et al., 1998; Gechtman et al., 1999).

Ectodomain shedding is accelerated under pathological conditions such as inflammation, apoptosis and arthritis, but the physiological mechanisms and signalling pathways that regulate this process are largely unknown (Hooper et al., 1997). Studies with mutant CHO cells support the idea that shedding of numerous proteins is regulated by similar mechanisms (Arribas et al., 1996). The most extensively studied inducer of shedding is phorbol 12-myristate-13-acetate (PMA), which activates protein kinase C and induces the cleavage of many proteins. Besides PMA, other agents that also induce shedding include calcium ionophores, chemotactic peptides, cytokines and growth factors (Hooper et al., 1997; Subramanian et al., 1997).

The enzymes implicated in cellular proteolysis belong to the metzincin family of zinc-dependent proteinases, which include the matrix metalloproteinases (MMPs) and the ADAMs (A Disintegrin and Metalloproteinase Domain). ADAMs have been hypothesized to be responsible for most protein ectodomain shedding events (reviewed by Schlondorff and Blobel, 1999). They are generally type I transmembrane glycoproteins containing both a metalloproteinase and a disintegrin domain. In addition to their proteolytic activities, ADAMs also mediate cell adhesion by binding to integrins and glycosaminoglycans (Almeida et al., 1995; Zhang et al., 1998; Nath et al., 1999; Nath et al., 2000; Iba et al., 1999). The most extensively studied adamalysin is the TNF-α converting enzyme (TACE, ADAM-17), which has been implicated in the shedding of numerous proteins including TNF-α, transforming growth factor-α (TGF-α) and L-selectin (Peschon et al., 1998). Interestingly, TACE knockout mice have severe phenotypic defects that resemble the EGFR and TGF-α knockout mice (Sibilia and Wagner, 1995; Sibilia et al., 1998; Peschon et al., 1998).

Interactions between different signalling systems are important in allowing cells to respond to varying physiological conditions. In recent years, EGFR has emerged as a key signal transducing molecule that can be activated not only by direct agonists but also by other receptor classes, including numerous G-protein coupled receptors (GPCRs), integrins and cytokine receptors (reviewed by Carpenter, 1999). Activation of EGFR upon ligand binding induces a variety of cellular responses,

including MAP kinase activation (Carpenter, 1999). Since transactivation of the EGFR also leads to MAP kinase activation, this receptor provides a link to the MAP kinase pathway in response to GPCR agonists and integrin ligation. Although the precise mechanisms that lead to receptor transactivation are not completely understood, a recent report suggests that EGFR transactivation by GPCRs involves the generation of soluble HB-EGF by a metalloproteinase (Prenzel et al., 1999).

EGFR is expressed ubiquitously by nonhaemopoietic cells and is therefore perfectly positioned to function as a major signal transducing molecule in response to a wide range of stimuli. In view of this and the observations that TACE knockout mice closely resemble the EGFR null mice, we wished to test whether EGFR activation can induce ectodomain shedding. To address this hypothesis, we examined the shedding of the hepatocyte growth factor receptor, Met. It is a tyrosine kinase receptor, which upon activation can mediate a variety of cellular responses, including cell growth and motility (Birchmeier and Gherardi, 1998). Met is initially synthesized as a single-chain intracellular precursor, and subsequently undergoes proteolytic processing at different stages during intracellular trafficking, leading to the presentation of an α/β heterodimer at the cell surface (Giordano et al., 1989). The 140 kDa β chain of the complex can be proteolytically cleaved by cells constitutively, and this may be enhanced by PMA treatment (Galvani et al., 1995).

Here, we report that EGF and LPA, which act through different cell surface receptors, activate ectodomain shedding of Met by a TIMP-3 sensitive metalloproteinase. This induced shedding is mediated by the Erk MAP kinase pathway and can be selectively modulated by different ECM-integrin interactions. These observations provide a mechanism by which ectodomain shedding can be induced by a diverse range of physiological stimuli.

MATERIALS AND METHODS

Reagents

Purified human recombinant EGF was purchased from Chemicon. Lysophosphatidic acid (LPA), phorbol 12-myristate-13-acetate (PMA) and the proHB-EGF inhibitor [Glu^{52}]*Diphtheria* toxin (CRM197) were from Sigma Chemicals (St Louis, USA). ^{35}S[Meth/Cys] radioactive mixture was purchased from Life Technologies. Dulbecco's modified Eagle's medium (DMEM), serum and other reagents for cell culture were from Gibco-BRL. Antibody against Met extracellular domain (clones DL-21 and DO-24), were purchased from Upstate Biotechnology (USA).

