Activation of Human Meprin-α in a Cell Culture Model of Colorectal Cancer Is Triggered by the Plasminogen-activating System*

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The activation of latent proenzymes is an important mechanism for the regulation of localized proteolytic activity. Human meprin-α, an astacin-like zinc metalloprotease expressed in normal colon epithelial cells, is secreted as a zymogen into the intestinal lumen. Here, meprin is activated after propeptide cleavage by trypsin. In contrast, colorectal cancer cells secrete meprin-α in a non-polarized way, leading to accumulation and increased activity of meprin-α in the tumor stroma. We have analyzed the activation mechanism of promeprin-α in colorectal cancer using a co-culture model of the intestinal mucosa composed of colorectal adenocarcinoma cells (Caco-2) cultivated on filter supports and intestinal fibroblasts grown in the companion dish. We provide evidence that meprin-α is activated by plasmin and show that the presence of plasminogen in the basolateral compartment of the co-cultures is sufficient for promeprin-α activation. Analysis of the plasminogen-activating system in the co-cultures revealed that plasmin activates proteases produced and secreted by fibroblasts converted plasminogen to active plasmin, which in turn generated active meprin-α. This activation mechanism offers an explanation for the observed meprin-α activity in the tumor stroma, a prerequisite for a potential role of this protease in colorectal cancer.

Human meprin is a metalloprotease of the astacin family of proteases (1–3). Meprins were first isolated from brush-border membranes of mammalian intestinal and kidney epithelial cells (4–7). The protease is a multidomain glycoprotein consisting of evolutionarily related α- and β-subunits with subunit masses of 85–110 kDa (8, 9). The subunits form disulfide-linked dimers, which can further oligomerize by noncovalent interactions, leading to homo- or hetero-oligomeric proteins (8–10). Meprins are synthesized as type I transmembrane proteins; however, only the β-subunit has been determined to be an integral membrane protein (11). The α-subunit is processed post-transcriptionally after the C-terminally located I-domain, resulting in detachment of its membrane-spanning anchor (12). Thus, when expressed individually, meprin-α is secreted from the cells; when both subunits are expressed, meprin-α associates with meprin-β at the cell surface (11).

Despite their similar protein domain structures, meprin-α and meprin-β show a clear difference in substrate specificity (13). According to a variety of known in vitro substrates, meprins may be implicated in normal and pathologic processes. They degrade proteins of the extracellular matrix (ECM)1 such as type IV collagen, fibronectin, laminin, and nidogen (14, 15) and process biologically active peptides, including bradykinin, angiotensins, parathyroid hormone, gastrin, the β-chain of insulin, growth factors, and cytokines (8, 13, 16).

Like other astacins, human meprin is initially synthesized as an inactive precursor protein that is activated after proteolytic processing of the N-terminal prosequence (17). In vitro, trypsin cleaves the propeptide of human meprin following an Arg residue (Arg60 in human meprin-α and Arg61 in human meprin-β) (18), and this is most probably the physiologic mechanism of promeprin activation in the intestinal lumen. However, possible other meprin-activating proteases must exist in vivo, especially in meprin-expressing tissues or cells, where trypsin is absent. Because meprin has also been detected in leukocytes of the subepithelial intestinal mucosa and in a variety of cancer cells and tissues (19, 20), knowledge of these proteases is an important factor to determine the physiologic significance of meprin activity under normal and pathologic conditions.

In this study, we demonstrate that plasmin activates promeprin-α. Plasmin is a trypsin-like protease present in the blood and the extracellular fluid as inactive plasminogen. Conversion of plasminogen to active plasmin is catalyzed by the urokinase-type plasminogen activator (u-PA) or tissue-type plasminogen activator (t-PA). Although t-PA is generally accepted to be primarily involved in thrombolysis, it is mainly u-PA that generates active plasmin in processes leading to ECM breakdown (21). Several proteases, including serine proteinases and metalloproteinases, are implicated in tumor growth, invasion into surrounding tissues, and metastasis. Plasmin, through the direct degradation of proteins of the basement membrane and the extracellular matrix (22–24) or the activation of zymogen forms of ECM-degrading metalloproteinases such as interstitial procollagenase (matrix metalloproteinase-1) and prostromelysin (matrix metalloproteinase-3) (25), plays a crucial part in these tissue-remodeling processes. Cell-surface activation of plasminogen is tightly controlled and is mediated through the concerted action of u-PA, the plasminogen activator receptor (u-PAR), and plasminogen activator inhibitors (PAI-1 and PAI-2) (26).

1 The abbreviations used are: ECM, extracellular matrix; u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; u-PAR, urokinase-type plasminogen activator receptor; PAI, plasminogen activator inhibitor; PET, polyethylene terephthalate; MDCK, Madin-Darby canine kidney; FABA peptide, N-benzy1-1-tyrosyl-p-aminobenzoic acid.

