Meprinα transactivates the epidermal growth factor receptor (EGFR) via ligand shedding, thereby enhancing colorectal cancer cell proliferation and migration.

Petra Minder¹, Elke Bayha¹, Christoph Becker-Pauly², Erwin E. Sterchi¹

¹University of Bern, Institute of Biochemistry and Molecular Medicine, Buehlstrasse 28, CH-3012 Bern, Switzerland,
²Christian-Albrechts-University, Institute of Biochemistry, Rudolf-Hoeber-Strasse 1, 24118 Kiel, Germany

*Running title: Meprinα triggers EGFR activation through ligand shedding

To whom correspondence should be addressed: Erwin E. Sterchi, Institute of Biochemistry and Molecular Medicine, University of Bern, Buehlstrasse 28, CH-3012 Bern, Switzerland, Tel.: (41) 31 631 41 99; E-mail: erwin.sterchi@ibmm.unibe.ch

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Background: EGFR signaling pathway activation is a crucial step in colorectal cancer tumor progression.

Results: Meprinα sheds the epidermal growth factor ligands EGF and TGFα. Phosphorylation of EGFR and ERK1/2 is increased and cell proliferation and migration is enhanced after stimulation with meprinα.

Conclusion: Meprinα transactivates the EGFR by proteolytic processing of TGFα and EGF.

Significance: Meprinα may be a therapeutic target in colorectal cancer treatment.

SUMMARY

Meprinα, an astacin-type metalloprotease is overexpressed in colorectal cancer cells and is secreted in a non-polarized fashion, leading to the accumulation of meprinα in the tumor stroma. The transition from normal colonocytes to colorectal cancer correlates with increased meprinα activity at primary tumor sites. A role for meprinα in invasion and metastatic dissemination is supported by its pro-angiogenic and pro-migratory activity.

In the present study, we provide evidence for a meprinα-mediated transactivation of the EGFR signaling pathway and suggest that this mechanism is involved in colorectal cancer progression. Using alkaline phosphatase-tagged EGFR ligands and an ELISA assay, we demonstrate that meprinα is capable of shedding epidermal growth factor (EGF) and transforming growth factor-alpha (TGFα) from the plasma membrane. Shedding was abrogated using actinonin, an inhibitor for meprinα. The physiological effects of meprinα-mediated shedding of EGF and TGFα were investigated with human colorectal adenocarcinoma cells (Caco-2). Proteolytically active meprinα leads to an increase in EGFR and ERK1/2 phosphorylation and subsequently enhances cell proliferation and migration. In conclusion, the implication of meprinα in the EGFR/MAPK signaling pathway indicates a role of meprinα in colorectal cancer progression.

The epidermal growth factor receptor (EGFR) signaling pathway has critical functions in normal cellular processes such as differentiation, proliferation, migration, and the modulation of apoptosis, but it is also crucial in the pathophysiology of hyperproliferative diseases such as cancer (1). Colorectal cancer is the third most prevalent cancer and the second leading cause of cancer related deaths, worldwide (2). The analysis of tumor samples by immunohistochemistry has shown that the EGFR protein is overexpressed in 65-75% of colorectal tumors (3). The EGFR is a transmembrane receptor that is activated after binding of specific extracellular protein ligands, including epidermal growth factor (EGF) (4), heparin-binding EGF-like growth factor (HB-EGF) (5), transforming growth factor-alpha (TGFα) (6), betacellulin (7), amphiregulin (8), epieregulin (9), and epigen (10). The ligands are structurally and functionally related type I trans-membrane proteins that are shed after their presentation on the cell surface by an extracellular metalloprotease (11,12). TACE/ADAM17 (a disintegrin and metalloprotease) has been identified as the main.
meprinα is activated by plasmin, which in turn, is activated by the fibroblast-derived urokinase-type plasminogen activator (36). Meprinα has been demonstrated to have pro-migratory and pro-angiogenic effects in colorectal cancer, and thus may be involved in the transition from benign growth (adenomas) to malignant primary tumors (37,38).

We investigated the molecular mechanisms by which meprinα may influence tumor progression. For the first time we demonstrate that meprinα is able to shed EGF from the plasma membrane, resulting in the transactivation of EGFR signaling pathway and enhancement of Caco-2 cell proliferation and migration. We also confirm the shedding of TGFα by meprinα.

**EXPERIMENTAL PROCEDURES**

**Antibodies and recombinant protein** – Antibodies specific for total EGFR (monoclonal rabbit antibody) and phospho-EGFR Y1068 (monoclonal rabbit antibody) were purchased from Epitomics (Burlingame, CA); antibodies specific for total ERK1/2 (monoclonal mouse antibody) and phospho-ERK1/2 (polyclonal rabbit antibody) were from Santa Cruz Biotechnology (Heidelberg, Germany). Horseradish peroxidase-linked anti-rabbit and anti-mouse secondary antibodies were obtained from Dako Cytomation (Denmark). Recombinant active human meprinα and recombinant human pro-meprinα were generated using a baculovirus expression system in insect cells as previously described (39,40).

**Reagents** - Cell culture media and all supplements were purchased from Invitrogen (Basel, Switzerland). All reagents for gel electrophoresis were obtained from Bio-Rad Laboratories (Reinach, Switzerland). Complete EDTA-free protease inhibitor cocktail tablets, PhosStop phosphatase inhibitor cocktail tablets, and NBT/BCIP ready-to-use tablets were purchased from Roche Applied Sciences (Rotkreuz, Switzerland). MEK inhibitor U0126 was obtained from Promega (Dübendorf, Switzerland). EGF and TGFα neutralizing antibodies were purchased from R&D (Abingdon, United Kingdom). All other reagents were purchased from Sigma.

