An Old Enzyme with a New Function: Purification and Characterization of a Distinct Matrix-degrading Metalloproteinase in Rat Kidney Cortex and Its Identification as Meprin

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Abstract. We have purified to homogeneity the enzyme in the kidney cortex which accounts for the vast majority of matrix-degrading activity at neutral pH. The purified enzyme has an apparent molecular mass of 350 kD by gel filtration and of 85 kD on SDS-PAGE under reducing conditions; and it degrades laminin, type IV collagen and fibronectin. The enzyme was inhibited by EDTA and 1,10-phenanthroline, but not by other proteinase inhibitors. The enzyme was not activated by organomercurials or by trypsin and was not inhibited by tissue inhibitors of metalloproteinases indicating that it is distinct from the other matrix-degrading metalloproteinases. Unexpectedly, the amino acid sequence of the NH_2-terminal and two internal peptides of the enzyme showed complete homology to those α subunits of rat meprin, an enzyme previously shown to degrade azocasein and insulin B chain but not known to degrade extracellular matrix components. Immunoprecipitation studies, Western blot analyses and other biochemical properties of the purified enzyme confirm that the distinct matrix-degrading enzyme is indeed meprin. Our data also demonstrate that meprin is the major enzyme in the renal cortex capable of degrading components of the extracellular matrix. The demonstration of this hitherto unknown function of meprin suggests its potential role in renal pathophysiology.

THE matrix-degrading metalloproteinases are a closely related group of zinc metal-dependent enzymes capable of degrading one or more of the extracellular matrix (ECM)[1] components at neutral pH. They have been classified into three major subclasses: collagenases, gelatinases, and stromelysins, which share several common structural and biochemical properties including inhibition by metal chelators (EDTA and 1,10-phenanthroline) and by the natural specific tissue inhibitors of metalloproteinases (TIMP) as well as activation by various agents including organomercurials (18). Because of their matrix-degrading ability, the metalloproteinases have been suggested to play critical roles in many biological processes such as tissue remodeling, embryonic development, inflammation, tumor invasion, and metastasis (17, 18, 19, 28).

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1. *Abbreviations used in this paper*: APMA, 4-aminophenylmercuric acetate; DE-52, DEAE-cellulose; E-64, L-*trans*-epoxysuccinyl-leucyl amido-(4-guanidino)-butane; ECM, extracellular matrix; EHS, Engelbreth-Holm-Swarm; PCMB, *p*-chloromercuric benzoate; SBTI, soybean trypsin inhibitor; TIMP, tissue inhibitors of metalloproteinases.

Alterations in the renal tubular basement membrane, a specialized form of extracellular matrix composed primarily of type IV collagen, laminin, and proteoglycans (1) occur in both acute (26) and chronic injury (23). Despite the potential importance of matrix-degrading enzymes in renal pathophysiology there is little information about these enzymes in the renal cortex. We recently reported that homogenates prepared from either renal cortex or isolated renal tubules contain a proteinase activity capable of degrading gelatin, laminin, and type IV collagen at neutral pH (16, 27). The matrix-degrading activity is markedly inhibited (>90%) by the metalloproteinase inhibitors, EDTA and 1,10-phenanthroline, but unaffected by inhibitors of serine, aspartate, and cysteine proteinases documenting that this metalloproteinase activity is responsible for the vast majority of the matrix degradation. This enzyme differs from the known matrix-degrading metalloproteinases in that it is not inhibited by TIMP and is not activated by organomercurial compounds indicating that this enzyme is distinct from the other well characterized matrix-degrading metalloproteinases. Thus, it was of interest to purify, characterize, and identify this distinct matrix-degrading enzyme.

In this study, we purified this matrix-degrading enzyme to homogeneity and also demonstrated the ability of the

purified enzyme to degrade extracellular matrix components including laminin, type IV collagen, and fibronectin. Unexpectedly, the amino acid sequence of the NH_2-terminal and two internal peptides of the enzyme showed complete homology to those of the α subunit of rat meprin, an enzyme previously shown to degrade azocasein and insulin B chain but not known to degrade ECM components. Additional studies including immunoprecipitation studies, Western blot analyses, and other biochemical properties of the purified enzyme were carried out to provide further evidence that the distinct matrix-degrading enzyme is indeed meprin.