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In the small intestine, both meprin-α and meprin-β are localized in the brush-border membranes of epithelial cells. In the colon, only meprin-α is expressed and is secreted apically into the colon lumen (27). We have previously shown that, in contrast to normal colonocytes, colorectal adenocarcinoma cells (Caco-2) secrete meprin-α in a non-polarized way to the apical and basolateral media when grown on Transwell filter dishes (20). Moreover, we have observed an increased activity of meprin-α in colorectal cancer tissue homogenates and a diffuse cellular localization in colorectal carcinoma cell lines by colon cancer tissue sections (20). Other authors have described an up-regulation of the expression of u-PA in colorectal carcinomas and its correlation with tumor invasion (28, 29). Using immunohistochemistry and in situ hybridization, u-PA and u-PAR expression was shown to be localized to colon epithelial cells (30), stromal fibroblasts, and endothelial and/or inflammatory cells (for a review, see Ref. 21). We therefore speculated that the plasminogen-activating system plays an important role in meprin-α activation in colorectal cancer tissue, in the subepithelial mucosa, or in other meprin-expressing cells and tissues in vivo. To test this hypothesis, we established a co-culture model of the intestinal mucosa by cultivating Caco-2 cells on Transwell filter inserts together with primary intestinal fibroblasts or HT-1080 fibrosarcoma cells in the companion dish. We analyzed meprin-α protein expression and secretion in differentiated Caco-2 cells and demonstrate that addition of the proenzyme plasminogen to the basolateral compartment of the co-cultures was sufficient for meprin-α activation. We examined components of the plasminogen-activating system in the co-cultures and propose a model for meprin-α activation in the subepithelial mucosa and colon cancer stroma in vivo.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and all supplements were obtained from Invitrogen (Bazel, Switzerland). Enzyme inhibitors, bovine fibrinogen, trypsin, and thrombin were purchased from Sigma. Human plasminogen, bovine and human plasminogen, trypsin, and thrombin were purchased from Sigma. Human plasminogen was added to the basolateral medium as indicated.

Fibronectin—Fibronectin (1 mg/ml) was added to the culture medium.

Co-culture—Caco-2 cells were cultured on Transwell filter inserts. Cells were treated with 10 μM of t-PA (Sigma) for 24 h. Following this treatment, the cells were collected and analyzed by Western blotting.

Immunohistochemistry—Immunohistochemistry was performed on paraffin-embedded sections of colorectal cancer tissue. Sections were stained with anti-u-PA and anti-u-PAR antibodies. The sections were counterstained with hematoxylin.

Western Blotting—Western Blotting was performed on cell lysates. The membranes were incubated with the indicated antibody (polyclonal anti-u-PA antibody, 1:2000–1:5000 dilution; or monoclonal anti-u-PA antibody, 1:1000 dilution), and then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:10,000 dilution) for 1.5 h at room temperature. Immunocomplexes were visualized using the ECL Plus Western blotting kit (Amersham Biosciences) and x-ray films.

Molecular Biochemicals, Basel). Sucrase-isomaltase activity was assayed at 37 °C. The gel was stained and fixed with 0.1% Coomassie Blue R-250 in methanol/acetic acid/water (40:10:50).

