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Marked Differences between Metalloproteases Meprin A and B in Substrate and Peptide Bond Specificity*

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Meprin A and B are highly regulated, secreted, and cell-surface metalloendopeptidases that are abundantly expressed in the kidney and intestine. Meprin oligomers consist of evolutionarily related α and/or β subunits. The work herein was carried out to identify bioactive peptides and proteins that are susceptible to hydrolysis by mouse meprins and kinetically characterize the hydrolysis. Gastrin-releasing peptide fragment 14–27 and gastrin 17, regulatory molecules of the gastrointestinal tract, were found to be the best peptide substrates for meprin A and B, respectively. Peptide libraries and a variety of naturally occurring peptides revealed that the meprin β subunit has a clear preference for acidic amino acids in the P1 and P1′ sites of substrates. The meprin α subunit selected for small (*e.g.* serine, alanine) or hydrophobic (*e.g.* phenylalanine) residues in the P1 and P1′ sites, and proline was the most preferred amino acid at the P2′ position. Thus, although the meprin α and β subunits share 55% amino acid identity within the protease domain and are normally localized at the same tissue cell surfaces, they have very different substrate and peptide bond specificities indicating different functions. Homology models of the mouse meprin α and β protease domains, based on the astacin crystal structure, revealed active site differences that can account for the marked differences in substrate specificity of the two subunits.

Meprin A and B are zinc metalloendopeptidases composed of evolutionarily related α and/or β subunits. They are members of the astacin family and are highly expressed in brush border membranes of the intestine and renal proximal tubules (1, 2). Meprins are particularly abundant in mouse juxtamedullary nephrons and constitute ~5% of total protein in renal brush border membranes (3). The meprin α and β subunits are expressed early in embryonic development of mouse kidney and intestine, by day 11, and have different patterns of expression in the suckling phase and after weaning (4). Homologous enzymes are found in rat and human kidney and intestine (1, 5, 6). Meprins are also expressed in leukocytes of intestinal lamina propria and in cancer cells and are consequently implicated in inflammation and cancer growth and metastasis (7, 8).

Mouse kidney meprin A (EC 3.4.24.18) is a homooligomer of α subunits, or a heterooligomer of α and β subunits (2, 9). Mouse kidney meprin B (EC 3.4.24.63) is a homooligomer of β subunits (10). The multidomain α and β meprin subunits are highly glycosylated and form disulfide-linked dimers and higher order oligomers by noncovalent interactions (11, 12). Meprins containing at least one β subunit remain membrane-bound by virtue of a transmembrane domain located near the carboxyl terminus of β subunits (1). Mature meprin α homooligomers contain no transmembrane domain and are found in mouse urine (13). The expression of the meprin α subunits in mice is strain-dependent (1). Random-bred mice (such as ICR) and many inbred strains of mice (*e.g.* C57BL/6) express both meprin α and β in the adult kidney, and these strains possess heterooligomeric forms of meprin A. Some inbred mouse strains (such as C3H/He) only express meprin β in the adult kidney and therefore have only meprin B. Furthermore, meprin β subunits in mouse kidney exist primarily in the proenzyme form, thus these subunits are latent (14). This is in contrast to the rat kidney enzyme, as well as meprins from mouse, rat, and human intestine where the propeptide is removed and the enzymes are fully active (1, 15). Therefore, meprin A and B isolated from adult mouse kidney are novel in that they can be used to determine the activities of the α and β subunits, respectively.

Identification of substrates for proteases is a valuable step toward elucidation of physiological function and provides a knowledge-based approach to inhibitor design. Previous studies have shown that a variety of biologically active peptides and proteins are hydrolyzed by meprins *in vitro*. For example, meprins cleave blood pressure regulators such as bradykinin, metabolism mediators such as parathyroid hormone, signaling molecules such as protein kinase A, and basement membrane proteins such as entactin (1, 2, 16, 17). There has been no systematic study, however, of the enzymatic differences between meprin A and B from any species or of the contributions of the individual subunits to activity. Enzymological data for meprin B are particularly lacking even though meprin B appears to be a more essential protease than meprin A as indicated by expression patterns in mammalian tissues. The work herein describes previously unidentified meprin substrates and provides the first detailed comparison of meprin α and β substrate and peptide bond specificity.

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EXPERIMENTAL PROCEDURES

Isolation of Meprin—Mouse meprin A and B were isolated from kidney brush border membranes of ICR and C3H/He male mice, respectively. For kinetic analyses, meprin B was activated using trypsin as previously described (18). Concentrations of meprins are based on a subunit molecular mass of 90 kDa. Meprin A contains active α subunits and inactive β subunits. Trypsin-treated meprin B has only β subunit activity.

Materials—Peptides, proteins, inhibitors, and reagents were purchased from Sigma Chemical Co. with the following exceptions. The nonsulfated cholecystokinin (CCK)[1] derivatives Boc-CCK8$_{NH2}$, CCK8$_{NH2}$, CCK8, CCK7$_{NH2}$, CCK6$_{NH2}$, and CCK4$_{NH2}$ were from Bachem. Somatostatin, gastrin-releasing peptide fragment 14–27 (GRP-(14–27)), sulfated-CCK (sCCK), sCCK8$_{NH2}$, cerulein, and human secretin were from American Peptide Co. Neurotensin, kassinin, and CCK5$_{NH2}$ were from Novabiochem. Recombinant mouse osteopontin and goat anti-mouse osteopontin antibody were obtained from R & D Systems. SuperSignal West Dura Extended Duration Substrate was from Pierce. The dodecamer peptide library was synthesized at the Tufts University Core Facility (Boston, MA).

Peptide Library Screen—An amino-terminally acetylated dodecamer peptide mixture (1 mM) consisting of a roughly equimolar mixture of the 19 naturally occurring L-amino acids, excluding cysteine, was incubated with either meprin A (33 nM) or B (10 nM) in 25 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$ at 37 °C until 5–10% of the peptides were digested. The mixture contained the inhibitors pepstatin, leupeptin, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane, 3,4-dichloroisocoumarin, and bestatin to prevent hydrolysis by trace amounts of protease impurities. Hydrolysis was terminated by heating to 100 °C for 2 min, and 10 μl of the mixture was subjected to Edman degradation-based amino-terminal peptide sequencing. The data in each sequencing cycle was normalized to the total molar amount of amino acids in that cycle so that a value of 1 indicates the average value. Undigested peptides and the amino-terminal fragments of digested peptides are amino-terminally blocked and therefore do not contribute to the sequenced pool. The relative amount of a given amino acid residue present in a particular sequencing cycle indicates the preference for that residue at a particular position relative to the cleavage site. Values for a given amino acid were corrected by renormalizing to the average relative amount of that residue present across the first nine sequencing cycles to correct for the distribution of amino acids in the starting mixture.

