Critical Amino Acids in the Active Site of Meprin Metalloproteinases for Substrate and Peptide Bond Specificity*

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SUMMARY

The protease domains of the evolutionarily-related α and β subunits of meprin metalloproteases are approximately 55% identical at the amino acid level, however, their substrate and peptide bond specificities differ markedly. The meprin β subunit favors acidic residues proximal to the scissile bond, while the α subunit prefers small or aromatic amino acids flanking the scissile bond. Thus gastrin, a peptide that contains a string of five Glu residues, is an excellent substrate for meprin β while it is not hydrolyzed by meprin α. Work herein aimed to identify critical amino acids in the meprin active sites that determine the substrate specificity differences. Sequence alignments and homology models, based on the crystal structure of the crayfish astacin, showed electrostatic differences within the meprin active sites. Site-directed mutagenesis of active site residues demonstrated that replacement of a hydrophobic residue by a basic amino acid enabled the meprin α protease to cleave gastrin. The meprin αY199K mutant was most effective; the corresponding mutation of meprin βK185Y resulted in decreased activity toward gastrin. Peptide cleavage site determinations and kinetic analyses using a variety of peptides extended evidence that meprin αTyr199/βLys185 are substrate specificity determinants in meprin active sites. These studies shed light on the molecular basis for the substrate specificity differences of astacin metalloproteinases.
INTRODUCTION

The meprin α and β subunits are zinc metalloendopeptidases of the ‘astacin family’ and ‘metzincin superfamily’ that can form homo- and heterooligomeric complexes (1, 2). The subunits derived from a common multidomain ancestor protein, but have evolved to have markedly different substrate and peptide bond specificities, structural properties, chromosomal locations, and membrane associations (3-5). Meprin β has a preference for hydrolysis of peptide bonds containing acidic amino acid residues, whereas meprin α prefers to cleave bonds flanked by small or hydrophobic residues. The meprin α subunit has a propensity to form large homooligomeric complexes containing 12 to 100 subunits, while meprin β alone forms only homodimers (5). Both subunits are synthesized as membrane-spanning type 1 proteins in the endoplasmic reticulum, however, while the meprin β subunit remains membrane-bound through the secretory pathway and at the plasma membrane, the meprin α subunit is proteolytically processed during biosynthesis and thus loses its transmembrane domain and is secreted unless it associates with meprin β (6). Meprin α is encoded on chromosome 17 in mice and 6 in humans near the histocompatibility complex; meprin β is encoded on chromosome 18 in mice and humans (7). While both meprin subunits are expressed in embryonic kidney proximal tubule cells and intestinal epithelial cells, the subunits are expressed differentially postnatally, and one or the other subunit appears to be upregulated in cancer cells (8-10). For example, meprin α is expressed and secreted in colon cancer cells, while meprin β is expressed in breast cancer cells. The different enzymatic properties,
expression profiles, and localization of the subunits indicate different functions for meprin α and β.

Both meprin subunits cleave a variety of peptides and proteins and have a preference for peptides larger than 6 amino acids, indicating extended substrate binding sites (3, 11). The best peptide substrates identified for meprin α were gastrin-releasing peptide, cholecystokinin, glucagon, substance P and valosin. The first three of the latter peptides are also substrates for meprin β, the last two are not (3, 4). Gastrin is by far the best substrate identified for meprin β, and it is not cleaved by mouse meprin α. Cytokines are also substrates for meprins implicating them in inflammatory and immune system processes (12). For example, monocyte chemoattractant protein-1 (MCP-1) is cleaved by meprin α, while osteopontin is a substrate for meprin β (3). Azocasein and gelatin are often used as substrates to assay both forms of meprin. Other protein substrates for both include extracellular proteins, such as collagen type IV, laminin 1 and 5, fibronectin, and nidogen. Meprins are known to play a critical role in development, and have been implicated in cancer metastasis, inflammatory bowel disease, and in kidney diseases (8, 12-16). The increasing knowledge accumulating about the regulation and substrate specificities of these enzymes will help to define the role of these proteinases in physiologic and pathological processes.