PD98059, SB203580, U0126 and AG1478 were from Calbiochem® (UK) and were used at 10 μM. PD98059 is an inhibitor of MAP kinase kinase (MEK) that acts by inhibiting the activation of MAP kinase and subsequent phosphorylation of MAP kinase substrates. U0126 is a specific inhibitor of MEK1 and MEK2. SB203580 specifically inhibits p38 kinase. AG1478 is a specific inhibitor of the tyrosine kinase activity of EGFR.

The broad-spectrum metalloproteinase inhibitor, BB94, was a generous gift from British Biotech and was used at the following concentrations: 6 μM, 2.5 μM and 1.0 μM. TIMP-1 was used at 0.5 μM, TIMP-2 at 1.0 μM and TIMP-3 at 0.3 μM.

Cell culture

A549 human lung adenocarcinoma cells were obtained from the European Collection of Cell Cultures. TACE$^{-/-}$ EC2 cells were a kind gift from Dr R. A. Black and colleagues (Immunex). The cells were maintained in DMEM supplemented with 10% bovine calf serum, 1% glutamine and 1% penicillin and streptomycin, in 5% CO_2. The TACE$^{-/-}$ cells were transfected using Fugene 6™ (Boehringer-Mannheim) according to the manufacturer's protocol.

Assays for Met cleavage

Immunoprecipitation

A549 cells were seeded in 6-well tissue culture plastic dishes at 8×10^5 cells per well in serum containing DMEM. Cells were then cultured overnight in methionine-, cysteine- and serum-free DMEM. To analyse the levels of transmembrane Met, cells were pulse-labeled with 140 μCi [^{35}S]methionine/cysteine for 2 hours; free label was removed by rinsing with cold medium and cells were allowed to incubate in regular DMEM alone or with EGF (40 ng/ml) or LPA (10 μM) for up to 6 hours. At specified time points after initiation of the chase, cells were lysed in 1% NP-40 lysis buffer containing 10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM phenylmethanesulfonyl fluoride. To measure cell-associated receptor, Met was immunoprecipitated in the cell extract using the anti-Met antibody, DO-24, and the ^{35}S-labeled Met was detected by SDS-PAGE and visualised by autoradiography. To measure the levels of soluble Met, the medium was collected at different points during the chase period and immunoprecipitations were performed using the DO-24 mAb. Soluble Met was detected by SDS-PAGE followed by autoradiography.

Western blot analysis

A549 cells were plated in 6-well tissue culture plastic dishes at 4×10^5 cells per well and grown overnight in serum containing DMEM. The next day, cells were switched to serum-free medium and then treated with EGF or LPA or with the inhibitors, as required. In some experiments, cells were grown on collagen. In these experiments, 6-well tissue culture plastic dishes were coated with the respective ECM proteins at a concentration of 10 μg/ml overnight in PBS. Wells were blocked with 1% heat-denatured BSA for 1 hour and then A549 cells were added at a density of 4×10^5 cells per well. The next day, cells were placed in serum-free medium and treated with either EGF or LPA. The MAP kinase inhibitors, PD98059 and SB203580, and the EGFR inhibitor, AG1478, were added to the cells for 10 minutes, prior to the addition of EGF or LPA.

Supernatants from these experiments were collected at the appropriate time points and concentrated eight- to tenfold using a Centricon-10 concentrator (Amicon). The amount of protein was determined using the BCA protein determination kit (Pierce) and soluble Met was detected by western blot analysis using DL-21 mAb.

For soluble Met detection, blots were first incubated with DL-21 monoclonal antibody (1:2500), overnight at 4°C and then with an HRP-conjugated sheep anti-mouse antibody (1:5000) for 1 hour at room temperature. The blots were developed using the Supersignal® chemiluminescence kit (Pierce).

RESULTS

EGF and LPA induce Met ectodomain shedding

To study regulation of Met ectodomain shedding, we used A549 cells, a human lung adenocarcinoma cell line. These cells were chosen because they remain viable in the absence of serum, express Met, and shed its ectodomain at a relatively low basal rate.

Two different methods of assaying the extracellular cleavage of Met with respect to time were compared. First, cellular

proteins were ^{35}S pulse-labeled for 2 hours and the time-dependent disappearance of cell-surface [^{35}S]Met (Fig. 1A) and appearance of soluble Met (Fig. 1B) were assessed over a subsequent 6 hour chase period by immunoprecipitation using a monoclonal antibody against the extracellular domain of Met. In the cell lysate, pulse-labeled Met appeared as a single 140 kDa mature form. Without stimulation, the mature form underwent negligible ectodomain cleavage (Fig. 1A, left). Concomitantly, soluble Met appeared in the medium as a single 90 kDa species (Fig. 1B, left). Using this assay we determined that EGF, a mitogenic growth factor acting through a transmembrane tyrosine kinase receptor, and lysophosphatidic acid (LPA), a serum phospholipid binding to a GPCR, stimulated the cleavage of transmembrane Met and ectodomain release in a time-dependent manner (Fig. 1A,B). As the duration of the chases increased from 1-6 hours, the relative amount of the 140 kDa mature protein immunoprecipitated decreased considerably from EGF- and LPA-treated cells (Fig. 1A). Simultaneously, the 90 kDa soluble Met increased in intensity with the same treatments (Fig. 1B).