Identification of Novel Meprin Substrates—Naturally occurring peptides (100 μM) were incubated with meprin A or B (2 nM) for 4 h. The incubation was carried out at 37 °C in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, containing the same inhibitors as above. The reaction was terminated by the addition of EDTA to 10 mM and trifluoroacetic acid (TFA) to 0.05% v/v. Samples were then subjected to high performance liquid chromatography (HPLC) analysis using a POROS R2H (4.6 × 100 mm) column and a Brownlee Newguard 7-μm RP-18 (3.2 × 15 mm) guard column. Peptides were eluted with a linear gradient of acetonitrile in 0.1% TFA at a constant flow rate of 3.2 ml min^{-1}. Peptides were monitored at A_{220}. Percent hydrolysis was then calculated by monitoring the decrease in the substrate peak area, compared with time zero.

Kinetic Constant Determination—Kinetics for peptide hydrolysis by meprins were determined by quantitative HPLC analysis of reaction mixtures using the same buffer conditions as the peptide screen. Meprin concentrations in reactions were between 1 and 4 nM depending on efficiency of proteolysis. Hydrolysis was limited to 20% and was calculated by monitoring loss of substrate peak area. Velocity (μM min^{-1}) was plotted against the average substrate concentration for the reaction ($[S]_{avg}$ (μM)) and fitted directly to the Michaelis-Menten equation by nonlinear regression analysis. $[S]_{avg}$ was calculated using the equation, $[S]_{avg} = ([S]_0 + [S]_f)/2$, where $[S]_0$ and $[S]_f$ equal initial and final substrate concentrations, respectively, as described previously (19). The K_m values of meprin A hydrolysis of neuropeptide Y and meprin B hydrolysis of neuropeptide Y, secretin, peptide YY, and kinetensin were too high for accurate measurement. Therefore, the k_{cat} and K_m constants were not individually determined. Instead the specificity constant k_{cat}/K_m was determined using the method of Fersht (20) where $k_{cat}/K_m = V_0/([E]\cdot[S])$, a simplification of the Michaelis-Menten equation that applies when $K_m \gg [S]$.

Identification of Cleavage Sites—Peptides (50 μM) were incubated with meprin A or B (2 nM) at 37 °C in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, in a final volume of 100 μl for 5 min to 6 h depending on the efficiency of hydrolysis. Total peptide was subjected to HPLC using a Spheri-5 ODS 5 micron (4.6 × 250 mm) column and a Brownlee Newguard 7-μm RP-18 (3.2 × 15 mm) guard column. Peptides were eluted with a linear gradient of acetonitrile in 0.1% TFA at a constant flow rate of 1.2 ml min^{-1}. Appropriate peptide peaks were extensively dried and then dissolved in a matrix solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.3% TFA. This was then spotted on a PerSeptive Biosystems stainless steel plate. Products and substrates were identified using a Perceptive Biosystems Linear Voyager matrix-assisted laser desorption ionization time of flight (MALDI-TOF) machine with continuous extraction. The accelerating voltage was set at 18,750, and the laser intensity was between 175 and 300 in positive mode except for sCCK8$_{NH2}$ detection, which was in the negative mode. Between 60 and 240 scans were averaged. The machine was calibrated using the PerSeptive Biosystems Sequazyme Peptide Mass Standards kit. Data were evaluated using Grams/386 software version 3.04 (Galactic Industrial Corp.). Where required, amino-terminal sequence analysis was performed to identify peptides. Neuropeptide Y and peptide YY and their products were not separated by HPLC due to poor resolution. Instead, total peptide was bound to a ZipTip (Millipore) in 1% TFA, washed extensively to remove nonpeptide impurities, and eluted in 70% acetonitrile. Total peptide was then dissolved in α-cyano-4-hydroxycinnamic acid solution and treated as above.

Meprin Activity against Extracellular Matrix Proteins—The extracellular matrix proteins gelatin, collagen I and IV, laminin, and fibronectin (20 μg) were incubated with meprin A or B (20 nM) for 18 h at 25 °C in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, with a final volume of 40 μl. Proteolysis was terminated by the addition of EDTA to 10 mM. The sample was boiled in SDS-PAGE sample buffer containing β-mercaptoethanol, and proteins were resolved on a 4–15% PAGE gradient gel. Protein was visualized with Coomassie Brilliant Blue.

Degradation of Osteopontin by Meprin—Recombinant mouse osteopontin (50 μg ml^{-1}) was incubated with meprin A or B (10 nM) at 37 °C in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, in a final volume of 40 μl. A 6-μl sample was removed at various times and immediately mixed with an equal volume of 20 mM EDTA to terminate hydrolysis. The sample was boiled in SDS-PAGE sample buffer containing β-mercaptoethanol and subjected to electrophoresis on a 4–15% gradient gel. Osteopontin was detected by Western blot analysis using goat anti-mouse osteopontin as a primary antibody (1:1000), rabbit anti-goat IgG peroxidase conjugate as a secondary antibody (1:5000), with SuperSignal West Dura Extended Duration Substrate used as a horseradish peroxidase substrate for detection purposes.

Inhibition Profiles of Meprins—The inhibition profiles of meprin A and B were compared using actinonin and Pro-Leu-Gly-hydroxamate (PLG-(NHOH)). Meprin A or B (2 nM) was preincubated with various concentrations of inhibitor for 20 min before addition of substrate. Reactions were performed in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, and sCCK8$_{NH2}$ (50 μM) was used as substrate. Substrate hydrolysis was limited to 25% and was determined by quantitative HPLC analyses. The concentrations of inhibitors ranged from 12.5 nM to 50 μM for actinonin and from 250 nM to 500 μM for PLG-(NHOH). The level of inhibition was determined by comparing the decrease in substrate concentration in the presence and absence of inhibitor.