Materials and Methods

[^{3}H]Acetic anhydride (50 mCi/mmol), [^{3}H]laminin (0.15 mCi/mg), and [^{3}H]collagen IV (0.14 mCi/mg) were purchased from New England Nuclear (Boston, MA). Phosphoramidon, gelatin-Sepharose, concanavalin A-Sepharose, L-*trans*-epoxysuccinyl-leucyl amido-(4-guanidino)-butane (E-64), *p*-chloromercuric benzoate (PCMB), 4-aminophenylmercuric acetate (APMA), bovine serum albumin, trypsin, soybean trypsin inhibitor (SBTI), papain, PMSF, leupeptin, pepstatin, aprotinin, gelatin, NP-40, Triton X-100, Sephadex G-200, Sephacryl S-300, and molecular weight standards for gel filtration were obtained from Sigma Chem. Co. (St. Louis, MO). Engelbreth-Holm-Swarm (EHS) laminin and type IV collagen were purchased from Collaborative Biomedical Products (Bedford, MA). Peptide *N*-glycosidase F and *N*-acetylglucosaminidase H were from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Hydroxylapatite was obtained from Bio-Rad Labs. (Richmond, CA) and DEAE-cellulose (DE-52) was from Whatman Chemical Separations, Ltd. (Hillsboro, Oregon). All chemicals for gel electrophoresis were purchased from Bio-Rad Labs. TIMP-2 was a generous gift from Dr. Keith Langley (Amgen Co., Thousand Oaks, CA). Stromelysin was the kind gift of Dr. Hideaki Nagase (University of Kansas Medical Center, Kansas City, Kansas).

Assay of the Matrix Degrading Activity

The matrix-degrading activity of the enzyme was determined by using [^{3}H]gelatin as the substrate. Gelatin was radiolabeled with [^{3}H]acetic anhydride according to the procedure of Montelaro and Reuckert (20) modified as described previously (16). The standard incubation mixture contained 30,000 cpm of [^{3}H]gelatin (2 μg protein), 50 mM Tris-HCl, pH 7.5, and various amounts of enzyme in a final volume of 0.2 ml. The reactions were incubated for 30 min at 37°C, and the supernatants containing the degraded products of [^{3}H]gelatin after TCA precipitation were counted to determine the amount of radioactivity released. When the effect of inhibitors was determined, the inhibitors were preincubated with the enzyme for 10 min before adding the substrate.

Preparation of Membranes from Kidney Cortex

In preliminary studies, we determined that the membrane fraction contained more than 90% of the total gelatin-degrading activity in renal cortical homogenates. In order to prepare the membranes, rat kidney cortices from 100 kidneys were homogenized in 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin by using three 30-S bursts in a Waring blender at maximum speed. The cortex homogenate was centrifuged at 9,000 *g* for 20 min and the supernatant was centrifuged at 100,000 *g* for 1 h to sediment the membranes. The membrane pellet was stored at −20°C until used.

Solubilization of Matrix Degrading Activity

The membranes were suspended in 50 mM Tris buffer, pH 7.2, containing 150 mM NaCl, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin, and centrifuged at 100,000 *g* for 1 h. The washed membranes were then suspended in solubilization buffer containing 50 mM Tris, pH 7.2, 1% NP-40, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin and homogenized with a Dounce homogenizer for 10 min. The resulting suspension was centrifuged at 100,000 *g* for 1 h. The supernatant was removed and saved. The pellet was again suspended in the solubilization buffer, homogenized, and centrifuged as before. The pooled supernatants were used for purification of the metalloproteinase activity.

Buffers

The various buffers used during purification studies are as follows: buffer A contained 10 mM Tris-HCl, pH 7.2, 0.1% NP-40, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. Buffer B contained 10 mM sodium phosphate, pH 7.0, 0.1% NP-40, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. Buffer C contained 10 mM Tris-HCl, pH 7.2, 0.1% NP-40, 250 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin.

Chromatography on DE-52 Column. The solubilized enzyme (290 ml) was applied to a 50 × 2.5-cm long DE-52 column equilibrated in buffer A. The column was washed with 500 ml of buffer A and the retained enzyme was eluted using a 500-ml linear gradient from 0 to 0.4 M NaCl in buffer A. With this gradient, the enzyme was eluted at 150 at 250 mM NaCl. Fractions containing enzyme activity were pooled, concentrated to ~250 ml by using an Amicon filtration apparatus, and extensively dialyzed overnight in buffer B.

Chromatography on Hydroxylapatite Column. The dialyzed fractions from the DE-52 column were applied to a 2.5 × 10-cm column of hydroxylapatite previously equilibrated with buffer B. The column was washed with buffer B until protein was no longer detected in the effluent. The retained proteins were eluted with 500 ml of a linear gradient of 10 to 250 mM potassium phosphate in buffer B. The gelatinolytic activity was eluted in the wash and was concentrated to ~5 ml.