In an alternative assay, ectodomain shedding was assayed by monitoring the appearance of the 90 kDa soluble fragment of the Met receptor in the culture medium by western blotting and enhanced chemiluminiscence (ECL) detection. Cells were either untreated, or stimulated by EGF and LPA over a period of time. Soluble Met was detected in the culture medium after 1 hour of stimulation with EGF or LPA (Fig. 1C) and continued to increase up to 6 hours. The basal amount of Met shedding seen in untreated cells was barely detectable after 2 hours, and remained at a low level compared to stimulated cells. Increasing concentrations of EGF and LPA significantly enhanced cleavage, with maximal shedding occurring at 10 ng/ml EGF and 10 μM LPA (Fig. 1D). Higher concentrations of EGF and LPA did not result in a further increase in shedding (data not shown).

Since the shed fragment of Met could be detected by western blotting and ECL detection and the sensitivity was comparable to the ^{35}S pulse-labelling experiments, we chose to use the former assay in all our future experiments.

These studies together demonstrate that stimulation of two structurally diverse receptor classes, a receptor tyrosine kinase and a GPCR, can induce cellular pathways leading to the cleavage of Met. It has been reported that a highly labile 55 kDa fragment of the Met β chain is left behind on the membrane following ectodomain cleavage, which can be stabilized by addition of the proteasome inhibitor, lactacystin (Jeffers et al., 1997). In our experiments, we were also able to detect the cell-associated 55 kDa fragment after lactacystin treatment of cells (data not shown).

Ectodomain shedding of Met is mediated by a metalloproteinase that is sensitive to TIMP-3

A large number of cell-surface proteins are proteolytically cleaved by metal-dependent proteinases, which are thought to be members of the ADAM family of proteins. The catalytic activity of these proteins, like those of the soluble matrix metalloproteinases (MMPs), can be inhibited by synthetic metalloproteinase inhibitors. To determine whether the EGF- and LPA-induced cleavage of Met was mediated by a metalloproteinase, the effects of BB94, a hydroxamic acid-based broad spectrum metalloproteinase inhibitor, was tested. Cells were pretreated with BB94 at varying concentrations, prior to stimulation with EGF and LPA. After 6 hours, medium was collected and analyzed for the presence of soluble Met (Fig. 2). BB94 at 1.0 μM markedly decreased both EGF and LPA induced shedding to below the basal level, whereas at higher concentrations of 2.5 μM and 6.0 μM there was complete inhibition (Fig. 2). Since BB94 inhibited shedding in a concentration-dependent manner, these results suggest that a metalloproteinase was involved in the cleavage of Met from the surface of A549 cells.