Homology Models of Meprin α and β Protease Domains—The protease domain structures of the mouse α and β subunits were determined by knowledge-based homology modeling using the program Modeller (21). The input consisted of a sequence alignment of the catalytic domains of meprin and astacin, and the coordinates obtained from the Rutgers Protein Data Bank for the crystal structure of astacin (accession number 1AST (22)). The catalytic zinc was modeled by coordinating it to the equivalent residues that were determined for astacin. The model quality was assessed using the program PROCHECK (23) and was found to be comparable in stereochemical quality to a low resolution crystal, typical of homology models. Surface representations were prepared using WebLab Viewer 3.7 (Molecular Simulations Inc., San Diego, CA).

[1] The abbreviations used are: CCK, nonsulfated cholecystokinin; GRP-(14–27), gastrin releasing peptide fragment 14–27; sCCK, sulfated-cholecystokinin; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; BMP-1, bone morphogenic protein-1; α-MSH, α-melanocyte stimulating hormone; LHRH, luteinizing hormone releasing hormone; PAGE, polyacrylamide gel electrophoresis; PLG-(NHOH), Pro-Leu-Gly-hydroxamate.

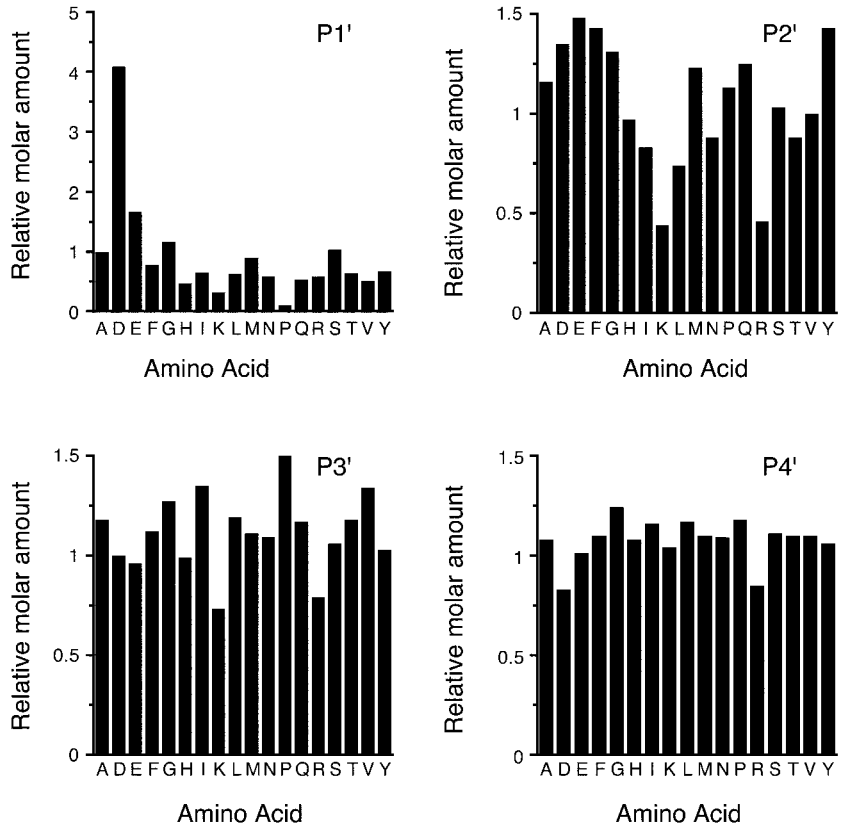

FIG. 1. **Preferred amino acids in substrate subsites P1′ to P4′ for meprin B.** An acetylated dodecamer peptide library mixture (1 mM) containing roughly equimolar amounts of all amino acids at each position, except cysteine, was incubated with meprin B (10 nM) until the library was between 5 and 10% digested. The incubation was in 25 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.4, at 37 °C. Amino-terminal sequencing of the resulting products allowed for the elucidation of amino acid preference for meprin B at each primed site. The data in each sequencing cycle was normalized to the total molar amount of amino acids in that cycle so that a value of 1 indicates the average value.

RESULTS

Meprin A and B Have Distinct Specificities for Preferred Amino Acids at or Near the Cleavage Site of Substrates as Determined by Peptide Libraries—Peptide libraries were used to identify preferred amino acids of substrates at or near the cleavage site and to map the primed subsite[2] binding region of meprins (25). A completely random dodecapeptide mixture acetylated at the amino terminus was partially digested with meprin A or B, and the digested peptides were subjected to amino-terminal peptide sequencing. For meprin B the strongest specificity was seen at the P1′ site of the substrate where acidic residues were preferred (Fig. 1). Aspartic acid at the amino terminus of peptide products was particularly prevalent with a signal 2.5- to 41-fold greater than other residues. Glutamic acid was also selected but to a lesser extent. The selectivity decreased further away from the scissile bond; with 41-, 3.4-, 2.1-, and 1.5-fold differences between the highest and lowest relative molar amounts for the P1′, P2′, P3′, and P4′ subsites, respectively. There was no clear preference for a single amino acid at the P2′ through P4′ sites, aside from a slight preference for proline in P3′. However, there was a selection against basic residues at P2′ and P3′ as well as a slight selection against aspartic acid and arginine side chains in P4′.

Meprin A had a completely different profile to meprin B for amino acid preference at the P1′ subsite (Fig. 2). There was a selection for small and aromatic residues (S > F > A > T > M > Y > G) at the P1′ position. The only similarity between the enzymes at the P1′ site was a strong selection against proline. The profiles at the P2′ subsite were also dissimilar for the two enzymes. Proline was the most preferred amino acid at the P2′ position for meprin A, with at least a 2-fold larger signal than other residues. There was also a slight selection against leucine, asparagine, and basic amino acids in the P2′ subsite. Proline was also the preferred residue at the P3′ site, although to a lesser extent than the P2′ subsite. Charged residues and tyrosine were slightly disfavored. The specificity signature at P3′ was strikingly similar to that of meprin B, and there was little or no specificity at P4′. The data indicated that, for both enzymes, at least three primed subsites contribute to substrate binding specificity (Figs. 1 and 2). In addition, meprin A and B have very different specificities in the P1′ and P2′ subsites.