**Expression vectors for AP-tagged EGFR ligands** - Constructs of alkaline phosphatase (AP)-tagged EGFR ligands were kindly provided by Shigeki Higashiyama (EGF, TGFα, HB-EGF, amphiregulin, epiregulin, betacellulin) (16,41) and Carl P. Blobel (epigen) (17). These vectors...
were constructed by inserting partial cDNAs for human TGFα, EGF, amphiregulin, epiregulin, betacellulin, and HB-EGF into the 3’ end of human placental AP cDNA in a pRe/CMV-based expression vector pAIPh (16, 41). Mouse epigen was constructed by inserting a partial cDNA for mouse epigen into the 3’ end of human placental AP in the CMV-based vector APTag-5 (17).

**Cell culture and transfection of AP-tagged EGFR ligands** – Cells were maintained at 37°C in a humified air/CO₂ (19:1) environment. Human colorectal adenocarcinoma cells (Caco-2) were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 20% (v/v) fetal bovine serum (FBS), 2 mM glutamine, 4.5 g/l D-glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, and non-essentials amino acids (100 µM each). Madin-Darby canine kidney cells (MDCK) were grown in minimal essential medium (MEM) supplemented with 5% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine.

Transfection was performed using PEI (Chemie Brunschwig, Basel, Switzerland). 1.5 x 10⁵ cells per well were seeded in a 12-well plate, 24 hours before transfection. Transfection mixture (100 µl of 150 mM NaCl containing 4 µl PEI plus 100 µl of 150 mM NaCl containing 1.5 µg DNA) was incubated for 30 minutes at room temperature, and then added to the cells.

**Conditioned medium and meprina activity assay** – Caco-2 cells, grown 7 days over confluency, were cultured in serum-free medium for 16 hours. The medium was collected (referred as conditioned medium) and accumulated meprina was activated using trypsin (20 µg/ml) for 2 hours at 37°C. Trypsin was inhibited using soybean trypsin inhibitor (50 µg/ml). Active meprina was inhibited using actinonin (100 nM, in excess), a meprin inhibitor.

**Ectodomain shedding assay** – 24 hours after transfection, MDCK/Caco-2 cells were stimulated with serum-free medium containing either 1 µg/ml recombinant active meprina or 1 µg/ml recombinant meprina inhibited with 100 nM actinonin. After stimulation, medium was collected and centrifuged for 30 minutes at 4°C at maximum speed. Cells were washed twice with phosphate-buffered saline (PBS) followed by lysis on ice in 0.5 ml lysis buffer (25 mM Tris-HCl pH 8, 50 mM sodium chloride, 1% IGEPAL, 1% sodium deoxycholate, with complete EDTA-free protease inhibitor cocktail tablets) for 30 minutes. Cells were scraped off and cell debris was removed by centrifugation. Supernatants and lysates were kept on ice at all the times. Each data point was generated from two consecutive AP-activity measurements shed from a single transfected well. (n=3 experiments).

**Detection of alkaline phosphatase** – For the spectrophotometric detection of alkaline phosphatase (AP), 100 µl of collected medium or lysate were mixed with 100 µl 4-nitro-phenyl phosphate (2 mg/ml) in AP buffer (100 mM Tris, 100 mM NaCl, 20 mM MgCl₂, pH 9.5) in a 96 well plate. After incubation at 37°C absorbance was measured at 405 nm in an ELISA reader. Absorbance was measured at different time points within a linear range (OD < 0.8) up to a maximum incubation time of 5 hours. The total amount of AP measured from a single well, was used to normalize the absorbance value obtained for the supernatant of a certain condition.

For in-gel detection, AP in cell culture supernatants was concentrated using ConA beads. After elution with 50 mM Tris, pH 8.0, 0.5 M α-D-methyl-mannopyranoside, the AP-tagged EGFR ligands were loaded on a SDS-polyacrylamide gel. The SDS-gel was incubated in 2.5% Triton-X-100 followed by incubation in AP buffer. AP was visualized using NBT/BCIP as substrate.

**EGF-ELISA** – Caco-2 cells were stimulated with medium, 1 µg/ml recombinant active meprina, or 1 µg/ml recombinant pro-meprina for 4 hours. Supernatants were collected and released EGF was measured via the human EGF quantikine ELISA Kit (R&D, Abingdon, United Kingdom).

**Phosphorylation of EGFR and ERK1/2** – Caco-2 cells, seeded at a density of 5 x 10⁵ cells per 6 cm dish, were stimulated for 0, 5, 15, 30, and 60 minutes with either control medium, 1 µg/ml recombinant active meprina, 1 µg/ml recombinant pro-meprina, or 100 ng/ml EGF (positive control). Phosphorylation induced by recombinant active meprina, recombinant pro-meprina, or EGF was inhibited with 2 µg/ml neutralizing EGF and TGFα antibodies, 10 µM EGFR inhibitor AG1478, or 10 µM MEK inhibitor U0126. Cells were pretreated with the inhibitors 30 minutes before stimulation. After stimulation, cells were washed once with PBS followed by lysis on ice for 30 minutes in 1 ml cell lysis buffer (Epitomics, Burlingame, CA) supplemented with protease and phosphatase...
inhibitors. Cell debris was removed by centrifugation and the protein content in the lysates was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, USA).

**Western blot analysis** - 10 µg protein was solubilized by boiling for 5 minutes in 2x Laemmli buffer. Samples were loaded on 7.5% (EGFR) or 10% (ERK1/2) SDS-polyacrylamide gels and subsequently electro-blotted onto a polyvinylidene difluoride (PVDF) membrane (Hybond P, Amersham Biosciences). Blocking was done overnight at 4°C using T-TBS (25 mM Tris-HCl pH 7, 150 mM sodium chloride, 2.5 mM potassium chloride, 0.1% Tween-20) containing 5% dry milk. The membrane was then incubated with the first antibody (pEGFR 1:5000, pERK1/2 1:200, EGFR 1:10000, ERK1/2 1:200) in T-TBS containing 2% dry milk for 1 hour at room temperature and with the appropriate horseradish peroxidase-conjugated secondary antibody (1:25000) for 1 hour at room temperature. Immune complexes were visualized using enhanced chemiluminescence (ECL Plus, Amersham Biosciences) on X-ray films. The blots were performed using Image J software (Wayne Rasband, National Institutes of Health, USA).