The aim of the study herein was to shed light on the molecular basis for the differences in the specificity of the subunits. Homology models and sequence alignments of the meprin active sites were examined for potential residues affecting peptide bond specificity of the subunits. Three amino acid residues in positions within the active sites were identified as potential contributors to the differences in specificities of meprins α
and β. These residues were studied by mutational and kinetic analyses of purified recombinant secreted forms of meprin α and β expressed in human 293 cells.
EXPERIMENTAL PROCEDURES

Reagents and Materials – Peptides, proteins, and reagents were purchased from Sigma Chemical Co. with the following exceptions: pcDNA 3.1+ plasmid, Dulbecco’s Modified Eagle Medium (DMEM), DMEM/F-12 and Geneticin (G418) were purchased from Invitrogen; QuickChange Site-Directed Mutagenesis Kit from Stratagene; HiTrap chelating column from Amersham Pharmacia Biotech.

Plasmid Construction and Mutagenesis – The cDNA for mouse meprin α truncated at 615 was constructed with a His-tag at the carboxy terminus. The primers 5’-GCAATAAGCTTACCATGGCAAGGAGGCTTGGGCAGATCC-3’ and 5’-GTCTGCTCGAGTCAATGATGATGATGATGCCGGTTCAGGTGGGTGAGGT C-3’ were used to amplify the truncated cDNA from the full length mouse meprin α cDNA. The restriction sites for Hind III and Xho I were utilized to ligate the PCR product into pcDNA 3.1+. The cDNA for the rat meprin β subunit was previously described (17). The active site mutants were constructed using the QuickChange Site-Directed Mutagenesis Kit. Mutagenic primers mouse meprin αY199K were 5’-GTTGGGATCAAATCATTACAGACAAGGAACATAATTTC-3’ and 5’-GAAATTATGTTCCTTGTCTGTAATGATTTGATCCCACC-3’. The αF161R mouse meprin primers were 5’-CCATTGGTGAGGGATGTGACTTTAAGGCCACCATTG-3’ and 5’-CAATGGTGCCCTAAAGTCACATCCCTACCCACCATTG-3’. Mouse meprin αP228K primers were 5’-GAGTCCCTGATGCACTACCGGCATTTCATGTTTAACAAAGACACAC-3’ and 5’-GGTCTTGTAAAAAGAAAATGCGCCGTAGTCATCCAGGACTC-3’. The primers
5′-GGGACAGGATCTTGTCCGGCTACGAAC ACAATTTCAACATC-3′ and 5′-GATGGTGAAATTGTGTTCGTAGCCGGACAAGATCCTGTCCC-3′ were used to mutate the His–tagged rat meprin β plasmid to construct the βK185Y mutant. All constructs were fully sequenced.

**Stable Line Production and Purification** – Human Embryonic Kidney 293 cells (ATCC 1573 CRL) were maintained in DMEM supplemented with 10% Fetal bovine serum, 50 units/ml penicillin and 50 µg/ml streptomycin (complete DMEM) in a 37 °C incubator with 5% CO₂. Meprin cDNAs were transfected into 293 cells by the calcium phosphate precipitation method using 10 µg of the appropriate expression plasmid in 100 mm tissue culture plates. Cells were treated with trypsin 24 h after transfection and split 1:500 for selection in 0.5 mg/ml G418. Stable clones were selected and grown in the presence of G418. Stable clones were split onto 500 cm² plates. Once the cells became 90% confluent, DMEM F12 supplemented with G418 lacking FBS was added. Meprin was detected by western blot analysis. Media were collected and the protein purified utilizing the His-tag as previously described (17). Purity was assessed by SDS-PAGE in the presence of 2-mercaptoethanol and Coomassie Brilliant Blue staining of gels.