Comparison of Naturally Occurring Peptides as Substrates of Meprins—Specific peptides were tested as meprin substrates to further define substrate preferences (Table I). The peptides were chosen based on the knowledge of meprin peptide bond specificity and the peptide localization *in vivo*. In an initial screen of potential substrates it was noted that peptides of the gastrointestinal tract (*e.g.* GRP-(14–27), $sCCK8_{NH2}$, and gastrin 17) were relatively good substrates of meprins. The screen employed 25 bioactive peptides that fell into four groups. The first group, GRP-(14–27), $sCCK8_{NH2}$, secretin, glucagon, neuropeptide Y, and cerulein were susceptible to hydrolysis by both meprin A and B. GRP-(14–27) was efficiently hydrolyzed by both meprins under the conditions used; the intact peptide was almost completely hydrolyzed after 4 h. The second group, bombesin, neurotensin, luteinizing hormone releasing hormone (LHRH), bradykinin, α-melanocyte stimulating hormone (α-MSH), substance P, valosin, parathyroid hormone fragment 13–34, vasoactive intestinal peptide, and angiotensin I were only susceptible to meprin A. The third group of peptides was susceptible to meprin B only; orcokinin and gastrin 17 were

[2] The nomenclature for the interaction of proteases with their substrates is from Schecter and Berger (24). The substrate amino acid residues are called P (for peptide), the subsites on the protease that interact with the substrate are called S (for subsite). The residues on the amino-terminal side (also known as the unprimed residues) of the scissile bond (bond that is cleaved during hydrolysis) are numbered P1 through P6 counting outward. The residues on the carboxyl-terminal side (also known as primed residues) of the scissile bond are numbered P1′ through P6′. Thus, hydrolysis occurs between the P1 and P1′ residues. The subsites on the protease are termed S1 through S6 and S1′ through S6′ to complement the substrate residues that interact with the enzyme.

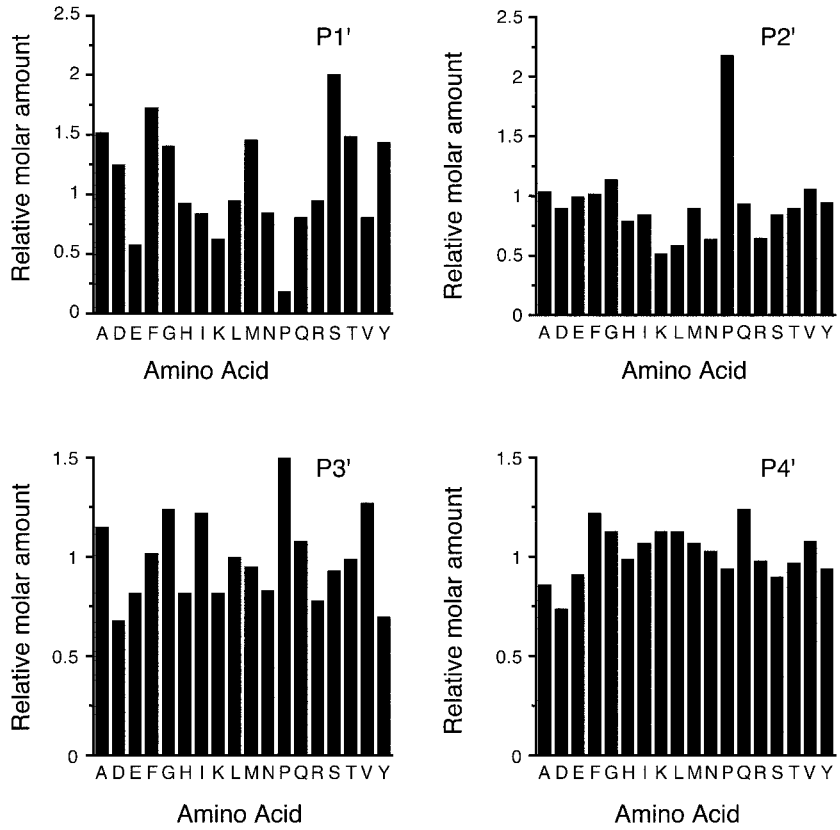

FIG. 2. **Preferred amino acids in substrate subsites P1′ to P4′ for meprin A.** The same acetylated dodecamer peptide library mixture (1 mM) described in Fig. 1 was incubated with meprin A (33 nM) until the library was between 5 and 10% digested. Amino-terminal sequencing of the resulting products allowed for the characterization of amino acid preference for meprin A at each primed subsite.

TABLE I

Percent hydrolysis of bioactive peptides by meprin A and B

Peptides (100 μM) were incubated with meprin A or B (2 nM) for 4 h in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, at 37 °C. Percent hydrolysis was determined by HPLC analyses as described under "Experimental Procedures."

Substrate	Meprin A	Meprin B
	%	
GRP-(14–27)	>95	>95
$sCCK8_{NH2}$	67	56
Secretin	43	35
Glucagon	41	56
Neuropeptide Y	31	55
Cerulein	13	60
Bombesin	87	ND[a]
Neurotensin	83	ND
LHRH	80	ND
Bradykinin	69	ND
α-MSH	68	ND
Substance P	51	ND
Valosin	45	ND
Parathyroid hormone fragment 13–34	43	ND
Vasoactive intestinal peptide	20	ND
Angiotensin I	16	ND
Orcokinin	ND	82
Gastrin 17	ND	65
Peptide YY	ND	32
Kinetensin	ND	16
[Lys^8]-vasopressin	ND	ND
Somatostatin	ND	ND
Kassinin	ND	ND
Oxytocin	ND	ND
α-Neurokinin	ND	ND

[a] ND, not detected.

particularly well hydrolyzed together with peptide YY and kinetensin. The final group of peptides was resistant to both enzymes; these peptides were [Lys^8]-vasopressin, somatostatin, kassinin, oxytocin, and α-neurokinin. The screen identified 11 peptides not previously known to be cleaved by meprins. Novel meprin A activity was seen against GRP-(14–27), $sCCK8_{NH2}$, secretin, glucagon, cerulein, bombesin, and vasoactive intestinal peptide. In addition, novel substrates of meprin B identified were GRP-(14–27), $sCCK8_{NH2}$, secretin, glucagon, neuropeptide Y, cerulein, orcokinin, peptide YY, and kinetensin.