**Cell treatment for proliferation and migration experiments** – For proliferation and migration experiments Caco-2 cells were stimulated with conditioned medium containing activated meprinα, inhibited meprinα, or 100 ng/ml EGF (positive control). Inhibitors were added to media containing active meprinα at the beginning of the treatment (2 µg/ml neutralizing EGF and TGFα antibodies, 10 µM EGFR inhibitor AG1478, or 10 µM MEK inhibitor U0126). Cells were pretreated with neutralizing antibodies and EGFR inhibitor for 2 hours.

**Alamar Blue cell proliferation assay** - Alamar Blue uses the natural reducing power of living cells to convert resazurin to the fluorescent molecule, resorufin (42). Caco-2 cells were seeded at a density of 1000 cells per well in a 96 well plate. After 48 hours of incubation, cells were washed twice using phenolred-free medium, followed by stimulation for 24 hours as described above. For the last 3 hours, Alamar Blue (43) solution was added to a final concentration of 10 µg/ml. Fluorescence at 595 nm was measured directly (0 hours) and 3 hours after addition in a multilabel plate reader (2300 EnSpire multilabel reader; Perkin-Elmer, Turku, Finland). Values obtained at time point 0 hours were subtracted from those obtained at time point 3 hours. (20 replicates/ condition, n=3 experiments).

**Cell Titer Glo cell viability assay** – This assay is a method to determine the number of viable cells in culture based on quantification of the ATP present. Consequently, ATP levels represent the number of metabolically active cells (44). 1000 Caco-2 cells/well were seeded in a 96 well plate. After 48 hours, cells were serum-starved overnight followed by 24 hours treatment with the different stimuli. Subsequently, 100 µl of Cell Titer Glo reagent were added to each well, cells were incubated for 10 minutes in the dark, and luminescence was measured in a 2300 EnSpire multilabel plate reader. (3 replicates/ condition, n=3 experiments).

**BrdU incorporation** - Cell proliferation was also determined by bromodeoxyuridine (BrdU) incorporation analysis. We used the In situ cell proliferation kit (Roche Applied Sciences; Rotkreuz, Switzerland) according to the manufacturer’s instructions. Briefly, Caco-2 cells were plated at a density of 2 x 10^5 cells per well of an 8 chambers culture slide (Lab-Tek). After 48 hours, cells were serum-starved overnight, followed by treatment for 24 hours with the different stimuli. During the last 90 minutes of the treatment, BrdU at a final concentration of 10 µM was added to the medium to allow BrdU incorporation. Cells were fixed in 70% ethanol for 45 minutes at room temperature, and incubated with anti-BrdU antibody in the presence of nuclease for DNA denaturation. Cells were counterstained with 5 µg/ml DAPI for 5 minutes. BrdU incorporation into cellular DNA was visualized by fluorescence microscopy. In three independent experiments a total number of 12 high-power fields (40x) and at least 800 cells per condition were analyzed. The proliferation rate was determined as a proportion of the total DAPI positive nuclei. The value for untreated control cells was arbitrarily set to 0.

**In vitro wound-healing assay** - 2 x 10^5 Caco-2 cells per well were seeded in a 12-well plate and grown to confluency. The cell monolayer was wounded by scratching, using a 200 µl pipette tip. After washing with PBS the cells were incubated with the corresponding stimuli. At time points 0 hours and 16 hours the same positions along the scratch wound were photographed using an inverted-phase-contrast microscope (Nikon
microscope TS100 fluorescence and video camera) and Adobe Photoshop was used for quantification of the scratch wound. Three measurements per scratch were performed. (2 replicates/ condition, n=3 experiments).

Transwell Migration Assay – $5 \times 10^4$ Caco-2 cells were seeded on top of transwell filters (polyethylene terephthalate (PET), 8 µM pores, 24-well format) from BD Biosciences. Cells were allowed to grow for 48 hours followed by serum starvation for 24 hours in medium containing 1% FBS. Then, medium in the lower chamber was replaced by conditioned medium containing 20% FBS and the stimuli. Medium in filter inserts was replaced by serum-free conditioned medium containing the corresponding stimuli. Cells were treated for 36 hours and at the end of the treatment cells were washed twice with PBS followed by fixation for 15 minutes using 4% paraformaldehyde. Cells on the upper side of the transwell filters were removed with a cotton swab and cells on the lower side were stained for 5 minutes with 5 µg/ml DAPI. Pictures were taken (Nikon microscope TS100) and migrated cells were counted using Image J software (Wayne Rasband, National Institutes of Health, USA). (3 replicates/ condition, n=3 experiments).

Statistical analysis - Data were analyzed using PRISM 5.0 software package (GraphPad, San Diego, CA). Results are shown as the mean ± SEM. Statistical differences between two groups were determined by unpaired Student's t-test. As significant differences considered were $P<0.05$.

RESULTS

EGF and TGFα are shed by meprina - To investigate the role of meprina in ectodomain shedding of EGF and TGFα, a cell-based assay using AP-tagged EGFR ligands was used. Shedding of EGF and to a lesser extent, TGFα was significantly stimulated in MDCK cells (p<0.001; p<0.05; Fig. 1A/B) as well as in Caco-2 cells (p<0.001; p<0.01; Fig. 1D/E) by meprina. Addition of the meprina inhibitor actinonin showed a significant decrease in ligand shedding in MDCK cells (p<0.01; p<0.05; Fig. 1A/B) and in Caco-2 cells (p<0.01; p<0.01; Fig. 1D/E). Actinonin did not influence constitutive shedding, suggesting that this is catalyzed by a different metalloproteinase (data not shown). Results obtained with the spectrophotometric assay were confirmed by in-gel detection of AP-tagged ligands, using NBT/BCIP as a substrate for the alkaline phosphatase (Fig. 1C/F).