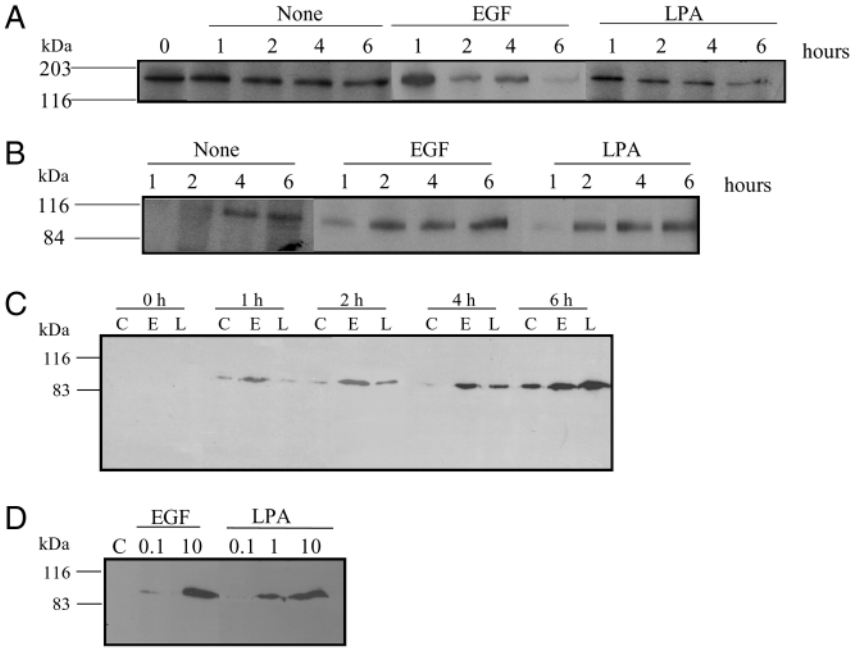

Fig. 1. Time-dependent stimulation of Met ectodomain shedding by EGF and LPA. (A) Immunoprecipitation and SDS-PAGE of ^{35}S pulse-labeled transmembrane Met in cell lysates at different time points after addition of EGF (40 ng/ml) or LPA (10 μM), or without any treatment (None). EGF and LPA induce a more rapid processing and disappearance of the 140 kDa transmembrane form of Met. (B) Immunoprecipitation and SDS-PAGE of ^{35}S pulse-labeled soluble Met in the medium at different time points after addition of EGF or LPA, or without any treatment (None). EGF and LPA induce a more rapid generation of the 90 kDa soluble form of Met. (C) Western blot analysis of soluble Met. A549 cells were either treated with EGF (E) at 40 ng/ml or LPA (L) at 10 μM, or remained untreated (C). Medium was collected at different times, concentrated and analyzed for the presence of soluble Met by western blot analysis, using the anti-Met mAb, DL-21. The proteolytically cleaved form of Met is detected as a 90 kDa band, which belongs to the β chain of the Met heterodimer. (D) Dose-dependent cleavage of Met. A549 cells were treated with varying doses of EGF (0.1 ng/ml and 10 ng/ml) and LPA (0.1 μM, 1 μM and 10 μM) or remained untreated (C), for 6 hours. Soluble Met was analyzed as described above. Molecular mass markers are shown on the left.

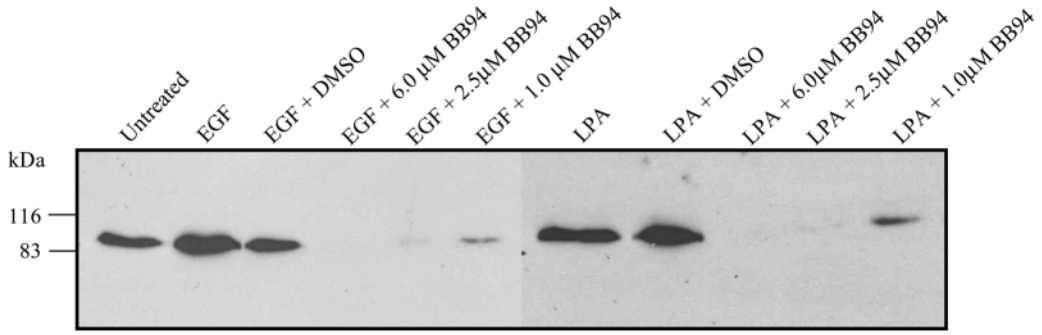

Fig. 2. Met shedding is mediated by a metalloproteinase. A549 cells were incubated with the hydroxamate inhibitor BB94 for 30 minutes at the indicated concentrations, before the addition of EGF (40 ng/ml) or LPA (10 μM). Untreated cells represent the basal level of shedding. After 6 hours, medium was concentrated and analyzed for soluble Met by western blotting using the DL-21 mAb. Met cleavage was inhibited to levels markedly below the basal level of shedding.

The TIMPs are natural inhibitors of matrix metalloproteinases (MMPs) and ADAMs. Out of the four TIMPs identified to date, only TIMP-3 inhibits the catalytic activity of TACE (Amour et al., 1998) and blocks cellular shedding of L-selectin and proTNF-α (Borland et al., 1999; Hargreaves et al., 1998). In contrast, ADAM-10 can be inhibited by TIMP-1 and only very poorly by TIMP-3 (Amour et al., 2000). These observations suggest that the involvement of particular ADAMs can be distinguished from TACE through their differing sensitivity to TIMPs.

We wanted to determine which TIMPs might affect the shedding of Met and whether any comparisons can be made to ADAM-mediated proteolytic events. A549 cells were treated with TIMP-1, -2 and -3 prior to stimulation with EGF and LPA. Out of the three TIMPs tested, TIMP-3 inhibited Met shedding to below the basal level, whereas TIMP-1 and -2 had no effect (Fig. 3A).

TACE-mediated cleavage events can be induced upon PMA treatment. Since the catalytic activity of TACE is sensitive to TIMP-3 we wished to assess whether the metalloproteinase mediating shedding of Met, had similar properties to TACE. A549 cells were treated with either PMA alone or in combination with TIMP-1, -2 and -3 and analyzed for the presence of soluble Met (Fig. 3A). TIMP-3 completely inhibited PMA-induced Met shedding, whereas TIMP-1 and -2 had no effect, hence following a similar inhibition pattern to EGF- and LPA-stimulated Met shedding.