Kinetic Parameters for Meprin Cleavage of Bioactive Peptides—A kinetic study was conducted to better characterize the substrates of meprins identified in the initial screen. The kinetic constants, k_{cat} and K_m as well as the specificity constant k_{cat}/K_m (Table II) were determined by directly fitting data to the Michaelis-Menten equation by nonlinear regression analysis as described under "Experimental Procedures." All peptides tested exhibited typical Michaelis-Menten kinetics (data not shown). Velocity was determined by monitoring the loss of substrate peak area rather than the appearance of substrate. This approach was taken due to the presence of multiple cleavage sites as evident by more than two product peaks for most substrates (data not shown). The K_m values for meprin A ranged from 116 for GRP-(14–27) to 425 μM for bradykinin; for meprin B the range was from 7.1 for gastrin and 211 μM for $sCCK8_{NH2}$. Meprin A had k_{cat} values between 11.8 for secretin and 88.3 s^{-1} for GRP-(14–27). The k_{cat} values of meprin B lay between 12.4 for gastrin and 26.8 s^{-1} for glucagon. Of all the peptides tested in this study, meprin B hydrolysis of gastrin 17 was found to give the highest specificity constant (17.5×10^5 M^{-1} s^{-1}). This was predominantly due to the low K_m value of 7.1 μM. This value is over 6-fold lower than any other K_m determined for either enzyme. The fluorogenic bradykinin analog substrate, 2-aminobenzoyl-RPPGFSPFRK-(dinitrophenyl)-G is commonly used to study meprin A activity (27). However, bradykinin is a relatively poor substrate due to a high K_m (425 μM (26)). This high K_m leads to the low specificity constant of 5.1×10^4 M^{-1} s^{-1}. Rates of hydrolysis for neuropeptide Y by meprin A and neuropeptide Y, secretin, peptide YY, and kinetensin by meprin B were linear with respect to substrate concentration at high peptide concentrations, therefore, the individual k_{cat} and K_m values were not directly determined.

TABLE II
Kinetic constants for meprin A and B against bioactive peptides

Kinetic constants were determined by quantitative HPLC analysis by directly fitting to the Michaelis-Menten equation as described under "Experimental Procedures." Peptides (2–750 μM) were incubated with meprin A or B (1–4 nM) in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, for between 5 and 40 min.

Peptide	Meprin A			Meprin B		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
	μM	s^{-1}	$\times 10^5$ (M^{-1} s^{-1})	μM	s^{-1}	$\times 10^5$ (M^{-1} s^{-1})
GRP-(14–27)	116	88.3	7.61	48.1	12.6	2.62
$sCCK8_{NH2}$	356	42.3	1.18	211	13.9	0.657
Glucagon	223	17.4	0.780	220	26.8	1.34
Secretin	217	11.8	0.544			0.133[a]
Neuropeptide Y			0.323[a]			0.609[a]
Substance P	118	27.8	2.36			
Valosin	120	18.8	1.57			
Bradykinin[b]	425	22.0	0.51			
Gastrin 17				7.10	12.4	17.5
Orcokinin				100	23.4	2.34
Peptide YY						0.513[a]
Kinetensin						0.363[a]

[a] Calculated by the method of Fersht (20).
[b] Data from Wolz and Bond (26).

Instead the specificity constant k_{cat}/K_m was determined directly. As expected, these values were the lowest seen for all substrates, the lowest value being 0.133×10^5 M^{-1} s^{-1} for meprin B hydrolysis of secretin.

Meprins Have Distinct Differences in Peptide Bond Specificity—Peptides that were relatively good substrates of meprins were further characterized to determine peptide bonds cleaved. The peptides were digested with either meprin B (Table III) or meprin A (Table IV), and products were identified by MALDI-TOF. Cleavage was evident at more than one site in all instances except meprin B hydrolysis of orcokinin, $sCCK8_{NH2}$, peptide YY, and kinetensin and meprin A hydrolysis of bradykinin (Tables III and IV). In general meprin B appeared to be a more specific protease than meprin A. Bonds with an acidic residue in the P1′ position were cleaved by meprin B in most instances (Table III). Out of 20 scissile bonds determined, 13 sites were amino-terminal to an acidic residue. Residues seen multiple times at P1′ were aspartic acid (9 times), glutamic acid (4 times), and glycine (twice), consistent with the peptide library data (Fig. 1). Meprin B cleaved gastrin 17, orcokinin, glucagon, $sCCK8_{NH2}$, secretin, peptide YY, and neuropeptide Y with an acidic residue at P1′ (Table III). However, GRP-(14–27) and kinetensin, which lack acidic residues, were also hydrolyzed. This demonstrated a distinct preference, but not an absolute requirement, for acidic residues in the P1′ site of substrates by meprin B. The data also indicated a lack of substrates with a basic residue in the P2′ position, which is consistent with peptide library data in which both lysine and arginine are strongly selected against (Fig. 1). Meprin B is able to accommodate all types of amino acids in P1 showing a lower stringency for specific residues in this subsite. Glutamic acid is seen the most often (6 out of 20 times). Proline was frequently seen in the unprimed region (8 times). Interestingly, meprin B was able to act as both an amino- and carboxypeptidase (*e.g.* orcokinin and $sCCK8_{NH2}$, respectively). In general, meprin A tended to cleave bonds that have small uncharged or hydrophobic residues in their P1 and P1′ positions (Table IV). The presence of a proline residue in meprin A substrates was frequent. Proline was seen at P4 (4 times), P3 (twice), P2′ (5 times), and P3′ (twice). Proline was never seen at P1 or P1′.

The Hydrolytic Efficiency of Meprin B Is Dependent on Peptide Length—Previous work indicated that meprin A has a preference for substrates with a minimum of eight amino acids (28). To test the peptide length requirement for efficient hydrolysis by meprin B, derivatives of $sCCK8_{NH2}$ were used as substrates (Table V). Meprin B cleaved $sCCK8_{NH2}$ at a single site toward the carboxyl terminus (Table III). The $sCCK8_{NH2}$ derivatives allowed the determination of the unprimed subsites contribution, peripheral to the scissile bond as well as the contribution of the carboxyl terminus toward substrate recognition. The data indicated that meprin B had a preference for a minimum of six amino acids. $CCK4_{NH2}$ and $CCK5_{NH2}$ were relatively poor substrates (17 and 15% hydrolyzed, respectively, compared with 56% for $sCCK8_{NH2}$), whereas $CCK6_{NH2}$ and larger peptides were relatively good substrates. The effects of modifications within $sCCK8_{NH2}$ were also examined. The absence of sulfation at the tyrosine residue resulted in a poorer substrate compared with the parent peptide (35 and 56% hydrolysis, respectively). Blockage at the amino termini (Boc-$CCK8_{NH2}$) increased the susceptibility to hydrolysis (59% hydrolyzed compared with 35% for $CCK8_{NH2}$). The presence or absence of amidation at the carboxyl terminus resulted in larger differences toward hydrolysis. The free acid (CCK8) was 76% hydrolyzed compared with 35% hydrolysis for $CCK8_{NH2}$. Cerulein, a CCK analog, was hydrolyzed 60% in 4 h. This peptide is identical to $sCCK8_{NH2}$ except for a methionine in place of a threonine residue, carboxyl-terminal to the sulfated tyrosine, and an amino-terminal extension of pyroglutamic acid and glutamine. The free acid was hydrolyzed to the greatest extent of all CCK derivatives tested.