Endogenous EGF released from Caco-2 cells into the medium was quantified by EGF ELISA (Fig. 1G). Stimulation of Caco-2 cells with recombinant active meprina resulted in a significant increase of soluble EGF compared to control values (p<0.001) and recombinant pro-meprina, the inactive form of meprina (p<0.001). Quantification of soluble TGFα upon meprina activation by ELISA has been shown before (22). Together these data suggest that meprina acts as a sheddase for EGF and TGFα.

Meprina induces EGFR and ERK1/2 phosphorylation - EGFR ligands, once released from the plasma membrane bind to the EGFR, which in turn is phosphorylated at several amino acid positions (Y992, Y1068, Y1086, Y1148, and Y1173) (45). To investigate the effect of meprina on the phosphorylation of EGFR via EGF or TGFα shedding, Caco-2 cells were stimulated for various periods of time with either medium alone, recombinant active meprina, recombinant pro-meprina, or with EGF (positive control). Figure 2A shows representative western blots of the phosphorylation experiment, and figure 2B shows the densitometric analysis of four individual experiments. In the latter, control values were subtracted and the values were normalized against total EGFR. After 5 minutes of treatment with meprina (Fig. 2A, lane 2), an increase in phosphorylation was observed. A maximum activation of EGFR was achieved between 15 minutes and 30 minutes of treatment (Fig. 2A, lane 3 and 4), followed by a decrease (Fig. 2A, lane 5). Non-active pro-meprina showed only a very slight increase in phosphorylation of the EGFR. Minor amounts of pro-meprina might be activated over time, for instance by plasmin or kallikreins (KLKs) (40,46). Treatment with recombinant EGF resulted in a similar EGFR activation pattern to meprina albeit with a stronger signal, which may have been due to the relatively high concentration of EGF used. These data indicate that the proteolytic activity of meprina is required for EGFR phosphorylation.

EGFR phosphorylation leads to the activation of intracellular pathways, such as the mitogen-activated protein kinase (MAPK) pathway. The classical MAP kinases, extracellular-signal-regulated kinases 1 and 2 (ERK1/2), are intracellular signaling molecules that are preferentially activated in response to growth factors and phorbol esters (47). To determine whether ERK1/2 are transactivated upon treatment of Caco-2 cells with meprina, the lysates obtained from the EGFR phosphorylation
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Phosphorylation was calculated by densitometric measurements (Fig. 2D). Control values were subtracted and data were normalized against total ERK1/2. We assume that this ERK1/2 phosphorylation after 5 minutes is a transient process that might be caused by the change of culture medium. Taken together, we conclude that active meprinα leads to the activation and phosphorylation of EGFR and consequently, transactivates the MAPK pathway, which culminates in ERK1/2 phosphorylation.

EGFR and ERK1/2 phosphorylation are meprinα dependent – To analyze whether the EGFR/MAPK signaling pathway is activated by meprinα via EGF and TGFα shedding, phosphorylation experiments using neutralizing EGF and TGFα antibodies were performed (Fig. 3A). Caco-2 cells were stimulated for 5 or 15 minutes with meprinα, pro-meprinα or EGF in the absence or presence of the neutralizing antibodies. In the absence of EGF and TGFα neutralizing antibodies, EGFR and ERK1/2 were phosphorylated when stimulated with meprinα or EGF but not with pro-meprinα. Cells treated with neutralizing EGF and TGFα antibodies showed EGFR and ERK1/2 phosphorylation reduced to control levels, after stimulation with meprinα. After stimulation with EGF, EGFR and ERK1/2 remained phosphorylated to a certain extent in the presence of neutralizing antibodies. This may be the result of ligand excess compared to the amount of antibodies used. EGFR and ERK1/2 phosphorylation remained the same after stimulation with pro-meprinα. Total EGFR and ERK1/2 were not affected by the neutralizing antibodies. We conclude, that EGFR transactivation by meprinα occurs via shedding of EGF and TGFα from the plasma membrane by meprinα.

We wondered if meprinα-induced ERK1/2 phosphorylation was entirely mediated by transactivation of EGFR. For this reason, we tested the effect of the EGFR inhibitor AG1478, (Fig. 3B). Phosphorylation of EGFR and ERK1/2 was detected after stimulation of Caco-2 cells with meprinα and EGF but not after stimulation with pro-meprinα. Inhibition of EGFR led to less EGFR phosphorylation while ERK1/2 phosphorylation was completely abrogated. Hence, meprinα activates ERK1/2 mostly via EGFR.

We further confirmed that the MEK inhibitor U0126, which was later used in our functional assays, is a potent agent that completely abrogates meprinα-induced ERK1/2 phosphorylation (Fig. 3C).

Meprina enhances Caco-2 cell proliferation - Activation of the EGFR pathway plays an important role in the regulation of cellular processes such as cell proliferation and migration. The functional relevance of meprinα-dependent EGFR/ERK1/2 signaling on cell proliferation was studied in three independent assays: Alamar Blue, Cell Titer Glo cell viability assay, and BrdU incorporation. Cells were stimulated with conditioned medium containing activated meprinα, inhibited meprinα, or EGF. In addition, cell proliferation, induced by meprinα, was analyzed in the presence of neutralizing antibodies for EGF and TGFα, or inhibitors against EGFR and MEK. The cell proliferation rate for Alamar Blue and Cell Titer Glo experiments is shown in figure 4A/B. A significant increase in proliferation was detected when cells were treated with active meprinα (p<0.001; p<0.001). Compared to that, inhibited meprinα reduced cell-proliferation to a level slightly above that of controls (p<0.001; p<0.01). This may be due to incomplete inhibition of meprinα by actinonin (unpublished data). EGF stimulation that was used as a positive control exhibited a comparable cell proliferation rate to meprinα. In both assays, the increase in cell proliferation monitored after stimulation with meprinα was significantly reduced in the presence of neutralizing EGF and TGFα antibodies (p<0.05; p<0.01), EGFR inhibitor (p<0.01; p<0.01) or MEK inhibitor (p<0.01; p<0.01). DNA synthesis was quantified by BrdU uptake in Caco-2 cells after treatment with the different stimuli. Representative photographs are shown in figure 4D and quantification of the results of 3 independent experiments are shown in figure 4C. Nuclear BrdU labeling was significantly increased after stimulation with active meprinα (p<0.001) and significantly decreased when treated with inhibited meprinα (p<0.001). BrdU uptake was significantly reduced in the presence of neutralizing EGF and TGFα antibodies (p<0.01), EGFR inhibitor (p<0.01), or MEK inhibitor (p<0.01). Altogether, these data demonstrate that the proliferative effect of meprinα in Caco-2 cells is regulated by...
through the EGFR/MAPK pathway.