**Activation, Enzymatic Assays and Identification of Cleavage Sites** – To activate the purified meprins, trypsin was added at a molar ratio of 1:20 trypsin to meprin in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5. After incubation at 25 °C for 30 min, soybean trypsin inhibitor (type II-S, Sigma) was added at 2-fold excess over trypsin. As a control, promeprins were incubated with trypsin preincubated with soybean trypsin inhibitor; no meprin proteolytic activity was observed under these conditions. Azocaseinase activity was measured as described previously (18). Kinetic analyses for peptide hydrolysis were
determined by quantitative HPLC analysis (3). Cleavage sites were determined as previously described using a Perceptive Biosystems Linear Voyager matrix-assisted laser desorption ionization time of flight (MALDI-TOF) machine with continuous extraction (3).

*Homology models of astacin family protease domains* - The protease domain structures of astacin family members were determined using knowledge-based homology modeling. The input consisted of a sequence alignment of the catalytic domains of the astacin family member to be modeled and the coordinates obtained from the Brookhaven Protein Data Bank for the crystal structure of astacin (accession number 1AST; 19). The model of the mouse α subunit has been described in detail elsewhere (3). For other members modeling was performed using SwissPdbViewer 3.2b and the online modeling program SWISS MODEL in the optimize mode (20). All representations were prepared using WebLab Viewer 4.0 (Molecular Simulations Inc., San Diego, CA).
RESULTS

Basic Amino Acid Differences within the Active Sites of Meprins α and β Revealed by Sequence Alignment and Homology Models - The protease domains of the meprin α and β subunits of mouse, rat and human have a high degree (approximately 55%) of amino acid sequence identity (Fig. 1). Six positions were identified in the protease domain in which the basic residues Arg or Lys were conserved within meprin β for all species, while the corresponding meprin α amino acid residues were not basic. At these sites, the equivalent residues are meprin αM111, a deletion at 150, αF/Y161, αQ/E194, αY199 and αP228. Because of the preference of meprin β for negatively charged substrates, it was reasoned that the identified basic amino acid residues may be important for peptide bond specificity.

Homology models of meprin protease domains, based on the crystal structure of astacin, were utilized to determine the positions of the potentially critical basic residues in meprin β implicated by the sequence alignments (Fig. 2). Three residues, denoted by arrows, which were electrostatically different in the subunits and located within the active site were proposed to be potentially critical to substrate interactions. The three residues are αF161, αY199, and αP228; the corresponding residues are βR147, βK185 and βK214. The localization of these basic amino acids in the meprin β subunit implicates these residues in acidic substrate specificity. The αF161 residue (βR147) is located at a potentially critical position within the central α-helix containing two of the His zinc ligands and the catalytic Glu residue. Residue αY199 (βK185) protrudes into the active site from the lower floor of the cleft. Residues αP228 and βK214, also at the floor of the
active site cleft, are located just two residues after a tyrosine residue in the SxMHY sequence of the ‘Met turn’, a structural feature of astacin proteases involved in transition state stabilization (19). These three potentially critical residues were mutated in mouse α to the corresponding basic meprin ß residue to determine whether they contribute to subunit substrate specificity differences.

*General Proteolytic Activity of Mutants* - The three mutants, αF161R, αY199K and αP228K, were expressed in 293 cells and purified utilizing a His-tag placed at the mature COOH- terminus (17, 21). To determine overall stability and activatibility, the mutant proteins were subjected to proteolysis by trypsin. Each mutant was enzymatically activated and decreased in mass by trypsin to the same extent as wild-type meprin (data not shown), indicating that the prosequence was removed; there was no evidence of extensive degradation of these mutants which has been observed to occur with unstable mutants (22). To determine whether the mutations affected overall proteolytic activity, hydrolysis of the protein substrate azocasein was examined (Table I). Mutants αY199K and αF161R degraded azocasein with specific activity similar to wild-type meprin α, while the αP228K mutant hydrolyzed the protein substrate less efficiently. This decreased activity may be due to an alteration in the orientation of Tyr residue involved in the transition state in the conserved ‘Met turn’ sequence thus affecting the catalytic efficiency (23, 24). Overall, however, these results indicate that the 3 meprin α mutants are proteolytically active and there are no major changes in stability of the proteins to trypsin.