These results together suggest that Met shedding is not mediated by a known MMP, since between TIMP-1 and -2, the catalytic activities of all known MMPs are inhibited. Shedding was inhibited by both BB94 and TIMP-3 to below the basal level, suggesting that a metalloproteinase activity could account for the constitutive and stimulated cleavage of Met. Transfection of Met cDNA into TACE$^{-/-}$ fibroblast cells led to Met shedding upon stimulation with PMA (Fig. 3B), suggesting that TACE is not the protease responsible for cleaving Met, at least in TACE$^{-/-}$ cells.

EGF and LPA induce Met shedding through EGFR tyrosine kinase activation

Most cellular responses to growth factors, including EGF, require the tyrosine kinase activity of their receptors. In addition, binding of LPA to its receptor results in activation of the intrinsic tyrosine kinase activity of the EGFR, which is required for downstream activation of the MAP kinases and cellular responses (Cunnick et al., 1998). Therefore, we investigated whether the kinase activity of EGFR was required to induce Met cleavage in response to EGF and LPA. We tested the effects of an EGFR kinase inhibitor, AG1478, tyrphostin, which prevents ligand-induced ATP hydrolysis by the stimulated EGFR. Cells were pretreated with this inhibitor at a concentration that precludes inhibition of other EGFR family tyrosine kinases, prior to EGF and LPA stimulation. Treatment with AG1478 markedly decreased both the EGF and LPA stimulation of Met shedding to below the basal level (Fig. 4). This experiment was done at the same time as the experiment shown in Fig. 2, and hence the control lanes (untreated, EGF, EGF+DMSO, LPA, LPA+DMSO) are identical in Figs 2 and 4. These results demonstrate that the tyrosine kinase enzymatic activity of EGFR is required to induce proteolytic cleavage of

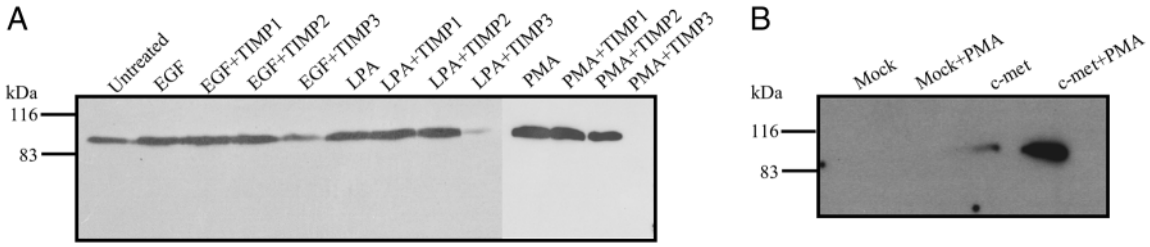

Fig. 3. Induced shedding is specifically inhibited by TIMP-3. (A) A549 cells were treated with TIMP-1 (0.5 μM), TIMP-2 (1.0 μM) or TIMP-3 (0.3 μM) for 30 minutes or remained untreated, before the addition of EGF (40 ng/ml), LPA (10 μM) or PMA (20 ng/ml). (B) TACE$^{-/-}$ EC2 cells were either mock transfected or transfected with c-met cDNA. 24 hours post-transfection, cells were stimulated with PMA (20 ng/ml) or untreated. After 6 hours, medium was concentrated and analyzed for soluble Met, by western blotting using the DL-21 mAb.

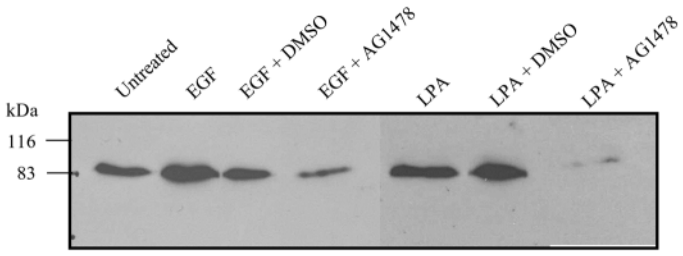

Fig. 4. Induction of Met shedding requires EGFR tyrosine kinase activity. A549 cells were either untreated, or treated with the specific EGFR tyrosine kinase inhibitor, AG1478 (10 μM), or its vehicle DMSO, for 10 minutes before the addition of EGF (40 ng/ml) or LPA (10 μM). After 6 hours, medium was collected, concentrated and analyzed for soluble Met by western blotting with the DL-21 mAb.

Met, and provide further evidence that the LPA/GPCR-mediated effects on ectodomain shedding proceed via EGFR function (Prenzel et al., 1999).