Migration of Caco-2 cells is enhanced by meprina activity - The effect of meprina on the migration behavior of Caco-2 cells was assessed using an in vitro wound-healing assay. A scratch was induced to confluent Caco-2 cells using a 200 µl pipette tip. We compared migration of Caco-2 cells treated with conditioned medium containing activated meprina, inhibited meprina, or EGF (positive control). Furthermore, the effect of inhibitors (neutralizing EGF and TGFα antibodies, EGFR inhibitor, or MEK inhibitor) on meprina-induced migration was analyzed. Representative photographs, taken at time point 0 hours and 16 hours of the identical location, are shown in figure 5A. Quantification of the results of six separate experiments is shown in figure 5B. Under all conditions a closing of the wound was observed. A significant enhancement in wound closure was detected in cells exposed to active meprina compared to inhibited meprina and control values (p<0.001, p<0.01). EGF and TGFα inhibition through neutralizing antibodies as well as EGFR inhibition revealed a significant reduction in meprina-induced wound closure (p<0.05, p<0.05), as did ERK1/2 inhibition (p<0.001).

The in vitro wound-healing assay represents a combination of cell migration and cell proliferation. To avoid the proliferative effect, we also performed a transwell migration assay. Caco-2 cells were cultivated on 8 µM pore size filters in a 24- well culture plate with the same conditions as used for the in vitro wound-healing assay. Migrated cells were found under all conditions, but a significant increase in migration was monitored after stimulation with meprina compared to control values (p<0.001). Inhibited meprina showed a significant decrease (p<0.01; Fig. 5C) compared to active meprina, and stimulation with EGF led to a slightly higher increase in migration compared to meprina. The increase in migrated cells monitored after stimulation with meprina was significantly reduced in the presence of neutralizing EGF and TGFα antibodies (p<0.01), and EGFR inhibitor (p<0.01). Inhibition of ERK1/2 led to less migration than the controls resulting in a negative value in the diagram (Fig. 5C). ERK1/2 is a key enzyme of many signaling cascades. Therefore, inhibition of ERK1/2 interferes with meprina-induced migration and most likely with supplemental pathways triggering cell migration.

In conclusion, we show that meprina activity enhances migration of Caco-2 cells and this effect is dependent on the transactivation of EGFR/MAPK signaling.

DISCUSSION

In this study, we set out to identify EGFR ligands as substrates for meprina. Our data demonstrate that human meprina is effectively capable of shedding EGF. Additionally, we could confirm the shedding of TGFα by meprina, which was shown previously in lung epithelial cells (22). We also demonstrate that active meprina transactivates the EGFR and ERK1/2 and subsequently increases Caco-2 cell proliferation and migration.

Shedding of EGF and TGFα was enhanced in cells treated with active recombinant meprina, and inversely, was reduced after inhibition of meprina by actinonin, indicating that the proteolytic activity is required for shedding of EGF and TGFα. Two other groups have demonstrated TGFα shedding by meprin in two individual assays (22,48). Back in 1991 Choudry et al., have identified the growth factor TGFα as an in vitro substrate for endopeptidase-2 (now known as meprin) (48). Using recombinant human TGFα and purified endopeptidase-2 from rat kidney, they showed that TGFα was processed in a time-dependent manner. In the presence of actinonin no hydrolysis was observed. Recently, Bergin et al. have analyzed shedding of TGFα by meprin in human bronchial epithelial cells (16HBE14o-cells) (22). Using an ELISA assay, they found elevated levels of soluble EGF in the medium of cells after treatment with recombinant meprina. This effect was also inhibited by the addition of actinonin. The authors suggested that meprina is activated by neutrophil elastase and, via TGFα precursor processing, induces IL-8 expression (22).

With our experimental setup using AP-tagged constructs of EGFR ligands we confirm TGFα shedding by meprina in MDCK and Caco-2 cells. Furthermore, we show that EGF, another EGFR ligand, is also shed by meprina. Accordingly, we found increased levels of soluble EGF in the media (ELISA). Compared to TGFα, EGF was shed by meprina to a higher extent, and cleavage of both ligands was abrogated when meprina was inhibited by actinonin. Other EGFR ligands were also analyzed as potential substrates for meprina. Epigen and betacellulin were not shed by meprina, HB-EGF, amphiregulin and epiregulin were shed but shedding was not inhibited by actinonin, indicating that another protease was involved (data not shown).

Differentiated Caco-2 cells express meprina endogenously, which makes them a preferred
cell culture system for the analysis of meprinα function (35). Therefore, we used Caco-2 cells in our studies to investigate the consequences of meprinα expression on cell behavior in the context of colorectal cancer. We carried out shedding experiments using MDCK cells to confirm our results acquired with recombinant meprinα in a second cell line. MDCK cells do not express endogenous meprinα. Nevertheless, the WT form in combination with recombinant meprin and stably transfected cell lines are widely used and established in meprin research (29,33).