*Mutations of Amino Acid Residues Identified Above Enable Meprin α Hydrolysis of Gastrin 17.* Several peptides were incubated with wild-type mouse meprin α,
αY199K, αF161R, and αP228K to further examine whether substrate specificity was altered by the mutations (Table II). The substrates tested had previously been determined to be either cleaved by meprin β only (gastrin and orcokinin), cleaved by both meprin α and β (cholecystokinin), or cleaved by meprin α only (neurotensin, LHRH, bradykinin). The most marked affect was that all three meprin α active site mutations were able to cleave the β-specific peptide gastrin 17. The meprin αY199K mutant was the most effective against gastrin. By contrast, another meprin β specific peptide, orcokinin, was not cleaved by any of the mutants. Meprin β has a much greater affinity for gastrin 17 compared to orcokinin (K_M of 7 µM compared to 100 µM, respectively), and this might contribute to the differences observed for the meprin α mutants (3). In general, the αY199K mutant was more effective against the other substrates tested (cholecystokinin, neurotensin, LHRH) than the wild-type enzyme or the other mutants. The αP228K mutant either hydrolyzed the peptides to the same extent as the wild-type protein, or it was 2-fold less effective. The αF161R mutant was more effective in cleaving cholecystokinin and less effective in cleaving neurotensin than the wild-type enzyme.

To better determine the interactions of peptide substrates with the mutant meprins, kinetic parameters were determined (Table III). Velocity was determined by evaluating the loss of substrate rather than the appearance of product due to the presence of multiple cleavage sites. For the αY199K mutant, the K_M for gastrin was 440 µM and k_cat was 10.8 x 10^5 M^-1 s^-1. This K_M is much higher than for meprin β (1 µM) while the k_cat values for αY199K and meprin β are comparable. The αY199K mutant was the only one for which the gastrin parameters k_cat and K_M could be determined due to the solubility limit of the
peptide. However, the specificity constant ($k_{cat}/K_M$) of αF161R was determined to be $0.07 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ by the method of Fersht (25); 3-fold lower than for αY199K.

The catalytic effectiveness of αY199K against cholecystokinin was approximately 7-fold greater than the wild-type enzyme and the αP228K mutant and 2-fold greater than αF161R (Table III). All the mutants had lower $K_M$ values than the wild-type enzyme, and the αF161R and αY199K mutants showed modest increases in $k_{cat}$ values. The increased catalytic efficiency of the mutants was primarily due to greater affinity for the substrate.

**Effects of Mutation of Meprin βK185Y** — Because the αY199K mutant was the most effective in enhancing activity of meprin α towards gastrin and other peptides, the equivalent residue in mepirn β (βK185Y) was mutated. The wild-type rat meprin β and the βK185Y mutant had similar activity against azocasein (Table IV). The βK185Y mutant also retained the ability to hydrolyze gastrin and cholecystokinin. In addition, this mutant hydrolyzed αMSH more rapidly than the wild-type meprin β, and was able to cleave neurotensin unlike the wild-type β protein.

