Phorbolel 12-Myristate 13-Acetate-induced Ectodomain Shedding and Phosphorylation of the Human Meprin-β Metalloprotease*

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Shedding of proteins localized at the cell surface is an important regulatory step in the function of many of these proteins. Human meprin (N-benzyol-D-tyrosyl-p-amino benzoic acid hydrolase, PPH, EC 3.4.24.18) a zinc-metalloendopeptidase of the astacin family is an oligomeric protein complex of α- and β-subunits and is expressed abundantly in the intestine and kidney as well as in leukocytes of the lamina propria and in cancer cells. In transfected cells intracellular proteolytic removal of the membrane anchor results in the secretion of the meprin α-subunit. In rats and mice, the β-subunit exists in a membrane-anchored form. In contrast, human meprin-β is constitutively secreted into a secretable form. We now show that phorbol 12-myristate 13-acetate (PMA) stimulates an increased release of hmeprin-β from transfected COS-1 cells, whereas hmeprin-α secretion is not influenced. This stimulatory effect is inhibited by the protein kinase C (PKC) inhibitor staurosporine, suggesting that activation of PKC mediates PMA-induced hmeprin-β shedding. The use of different protease inhibitors shows that two different metalloprotease activities are responsible for the constitutive and the PMA-stimulated hmeprin-β shedding. We identified tumor necrosis factor-α-converting enzyme (TACE or ADAM17) as the protease that mediates the PMA-induced release. We also demonstrate that hmeprin-β is phosphorylated by PMA treatment on Ser487 within a PKC consensus sequence in the cytosolic domain of the protein. The phosphorylation of hmeprin-β is not, however, implicated in the enhanced secretion by PMA treatment.

Meprins, membrane-bound zinc-metalloendopeptidases of the astacin family and the metzincins superfamily were first identified in brush-border preparations of rodent and human kidney and intestinal epithelial cells (1–5). The enzymes are homo- or heteromeric glycoproteins composed of two related subunits (α and β) with a multidomain structure, which form covalent and non-covalent dimers and/or tetramers (6–8). Meprin is capable of hydrolyzing many substrates in vitro, such as extracellular matrix proteins, hormones, and small peptides like gastrin 17, cerulein, and sCCK 8 of the gastrointestinal tract (6, 9, 10). Meprin may therefore be involved in the modulation of the activity of important cellular and extracellular proteins and peptides. We have observed elevated levels of meprin in patients with Crohn’s disease† and colon cancer (11). In addition we have found expression of meprin in leukocytes in the lamina propria of inflamed intestine, where it may be involved in cytokine processing (12). Moreover a novel mRNA isoform (meprin-β’ ) was identified in a variety of human cancer cell lines like MCF7, SK-BR-3, U2OS, and BxPC3 (13).

Studies of recombinant forms of meprin expressed in mammalian cells have yielded important information about the biosynthesis of both subunits (14–19). In cells transfected with meprin-α, cDNA proteolytic processing of the subunit occurs within the α-specific insertion domain. Cytosolic and transmembrane domains are involved in retention and proteolytic processing of meprin-α in the endoplasmic reticulum and are essential for subsequent intracellular transport (19). The cleaved α-subunit is constitutively secreted into the medium when expressed alone and is largely retained at the cell surface in the presence of the β-subunit. Whereas mouse meprin-β is localized only at the cell surface (5, 8), human meprin-β is found at the cell surface and in a processed form in the culture medium of transfected cells and of cultured intestinal explants (12, 18).

We have recently shown that the soluble form of hmeprin-β is released from transfected cells by cleavage in front of the epidermal growth factor-like domain. A peptide sequence 13 amino acids long is necessary for this cleavage (20). The enzyme(s) involved and the regulation of this release are still unknown. Soluble forms of transmembrane proteins diverse in structure and function are generated by a process referred to as “ectodomain shedding.” The enzymes catalyzing this shedding process are named “membrane protein secretases,” “membrane protein convertases,” or “sheddases” and belong to the superfamily of zinc-dependent proteases that include MMPs (matrix metalloproteases) and ADAMs (a disintegrin and metalloprotease). The release of membrane-bound proteins like β-amyloid precursor (21, 22), angiotensin-converting enzyme (23, 24), transforming growth factor-α (25, 26), tumor necrosis factor-α, Fas ligand (27), tumor necrosis factor receptor (28), CD30 (29),