Meprin consists of two homologous isoforms, meprinα and meprinβ. We have previously shown that meprinβ cleaves and releases E-cadherin, which is considered to act as a tumor suppressor (49). Thus, we also considered meprinβ as a potential sheddase for EGFR ligands. In contrast to meprinα, meprinβ is not implicated in the shedding of EGFR ligands (data not shown). Although, both isoforms have related cleavage sites, different substrate specificities have been described (50,51) (Jefferson et al., Cellular and Molecular Life Sciences, in press), which most likely is the reason for their different behavior towards EGFR ligands. Additionally, meprinα and β exhibit remarkable differences in their activation (46) and regulation by inhibitors (52) (Jefferson et al., Cellular and Molecular Life Sciences, in press). This certainly contributes to the different functions in cell proliferation and migration as observed previously (40). In the large intestine, only minor amounts of meprinβ are expressed. Hence, most meprinα is released into the gut lumen in vivo and may be rapidly diluted. However in colorectal cancer, meprinα accumulates in the tumor stroma and persists close to the cell plasma membrane (34,35).

Two membrane-anchored metalloproteases, ADAM10 and ADAM17, were found to have critical roles in the release of EGFR ligands (14-17,53). Most likely the basal amounts of shed EGF and TGFα that we found in the ectodomain shedding assay are due to these enzymes. We inhibited ADAMs to exclude that these ADAMs interfere with the shedding of TGFα and EGF (data not shown). The inhibition of ADAMs led to a minimization of the constitutive shedding without affecting the shedding by meprinα. In addition, meprinα generates a slightly smaller TGFα fragment (Fig. 1C/F) to that obtained with ADAM17, indicating that the cleavage site of meprinα differs from that of ADAM17.

Mice lacking ADAM17 expression die perinatally and have a similar phenotype to TGFα -/- mice (54). This points to ADAM17 as the main sheddase for TGFα. In mouse cells ADAM10 is responsible for EGF shedding. Mice lacking ADAM10 die very early during embryogenesis and hence, determination of the physiological contribution to EGF signaling in animals remains to be determined (55). In cell-based assays, ADAM10 has been identified as a sheddase that can release TGFα almost as efficiently as its primary sheddase, in ADAM17 -/- cells (16,18). This implies that the function of ADAMs may be replaced by other metalloproteases in tissues where ADAMs are not expressed or are not stimulated. We therefore propose a physiological role for meprinα in the local transactivation of the EGFR pathway.

Aberrant expression and/or activities of EGF family members and their receptors have been reported in solid tumors including colorectal cancer (56-59). We have previously analyzed meprinα mRNA levels, protein expression, and proteolytic activity in colonic adenomas, primary tumors and liver metastases from colorectal cancer patients. Varied levels of meprinα mRNA were detected in all specimens. While expression of meprinα protein was very weak in adenomas, it was detected in primary tumor tissue as well as in liver metastases. In advanced primary tumors (UICC stages III and IV), subpopulations of cells were detected with a strong expression of meprinα protein. The activity of meprinα correlated mostly with protein expression except for liver metastases where activity was as low as in adenomas. This implies that the spreading of cancer cells correlates with increased meprinα protein as well as meprinα activity (37).

To further investigate the mechanism that leads to the spreading of colorectal cancer in response to meprinα, we analyzed the ability of meprinα to transactivate the EGFR. Stimulation of Caco-2 cells with active meprinα showed a significant increase in EGFR phosphorylation compared to pro-meprinα (Fig. 2A/B). Ligand binding to the EGFR activates two main intracellular pathways known to play a role in colorectal cancer: the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol-3-kinase- (PI3K-) protein kinase (AKT) pathway (60). In mammals five distinct groups of MAPKs have been characterized, with the most prominent being ERK1/2. Our data using neutralizing EGF and TGFα antibodies, as well as EGFR and ERK1/2 inhibitors (Fig. 3), imply that EGFR phosphorylation occurs after shedding of EGF or
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TGFrα by meprinα and that this transactivation of EGFR leads to the activation of the MAPK signaling cascade and consequently to the phosphorylation of ERK1/2 (Fig. 2C/ Fig. 3). ERK1/2 are preferentially activated in response to growth factors and have been implicated in cell migration, invasion, proliferation, angiogenesis, cell differentiation, and cell survival (60).

Proliferation and migration experiments were performed using conditioned media from Caco-2 cells, which endogenously express meprinα. Meprinα is secreted as a zymogen into culture media due to constitutive proteolytic removal of the C-terminal transmembrane and cytosolic domain (34). Trypsin activation on cells is difficult to achieve as meprinα is removed with each washing step. Therefore culture medium was collected and accumulated meprinα was activated (see materials and methods).

Cell proliferation is a cellular response known to be enhanced in colorectal cancer upon EGFR activation (61). In three independent assays we demonstrate that Caco-2 cell proliferation is significantly increased in response to meprinα. Further we demonstrate that inhibition of EGF and TGFrα, EGFR or ERK1/2 leads to a significant reduction in meprinα-induced proliferation. Therefore, we conclude that the increase in cell proliferation occurs via transactivation of the EGFR/MAPK signaling pathway by a meprinα-dependent mechanism.

We have previously demonstrated a pro-migratory effect induced by meprinα in MDCK cells using videomicroscopy (37). In that study plasmin-activated meprinα was used and migration was induced by hepatocyte growth factor (HGF). In the present study, we demonstrate increased migration of colorectal cancer cells in response to meprinα in an in vitro scratch assay and in a transwell migration assay (Fig. 5). Caco-2 cells endogenously express meprinα, and represent a more natural environment for meprinα function. Inhibition of EGF and TGFrα, EGFR, or ERK1/2 showed a significant reduction in meprinα-induced migration in both assays. Altogether, both approaches highlight that meprinα enhances cell migration via EGFR/MAPK signaling pathway.