Mutation of the βK185 residue to Tyr significantly reduced the specificity constant for gastrin from 105 to $8.9 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ (Table V). The $k_{cat}$ for gastrin hydrolysis was not substantially affected, but the $K_M$ was increased 20-fold by the single amino acid replacement reflecting decreased binding affinity. To more fully examine the electrostatic interactions, salt concentrations were varied in the gastrin reactions. The increase in NaCl from 150 mM to 500 mM considerably decreased the $k_{cat}/K_M$ (18-fold) for meprin β, while the βK185Y kinetic constants were only mildly affected by this difference in salt concentration. In the presence of 500 mM NaCl, the $k_{cat}$ values for the
meprin β hydrolysis of gastrin were unaffected, but the $K_M$ value increased 18-fold. By contrast, the $K_M$ of the βK185Y mutant was increased less than two-fold in the presence of 500 mM NaCl. The Lys substitution and increase in NaCl concentration increased the meprin β $K_M$ for hydrolysis to approximately the same extent indicating that βK185 is critical in the electrostatics governing substrate interactions with meprin.

The kinetic constants for hydrolysis of CCK8s and αMSH were also determined for meprin β and the βK185Y mutant. The amino acid replacement resulted in a 1.6-fold increase in $k_{cat}/K_M$ for CCK8s and a 13-fold increase in this value for αMSH. Therefore, while the βK185Y mutant cleaved gastrin less effectively than the wild-type protein, it was more effective for other substrates.

Cleavage Site Analysis Demonstrates the Changes in Substrate Specificity for the Active Site Mutants. Determination of cleavage sites within the peptides gastrin, CCK8s and αMSH were determined through the separation of products by HPLC and identification by MALDI-TOF (Table VI). This analysis revealed that the αY199K mutant hydrolyzed some of the same bonds within the gastrin peptide as meprin β, however, there was a cleavage site COOH-terminal to the Asp residue rather than NH₂ terminal to this residue. The bond COOH-terminal to this Asp residue is also hydrolyzed by the αF161R mutant, but this is the only cleavage site for this mutant.

The cleavage site analysis of CCK8s revealed that meprin α and the three α mutants hydrolyze this peptide between a Trp and Met residue and between an Asp and Phe at the COOH-terminus. Although the cleavage sites remain unchanged, the amount of hydrolysis at each site differs (Fig. 3). There is an approximately equal distribution of CCK8s hydrolysis product peaks for the wild-type meprin α and for the αP228K mutant.
However, the product distribution was significantly different for the αY199K and αF161R meprin mutants. The αF161R mutant favored cleavage of the Trp-Met bond, while the αY199K mutant favored cleavage of the Asp-Phe bond. Meprin β and the βK185Y mutant both hydrolyzed the peptide between the Met-Asp in the COOH-terminal portion of the peptide. These results in combination with the cleavage site analysis indicate that the L185 residue interaction with a negatively charged amino acid in the substrate enables hydrolysis at P1 or P1’.
DISCUSSION

The studies herein provide insight into the molecular basis for the differences in the peptide bond preferences of the meprin α and β subunits. More specifically, they establish the importance of positively charged amino acid residues, particularly βK185, in the meprin β subunit active site. Mutation of this residue to a hydrophobic amino acid increased the $K_M$ for gastrin 20-fold, as did increasing the salt concentration, indicating electrostatic interactions between gastrin and the enzyme are important for affinity. This conclusion is consistent with the observation that when a Lys residue was substituted into meprin α (αY199K) at the equivalent position (βK185), meprin α gained the ability to cleave gastrin at the same sites as meprin β, and enhanced cleavage of CCK8s at a peptide bond COOH-terminal to an Asp residue. The results of this study indicate that amino acid βK185 interacts with substrates at both P1 and P1’, while βR147 appears to interact at P1. The αY199K mutant had a much higher $K_m$ value for gastrin than did meprin β, indicating that there are multiple factors in the meprin β active site that determine affinity. This is consistent with results of previous studies that had shown that meprins have a preference for substrates with a minimum of 6 to 8 amino acids (3, 26) indicating an extended binding site.