1 D. Lottaz and E. E. Sterchi, unpublished data.

2 The abbreviations used are: MMP, matrix metalloprotease; ADAM, a disintegrin and metalloprotease; COS-1 cells, African green monkey kidney cells; MeSO₂, dimethyl sulfoxide; Endo H, endoglycosidase H; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TACE, tumor necrosis factor-α converting enzyme; TAPI, tissue inhibitor of metalloprotease-1; TIMP, tissue inhibitor of metalloprotease.
and interleukin 6 receptor (30–32) is usually enhanced by phorbol 12-myristate 13-acetate (PMA), indicating common mechanisms. PMA is thought to activate protein kinase C (33, 34), a family of isoenzymes (35, 36) by substituting 1,2-diacylglycerol in its binding domain or by binding simultaneously to the C1 region of the kinase (37–39). These serine/threonine kinases are activated in response to numerous hormones, mitogens, and neurotransmitters (40–42). In contrast to the α-subunit and the homologous mouse meprin β subunit, human meprin β has potential regulatory elements in the cytosolic domain including two phosphorylation sites (Ser⁶⁸⁷ and Ser⁶⁸⁸) in PKC consensus sequences (18, 43). We have now analyzed the regulation of meprin β secretion. Our results provide evidence that hmeprin β cleavage at the cell surface can be markedly enhanced by PMA via the PKC pathway and that the constitutive and inducible release is mediated by two different metalloprotease activities. Furthermore, we provide evidence that tumor necrosis factor–α converting enzyme (TACE or ADAM) is the sheddase responsible for the PMA-induced hmeprin β release. We also find that PMA induces phosphorylation of hmeprin β and we have identified Ser⁶⁸⁷ as the main phosphorylation site. This phosphorylation step is not directly involved in the PMA-induced shedding of hmeprin β. It does, however, provide the potential for a coupling between signaling pathways and meprin activity. The potential of regulated shedding of hmeprin β in contrast to the constitutive secretion of hmeprin from the cell surface suggests different biological roles for the two meprin subunits in man.

**EXPERIMENTAL PROCEDURES**

**Materials**—[35S]Methionine (1,000 Ci/mmol) was from PerkinElmer Life Sciences, [32P]orthophosphate (carrier-free) from Amersham Biosciences. Cell culture media, penicillin, streptomycin, fetal calf serum, and LipofectAMINE were obtained from Invitrogen. Phenylmethane-sulfonfluride, pepstatin, aprotinin, leupeptin, benzamidine, diethyl sulfoxide (Me₂SO), and molecular weight standards were from Sigma. PMA and staurosporine, both from ALEXIS Corp. (Läufelfingen, Switzerland), were resolved in Me₂SO and kept as stock solutions of 1 mg/ml and 10 μM, respectively. TIMP1, TIMP2, and TIMP3 were obtained from R&D Systems (Minneapolis, MN). TAPI was a gift from Immunex, RO111-3456 a gift from Roche Diagnostics, and BB94 a gift from British Biotech. Endo-β-N-acetylglucosaminidase, N-glycosidase F, neuraminidase, and O-glycosidase were purchased from Roche Diagnostics. The protein A-Sepharose beads were obtained from Amersham Biosciences. All other chemicals were analytical grade from Merck (Dietikon, Switzerland). Primers used for mutagenesis were synthesized by MWG Biotech (Ebersberg, Germany).

**Construction of Hmeprin Mutants**—The potential phosphorylation sites in hmeprin β for protein kinase C: Ser⁶⁸⁷ and Ser⁶⁸⁸ were mutated into Arg⁶⁸⁷ and Ala⁶⁸⁸ (the respective amino acids in mouse meprin β) by the method of recombinant PCR (44). The mammalian expression vector pFPDH (15) was used as a template. Two PCR rounds were performed with the following primers (Ser⁶⁸⁷ mutant): lower primer, 5′-GCGTATGTCACATCAGC-3′; PPH″ArgL, 5′-TGGTGCAGTTGATCTC-ATCCTTTCCAGC-3′; and PPH″ArgU, 5′-TCGTAAGAGATGAGATCA-AATCGACC-3′; lower primer: 5′-TAATACGACTCACTATAGGGCG-3′.