Autocrine release of HB-EGF is required for LPA-stimulated activation of Met shedding

Recent studies suggest that transactivation of EGFR can occur via an autocrine/paracrine mechanism involving the release of soluble EGF-like ligands such as HB-EGF from the cell surface by a metalloproteinase (Prenzel et al., 1999; Carpenter, 1999). Transmembrane HB-EGF (proHB-EGF) is the receptor for *Diptheria* toxin. Thus, binding of *Diptheria* toxin to the extracellular HB-EGF domain potently and specifically inhibits its mitogenic activity. To determine whether soluble HB-EGF contributes to LPA-mediated activation of Met shedding through EGFR, we assessed whether treatment with CRM197, a catalytically inactive [Glu^{52}] mutant of *Diptheria* toxin, affected LPA-induced Met shedding. As shown in Fig. 5, preincubation with CRM197 markedly attenuated LPA-induced Met shedding. As expected, the EGF-induced shedding of Met was not affected because direct application of the EGFR ligand circumvented the requirement for the release of endogenous HB-EGF. These data further support the idea that LPA-mediated transactivation of EGFR proceeds via activation of a metalloproteinase that cleaves proHB-EGF from the surface to yield soluble HB-EGF. Together our results demonstrate that LPA treatment leads to at least two metalloproteinase cleavage events, one resulting in pro HB-EGF cleavage and the other leading to Met shedding.

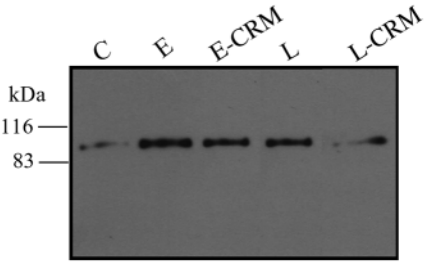

Fig. 5. Role of HB-EGF shedding in transactivation of EGFR by LPA. A549 cells were either untreated (C), or preincubated for 1 hour with CRM197 (10 μg/ml) and then stimulated with EGF (40 ng/ml) or LPA (10 μM). After 6 hours, medium was collected, concentrated and analyzed for soluble Met by western blotting with the DL-21 mAb.

Inhibition of ERK kinase abrogates EGF and LPA stimulated Met shedding

Cellular responses induced upon growth factor stimulation involve the activation of intracellular signalling cascades, and the MAP kinase cascade has been extensively studied. In particular, the Erk subfamily of this pathway, which becomes activated in response to growth factors like EGF through their tyrosine kinase receptors (Carpenter, 1999), or through GPCR-agonists like LPA (Cunnick et al., 1998; Della Rocca et al., 1999; Moolenaar et al., 1997), has been studied. Since our data (Fig. 4), show that phosphorylation of EGFR is required for EGF- and LPA-stimulated Met shedding, we wanted to determine whether the MAP kinases were involved in inducing a metalloproteinase activity. This was tested using the signalling inhibitors, PD98059 and SB203580, which inhibit the activity of ERK kinase (MEK, MAPKK) and p38 kinase, respectively. Cells were treated with these inhibitors at concentrations that maintain their selectivity, prior to and during stimulation with EGF and LPA (Fig. 6). SB203580 had no effect on either EGF or LPA stimulated Met shedding. In contrast, PD98059 completely inhibited LPA-induced shedding and decreased EGF-stimulated shedding to the basal level (Fig. 6). A similar decrease in shedding was observed with U0126, another inhibitor of MEK (data not shown). These results suggest that the EGF- and LPA-induced shedding of Met requires activation of the ERK MAP kinase pathway.

Effect of extracellular matrix interactions on Met shedding

Ligation of integrins can lead to activation of the MAP kinase pathway through transactivation of growth factor receptors (Moro et al., 1998). Such integrin-dependent activation occurs in the absence of growth factors and can also be mimicked in vitro by plating cells on immobilized anti-integrin antibodies or on ECM proteins. Given that activation of the MAP kinase pathway leads to an induction in Met cleavage, we wished to test the effect of integrin ligation on ectodomain shedding. A549 cells were grown on collagen, or on uncoated plastic, and

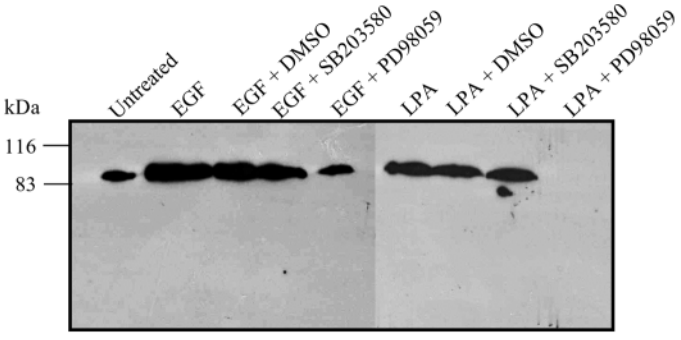

Fig. 6. Erk MAP kinase signalling mediates EGF- and LPA-induced Met shedding. A549 cells were either untreated, or treated with PD98059 (10 μM), a specific inhibitor of Erk kinase, or SB203580 (10 μM), a specific inhibitor of p38 kinase, or the vehicle DMSO, for 10 minutes before the addition of EGF (40 ng/ml) or LPA (10 μM). After 6 hours, medium was collected, concentrated and analyzed for soluble Met by western blotting with the DL-21 mAb.