The substrate specificity of many members of the metzincin superfamily have been studied, and the great majority of these metalloproteinases have preferences for small or hydrophobic amino acids flanking the scissile bond. For example, ADAM 17 (tumor necrosis factor-alpha converting enzyme), stromelysin, matrylsin, collagenase, and gelatinases cleave bonds such as A-V, A-M, G-I (27). There are some exceptions to
this generallity, e.g. aggrecanases cleave at Glu residues (28). Two other members of the astacin family beside meprin β that cleave peptide bonds flanked by negatively charged amino acids are BMP-1, a mammalian procollagen C-proteinase, and flavastacin, an astacin in flavobacteria (4, 29, 30). Homology models of the protease domains of these enzymes, implicate equivalent basic amino acids as substrate specificity determinants as in meprin β (Fig. 3). The models show the presence of prominent basic residues located on the lower active site cleft that resides in the putative primed substrate binding regions. Meprin α, crayfish astacin and a fish hatching enzyme (HCE-1) prefer small or hydrophobic amino acids at P1’ and these peptidases lack the basic residue at the base of the active site cleft (31-33). Thus, the results of the studies herein with meprins are applicable to other astacins.

Insights into the meprin active site residues that affect affinity for substrates will be applicable to the design of inhibitors of these proteases. Meprins are not inhibited by any known naturally occurring metalloproteinase inhibitors (such as tissue inhibitors of metalloproteinases, TIMPs), and specific inhibitors for the subunits are not yet available (3, 4, 17). Yet these proteinases are induced in several types of cancer cells (e.g., colon and pancreatic cancer) and in leukocytes at inflammatory sites (such as in inflammatory bowel disease) (9, 10, 12). Specific inhibitors of meprins have the potential to decrease the growth and metastasis of certain tumors and decrease adverse consequences of inflammation, without affecting normal physiological processes that involve multiple metalloproteinases.
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FOOTNOTES

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The abbreviations used are: MCP-1, monocyte chemoattractant protein-1; DMEM, Dulbecco’s Modified Eagle Medium; HPLC, high performance liquid chromatography; MALDI-TOF, matrix assisted laser desorption ionization time of flight; LHRH, luteinizing hormone releasing hormone; α-MSH, α-melanocyte stimulating hormone; CCK8s, sulfated-cholecystokinin; BMP-1, bone morphogenic protein-1.
LEGENDS TO FIGURES

FIG. 1. Electrostatic differences in the protease domain of meprins α and β.
Sequence alignment of the amino acids in the meprin (mep) protease domains of mouse (m), rat (r) and human (h) meprins β (b) and α (a), respectively. The numbering system is based on the nascent mouse meprin α sequence and begins with the N-terminal amino acid of the protease domain. Residues conserved among mouse, rat and human meprin β are colored blue. Similar amino acids conserved among all six sequences are colored red in meprin α. Basic residues conserved throughout the species in the β subunits that are nonbasic in α are denoted by * below the sequences. Amino acids that were mutated in mouse α to the corresponding basic β residues are boxed.

FIG. 2. Critical electrostatic differences within the active site of meprins α and β.
The top panels are homology models in space filling (left) and ribbon (right) representations of the protease domain of mouse meprin α based on the crystal structure of crayfish astacin. For the space filling representations acidic residues, Asp and Glu, are red, basic residues Lys and Arg, are blue; all other residues are white. The zinc located in the center of the active site is green. For the ribbon representation α-helices are red and β-sheets are cyan. The bottom panels contain the corresponding representations for mouse meprin β. Active site electrostatic differences are denoted by arrows in space filling models and are numbered in ribbon representation.
Fig. 3. Peptide bonds hydrolyzed in cholecystokinin by meprin mutants. Chromatograms of CCK8s treated with wild-type merpin $\alpha$, $\alpha$Y199K, $\alpha$F161R and $\alpha$P228K mutants separated on a reverse phase HPLC column. The hydrolysis products were identified by MALDI-TOF and correspond to the W-M and D-F hydrolysis sites.