**Ectodomain Shedding of Hmeprin β**

![Fig. 1. Effect of phorbol 12-myristate 13-acetate on secretion of human meprin](image-url)
In a second round these two PCR products were combined and amplified using the upper and lower primer. Finally the mutated fragment was cut with StuI and NotI and ligated back into the corresponding sites of pPPHβ. The mutations were confirmed by sequencing. The Ser⁶⁸⁷ mutation and the Ser⁶⁸⁷-Ser⁶⁸⁸ mutation were identically done with the following primers: PHβAlaL, 5'-TGGTCGATTTGCGCTCATCCTTTCA-3' and PHβArg/AlaL, 5'-TGGTCGATTTGCTCTCATCCTTTCA-3' and PHβArg/AlaU, 5'-TCGTGAAAGGATGAGAGCAAATCGACC-3'.

**Western Blot Analysis**—COS-1 cells transfected with hmeprinβ were stimulated with PMA (10 ng/ml) for 30 min before analyzing the effects of potential protease inhibitors on hmeprinβ shedding. After incubation of cells for 6 h in the presence of the inhibitors, the media and cells were collected. The cells were lysed in 1% deoxycholate, 1% Nonidet P-40 in homogenization buffer (50 mM NaCl, 25 mM Tris-HCl, pH 8) containing complete Mini EDTA-free protease inhibitor mixture (Roche) and centrifuged (13,000 rpm, 4 °C, 5 min). Aliquots of the media and cells were removed and subjected to 7.5% SDS-PAGE followed by transfer to Hybond-P membrane (Amersham Biosciences). The membrane was blocked in TBS/T containing 5% blocking agent NIF833 (Amersham Biosciences), followed by incubation with hmeprinβ antibody raised against its intervening region in blocking solution for 2 h at room temperature. After four washes in TBST, the membrane was incubated with anti-rabbit horseradish peroxidase (1:30000) in blocking solution. The ECL plus detection system (Amersham Biosciences) was used according to the guidelines to detect horseradish peroxidase activity.

**Cell Culture and Transfection**—COS-1 cells were grown in Eagle's minimal essential medium supplemented with 10% (v/v) fetal calf serum, 1% deoxycholate, 1% Nonidet P-40 in homogenization buffer (50 mM NaCl, 25 mM Tris-HCl, pH 8) containing complete Mini EDTA-free protease inhibitor mixture (Roche) and centrifuged (13,000 rpm, 4 °C, 5 min). Aliquots of the media and cells were removed and subjected to 7.5% SDS-PAGE followed by transfer to Hybond-P membrane (Amersham Biosciences). The membrane was blocked in TBS/T containing 5% blocking agent NIF833 (Amersham Biosciences), followed by incubation with hmeprinβ antibody raised against its intervening region in blocking solution for 2 h at room temperature. After four washes in TBST, the membrane was incubated with anti-rabbit horseradish peroxidase (1:30000) in blocking solution. The ECL plus detection system (Amersham Biosciences) was used according to the guidelines to detect horseradish peroxidase activity.

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**Immunoprecipitation and SDS-PAGE**—Immunoprecipitation was conducted as described previously (18, 19). In [³²P]phosphate-labeled
cells, lysis was performed in the presence of 10 mM NaF, 20 mM calyculin, and 1 mM Na3VO4 to inhibit phosphatases, followed by centrifugation in a Microspin column P-30 (Bio-Rad) to remove unincorporated nucleotides before immunoprecipitation. Immunoprecipitated proteins were analyzed on 7.5% polyacrylamide gels under reducing conditions.

Electrophoresis, fixation, and fluorography were carried out as described before (19).

**Glycosidase Treatments**—After immunoprecipitation the samples were treated with endoglycosidase H essentially as described before (18, 19) and prepared for electrophoresis. For N-glycosidase F and combined O-glycosidase/neuraminidase/N-glycosidase F treatment the immunoprecipitated proteins were boiled in 25 μl of 20 mM sodium phosphate, pH 7.2, 0.5% SDS. The samples were diluted in 100 μl of 20 mM sodium phosphate, pH 7.2, 1% Nonidet P-40, 50 mM EDTA and divided into equal aliquots. N-Glycans were digested by treatment with 1 unit of N-glycosidase F for 24 h at 37 °C. Combined digestion of O-linked oligosaccharides and N-glycans was obtained by treatment with 2 units of neuraminidase, 2.5 units of O-glycosidase, and 1 unit of N-glycosidase F for 24 h at 37 °C.