Reverse Transcription-PCR—Total RNA was isolated from cultured cells using the acid guanidium thiocyanate method according to the manufacturer's recommendations (Trizol reagent, Invitrogen). First-strand cDNA synthesis was carried out with 2 μg of RNA and random hexanucleotide primers using the GeneAmp PCR kit (Applied Biosystems). The amount of cDNA in each sample was quantified using PCR with primers for the glyceraldehyde-3-phosphate dehydrogenase gene as an internal standard. The oligonucleotide primers employed for the PCRs were as follows: meprin-α(5'-3'), 5'-GGGATCCATCTTCTGGAGAACATCCC-3'; meprin-β(5'-3'), 5'-CACCGAGTCTCCTCGAACAGCT-3'; u-PA(5'), 5'-GGGAGCCACAGCTTTGGAATAG-3'; u-PA(3'), 5'-AAGGCAATGTCCTTGTTGGTGAAC-3'; t-PA(5'), 5'-TGGAGGACCTTCTCCTGTCGCC-3'; t-PA(3'), 5'-CCCAACACCATGAGATAATAGC-3'; u-PAR(5'), 5'-TGTAGACCCAGCGAGGATTGC-3'; u-PAR(3'), 5'-CGTGGTGTGACACATGTTACCC-3'; PAI-2(5'), 5'-TTTGTGCTGATGGGACACC-3'; PAI-2(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PA(3'), 5'-CAGGGGATGTTATCTCTGGACAC-3'; t-PA(3'), 5'-TTTGTGCTGATGGGACACC-3'; t-PA(3'), 5'-CCCAACACCATGAGATAATAGC-3'; u-PAR(5'), 5'-TGTAGACCCAGCGAGGATTGC-3'; u-PAR(3'), 5'-CGTGGTGTGACACATGTTACCC-3'; PAI-2(5'), 5'-TTTGTGCTGATGGGACACC-3'; PAI-2(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PA(3'), 5'-CAGGGGATGTTATCTCTGGACAC-3'; t-PA(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PAR(5'), 5'-TGTAGACCCAGCGAGGATTGC-3'; u-PAR(3'), 5'-CGTGGTGTGACACATGTTACCC-3'; PAI-2(5'), 5'-TTTGTGCTGATGGGACACC-3'; PAI-2(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PA(3'), 5'-CAGGGGATGTTATCTCTGGACAC-3'; t-PA(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PAR(5'), 5'-TGTAGACCCAGCGAGGATTGC-3'; u-PAR(3'), 5'-CGTGGTGTGACACATGTTACCC-3'; PAI-2(5'), 5'-TTTGTGCTGATGGGACACC-3'; PAI-2(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PA(3'), 5'-CAGGGGATGTTATCTCTGGACAC-3'; t-PA(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PAR(5'), 5'-TGTAGACCCAGCGAGGATTGC-3'; u-PAR(3'), 5'-CGTGGTGTGACACATGTTACCC-3'; PAI-2(5'), 5'-TTTGTGCTGATGGGACACC-3'; PAI-2(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PA(3'), 5'-CAGGGGATGTTATCTCTGGACAC-3'; t-PA(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PAR(5'), 5'-TGTAGACCCAGCGAGGATTGC-3'; u-PAR(3'), 5'-CGTGGTGTGACACATGTTACCC-3'; PAI-2(5'), 5'-TTTGTGCTGATGGGACACC-3'; PAI-2(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PA(3'), 5'-CAGGGGATGTTATCTCTGGACAC-3'; t-PA(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PAR(5'), 5'-TGTAGACCCAGCGAGGATTGC-3'; u-PAR(3'), 5'-CGTGGTGTGACACATGTTACCC-3'; PAI-2(5'), 5'-TTTGTGCTGATGGGACACC-3'; PAI-2(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PA(3'), 5'-CAGGGGATGTTATCTCTGGACAC-3'; t-PA(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PAR(5'), 5'-TGTAGACCCAGCGAGGATTGC-3'; u-PAR(3'), 5'-CGTGGTGTGACACATGTTACCC-3'; PAI-2(5'), 5'-TTTGTGCTGATGGGACACC-3'; PAI-2(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PA(3'), 5'-CAGGGGATGTTATCTCTGGACAC-3'; t-PA(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PAR(5'), 5'-TGTAGACCCAGCGAGGATTGC-3'; u-PAR(3'), 5'-CGTGGTGTGACACATGTTACCC-3'; PAI-2(5'), 5'-TTTGTGCTGATGGGACACC-3'; PAI-2(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PA(3'), 5'-CAGGGGATGTTATCTCTGGACAC-3'; t-PA(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PAR(5'), 5'-TGTAGACCCAGCGAGGATTGC-3'; u-PAR(3'), 5'-CGTGGTGTGACACATGTTACCC-3'; PAI-2(5'), 5'-TTTGTGCTGATGGGACACC-3'; PAI-2(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PA(3'), 5'-CAGGGGATGTTATCTCTGGACAC-3'; t-PA(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PAR(5'), 5'-TGTAGACCCAGCGAGGATTGC-3'; u-PAR(3'), 5'-CGTGGTGTGACACATGTTACCC-3'; PAI-2(5'), 5'-TTTGTGCTGATGGGACACC-3'; PAI-2(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PA(3'), 5'-CAGGGGATGTTATCTCTGGACAC-3'; t-PA(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PAR(5'), 5'-TGTAGACCCAGCGAGGATTGC-3'; u-PAR(3'), 5'-CGTGGTGTGACACATGTTACCC-3'; PAI-2(5'), 5'-TTTGTGCTGATGGGACACC-3'; PAI-2(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PA(3'), 5'-CAGGGGATGTTATCTCTGGACAC-3'; t-PA(3'), 5'-TTTGTGCTGATGGGACACC-3'; u-PAR(5'), 5'-TGTAGACCCAGCGAGGAT-
of the free chromophore p-nitroaniline generated after hydrolysis of carbenzoxyl-L-Glu-(t-buty1-ester)-Gly-Arg-p-nitroaniline (SPEC- TROZYME® UK, no. 244, American Diagnostica). For the assay, cells were grown for 20 h in serum-free medium (free of colored matter). Enzyme activity was measured in sterile-filtered medium according to the manufacturer's instructions using high molecular mass u-PA as an activity standard.

**N-terminal Sequencing of Plasmin-activated Meprin-a**—Purification of plasmin-activated meprin-a was achieved according to the procedure described for purification of trypsin-activated meprin-a (36) with a slight modification. Recombinant promeprin-a (500 µg), purified by gel filtration from overexpressing High Five insect cell culture medium, was incubated with 10 nM human plasmin in 0.1 M NaCl, 0.1% polyethylene glycol 8000 (pH 7.5) for 5 h at room temperature. The activated form of meprin-a was loaded onto an affinity column carrying the immobilized inhibitor Pro-Leu-Gly-hydroxamate, which selectively binds active meprin-a. After elution, protein fractions with the highest meprin-a activity were pooled, precipitated, and subjected to SDS-PAGE. Following transfer to a polyvinylidene difluoride membrane, the Coomassie Blue-stained meprin-a band was excised and subjected to N-terminal amino acid sequencing (SeqLab).