Meprin Degradation of Extracellular Matrix Proteins—To determine the ability of meprins to degrade extracellular matrix components, gelatin, fibronectin, collagens I and IV, and laminin were incubated with meprins, and the products were subjected to SDS-PAGE (Fig. 3, *upper panel*). Gelatin proved to be the best substrate under the conditions used. After an 18-h incubation with meprin A or B, intact gelatin was extensively hydrolyzed by both enzymes. Fibronectin was sensitive to both enzymes, yielding similar patterns of hydrolysis. The major protein bands above 200 kDa seen in the control were hydrolyzed by both meprin A and B, yielding bands that migrated slightly faster and reproducibly in both cases. Collagens I and IV and laminin were resistant to hydrolysis at 25 °C (data not shown). Collagen I was sensitive to hydrolysis by meprin B at 37 °C, possibly due to some local unfolding of the triple helix (data not shown).

Meprin B Hydrolysis of Osteopontin—To test the ability of meprin B to hydrolyze proximal to acidic residues in the context of a protein rather than a short peptide, osteopontin was used as a substrate (Fig. 3, *lower panel*). This 294-amino acid protein has a high percentage of acidic residues (23%). Meprin B effectively degraded the protein in a time-dependent fashion.

TABLE III
Cleavage sites in peptides by meprin B

Peptides (50 μM) were incubated with meprin B (2 nM) in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, for between 5 and 300 min. Peptide products were separated by HPLC, collected, and identified by MALDI-TOF and amino-terminal sequencing. Cleavage sites are indicated by an *arrow* (↓). In instances of multiple cleavage sites *double arrows* (⇓) represent the major site(s) of cleavage. The subscript "NH2" represents amidation at the carboxy terminus; the asterisk represents sulfation at the tyrosine; p represents pyro.

Peptide	Sequence and cleavage sites
Gastrin 17	pEGPWL ↓ E ↓ E ↓ E ↓ EEAYGWMDF$_{NH2}$
GRP-(14–27)	M ↓ YP ↓ R ↓ GNHWAVGHLM$_{NH2}$
Orcokinin	NF ↓ DEIDRSGFGFN
Glucagon	HSQG ↓ TFTS ↓ DYSKYL⇓DSRR ↓ AQ ↓ DFVQWLMNY
sCCK8$_{NH2}$	DY*MGWM ↓ DF$_{NH2}$
Secretin	HS ↓ D ↓ GTFTSELSRLREGARLQRLLQGLV$_{NH2}$
Peptide YY	YPIKPEAPGE ↓ DASPEELNRYYASLRHYLNLVTRQRY$_{NH2}$
Neuropeptide Y	YPSKPDNPGE ↓ DAPAE ↓ DMARYYSALRHYLNLITRQRY$_{NH2}$
Kinetensin	IARRHPY ↓ FL

TABLE IV
Cleavage sites in peptides by meprin A

Peptides (50 μM) were incubated with meprin A (2 nM) in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, for between 5 and 300 min. Peptide products were separated by HPLC, collected, and identified by MALDI-TOF and amino-terminal sequencing. Cleavage sites are indicated by an *arrow* (↓). In instances of multiple cleavage sites *double arrows* (⇓) represent the major site(s) of cleavage. The subscript "NH2" represents amidation at the carboxy terminus; the asterisk represents sulfation at the tyrosine; p represents pyro; Ac- represents *N*-acetylated at the amino terminus.

Peptide	Sequence and cleavage sites
GRP-(14–27)	MYPRGN⇓H ↓ W ↓ A ↓ VGH ↓ LM$_{NH2}$
sCCK8$_{NH2}$	DY*MGW ↓ MD ↓ F$_{NH2}$
Glucagon	HS ↓ QGTF ↓ T ↓ S ↓ D ↓ Y ↓ S ↓ K ↓ Y ↓ L ↓ D ↓ SR ↓ RAQ ↓ D ↓ FVQW ↓ LMNY
Secretin	HSDGT ↓ F ↓ T ↓ SELS ↓ R ↓ LR ↓ E ↓ G ↓ AR ↓ LQ ↓ R ↓ LLQ ↓ GLV$_{NH2}$
Substance P	RPKPQQ ↓ F ↓ F⇓G ↓ LM$_{NH2}$
Valosin	VQ ↓ YPVEHPDKF ↓ L ↓ K ↓ F ↓ G⇓M⇓TPSKGVL ↓ FY
Bradykinin	RPPGF ↓ SPFR
α-MSH	Ac-SYS⇓MEHFRWG ↓ KPV$_{NH2}$
LHRH	pEHW ↓ S ↓ Y ↓ G ↓ L ↓ RPG$_{NH2}$

TABLE V
Percent hydrolysis of cholecystokinin derivatives by meprin B

Cholecystokinin (CCK) derivatives (100 μM) were incubated with meprin B (2 nM) for 4 h in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, at 37 °C. Percent hydrolysis was determined by quantitative HPLC analyses as described under "Experimental Procedures." The lengths of the CCK derivatives are indicated counting from the carboxyl terminus. The subscript "NH2" represents amidation at the carboxyl terminus; Boc represents amino terminus blocked with a butyloxycarbonyl group; S represents sulfation at the tyrosine.