**Densitometric Measurements and Molecular Weight Determination**—

**Fig. 2. Effect of protease inhibitors on the release of human meprinβ in transfected COS-1 cells.** A, representative Western blot of aliquots from cells and media of COS-1 cells transfected with hmeprinβ. The cells were incubated for 6 h in the presence of protease inhibitors as indicated (lanes 1–7). Hmeprinβ was detected with a specific antibody using ECL plus (Amersham Biosciences). Media were ultracentrifuged (100,000 × g) to remove insoluble cell components. B, TACE(−/−) cells transfected with hmeprinβ cDNA were metabolically labeled for 1 h. The PMA-stimulated cells were incubated with/without 50 μM TAPI (lanes 2 and 3). After a chase of 6 h hmeprinβ was immunoprecipitated from cells and media and analyzed by SDS-PAGE. C, Western blot of COS-1 cells transfected with hmeprinβ. The cells were stimulated with PMA for 30 min followed by treatment with TIMP1, TIMP2, and TIMP3 for 6 h. Hmeprinβ was detected with a specific antibody using ECL plus (Amersham Biosciences).
FIG. 3. Pulse-chase labeling of human meprinβ in transfected COS-1 cells in the presence and absence of phorbol 12-myristate 13-acetate. Representative fluorographs of COS-1 cells transiently transfected with hmeprinβ. The cells were processed as described in the legend to Fig. 1, followed by a chase of 0 h (A) and 3 h (B). After immunoprecipitation, protein samples were divided and the aliquots were treated with the indicated enzyme(s) before separation on 7.5% SDS-PAGE and analysis by fluorography. C, fluorographs shown in A and B were densitometrically scanned. Protein bands of uncleaved (□) and cleaved (■) hmeprinβ in cell extracts were added and set as 100% of total protein, before the ratio of cleaved and uncleaved protein was calculated.
Densitometric measurements were carried out with Lumi Analyst version 3.0 from Roche. Fluorographic signals were scanned densitometrically with the Lumi Image F1 from Roche using Roche’s Lumi Analyst software version 3.0. Total protein amount (100%) was calculated by software version 3.0 from Roche. Fluorographic signals were scanned densitometrically.

**RESULTS**

**Up-regulation of Hmeprinβ Shedding by PMA**—Expression and secretion of hmeprina and β were analyzed in transfected COS-1 cells using a pulse-chase protocol (Fig. 1). The cell-associated fraction of hmeprina consisted of two bands with apparent molecular masses of 100 and 90 kDa (Fig. 1A, lane 1–6). The 100-kDa band represents the uncleaved, and the 90-kDa band the cleaved form of hmeprina as demonstrated previously (14, 19, 46). A 95-kDa form was detected in filtered medium after 1 h of chase (Fig. 1A, lane 7). Treatment with PMA had no effect on hmeprina secretion (Fig. 1A, lanes 8 and 10) as demonstrated also by densitometric analysis of the fluorographic signals (Fig. 1B).

In COS-1 cells expressing hmeprinβ the cell fraction consisted of two bands with apparent molecular masses of 105 and 95 kDa (Fig. 1C, lane 1–6). Treatment with PMA resulted in an enhanced secretion of the major species of hmeprinβ of 95 kDa (Fig. 1C, lane 10 and 12), which was matched by a corresponding decrease of cell-associated protein. PMA-induced stimulation of hmeprinβ secretion was verified by densitometric analysis (Fig. 1D). In untreated COS-1 cells most of the synthesized hmeprinβ remained associated with the cellular membrane and only a minor fraction was secreted. After 3 h of chase the amount of hmeprinβ secreted into the medium was ~6.5-fold higher in PMA-treated cells compared with untreated cells.