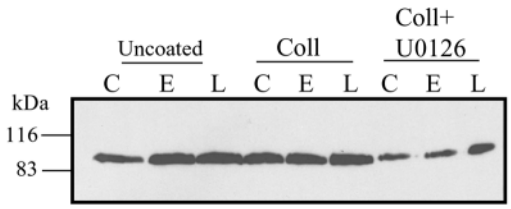

Fig. 7. Cell-matrix interactions modulate Met shedding through activation of Erks. A549 cells were grown to confluency on wells coated with 10 μg/ml collagen (Coll) or on uncoated wells. Cells were either untreated (C), or treated with EGF (E, 40 ng/ml) or LPA (L, 10 μM**)**. To test whether Erk activation was mediating increased Met shedding, cells were preincubated with the MEK inhibitor U0126 (10 μM) for 10 minutes prior to stimulation with EGF or LPA. After 6 hours, medium was concentrated and analyzed for soluble Met by western blotting with DL-21 mAb.

were either untreated or stimulated with EGF and LPA. As shown in Fig. 7, the basal level of Met shedding was significantly higher from cells plated on collagen compared to cells on uncoated wells. However, stimulated shedding occurred at comparable levels whether the cells were plated on ECM proteins or on uncoated plastic. To determine whether the ECM affected the Erk signalling pathway, the phosphorylation status of Erk1 and Erk2 was examined and the level of phosphorylated Erk-2 was found to be markedly increased in cells adhering to ECM (data not shown), in accordance with previous observations (Moro et al., 1998). To unequivocally demonstrate that increased Erk activation contributes to the increase in basal shedding, the effects of the MEK inhibitor, U0126 were examined. As shown in Fig. 7, treatment with U0126 markedly inhibits both basal and stimulated Met shedding on collagen. These experiments were performed at least three times and consistently demonstrate that basal shedding of Met is increased upon integrin ligation through activation of Erks.

Taken together, our results indicate that increased activation of Erk-2 in cells adhering to ECM leads to an increase in Met shedding. Thus, different cell-matrix or cell-integrin interactions can modulate ectodomain shedding, possibly through activation of growth factor receptor signalling pathways.

DISCUSSION

In this study we have examined the physiological mechanisms that induce ectodomain shedding, using the Met receptor as a model system. Several important observations have emerged from our investigations. Firstly, two diverse receptor classes, growth factor receptors and GPCRs, activate pathways leading to ectodomain shedding, and both require the tyrosine kinase activity of the EGFR. Secondly, both LPA- and EGF-stimulated shedding appear to be mediated by the activation of the Erk subfamily of the MAP kinase signalling pathway. Thirdly, integrin ligation results in an increase in the basal level of Met shedding.

Recent reports suggest that growth factor stimulation of ectodomain shedding may be a general phenomenon. Transmembrane proteins including syndecans 1, 4 (Subramanian et al., 1997), Tie receptors (Yabkowitz et al., 1999), TGF-α and L-selectin (Fan and Derynck, 1999) can be released rapidly from the cell surface upon growth factor treatment. In the case of TGF-α, cellular activation by PDGF and FGF results in ectodomain shedding, proceeding via the activation of their respective receptors (Fan and Derynck, 1999). In our work we provide evidence of the ability of ECM-integrin interactions, as well as cooperation between the EGFR and LPA receptor, to activate a metalloproteinase activity.

Our initial observation was that either LPA or EGF results in generation of soluble Met to levels that are considerably higher than the basal level of shedding. Treatment with AG1478, a drug that inhibits EGFR kinase (Levitzki and Gazit, 1995), completely inhibited shedding (Fig. 4), suggesting that (1) the kinase activity of the EGFR is required for induction of metalloproteinase activity and (2) the LPA response proceeds via the EGFR. Furthermore, LPA-induced EGFR transactivation proceeds via generation of soluble HB-EGF through a metalloproteinase-mediated cleavage (Fig. 5). These results suggest that the LPA-mediated shedding of Met might involve two different metalloproteinases, the first metalloproteinase operating inbetween the GPCR and EGFR to yield soluble HB-EGF, and the second acting downstream of the EGFR. Interestingly, cleavage of proHB-EGF can occur within 5 minutes of GPCR stimulation, whereas c-met shedding can be observed much later. One possible explanation might be that the relatively low level of soluble HB-EGF produced upon GPCR activation may not be sufficient to result in complete activation of the EGFR.