Peptide	Meprin B
	%
sCCK8$_{NH2}$	56
CCK8$_{NH2}$	35
CCK8	76
Cerulein	60
Boc-CCK8$_{NH2}$	59
CCK7$_{NH2}$	57
CCK6$_{NH2}$	48
CCK5$_{NH2}$	15
CCK4$_{NH2}$	17

Over a 30-min incubation the intact protein decreased markedly as detected by Western blot analysis. Meprin A showed little if any ability to degrade osteopontin under the conditions used; the 60-min time point shows slightly less protein than the control.

Inhibitor Profiles of Meprins—The active sites of meprins were further mapped using the inhibitors PLG-(NHOH) and actinonin. The crystal structure of astacin complexed with PLG-(NHOH) has shown that the inhibitor binds the unprimed subsite region (accession number 1QJJ (29)). The hydroxamate moiety ligates the zinc, whereas the peptide moiety binds the unprimed subsites and proline binds P3, leucine P2, and the glycine P1. The IC_{50} values toward meprin A and B were similar to one another with values of 30 and 50 μM, respectively (Fig. 4). This was in the same range as the reported K_i value toward astacin, which has a K_i of 16 μM (29). Unexpectedly, actinonin was a much more potent inhibitor of both meprin A and B compared with astacin, even though it has a very similar structure to PLG-(NHOH). The IC_{50} values were over 300- and 125-fold higher against meprin A and B, respectively, at 100 and 400 nM, respectively. In contrast, the K_i toward astacin is 8-fold lower at 130 μM (30).

DISCUSSION

The work herein clearly demonstrates marked differences in the preferences of meprin A and B for the P1′ and P2′ subsites of substrates. Meprin B is predominantly an Asp/Glu-N peptidase as shown by the peptide library studies and individual substrate data, selecting acidic residues in the P1′ site (Fig. 1 and Table III). By contrast, meprin A selects a variety of small and hydrophobic amino acids in the P1′ site and clearly prefers proline residues in the P2′ sites (Fig. 2 and Table IV). Both proteases have extended binding sites and prefer substrates of at least 6 amino acids (Ref. 28 and Table V). The different specificities of the two subunits implicate diverse functions.

Amino acid sequence analyses and homology models of the α and β protease domains yield insights into the substrate specificity differences and activities of meprins compared with other astacin family members, such as crayfish astacin and mouse bone morphogenic protein-1 (BMP-1; Fig. 5 and Ref. 1). There are nine basic residues in the meprin β sequence that are not basic in the equivalent positions in the meprin α protease domain. Three of the basic residues are within the active site cleft of mouse meprin β (Arg-147, Lys-185, and Lys-214; Fig. 5) and have the potential to form a salt bridge with acidic residues of substrates. For example, Phe-161 in meprin α, six amino acids carboxyl-terminal to the zinc HE*XX*H binding site, is equivalent to the Arg-147 position in meprin β. Astacin and meprin α have aromatic residues at this position, whereas meprin β and BMP-1 have basic residues. Meprin β and BMP-1 have a preference for acidic residues in P1′ of substrates (Fig. 1, Table III, and Ref. 31). BMP-1 hydrolyzes several proteins

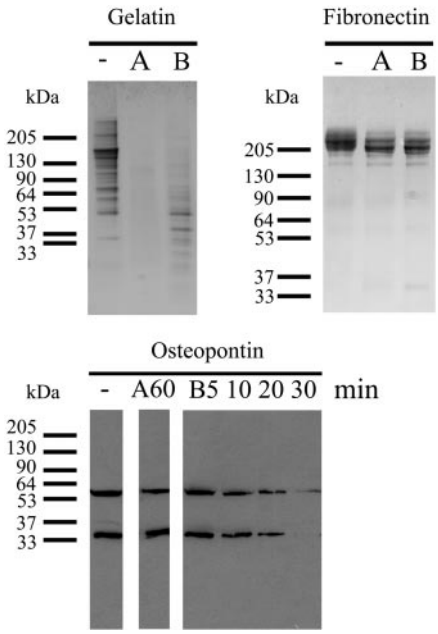

FIG. 3. **Degradation of proteins by meprins.** *Upper panel*, the extracellular matrix proteins gelatin and fibronectin (20 μg) were incubated with meprin A or B (20 nM) or no enzyme (−) in a final volume of 40 μl. The reactions were conducted in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, at 25 °C for 18 h. The reaction was terminated by addition of EDTA (10 mM), and samples subjected to electrophoresis on a 4–15% reducing denaturing gradient SDS-PAGE gel. Proteins were visualized with Coomassie Brilliant Blue. For control lanes (−) substrates were incubated without meprins: *lanes A*, meprin A-treated; *lanes B*, meprin B-treated. *Lower panel*, mouse osteopontin (50 μg ml^{-1}) was incubated with meprin A or B at 37 °C in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5 for various times as indicated. Meprins were at 10 nM. Remaining osteopontin was detected by Western blot analysis after SDS-PAGE on a 4–15% gradient gel. Representative data from at least three independent experiments are shown.

	IC50 (μM)		K_i (μM)
	Meprin A	Meprin B	Astacin
PLG-(NHOH)	30	50	16
Actinonin	0.10	0.40	130

FIG. 4. **Inhibition of meprins by hydroxamate inhibitors.** Meprin A or B (2 nM) was preincubated with inhibitor before addition of $sCCK8_{NH2}$ (50 μM) as substrate. Reactions were performed in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5. Substrate hydrolysis was determined by quantitative HPLC analyses. The concentrations of hydroxamate inhibitors ranged from 12.5 nM to 50 μM for actinonin and from 250 nM to 500 μM for PLG-(NHOH). The level of inhibition was determined by comparing the decrease in substrate concentration in the presence and absence of inhibitor. IC_{50} values of inhibition toward meprins as well as structures of actinonin and PLG-NHOH are shown. K_i values for astacin are shown for comparison (29, 30).

with a P1′ aspartic acid. Thus, it appears that meprin β and BMP-1 are similar proteins with respect to activity. Residues Tyr-199 and Lys-185 of mouse meprin α and β, respectively, form the floor of the proposed S1′ subsites (Fig. 5). Thus, this is a potential site of interaction between meprin β and acidic side chains of substrates such as gastrin. The CCK free acid, CCK8, is a better substrate for meprin B than $CCK8_{NH2}$, which is consistent with the preference of meprin β for an acidic moiety in or around the P2′ position of substrates.