**Role of Metalloproteases in Secretion of Hmeprinβ**—To characterize the enzymes responsible for constitutive and PMA-induced shedding of hmeprinβ, we have analyzed the effects of several protease inhibitors on the shedding of hmeprinβ in transfected COS-1 cells (Fig. 2). The possibility of autocatalytic cleavage can be ruled out; in transfected cells hmeprinβ is expressed as a zymogen and therefore enzymatically inactive. Besides the general metalloprotease inhibitors EDTA, 1,10-phenanthroline, or dithiothreitol (data not shown) shedding was also inhibited by the MMP- and ADAM-targeting hydroxamate inhibitors RO111-3456 (a derivative of RO31-970), BB94, and TAPI (Fig. 2A, lanes 4–6). The natural tissue inhibitors of MMPs, TIMP1, TIMP2, and TIMP3 had no effect on hmeprinβ release (Fig. 2A, lanes 1–3). The activity of the TIMPs was ascertained in a MMP-2 control assay (data not shown). The inhibition of hmeprinβ shedding by RO111-3456, BB-94, and TAPI was accompanied by an accumulation of the mature cell-associated form of hmeprinβ (105 kDa) (Fig. 2A, lanes 4–6). These results practically exclude an involvement of MMPs in the constitutive shedding of hmeprinβ.

The hydroxamate-type metalloprotease inhibitor TAPI (47) also inhibited hmeprinβ release. To test a possible involvement of TACE in hmeprinβ shedding, TACE(−/−) cells were transfected with hmeprinβ cDNA. These transfected TACE(−/−) cells still secreted hmeprinβ (Fig. 2B, lane 1) but this secretion was unaffected by PMA treatment (Fig. 2B, lane 2). This and the inhibition of hmeprinβ secretion in TACE(−/−) cells by TAPI (Fig. 2B, lane 3) indicates that TACE function is required for PMA inducible shedding but not for the constitutive hmeprinβ shedding. The constitutive shedding is mediated by a yet unknown metalloprotease. In contrast to unstimulated conditions where TIMP3 had no effect on hmeprinβ release, the stimulatory effect of PMA in secretion of hmeprinβ was inhibited by TIMP3 (Fig. 2C, lane 4) (48). As TIMP3 has also been shown to inhibit TACE, these data support the involvement of TACE in the PMA-induced shedding of hmeprinβ.

**PMA Treatment Does Not Affect the Ratio of Intracellular Cleavage of Human Meprinβ**—To study the effect(s) of PMA treatment on proteolytic processing and secretion of the meprin β-subunit, pulse-chase experiments in the presence or absence of PMA followed by subsequent digestion with different endoglycosidases were carried out. The two 105- and 95-kDa forms were detected after a pulse of 1 h with [35S]methionine (Fig. 3A, lanes 1 and 2). The 95-kDa band was sensitive to Endo H treatment and shifted to 78 kDa (Fig. 3A, lanes 3 and 4), indicating that this was the high mannose form of hmeprinβ. The 105-kDa band was partially resistant to Endo H digestion and represents a complex glycosylated form of hmeprinβ that still contains some high-mannose glycans. After digestion with N-glycosidase F we observed three bands with molecular masses of 83, 78, and 70 kDa, respectively (Fig. 3A, lanes 5 and 6). These represent uncleaved and cleaved forms of hmeprinβ with differing glycosylation status. Complete deglycosylation with N-glycosidase F/neuraminidase/O-glycosidase yielded two forms at 78 and 68 kDa, respectively (Fig. 3A, lanes 7 and 8). The same molecular weight pattern was observed after PMA treatment of these cells. After 3 h of chase secreted hmeprinβ was detectable in the culture medium (Fig. 3B, lanes 9 and 10). This secreted form did not contain O-glycans as seen by the insensitivity to O-glycosidase digestion (Fig. 3B, lanes 15 and 16).