**RESULTS**

**Expression and Non-polarized Secretion of Meprin-a in Post-confluent Caco-2 Cells**—We have previously shown a non-polarized secretion of meprin-a in Caco-2 cells (20). To analyze this further, Caco-2 cells were grown on PET membrane inserts over a period of 21 days post-confluence. During this period, Caco-2 cells differentiate spontaneously to a polarized phenotype (37). Establishment of tight monolayers was judged by light microscopy and measurement of the transepithelial electrical resistance. At 7 days post-confluence, the transepithelial electrical resistance reached a level of 700–750 ohms cm², a value that is generally accepted as proof of a tight cell layer. Apical and basolateral media as well as cell lysates at different days post-confluence were analyzed for meprin-a protein expression by immunoblotting (Fig. 1). A plateau of maximal meprin-a expression was reached at 7 days post-confluence (Fig. 1A, lane 3). The higher molecular mass form of 100 kDa in cell extracts represents the endoplasmic reticulum resident precursor form of meprin-a (18), whereas the 90-kDa protein corresponds to the C-terminally processed form of meprin-a (38). We confirmed that the 95-kDa secreted form of meprin-a was directed in a non-polarized way to the apical and basolateral compartments (Fig. 1B). In accordance with expression levels, a maximal level of total meprin-a secretion was reached at 7 days post-confluence (Fig. 1B, lanes 5 and 6). More than 50% of the enzyme reached the basolateral compartment at this time, as verified by densitometric scanning analysis. Although secretion of meprin-a into the apical medium remained constant during the residual culture period, the amount of basolaterally secreted meprin-a diminished in the later phases of cell culture (Fig. 1B, lanes 10 and 12). This is most likely the result of hindrance of basolateral transport due to the formation of cell multilayers, a phenomenon that has been described for prolonged cultivation of Caco-2 cells on PET filters (39). When Caco-2 cells were grown on polycarbonate membranes (Millipore), 50% of meprin-a was secreted basolaterally throughout the 21-day culture period (Fig. 1C, lanes 8, 10, and 12). Thus, cells cultivated for >10 days post-confluence on PET filters were prone to “filter clogging.”

To analyze the phenotypic development of cell polarization during the culture period, the activity of two marker enzymes of the brush-border membrane, alkaline phosphatase and sucrase-isomaltase, were assayed in the Caco-2 cell extracts. Alkaline phosphatase and sucrase-isomaltase activities both increased progressively, reaching a 3-fold increase at 7 days post-confluence, further demonstrating that cells had reached a differentiated state (data not shown). In conclusion, meprin-a protein expression is induced in differentiated Caco-2 cells, and targeting and secretion of the enzyme are clearly independent of cell polarization. Taking all these results into account, plus the fact that cells may be monitored by light microscopy on PET filters, we used 7-day post-confluent Caco-2 cells grown on these filter inserts for the subsequent co-cultures experiments.

**Activation of Promeprin-a by Plasmin**—Following zymogen activation by limited trypsin treatment, meprin-a hydrolytic activity was measured using the PABA peptide as a substrate (8). To investigate whether plasmin activates promeprin-a, the medium of MDCK cells stably transfected with meprin-a cDNA (32) was incubated with various concentrations of plasmin (Fig. 2). Plasmin showed no hydrolytic activity for the PABA peptide itself (not shown). The activities of trypsin-treated meprin-a were higher than those after plasmin treatment, indicating that activation by trypsin was more efficient.

Processing of promeprin-a by plasmin was also assessed by immunoblotting. The zymogen was incubated with pure human plasmin or trypsin (50 nM) for 0–6 h at 37 °C. Immunoanalysis using an anti-meprin-a antibody directed against both the zymogen and mature enzyme (Fig. 2B, upper panel) revealed a protein band corresponding to the zymogen (95 kDa) and an additional slightly smaller band after 3 and 6 h of incubation with plasmin. Detection with an antibody recognizing only the zymogen (Fig. 2B, lower panel) showed that this form disap-
stopped the activation process. Meprin-a overexpression was confirmed by N-terminal sequence analysis. Recombinant meprin-a isolated from the culture medium of meprin-a-expressing High Five insect cells was incubated with human plasmin (10 nM) for 5 h at 37 °C. Addition of a 10-fold molar excess of aprotinin or soybean trypsin inhibitor, respectively, stopped the activation process. Meprin-a activity was measured by the colorimetric PABA peptide assay. The data represent averages of three independent activity assays (z: maximum/minimum). B, MDCK cells cultured with pure human plasmin (50 nM) or trypsin (50 nM) for the indicated times, and aliquots were subjected to immunoblotting. Anti-meprin-a antibody recognizing the zymogen (z) and mature (m) forms was used in the upper panel; an antibody detecting predominantly the zymogen form was applied in the lower panel.

Fig. 2. Activation of meprin-a by human plasmin. A, aliquots of sterile-filtered medium of confluent MDCK cells that had been stably transfected with human meprin-a cDNA were incubated with human plasmin or trypsin at 10–500 nM for 2 h at 37 °C. Addition of a 10-fold molar excess of aprotinin or soybean trypsin inhibitor, respectively, stopped the activation process. Meprin-a activity was measured by the colorimetric PABA peptide assay. The data represent averages of three independent activity assays (z: maximum/minimum). B, MDCK cells cultured medium was incubated with pure human plasmin (50 nM) or trypsin (50 nM) for the indicated times, and aliquots were subjected to immunoblotting. Anti-meprin-a antibody recognizing the zymogen (z) and mature (m) forms was used in the upper panel; an antibody detecting predominantly the zymogen form was applied in the lower panel.

peared with incubation time, confirming that promeprin-a was converted to mature meprin-a by plasmin treatment. Activation of meprin-a with trypsin (50 nM) for 3 h led to a quantitative conversion of promeprin-a to the mature form.