The identity of the metalloproteinase mediating Met cleavage remains to be determined. Our studies show that shedding induced by EGF, LPA and PMA can be efficiently inhibited by TIMP-3, but not by TIMP-1 and -2 (Fig. 3A). Given that between TIMP-1 and -2 the catalytic activities of all known MMPs are inhibited, our results indicate that either an unknown MMP or an ADAM might be mediating Met shedding. To date, TACE is the only adamalysin that is known to be inhibited by physiological levels of TIMP-3 (Amour et al., 1998), resulting in inhibition of PMA induced L-selectin and TNF-α shedding (Borland et al., 1999). We have shown recently that ADAM-10 can be strongly inhibited by TIMP-1, but only weakly by TIMP-3 (Amour et al., 2000). This suggests that the ADAM cleaving Met, is similar to TACE but not ADAM-10. However, in murine $TACE^{-/-}$ fibroblasts cells transfected with Met cDNA, shedding of Met occurred on PMA stimulation (Fig. 3B), suggesting that the adamalysin mediating Met cleavage at least in these cells is distinct from TACE. These results, however, do not rule out the possibility that TACE can cleave Met in the parental A549 cells, and further experiments using dominant-negative constructs will allow us to determine the identity of the ADAM(s) mediating Met cleavage initiated by different stimuli. TIMP-3 is unique among the TIMPs in that it contains a heparin-binding motif by which it is largely bound to the extracellular matrix, thereby potentiating its inhibitory activity. It is expressed by a large number of tissues and cells and is induced by treatment of cells with cytokines, growth factors and anti-inflammatory agents, suggesting that it may regulate shedding under a variety of different conditions. Recently we have shown that TIMP-3 has an apoptotic effect on a number of cell types but the precise mechanism for this is not known (Bond et al., 2000). Since its

metalloproteinase inhibitory functions are required, it is interesting to speculate that the inhibition of specific membrane shedding events may be involved in regulating cellular fate.

Our results show that activation of Erk MAP kinase is required for metalloproteinase-mediated activation of Met shedding. A549 cells, pre-incubated with the MEK inhibitor, PD98059, blocked both EGF- and LPA-stimulated shedding (Fig. 6). Upon EGF stimulation, Erk2 activity reached a peak at 15 minutes and sustained activity until 60 minutes when soluble Met release was detectable (data not shown). Thus Erk activation precedes Met shedding. Our results are consistent with recent reports showing that PMA-induced shedding of HB-EGF and growth factor-stimulated shedding of TGF-α require MAP kinase activation (Gechtman et al., 1999; Fan and Derynck, 1999).

Our work has also identified integrin interactions as a novel regulator of Met shedding. Recent evidence demonstrates that HB-EGF shedding occurs only in adherent cells and not in cells in suspension (Gechtman et al., 1999). The process is reversible in that, if adherent cells are placed in suspension and then allowed to adhere to ECM, their ability to shed HB-EGF is restored. Furthermore, fibroblasts and endothelial cells plated on fibronectin matrix as well as anti-integrin antibodies were shown to induce rapid and transient phosphorylation of the EGFR and Erk MAP kinase activation (Moro et al., 1998). However, integrins induce only a partial activation of the EGFR, which can be increased strongly by the addition of EGF. Given that integrins can associate with EGFR as well as bind to ADAMs, we hypothesized that integrin interactions may be able to influence ectodomain shedding. Our results show that basal shedding of Met is considerably increased in cells adhering to collagen due to increased Erk activation (Fig. 7).

The ability of EGFRs to activate a metalloproteinase via the Erk MAP kinase signalling pathway suggests that a large number of stimuli and changes in cellular environment can lead to ectodomain shedding. Given that the EGFR can be activated by many different protein receptors as well as by stress stimuli, and that ERK MAP kinases can be induced by growth factors and oncogenes, this further broadens the context in which ectodomain shedding can be induced. These observations support the idea that a small number of metalloproteinases can mediate ectodomain shedding of a large number of different proteins, functioning upstream and downstream of the EGFR.

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Note added in proof

It has recently been shown that the catalytic activity of ADAM12-s is inhibited by TIMP-3 (Loechel et al., 2000). ADAM12-s cleaves IGFBP-3 and IGFBP-5 and is inhibited by TIMP-3. *Biochem. Biophys. Res. Commun.* (2000). **278**, 511-515.