Fig. 4. Protease domains of several proteases of the astacin family. Homology models of the protease domains of rat meprin $\beta$ (rmepr$\beta$), mouse bone morphogenetic protein-1 (mBMP-1; procollagen C-proteinase), flavastacin (FLAV), mmep$\alpha$ (mouse meprin $\alpha$), crayfish astacin (AST) and high choriolytic enzyme-1 (HCE-1) were produced as described in Experimental Procedures.
Table I

*Proteolytic activity of wild-type meprin α and mutants against the protein substrate azocasein*

Meprins were activated with trypsin as described in “Experimental Procedures”. Azocaseinase activity is expressed in U/mg of meprin (mean +/- SD) for at least 3 measurements for each sample.

| Azocaseinase Activity (U/mg) |            |
|-----------------------------|------------|
| Wild-type meprin α          | 12,407 ± 255|
| αY199K                      | 15,381 ± 3,678|
| αF161R                      | 15,704 ± 1,690|
| αP228K                      | 3,641 ± 1,002|
TABLE II

**Percent hydrolysis of bioactive peptides by meprin α and active site mutants**

Peptides (100 µM) were incubated with meprin α and mutants (5 nM) for 2 h in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, at 37 ºC. Percent hydrolysis was determined by quantitative HPLC analyses. Peptide sequences of the substrates are listed below each peptide.

| Peptide | % Hydrolysis / 2 h |
|---------|-------------------|
|         | wt|mmpe| αY199K | αF161R | αP228K |
| β Specific |      |     |       |       |       |
| Gastrin 17 | ND | 42  | 10  | 3   |
| pEGPWLEEEAYGWMDF
| ND | ND | ND | ND |
| Orcokinin | ND | ND | ND | ND |
| NFDEIDRSGFGFN |       |     |       |       |       |
| α and β |      |     |       |       |       |
| Cholecystokinin 8 sulfate | 23 | 70  | 56  | 17  |
| DY*MGWMDF
| ND | ND | ND | ND |
| α Specific |      |     |       |       |       |
| Neurotensin | 54 | 100 | 22  | 23  |
| ELYENKPRRPYIL |       |     |       |       |       |
| LHRH*1 | 45  | 65  | 48  | 23  |
| pEHWSYGLRPG
| ND | ND | ND | ND |
| Bradykinin | 43  | 46  | 49  | 20  |
| RPPGFSPFR |       |     |       |       |       |

ND, Not detected; *, sulfated at the tyrosine; *1, Luteinizing hormone releasing hormone.
Table III

Kinetic constants for wild-type meprin α and mutants against the bioactive peptides gastrin and cholecystokinin.

Kinetic constants were determined by quantitative HPLC analysis by directly fitting to the Michaelis-Menten equation by nonlinear regression analysis. Peptides (2-750 µM) were incubated with mouse meprin α, β, and mutants (1-20 nM) in 20 mM Tris/HCl, 150 mM NaCl, pH 7.5 at 37 °C for 8 to 40 min. ND: Not Detected

|                | Gastrin          |             |             |
|----------------|------------------|-------------|-------------|
|                | K$_M$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_M$ (x10$^5$ M$^{-1}$ s$^{-1}$) |
| Wt meprin α    | ND               | ND          | ND          |
| αY199K         | 440              | 10.8        | 0.24        |
| Wt meprin β    | 1.04             | 11.0        | 105.8       |

|                | Cholecystokinin  |             |             |
|----------------|------------------|-------------|-------------|
|                | K$_M$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_M$ (x10$^5$ M$^{-1}$ s$^{-1}$) |
| Wt meprin α    | 490              | 8.6         | 0.18        |
| αY199K         | 110              | 14.2        | 1.29        |
| αF161R         | 230              | 13.4        | 0.58        |
| αP228K         | 290              | 3.8         | 0.13        |
| Wt meprin β    | 211              | 13.9        | 0.66        |
TABLE IV
Proteolytic activity of meprin β and the βK185Y mutant