To map the cleavage site, plasmin-activated meprin-a was subjected to N-terminal sequence analysis. Recombinant meprin-a (500 µg) isolated from the culture medium of meprin-a-overexpressing High Five insect cells was incubated with human plasmin (10 nM) for 5 h at room temperature. The activated form of meprin-a was then purified by affinity chromatography, followed by SDS-PAGE and transfer to a polyvinyldiene difluoride membrane as described under “Experimental Procedures.” The N-terminal sequence obtained corresponded to the sequence previously reported for trypsin-activated meprin-a (18). Hence, plasmin and trypsin cleave promeprin-a at the same site, between Arg66 and Asn66, thereby generating mature enzymes with identical N termini.

Co-culture of Caco-2 Cells and Intestinal Fibroblasts: Presence of Plasminogen in the Basolateral Compartment Results in Activation of Meprin-a—Non-polarized secretion of meprin-a from colon cancer cells, increased meprin-a activity measured in colon cancer tissue homogenates (20), and up-regulation of components involved in plasminogen activation described in colon adenocarcinomas (28) led us to speculate that the plasminogen-activating system may play a role in the activation of basolaterally secreted meprin-a in colon cancer tissue. Addressing this issue, we established an in vitro co-culture model of the intestinal mucosa composed of Caco-2 cells grown on filter supports and primary intestinal fibroblasts grown to confluence in the bottom companion dish (see model in Fig. 8). We investigated whether basolaterally secreted meprin-a was activated if plasminogen was added to the lower compartment of the co-cultures (Fig. 3). In addition to primary intestinal fibroblasts that were isolated from an intestinal mucosal biopsy, HT-1080 fibrosarcoma cells were used in the co-culture experiments. The latter are known to express elevated levels of plasminogen activators (40). The presence of plasminogen in the basolateral medium of the co-cultures resulted in a dose-dependent activation of meprin-a in this compartment, whereas no activity was measured in the absence of plasminogen (Fig. 3A). Addition of plasminogen to the apical compartment did not lead to activation of apically secreted meprin-a. This, plus the fact that addition of plasminogen to the basolateral medium of Caco-2 cells cultured alone did not lead to promeprin-a activation, clearly indicated that both Caco-2 cells and fibroblasts were required for the generation of active meprin-a in the basolateral medium. Treatment of the apical and basolateral media of Caco-2 cells cultured alone for 20 h in serum-free medium were incubated for an additional 2 h with 100 nM trypsin (lane 9). A, meprin-a activity measured in 100-µl aliquots of apical and basolateral media using the PABA peptide as a substrate; B, immunoblot analysis of the basolateral medium (80 µl) using an anti-meprin-a antibody detecting zymogen (z) and mature (m) meprin-a.

Reverse Transcription-PCR Analysis of Components of the Plasminogen-activating System—Plasminogen activation de-
were as follows: 2 min at 95°C, 35 cycles for 0.5 min at 95°C, 0.5 min at 56–58°C, and 1.5 min at 72°C; and 10 min at 72°C. The sizes of the PCR products are shown on the left.

depends on the concerted action of plasminogen activators (u-PA and t-PA), u-PAR, as well as PAIs. To characterize the coculture model with respect to these components, semiquantitative mRNA analysis was carried out (Fig. 4). The presence of meprin-α mRNA was analyzed in the three cell types. Amplification products of u-PA, t-PA, u-PAR, PAI-1, and PAI-2 were obtained from cDNAs of HT-1080 cells and intestinal fibroblasts (Fig. 4, lanes 2 and 3). No meprin-α mRNA was detected in these cells. In contrast, Caco-2 cells expressed meprin-α m-RNA, low levels of u-PA and u-PAR, and trace amounts of PAIs. These results confirm the expression of the plasminogen-activating system predominantly in HT-1080 cells and in intestinal fibroblasts.

*Urokinase-type Plasminogen Activators Are Secreted by Fibroblasts*—To detect plasminogen activators in the coculture model, fibrin zymography was carried out. u-PA activity was detected in the conditioned media of primary intestinal fibroblasts and of HT-1080 cells cultured alone (Fig. 5A, lanes 3 and 4), but not in the apical or basolateral medium of Caco-2 cells (lanes 1 and 2). In co-cultures of Caco-2 and HT-1080 cells or Caco-2 cells and intestinal fibroblasts, u-PA was detected only in the basolateral compartment, indicating that u-PA was secreted predominantly by fibroblasts (Fig. 5A, lanes 5–8). Pure human high molecular mass u-PA and human t-PA were loaded as size references and activity controls (Fig. 5A, lanes 9 and 10). Cultivation of the three cell types individually or in co-culture in the presence of 50 nM human plasminogen resulted in an increase in u-PA activity (Fig. 5B and Table 1), which may be explained by the conversion of pro-u-PA to high and low molecular mass u-PA. Generation of both molecular mass forms of u-PA was confirmed by immunodetection of u-PA in the conditioned medium of plasminogen-treated fibroblasts (Fig. 5C, lanes 7 and 8). Analysis of cell extracts by fibrin zymography showed that u-PA was detected in all three cell types (Fig. 6). However, the amount of cellular u-PA in Caco-2 cells was much lower than in HT-1080 cells and intestinal fibroblasts.

u-PA activity in the medium of untreated or plasminogen-treated cells, cultured alone or in co-culture, was analyzed additionally by an amidolytic assay using the chromogenic substrate carbobenzoxy-L-Glu(α-t-buty1-ester)-Gly-Arg-p-nitroanilide. As shown in Table 1, low u-PA activities were detected in the media of HT-1080 cells and primary intestinal fibroblasts not treated with plasminogen as well as in the basolateral medium of untreated co-cultures. Increased u-PA activity was observed in the media of HT-1080 cells and intestinal fibroblasts and in the basolateral co-culture medium upon addition of plasminogen, confirming the zymography results (Fig. 5B).