To analyze the ratio of cleaved and uncleaved hmeprinβ in cell extracts in the presence and absence of PMA only the signals after complete deglycosylation (Fig. 3, A and B, lanes 7 and 8) were compared densitometrically (Fig. 3C). PMA-treated cells thus showed no significant differences in the ratio of proteolytic processing of hmeprinβ. These results indicate that the intracellular ratio of uncleaved and cleaved protein is unchanged by PMA treatment, whereas the ectodomain shed-
Involvement of PKC in the PMA-induced Release of Human Meprin—The involvement of protein kinase in the PMA-induced generation of soluble hmeprin/H9252 was validated using the general protein kinase inhibitor staurosporine, one of the most potent protein kinase C inhibitors (49, 50) (Fig. 4). Unstimulated secretion of hmeprin/H9252 occurred at a low level (Fig. 4, lanes 10 and 13). This secretion was strongly enhanced by PMA (Fig. 4, lanes 11 and 14), and the induced release was reduced to basal levels of secreted protein by staurosporine (Fig. 4, lane 12 and 15). These findings suggest that activation of a protein kinase, presumably PKC, mediates PMA-induced hmeprin/H9252 shedding.

PMA Treatment Induces Phosphorylation of Human Meprin—To clarify the action of PMA, we investigated the phosphorylation of hmeprin in transfected COS-1 cells. The cytoplasmic domain of hmeprin contains PKC consensus sequences with two potential PKC phosphorylation sites (Ser687 and Ser688). COS-1 cells were transiently transfected with hmeprin and metabolically labeled with [32P]orthophosphate in the presence of PMA followed by immunoprecipitation with a polyclonal antibody that recognizes both meprin subunits (Fig. 5). In COS-1 cells expressing hmeprin, the cytoplasmic tail of hmeprin lacks a PKC phosphorylation sequence) no incorporation of [32P] was observed (Fig. 5, lane 6). In contrast, COS-1 cells transiently transfected with the β-subunit of hmeprin led to a 105-kDa precipitable phosphorylated protein after PMA treatment (Fig. 5, lane 7). By labeling COS-1 cells transfected with mutant constructs lacking the potential phosphorylation sites, we identified Ser687 as the main 32P-acceptor. These data provide evidence that the cytoplasmic domain of hmeprin can function as a PKC substrate.

Up-regulation of Hmeprin Release Is Independent of the Cytoplasmic Tail or Its Phosphorylation—To study if the phosphorylation of hmeprin is associated with the regulation of its shedding, we analyzed the potential of PMA to induce secretion of hmeprin containing the mutated phosphorylation sites. Elimination of Ser687 and Ser688 had no considerable influence on the enhanced shedding of hmeprin following PMA stimulation (Fig. 6, A and B). This result supports the view that the release of human meprin is independent of its phosphorylation status and that shedding occurs by an ADAM (in all probability TACE) whose activity is stimulated by PMA via the PKC pathway. PMA treatment of cells transfected with a tail switch mutant where the transmembrane and cytosolic domains were replaced by the homologous counterparts from the H9251 subunit (19), also resulted in induced shedding of the hmeprin-ectodomain into the medium (data not shown). This clearly shows that neither the cytoplasmic domain nor the phosphorylation site contained herein is involved in the PMA induced up-regulation of hmeprin secretion.

**Fig. 5.** Detection of phosphorylated human meprinβ after phorbol 12-myristate 13-acetate treatment. A, COS-1 cells, transiently transfected with hmeprinα, β, and phosphorylation mutants were metabolically labeled with 0.4 mCi of [32P]orthophosphate for 3 h in the presence of phorbol 12-myristate 13-acetate (10 ng/ml). Immunoprecipitates of cell lysates were prepared in the presence of phosphatase inhibitors (10 mM NaF, 20 nM calyculin, 1 mM Na3VO4) and separated by 7.5% SDS-PAGE before analysis by fluorography. For control reasons immunoprecipitations were also done of COS-1 cells transfected with the same constructs and labeled overnight with [35S]methionine (shown on the left). B, sequence comparison of the cytosolic tails of human meprinβ, the phosphorylation mutants, and mouse meprinβ.
We have previously reported that meprin/\(H9251\) and meprin/\(H9252\) are secreted in a polarized fashion from transfected Madin-Darby canine kidney cells (14, 18). Secretion of the meprin/\(H9252\) subunit was specific to the human enzyme, as mouse meprin/\(H9252\) was not secreted. Proteolytic processing of hmeprin/\(H9252\) occurs intracellularly and at the cell surface and requires a stretch of 13 amino acids (QIQLTPAPSVQDL) in the intervening sequence (20). This is in contrast to hmeprin/\(H9251\), which is cleaved in the endoplasmic reticulum by a process that is dependent on the \(\alpha\)-spe-