The identification of EGFR ligands as substrates for meprinα and the known pro-migratory, pro-proliferative, and pro-angiogenic effects of meprinα in vitro, lay the foundation for further analysis on the role of meprinα in colorectal cancer. Experiments, including the use of a transgenic meprinα KO mouse model, will be necessary to address the biological relevance of meprinα in shedding of endogenous forms of EGFR ligands in vivo. Further, in vitro experiments focusing on the role of meprinα in metastasis of colorectal cancer cells would be of great interest. The transactivation of the EGFR may be critical for the transition from benign growth to malignant primary tumors in colorectal cancer and thus meprinα may be an interesting target for the design of drugs that modulate its activity.
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FOOTNOTES

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1To whom correspondence may be addressed: Institute of Biochemistry and Molecular Medicine, University of Bern, Buehlstrasse 28, CH-3012 Bern, Switzerland, Tel.: (41) 31 631 41 99; E-mail: erwin.sterchi@ibmm.unibe.ch

2The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; TGFα, transforming growth factor-alpha; ERK, extracellular-signal-regulated; AP, alkaline phosphatase; MDCK, Madine-Darby canine kidney, PAPA peptide, N-benzoyl-L-tyrosyl-p-aminobenzoic acid

FIGURE LEGENDS

FIGURE 1. EGF and TGFα are shed by meprin. MDCK or Caco-2 cells, transiently transfected with AP-EGF or AP-TGFα, were stimulated for 1 hour with serum-free medium, recombinant active meprinα, or recombinant inhibited meprinα. Alkaline phosphatase (AP) in cell culture supernatant was detected using 4-nitro-phenyl phosphate as substrate. Absorbance value obtained for EGF and TGFα shedding are shown for MDCK cells (A/B) and for Caco-2 cells (D/E). C and F show in-gel detection of AP from shed EGFR ligands after renaturation in SDS gel in MDCK cells (C) and Caco-2 cells (F). Lanes 1: protein molecular weight marker. Lanes 2: AP-tagged forms of EGF and TGFα released in cell culture supernatant after 1 hour of stimulation with serum-free medium (control). Lanes 3: EGFR ligands released after 1 hour from the same well after stimulation with recombinant active meprinα. Lanes 4: EGFR ligands released from a separate well after stimulation for 1 hour with recombinant inhibited meprinα. Quantification of shed endogenous EGF is shown in (G). Caco-2 cells were stimulated with media, recombinant active meprinα, or recombinant inhibited meprinα and the media were analyzed using ELISA. n=3, ±SEM; Student’s t-test ***P≤0.001, **P≤0.01, *P≤0.05.

FIGURE 2. Meprinα induces EGFR and ERK1/2 phosphorylation. A time course (0, 5, 15, 30, and 60 minutes) of EGFR and ERK1/2 phosphorylation is shown for Caco-2 cells treated with either control media, 1 µg/ml recombinant active meprinα, 1 µg/ml recombinant pro-meprinα, or 100 ng/ml EGF (positive control). A and C show representative western blots for EGFR and ERK1/2 respectively. Immunoblots of total EGFR or ERK1/2 were used as loading control. Densitometric quantifications of phospho-EGFR relative to total EGFR are shown in (B) and densitometric quantifications of phospho-ERK1/2 relative to total ERK1/2 are shown in (D). Control values were subtracted from the other values at the corresponding time points. n=4 for all densitometric quantifications, ± SEM.

FIGURE 3. EGFR and ERK1/2 phosphorylation are meprinα-dependent. Caco-2 cells were stimulated for 5 or 15 minutes with 1 µg/ml recombinant active meprinα, 1 µg/ml recombinant pro-meprinα, or 100 ng/ml EGF in the presence or absence of neutralizing EGF and TGFα antibodies (nAB EGF/ nAB TGFα) (A), EGFR inhibitor AG1478 (B), or MEK inhibitor U0126 (C). Cells were pretreated with the inhibitors for 30 minutes. Phosphorylation of EGFR and ERK1/2 were determined using antibodies detecting the phosphorylated form of EGFR and ERK1/2. Total EGFR and ERK1/2 were used as loading control and were not affected by neutralizing EGF and TGFα antibodies, or EGFR and ERK1/2 inhibitor. n=3.

FIGURE 4. Meprinα enhances Caco-2 cell proliferation. Caco-2 cells were treated with conditioned medium containing activated meprinα, inhibited meprinα, or 100 ng/ml EGF (positive control). Further, the effect of inhibitors (neutralizing EGF and TGFα antibodies (nAB EGF/ nAB TGFα), EGFR inhibitor AG1478, or MEK inhibitor U0126) on meprinα-induced proliferation was analyzed. Proliferation was measured by three independent assays: Alamar Blue (A), Cell Titer Glo cell viability...
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(B), and BrdU incorporation (C/D). Figure D shows representative pictures for each condition and in figure C quantification of three separate experiments is shown. For all experiments, Caco-2 cell proliferation in response to the corresponding stimuli is shown as percentage increase. Control values were set as 0. n=3; ±SEM, Student’s t-test ***P≤0.001, **P≤0.01, *P≤0.05.

FIGURE 5. Migration of Caco-2 cells is increased through meprinα activity. Cells were treated with conditioned media containing activated meprinα (in the presence or absence of neutralizing EGF and TGFα antibodies (nAB EGF/ nAB TGFα), EGFR inhibitor AG1478, or MEK inhibitor U0126), inhibited meprinα, or EGF. (A/B) In vitro wound-healing assay. One representative of three independent experiments is shown in (A), and in (B) quantification of the results of three separate assays are shown. (C) Transwell migration assay of Caco-2 cells. Cells fixed and stained with DAPI were counted. In figure 5C the percentage increase compared to control values is shown and the control values were set as 0. n=3; ±SEM, Student’s t-test ***P≤0.001, **P≤0.01, *P≤0.05.
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Figure 1