*Meprin-α Is Activated Directly by Plasmin in Plasminogen-treated Co-cultures*—Because plasmin is known to activate sev-
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**FIG. 6. Detection of cellular plasminogen activators.** Plasminogen activator activity in cell extracts was analyzed by fibrin zymography. After copolymerization of bovine fibrin (1.2 mg/ml) and plasminogen (15 µg/ml) on a 10% SDS-polyacrylamide gel, 1 µg of cell extracts from Caco-2 cells (Ca), HT-1080 cells (HT), or intestinal fibroblasts (IF) was separated under nonreducing conditions, followed by proteolysis as described under “Experimental Procedures.” Purified u-PA and t-PA were loaded as references as described in the legend to Fig. 5. HMW- and LMW-u-PA, high and low molecular mass u-PA, respectively.

**FIG. 7. Promeprin-α is processed directly by plasmin in plasminogen-treated co-cultures.** Co-cultures of Caco-2 cells and fibroblasts were treated basolaterally with 100 ng plasminogen for 20 h in serum-free medium. 1,10-Phenanthroline (Ph; 100 µg/ml) or aprotinin (Apr; 100 µg/ml) was added to the basolateral medium as indicated. A, aliquots of conditioned medium (80 µl) were analyzed by immunoblotting using anti-meprin-α antibody. B, meprin-α activity was measured using the PABA peptide assay. Ca, Caco-2 cells; Ca/HT, co-culture of Caco-2 and HT-1080 cells; Ca/IF, co-culture of Caco-2 cells and primary intestinal fibroblasts; z, zymogen; m, mature meprin-α.

**TABLE I**

| Enzyme activity | Caco-2 | HT-1080 | IF | Co-cultures |
|-----------------|--------|---------|----|-------------|
| u-PA range (IU/ml) | AP | BL | | |
| Untreated | 0 | 0 | 14.7 (13.4–16.8) | 4.0 (1.9–4.6) |
| Plasminogen-treated | 0.5 (0.2–0.6) | 3.8 (3.4–4.0) | 69.2 (51.2–88.2) | 17.1 (11.9–22.7) |

a u-PA activities in the media of untreated individual all cultures and co-cultures.

b u-PA activities in the media of individual all cultures and co-cultures treated with human plasminogen (50 nM) in the basolateral compartment.

**DISCUSSION**

Meprin is expressed in normal human intestine either as meprin-α/meprin-β heterodimers (small intestine) or as meprin-α homodimers (colon) (8, 27). In the colon, meprin-α is secreted into the lumen due to constitutive proteolytic removal of the C-terminal transmembrane and cytosolic domain (27). Activation of meprin is induced in the intestinal lumen by pancreatic trypsin (18). Several previous observations led us to investigate the role of the plasmin-activating system in meprin-α activation in human colorectal cancer cells. (i) Colon adenocarcinoma cells (Caco-2) endogenously express meprin-α and, in contrast to normal colonocytes, secrete the protein in a non-polarized way from the apical and basolateral membrane domains when grown on Transwell filters (20). (ii) Increased meprin-α activity was measured in colorectal tissue homogenates compared with the normal colon mucosa of the same patients (20), suggesting activation of meprin-α by a hitherto unknown protease. (iii) An increase in the expression of components involved in plasminogen activation (plasminogen, u-PA, u-PAR, and PAIs) was described in colorectal adenocarcinomas (21, 28, 41). To address the issue, we have used the co-culture model of Caco-2 cells with fibroblasts described herein.

We have shown that human meprin-α was activated by plasmin in addition to trypsin. Plasmin-mediated meprin-α activation was achieved by cleavage of the propeptide, which is consistent with the described activation mechanism for the members of the astacin family of proteases (17, 42). Although plasmin and trypsin cleaved at the same peptide bond in *vitro*, trypsin-activated meprin-α showed a slightly lower molecular mass than plasmin-activated meprin-α (Fig. 2B), indicating that an additional cleavage by trypsin resulted in the removal of a small peptide from the C terminus. Activation of meprin-α by plasmin was less efficient than by trypsin, which may be due to a lower specific activity of plasmin compared with trypsin.