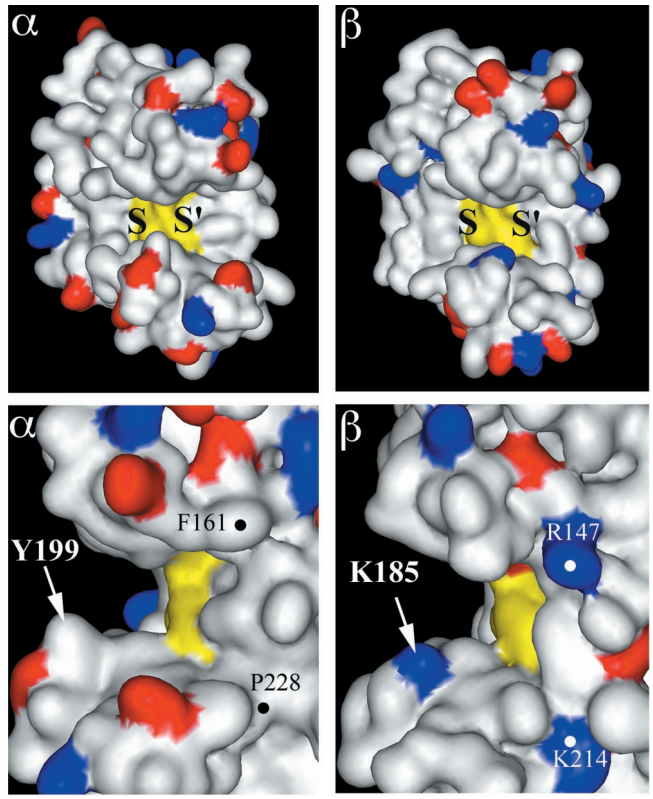

FIG. 5. **Active site clefts of the protease domains of mouse meprin α and β.** Homology models of the protease domains of mouse meprin α and β were produced using Modeller as described under "Experimental Procedures." *Upper panels*, *left* and *right*, surface representations of the front of the protease domains of mouse meprin α and β protease domains, respectively. The unprimed (*S*) and primed (*S′*) binding regions are shown. *Lower panels*, *left* and *right*, enlarged views of the mouse meprin α and β active site clefts, respectively. This view is from the perspective of the primed subsite region through the active site clefts toward the unprimed subsite region of the domains. *Yellow* indicates the catalytic center. *Blue* and *red* indicates basic and acidic residues respectively, and all other residues are colored *gray*. Key residues are shown and are numbered according to the full-length sequence starting from the initiator methionine.

The Tyr-199 residue of mouse meprin α protrudes into the active site cleft. This may explain why prolines are preferred at proximal sites to the scissile bonds in substrates. Prolines impart rigidity and stability to peptide backbones and have the ability to form cis peptide bonds within the backbone of substrates. This may allow the peptide to kink around the protruding tyrosine, thus allowing the peptide to fit in the cleft. Consequently, the cis conformation of a peptide bond may be preferred by meprins.

The inhibition of meprins by PLG-(NHOH) was expected, because both enzymes select for prolines in the unprimed substrate sites (*e.g.* bradykinin and gastrin 17 for meprin A and B, respectively). The crystal structure of astacin complexed with PLG-(NHOH) shows that the inhibitor binds along a β-edge strand, which consists of Trp-114 through Tyr-116 (accession number 1QJJ (29)). The tyrosine is not conserved in the meprins; Met-143 and Ser-128 are in the equivalent positions in meprin α and β, respectively. Therefore the pocket in astacin is likely smaller than in both meprins. Actinonin is bulkier than PLG-(NHOH) in the region that is predicted to bind along the β-edge strand. Actinonin is a much better inhibitor of meprins than it is of astacin, perhaps due to steric hindrance with astacin that would not occur with the meprins. The improved inhibition of meprins by actinonin over PLG-(NHOH) could reflect the presence of an additional methylene group within the carboxyl-terminal residue of the peptide backbone in acti-

nonin. This may provide more optimal spacing between the metal chelating hydroxamate moiety and the peptide backbone and side chains of the inhibitor.

Meprins are the only known endopeptidases in brush border membranes that degrade proteins; other proteases in these specialized cell surface membranes either degrade only small polypeptides (*e.g.* neprilysin) or are exopeptidases such as angiotensin converting enzyme or leucine aminopeptidase (32). Thus, meprins may initiate degradation of proteins in the lumen of the kidney proximal tubule, act as sheddases in this location, or activate/inactivate polypeptide hormones in the glomerular filtrate. In the intestine, meprins are most active in the ileum where there is an active immune system (33). The location at this site and proteolytic capacity of meprins implicates them in the hydrolysis of proteins and formation of peptides that are presented to antigen-producing cells. The presence of meprins in leukocytes and cancer cells also implies functions for meprins in cytokine activation or degradation and in hydrolysis of basement membrane components.

Peptides of the gastrointestinal tract appear to be among the best substrates identified for meprins and are of particular interest because of the expression of meprins in the intestine. Gastrin 17, cerulein, and $sCCK8_{NH2}$ have an identical stretch of five amino acids at their carboxyl termini, the four terminal amino acids confer the total biological activity of these peptides (34). Therefore meprins have the ability to inactivate these important regulatory molecules. The gastrointestinal peptides regulate the movement, secretory activity, and growth of the intestinal tract and pancreas, and thus the concentration of these peptides must be highly regulated.

The kidney is known to play a major role in the clearance of many plasma polypeptides such as glucagon and $sCCK8_{NH2}$ (*e.g.* Ref. 35). Patients with chronic renal failure also have elevated levels of peptides in the blood that are involved in gut motility, hunger, and satiety. Neurotensin, peptide YY, substance P, vasoactive intestinal peptide, GRP-(14–27), and gastrin are all elevated during chronic renal failure probably due to a decrease in metabolism of circulating peptide (36, 37). This points toward a role of renal brush border proteases, including meprins, in the catabolism of circulating peptides and thus the recapture of amino acids and/or the alteration of urinary peptides. The finding that osteopontin is a substrate for meprin B is intriguing. Experimental hydronephrosis results in an accumulation of osteopontin protein within the lumen of the proximal tubule at a time that meprin protein is markedly decreased (38, 39). The absence of meprins may account for some of the accumulation of peptides and proteins that contribute to a cascade of events that lead to fibrosis and end-stage renal disease.

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Marked Differences between Metalloproteases Meprin A and B in Substrate and Peptide Bond Specificity

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