**Fig. 6.** Influence of phosphorylated human meprin\(\beta\) on its secretion after phorbol 12-myristate 13-acetate treatment. A, representative fluorograph showing immunoprecipitated proteins from COS-1 cells, transiently transfected with hmeprin/\(\beta\) wild type and phosphorylation mutants. The cells were metabolically labeled with 50 \(\mu\)Ci of \([35S]\)-methionine for 1 h, treated with (+) or without (−) phorbol 12-myristate 13-acetate (10 ng/ml) for 30 min, and chased in complete culture medium for 4 h. Immunoprecipitated proteins from the cells and the cell culture media were submitted to 7.5% SDS-PAGE and analyzed by fluorography. (To notice, the apparent increase in secretion of hmeprin\(\beta\) Ser\(^{687}\)/Arg\(^{687}\) in PMA-stimulated cells is because of the higher concentration of hmeprin\(\beta\) present in the cells.) B, fluorographs of independent experiments were densitometrically scanned. Protein bands in cell extracts and in the corresponding media were added and set as 100% of total protein. The distribution of cellular and media forms were estimated before calculating the \(x\)-fold induction of secretion of hmeprin\(\beta\) upon PMA stimulation. Shown are the means of two, respectively, of three independent experiments.

**DISCUSSION**

We have previously reported that meprin\(\alpha\) and meprin\(\beta\) are secreted in a polarized fashion from transfected Madin-Darby canine kidney cells (14, 18). Secretion of the meprin\(\beta\) subunit was specific to the human enzyme, as mouse meprin\(\beta\) was not secreted. Proteolytic processing of hmeprin\(\beta\) occurs intracellularly and at the cell surface and requires a stretch of 13 amino acids (QIQLTPAPSVQDL) in the intervening sequence (20). This is in contrast to hmeprin\(\alpha\), which is cleaved in the endoplasmic reticulum by a process that is dependent on the \(\alpha\)-spe-
specific insertion domain and the transmembrane region (15–17, 19, 51). In the present study we show that secretion of hmeprin\(\beta\), in contrast to the \(\alpha\)-subunit, is affected by phorbol ester treatment. PMA treatment dramatically increases the amount of secreted hmeprin\(\beta\), whereas secretion of hmeprin\(\alpha\) is not influenced. These results clearly support the notion that processing and secretion of the two subunits are different. A large number of secreted proteins are derived from integral plasma membrane protein precursors (52–56) and are released upon treatment of cells with phorbol esters such as PMA, presumably by activating protein kinase C, a cytosolic protein that becomes membrane associated after activation and rapidly phosphorylates intracellular target proteins (42). To clarify the involvement of a kinase in the regulation of hmeprin\(\beta\) shedding, staurosporine inhibition experiments were carried out. The effect of PMA-induced shedding of hmeprin\(\beta\) was antagonized by staurosporine, confirming that protein kinase C is involved in the regulation of hmeprin\(\beta\) shedding.

Shedding appears to occur at or near the cell surface. The proteases involved are often ADAMs. They have been shown to play important roles in the phorbol ester-stimulated shedding or release of membrane-anchored precursor proteins (57). The observation that TIMP1, TIMP2, nor TIMP3 inhibit constitutive hmeprin\(\beta\) shedding practically rules out an involvement of MMPs in this process. This notion is further supported by the data with TAPI that showed an inhibition of hmeprin\(\beta\) shedding. One metalloprotease that is inhibited by TAPI is ADAM 17 (TACE), identified by its ability to release the inflammatory cytokine tumor necrosis factor \(\alpha\) (47). Other structurally unrelated proteins like transforming growth factor \(\alpha\) and L-selectin are also shed by TACE (58). This prompted us to analyze the ability of hmeprin\(\beta\)-transfected TACE\((\sim\sim)\) cells to secrete hmeprin\(\beta\). An diminished secretion of hmeprin\(\beta\) was observed in these cells not stimulated by PMA, indicating that constitutive and stimulated hmeprin\(\beta\) shedding are mediated by different metalloproteases. The protease responsible for the induced shedding is in all probability TACE. This is supported by the fact, that TIMP3, an inhibitor of TACE, has no effect on the constitutive shedding but inhibits the PMA-stimulated shedding of hmeprin\(\beta\) in transfected COS-1 cells. Such observations have also been made by others. Garton et al. (59) demonstrated that shedding of fractalkine (CX3CL1) may be stimulated by PMA and that TACE is the shedding enzyme that mediates the PMA-induced but not the constitutive release. The constitutive sheddase of hmeprin\(\beta\) remains to be determined. It may be an ADAM or another TIMP3-sensitive metalloprotease.