We found that meprin-α expression was strongly induced in post-confluent, spontaneously differentiated Caco-2 cells, and we confirmed that secretion of meprin-α by these polarized cells occurred in a non-polarized fashion. Misguiding meprin-α to...
the basolateral domain in Caco-2 cells is therefore a feature of transformed colon cancer cells, as in the normal colon, meprin-α is secreted apically into the intestinal lumen and does not accumulate basolaterally (27). Meprin-α also shows an exclusively apical targeting in transfected MDCK cells, non-transformed canine kidney epithelial cells, which are widely used as a model for studies of polarized transport (32). Caco-2 cells are known to sort apical proteins either directly from the trans-Golgi network to the apical surface or indirectly via the basolateral membrane by transcytosis (43, 44). The transcytotic pathway depends on anchorage of the protein to the basolateral plasma membrane, as secretory mutants of aminopeptidase N have been shown to be secreted from both membrane domains in Caco-2 cells (45). As the transmembrane anchor of meprin-α is intracellularly cleaved (12), the protein targeted to the basolateral membrane is no longer available for transcytosis to the apical membrane. Instead, meprin-α is secreted into the basolateral compartment of Transwell cultures. This non-polarized secretion of meprin-α from colon cancer cells offers an explanation for the accumulation of the enzyme in the stroma of tumor tissue in vivo, as demonstrated by Lottaz et al. (20).

Basolateral meprin-α secretion from Caco-2 cells was dependent on the filter inserts used. On polycarbonate filters, approximately half the meprin-α was secreted to the apical medium and half to the basolateral medium during the 21-day post-confluent culture. In cells grown on PET filters, the level of basolateral secretion decreased over the same culture period, whereas apical secretion remained constant. Using confocal laser scanning microscopy, Rothen-Rutishauser et al. (39) have shown that prolonged culture of Caco-2 cells on PET membranes resulted in the buildup of multilayers, whereas only monolayers were observed when cells were cultivated on polycarbonate membranes. The apparently reduced basolateral secretion of meprin-α from PET filter-grown Caco-2 cells is probably due to the formation of cell multilayers, resulting in filter clogging. For this reason, we have chosen a 7-day post-confluence time point for our co-culture experiments. At this time point, the expression of sucrase-isomaltase and alkaline phosphatase, both marker enzymes of the apical brush-border membrane, was enhanced 3-fold, and a transepithelial electrical resistance of 700 ohms cm² was obtained, indicating that the cell layer was tight and that the cells had reached a differentiated phenotype. The phenomenon of filter clogging due to the formation of cell multilayers of Caco-2 cells on PET filters must be taken into account when studying polarized expression of proteins in these cells.

Although the Caco-2 cell line was derived from a primary colon tumor, the cells have often been used to study enteroctytic functions of the normal small bowel because the cells differentiate post-confluence to a polarized phenotype with properties of fetal enterocytes (46, 47). However, they represent a heterogeneous population of cells. Engle et al. (47) have shown that they express proteins characteristic of both colonocytes and enterocytes immediately after confluence. After prolonged post-confluent culture, proteins characteristic of colonocytes decrease, whereas enterocyte-specific proteins increase. The authors suggested that, under certain conditions, the colonic/tumor phenotype of Caco-2 cells may not revert to an enterocyte-like phenotype. Indeed, Pandrea et al. (48) have shown that differentiated Caco-2 cells grown up to 30 days post-confluence retain (i) a tumorigenic capacity in nude mice and (ii) the ability to dedifferentiate and to migrate in wound healing assays. A differentiated, apparently enterocyte-like phenotype is therefore compatible with the use of Caco-2 cells in a model of colon tumors. In fact, several colon tumors have been shown to contain, in various proportions, differentiated cell types that express markers of a fetal enterocyte-like phenotype, including sucrase-isomaltase, dipeptidylpeptidase IV, and alkaline phosphatase (49–51).

The tumorigenic and/or metastatic potential of Caco-2 cells may be influenced by a variety of factors. In the context of meprin-α, protease-activating systems contributed by other cells in the lamina propria are of prime importance. The co-culture model of Caco-2 cells and fibroblasts is well suited to analyze the pericellular interplay of proteases, activators, or inhibitors, expressed and secreted from different cell types, and represents a step toward imitating the complex situation in the colon mucosa and colorectal cancer tissue in vivo. Using this co-culture model, we have clearly demonstrated that the presence of plasminogen in the lower compartment resulted in activation of basolaterally secreted meprin-α (Fig. 3). No meprin-α activity was measured in the lower compartment when Caco-2 cells were cultured alone (on filters) in the presence of plasminogen. This indicated that fibroblasts were responsible for the production of plasminogen activators that mediated the conversion of plasminogen to active plasmin, which in turn resulted in the activation of promeprin-α.
Activation of Human Meprin-α in Colorectal Cancer

Determination of plasminogen activator mRNA in cells and measurement of plasminogen activator activity in the lower compartment of cocultures confirmed that fibroblasts were largely responsible for plasminogen activator expression and secretion. Fibrin zymography revealed that all plasminogen activator activity in the basolateral medium was u-PA. An increase in u-PA activity was found in the culture medium of fibroblasts cultured alone in the presence of plasminogen. Pro-u-PA was activated to high and low molecular mass u-PA as verified by immunoblotting (Fig. 5C). Plasminogen was initially present in the medium as an inactive proenzyme, which raises the question of how the activation of pro-u-PA is started. Trace amounts of plasmin were shown to be sufficient to activate pro-u-PA, thereby generating a self-maintained activation mechanism of pro-u-PA and plasminogen (52). Thus, traces of plasmin present in the plasminogen preparation may lead to initiation of the activation cascade. Alternatively, plasmin-independent pro-u-PA activation has been described by binding of pro-u-PA to its cellular receptor u-PAR (53). A role of receptor-bound u-PA in the generation of plasmin at cell surfaces followed by ECM degradation has been demonstrated in several physiologic and pathologic settings (54, 55). The low u-PA activities measured by spectrophotometry in the untreated medium of fibroblasts and in the basolateral medium of cocultures were probably due to low intrinsic pro-u-PA activity, which has been described previously (56). The finding that small amounts of u-PA were also detected in Caco-2 cell extracts (Fig. 6) is in agreement with other studies describing low u-PA expression by Caco-2 cells (57, 58). Basolateral secretion of u-PA has been described in Caco-2 cells after a mild acid treatment, leading to dissociation of u-PA from its receptor (58, 59).