A

B

C

D

E

F

G

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Figure 2

A

|       | 0   | 5   | 15  | 30  | 60  |
|-------|-----|-----|-----|-----|-----|
| pEGFR (170 kDa) |     |     |     |     |     |
| EGFR (170 kDa) |     |     |     |     |     |
| pEGFR (170 kDa) |     |     |     |     |     |
| EGFR (170 kDa) |     |     |     |     |     |
| pEGFR (170 kDa) |     |     |     |     |     |
| EGFR (170 kDa) |     |     |     |     |     |
| pEGFR (170 kDa) |     |     |     |     |     |
| EGFR (170 kDa) |     |     |     |     |     |
| pEGFR (170 kDa) |     |     |     |     |     |
| EGFR (170 kDa) |     |     |     |     |     |
| pro-Meprina |     |     |     |     |     |
| Meprina     |     |     |     |     |     |

B

C

|       | 0   | 5   | 15  | 30  | 60  |
|-------|-----|-----|-----|-----|-----|
| pERK1/2 (42/44 kDa) |     |     |     |     |     |
| ERK1/2 (42/44 kDa) |     |     |     |     |     |
| pERK1/2 (42/44 kDa) |     |     |     |     |     |
| ERK1/2 (42/44 kDa) |     |     |     |     |     |
| pERK1/2 (42/44 kDa) |     |     |     |     |     |
| ERK1/2 (42/44 kDa) |     |     |     |     |     |
| pERK1/2 (42/44 kDa) |     |     |     |     |     |
| ERK1/2 (42/44 kDa) |     |     |     |     |     |
| pERK1/2 (42/44 kDa) |     |     |     |     |     |
| ERK1/2 (42/44 kDa) |     |     |     |     |     |
| pro-Meprina |     |     |     |     |     |
| Meprina     |     |     |     |     |     |

D

|       | 0 min | 5 min | 15 min | 30 min | 60 min |
|-------|-------|-------|--------|--------|--------|
| pERK1/2/ERK1/2 |     |       |        |        |        |
| Meprina     |     |       |        |        |        |
| pro-Meprina |     |       |        |        |        |
| EGF         |     |       |        |        |        |

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Figure 3

A

Meprina
nAB EGF/ nAB TGFα

- + + + + + + + + +

- - - + + + + + + +

pEGFR (170 kDa)
EGFR (170 kDa)
pERK1/2 (42/44 kDa)
ERK1/2 (42/44 kDa)

- - + + + + + + +

- - + + + + + + +

EGFR (170 kDa)
EGFR (170 kDa)
pERK1/2 (42/44 kDa)
ERK1/2 (42/44 kDa)

EGF
nAB EGF/ nAB TGFα

- + + + + + + + + +

- - + + + + + + +

pEGFR (170 kDa)
EGFR (170 kDa)
pERK1/2 (42/44 kDa)
ERK1/2 (42/44 kDa)

- + + + + + + + + +

- + + + + + + + + +

EGFR (170 kDa)
EGFR (170 kDa)
pERK1/2 (42/44 kDa)
ERK1/2 (42/44 kDa)

B

Meprina
AG1478

- + + + + + + + + +

- - - + + + + + + +

pEGFR (170 kDa)
EGFR (170 kDa)
pERK1/2 (42/44 kDa)
ERK1/2 (42/44 kDa)

- + + + + + + + + +

- + + + + + + + + +

EGFR (170 kDa)
EGFR (170 kDa)
pERK1/2 (42/44 kDa)
ERK1/2 (42/44 kDa)

EGF
AG1478

- + + + + + + + + +

- - + + + + + + +

pEGFR (170 kDa)
EGFR (170 kDa)
pERK1/2 (42/44 kDa)
ERK1/2 (42/44 kDa)

- + + + + + + + + +

- - + + + + + + +

EGFR (170 kDa)
EGFR (170 kDa)
pERK1/2 (42/44 kDa)
ERK1/2 (42/44 kDa)

C

Meprina
U0126

- + + + + + + + + +

- - - + + + + + + +

pERK1/2 (42/44 kDa)
ERK1/2 (42/44 kDa)

- + + + + + + + + +

- - - + + + + + + +

pERK1/2 (42/44 kDa)
ERK1/2 (42/44 kDa)

EGF
U0126

- + + + + + + + + +

- - + + + + + + +

pERK1/2 (42/44 kDa)
ERK1/2 (42/44 kDa)
Meprin triggers EGFR activation through ligand shedding.

Figure 4

A

B

C

D

BrdU

DAPI

Media + + + + + + + +
Meprin - + - + + + + +
Actinomycin - - - - - - - -
EGF - - - - - - - -
αAB EGF/αAB TGFα - - - - - - - -
AG1478 - - - - - - - -
U0126 - - - - - - - -
Meprin triggers EGFR activation through ligand shedding

Figure 5

A

| Treatment                        | 0 h   | 16 h  |
|----------------------------------|-------|-------|
| Media                            | +     | +     |
| Meprin                           | +     | -     |
| Actinoin                         | -     | -     |
| EGF                              | -     | -     |
| nAB EGF/ nAB TGFα                | -     | -     |
| AG1478                           | -     | -     |
| U0126                            | -     | -     |

B

| Treatment                        | 0 h   | 16 h  |
|----------------------------------|-------|-------|
| Media                            | +     | +     |
| Meprin                           | +     | -     |
| Actinoin                         | -     | -     |
| EGF                              | -     | -     |
| nAB EGF/ nAB TGFα                | -     | -     |
| AG1478                           | -     | -     |
| U0126                            | -     | -     |

C

| Treatment                        | 0 h   | 16 h  |
|----------------------------------|-------|-------|
| Media                            | +     | +     |
| Meprin                           | +     | -     |
| Actinoin                         | -     | -     |
| EGF                              | -     | -     |
| nAB EGF/ nAB TGFα                | -     | -     |
| AG1478                           | -     | -     |
| U0126                            | -     | -     |
Meprinα transactivates the epidermal growth factor receptor (EGFR) via ligand shedding, thereby enhancing colorectal cancer cell proliferation and migration

Petra Minder, Elke Bayha, Christoph Becker-Pauly and Erwin E. Sterchi

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