Peptides (100 µM) were incubated with meprin α and mutants (5 nM) for 2 h in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, at 37º C. Percent hydrolysis was determined by quantitative HPLC analyses.

|                          | Azocaseinase Activity |
|--------------------------|-----------------------|
|                          | (U/ mg)               |
| Wild-type meprin β       | 5,437 ± 916           |
| βK185Y                   | 5,164 ± 1,177         |

| Peptide                  | % Hydrolysis / 2 h    |
|--------------------------|-----------------------|
| Wild-type meprin β       |                       |
| βK185Y                   |                       |
| Gastrin                  | 100                   |
| αMSH*¹                   | 15                    |
| Cholecystokinin 8 sulfate| 82                    |
| Neurotensin              | ND                    |
|                          | 100                   |
|                          | 55                    |
|                          | 87                    |
|                          | 9                     |

*¹ αMSH, α-melanocyte stimulating hormone; ND, Not detected.
**TABLE V**

*Kinetic constants for meprin β and βK185 against the peptides gastrin, cholecystokinin (CCK8s) and α-melanocyte stimulating hormone*

Kinetic constants were determined by quantitative HPLC analysis by directly fitting to the Michaelis-Menten equation. Peptides (2-750 µM) were incubated with rat meprin β and the βK185Y mutant (1-20 nM) in 20 mM Tris/HCl, 150 mM NaCl, pH 7.5, 37 ºC for 8 to 40 min.

| Peptide                | Wild-type meprin β | βK185Y |
|------------------------|--------------------|--------|
|                        | K_M (µM) | k_cat (s⁻¹) | k_cat/K_M (x10⁵ (M⁻¹s⁻¹)) | K_M (µM) | k_cat (s⁻¹) | k_cat/K_M (x10⁵ (M⁻¹s⁻¹)) |
| Gastrin (150mM NaCl)   | 1.04     | 11.0      | 105.8          | 20.0     | 17.8      | 8.9            |
| Gastrin (500 mM NaCl)  | 18.5     | 11.1      | 6.0            | 29.6     | 10.5      | 3.5            |
| CCK8s                  | 211      | 13.9      | 0.66           | 220      | 22.7      | 1.03           |
| αMSH                   | --       | --        | 0.04           | 282      | 15.0      | 0.53           |
TABLE VI  
Cleavage sites in peptides by meprins

Peptides (100 µM) were incubated with meprins (1-5 nM) in 20 mM Tris/HCl, 150 mM NaCl, pH 7.5, 37 ºC for 10 to 40 min. Peptide products were separated by HPLC, collected and identified by MALDI-TOF. Cleavage sites are indicated by an arrow (↓).

### Gastrin 17

| Peptide | Cleavage Sites |
|---------|----------------|
| αY199K  | pEGPGWL↓E↓E↓E↓EEAYGWMD↓F_{NH2} |
| αF161R  | pEGPGWLEEEEEAYGWMD↓F_{NH2} |
| wtrmepβ | pEGPGWL↓E↓E↓E↓EAYGWMD↓DF_{NH2} |
| βK185Y  | pEGPGWL↓E↓E↓E↓EAYGWMD↓DF_{NH2} |

### Cholecystokinin

| Peptide | Cleavage Sites |
|---------|----------------|
| meprin α | DY*MGW↓MD↓F_{NH2} |
| αY199K  | DY*MGW↓MD↓F_{NH2} |
| αF161R  | DY*MGW↓MD↓F_{NH2} |
| αP228K  | DY*MGW↓MD↓F_{NH2} |
| wtrmeprin β | DY*MGWM↓DF_{NH2} |
| βK185Y  | DY*MGWM↓DF_{NH2} |

_{NH2}. Amidated at the carboxy terminus; *, sulfated at the tyrosine; p, pyro.
Critical amino acids in the active site of meprin metalloproteinases for substrate and peptide bond specificity

James P. Villa, Greg P. Bertenshaw and Judith S. Bond

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