The cell types responsible for the expression of u-PA, u-PAR, or PAIs in colon adenocarcinoma tissue sections have been analyzed by immunohistochemistry and in situ hybridization (for a review, see Ref. 21). Taking into account some discrepancies between the different groups, a general finding was that each of the components is expressed not only by a single cell type, but may be expressed by cancer cells, fibroblasts, and inflammatory or endothelial cells (60–62). It has been suggested that secreted components like u-PA and PAI-1 produced by stromal, inflammatory, or endothelial cells are delivered for the assembly of a functional plasminogen-activating system to the surface of cancer cells by binding to cellular u-PAR (53). Our results described here indicate that a similar pathway exists in the co-culture model. Fibroblasts are responsible for the production and secretion of pro-u-PA that is converted to its active forms (high and low molecular mass u-PA) in the presence of plasminogen. Because Caco-2 cells and fibroblasts have been shown to express u-PAR (Fig. 4) (40, 57), soluble u-PA is partly delivered to and binds to the cell surface of both cell types. Moreover, u-PA expressed by Caco-2 cells binds to u-PAR on their basolateral cell surface. Meprin-α is then activated either directly in the basolateral medium by soluble plasmin or by interaction with plasmin bound to the u-PAR-u-PA PAR complex at the surface of Caco-2 cells and fibroblasts. Further experiments are required to determine whether there is an assembly of such a cell surface-associated complex triggering promeprin-α activation. This could lead to a concentration of meprin-α proteolytic activity at the surface of cancer cells. HT-1080 cells have been described to shed membrane vesicles containing active forms of u-PA associated with the vesicle surface (63). In the basolateral medium, plasminogen may therefore be activated by binding to vesicle-bound u-PA. Whether primary intestinal fibroblasts shed vesicles containing u-PA remains to be investigated. u-PA activity is also influenced by serpin inhibitors (PAI-1/2). These are expressed and secreted primarily by fibroblasts (Fig. 4) (21) and competitively bind to u-PAR-bound u-PA. The PAI-1-u-PA/PAI complex is then rapidly endocytosed and degraded (64).

The results obtained from the co-culture experiments reflect the importance of the plasminogen-activating system for meprin-α activity in the stroma of colorectal cancer tissue. We propose the following mechanisms that lead to increased meprin-α proteolytic activity in colon cancer tissue. (i) Non-polarized secretion of meprin-α by transformed colon cancer cells localizes the enzyme to the stroma of the tumor, where it accumulates. (ii) Stromal fibroblasts and other cell types present in the tumor tissue produce and secrete u-PA and enable efficient activation of plasminogen in the extracellular compartment or at the surface of colon cancer cells by binding of u-PA to u-PAR. (iii) The presence of plasminogen and elevated u-PA activity in the stroma of colorectal cancer tissue results in efficient plasmin generation from plasminogen, followed by activation of meprin-αzymogens. A model for promeprin-α activation in colorectal cancer is illustrated in Fig. 8.

As meprin-α degrades a series of ECM and basement membrane proteins such as type IV collagen, laminin, nidogen, and fibronectin, it is proposed that meprin-α acts in concert with matrix metalloproteases in tissue-remodeling processes by facilitating cancer cell migration and tumor cell invasion into surrounding normal tissues. Invasion and migration assays based on the already established co-culture model will clarify this issue in future experiments. Although Caco-2 cells appear to be noninvasive in nude mice (65), secretory products of cells in the vicinity may interact at the surface of the cancer cells and by this increase their invasive potential. For instance, migration of Caco-2 cells in wounded cell layers was stimulated 2–3-fold by exogenous u-PA, an effect dependent on binding to u-PAR (57). Thus, the mechanism of meprin-α activation by plasmin may be especially important in meprin-α-expressing tissues, where components of the plasminogen-activating system are co-localized. In addition to colorectal cancer, we have found expression of meprin in leukocytes of the lamina propria in the intestine (27). Activation of leukocyte meprin by the plasminogen-activating system may thus play an important role in inflammatory diseases of the intestine such as Crohn’s disease, ulcerative colitis, and celiac disease.

In addition to plasmin and trypsin, other serine proteases may be involved in activation of meprin. Matriptase and tryptase are potential candidates due to their trypsin-like activity and their expression in the intestinal mucosa (66, 67). Moreover, a series of cancer cell lines, including colorectal cancer cells (68), produce isoforms of pancreatic trypsinogen (mainly trypsinogens I and II). Caco-2 cells and other colon cell lines secrete trypsinogen at low concentrations into the culture medium (69). As extrapancreatic trypsinogen has been implicated in proliferation and migration (70), it will be interesting to investigate whether one of these proteases is capable of meprin-α activation in colorectal cancer.

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