As we have shown recently, shedding of hmeprin\(\beta\) is also influenced by its glycosylation status (60). In contrast to hmeprin\(\alpha\) and mouse meprin\(\alpha\) and meprin\(\beta\), hmeprin\(\beta\) bears O-glycan side chains. If the formation of O-glycan side chains during post-translational processing of hmeprin\(\beta\) in transfected Madin-Darby canine kidney cells was prevented by benzyl-N-acetyl-\(\alpha\)-\(\beta\)-galactosamide an increase in secretion of the enzyme was observed, suggesting that these carbohydrate chains have a "protective" role against proteolytic cleavage within the intervening region of hmeprin\(\beta\). Two O-glycosylation sites at Thr\(^{599}\) and Ser\(^{603}\) were identified within the 13-amino acid peptide found essential for hmeprin\(\beta\) shedding. Transfection of COS-1 cells with a mutant enzyme in which the 13 amino acids had been deleted resulted in a protein that was (a) not O-glycosylated and (b) not secreted. As the secreted ectodomain of hmeprin\(\beta\) (wt) was not O-glycosylated (Fig. 3, lane 16), these two O-glycosylation sites must be in the C-terminal stub remaining bound to the plasma membrane. As the meprin\(\beta\) mutant lacking this 13-amino acid peptide sequence is not shed (60), the cleavage site leading to the release of the enzyme may be narrowed down further and must be localized between Thr\(^{584}\) and Thr\(^{599}\) (Fig. 7).

Human meprin\(\beta\), in contrast to hmeprin\(\alpha\), has two potential phosphorylation sites (Ser\(^{687}\) and Ser\(^{688}\)), within the protein kinase C phosphorylation site motives in the cytosolic tail: XR/K\(^{687}\)XS\(^{687}\)/XR/K\(^{687}\) and R/K\(^{688}\)XS\(^{688}\)/T (18, 43, 61). This suggests a role of hmeprin\(\beta\) in signal transduction and/or regulation of proteolytic activity. This phosphorylation motif is present exclusively in human meprin\(\beta\), but not in mouse meprin\(\beta\), indicating a possible involvement of phosphorylation/dephosphorylation reactions in the species-specific function of this subunit. We have now demonstrated phosphorylation of hmeprin\(\beta\) in transfected COS-1 cells after stimulation with PMA. This is the first report showing phosphorylation of a member of the astacin family. Serine 687 was identified as the main \(\text{Y}^{\text{P}}\) acceptor site. Phosphorylation of proteins on serine and threonine residues is seen to trigger changes in allosteric conformations (62, 63). A conformational change in hmeprin\(\beta\) because of phosphorylation events may mediate easier access of an appro-
priate shedding enzyme to the ectodomain-cleavage site. To address this issue, the amounts of secreted hmeprin in COS-1 cells, transfected with the mutants, were compared. However, neither the single nor the double mutants showed any significant difference in secretion, ruling out a direct influence of the phosphorylated cytoplasmic tail in the secretion of hmeprin. The role of phosphorylation of hmeprin thus remains open to speculation. Indirect evidence has been presented for the regulation of catalytic activity by phosphorylation, of AB-M2 (testase1), a plasma membrane-anchored sperm protease (64). It is conceivable that phosphorylation may, under certain physiological conditions, affect the activity of hmeprin. The only direct activation step, known so far for meprins is the removal of the pro-domain by trypsin or plasmin (Fig. 7) (14, 65). Another intriguing possibility is that modification of hmeprin of the pro-domain by trypsin or plasmin (Fig. 7) (14, 65). An- other intriguing possibility is that modification of hmeprin expression of soluble and membrane-bound meprin expressed in leukocytes of the lamina propria (12). Differential thus shedding probably plays a subordinate role in intestinal secretion in relation to potential substrates in a given cell or tissue, may have consequences that lead to a pathological situation.

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