Chromatography on Concanavalin A-Sepharose Column. About 3 ml of concanavalin A-Sepharose was washed with 50 ml of buffer C and gently mixed with 4–5 ml of the enzyme from the hydroxylapatite step. The mixture was placed on a platform rocker and gently rocked for about 4 h at 4°C. The suspension was then transferred to a small column (1.5 × 6 cm), and was washed with at least 10 column volumes of buffer C. The bound enzyme activity was eluted with a repeated wash of 0.5 M methyl α-mannoside in buffer C. The active fractions from each elution were pooled, concentrated to ~20 ml, and dialyzed overnight with buffer A.

Chromatography on Gelatin-Sepharose. The dialyzed and concentrated enzyme from the concanavalin A-Sepharose column was applied to a 1 × 20-cm gelatin-Sepharose column that had been equilibrated with buffer A. The column was washed with 200 ml of buffer A and the retained enzyme activity was eluted using a linear gradient of 0 to 0.5 M NaCl in buffer A. The active fractions were pooled, concentrated to about 10 ml, and dialyzed overnight with buffer A.

Polyacrylamide Gel Electrophoresis and Gelatin-Zymography

SDS-PAGE was performed according to Laemmli (14) in 8% polyacrylamide gels. Zymography was carried out essentially as described by Chin et al. (4) by copolymerizing gelatin (1 mg/ml) into 8% gels. Samples were prepared in 2% SDS in sample buffer without 2-mercaptoethanol. After electrophoresis, the gels were washed in 200 ml of 50 mM Tris-HCl, pH 7.5, containing 2.5% Triton X-100 and 10 mM $CaCl_2$ at room temperature for 30 min. The gels were then incubated at 37°C in this buffer without Triton X-100 for 24 h. The gels were stained with 0.05% Coomassie blue in 10% acetic acid containing 10% isopropanol and destained in the same solvent without Coomassie blue.

Effect of Endo β-N-acetylglucosaminidase H (Endo H) and Peptide N-glycosidase F

To examine the presence of asparagine-linked oligosaccharide chains, ~10 μg of the purified enzyme was treated with Endo H and peptide *N*-glycosidase F as described (11) and the deglycosylated enzyme was analyzed by SDS-PAGE.

Degradation of Type IV Collagen, Laminin, and Fibronectin

[^{3}H]collagen type IV (100,000 cpm) was incubated for various times with 1 μg of purified enzyme in 50 mM Tris-HCl, pH 7.5, containing 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin in a final volume of 200 μl. At the end of incubation, the unhydrolyzed collagen was precipitated by adding 100 μl of 50% TCA and 50 μl of 1 mg/ml bovine serum albumin as carrier. The supernatants containing the degraded products were counted to determine the radioactivity. EHS laminin and fibronectin were incubated for various times with the purified enzyme in the same buffer as described for type IV collagen. The reaction products were resolved by SDS-PAGE and the protein bands were visualized by staining with Coomassie blue.

Amino Acid Sequencing of the Purified Enzyme

The purified enzyme was subjected to SDS-PAGE and the protein band was electrophoretically transferred to Immobilon P as described (24). Immobilon P membrane was stained with Ponceau S to detect the protein. The Ponceau S-stained portion of the membrane was excised, thoroughly washed with water, and used for peptide sequence analyses. Sequence analysis of the NH_2-terminal as well as the internal peptides was performed at the Protein Sequencing Facility of Harvard Microchem using ABI 477A Protein Sequencer with 120A Online PTH-AA Analyzer.

Purification of Meprin from Rat Kidney Cortex

Meprin was purified from rat kidney cortex as described by Kenny and Ingram (12).

Preparation of Polyclonal Antibodies

The purified enzyme (120 μg) in 0.5 ml of phosphate buffer saline was emulsified with an equal volume of Freund's complete adjuvant and injected intradermally at multiple sites along the back of a female New Zealand white rabbit. This was followed by two booster injections of 100 μg of the enzyme protein, emulsified with Freund's incomplete adjuvant at 4-wk intervals. 2 wk after the last injection, the serum was collected and stored at −80°C until used. A polyclonal antibody to meprin was also raised.

Immunoprecipitation of the Enzyme Activity

Serial dilutions of the antibody were made with phosphate buffered saline and various amounts of antibody (50 μl total volume) were incubated with solubilized renal cortical membranes in Eppendorf tube at 4°C for 4 h. Control tubes were set up with preimmune serum. At the end of incubation, 50 μl of *Staphylococcus aureus* (1 g of cells in 10 ml of H_2O) was added and mixed on a platform shaker for 2 h at 4°C. The mixture was then centrifuged to pellet the precipitated antigen-antibody complex and the metalloproteinase activity in the supernatant was tested.

Western Blot Analysis

Polyclonal antibodies to purified matrix-degrading metalloproteinase and purified meprin were used for Western blot analysis as previously described (9).

Results

Purification of the Matrix-degrading Enzyme

The gelatin-degrading activity was purified from solubilized renal cortical membranes. The solubilized extract was first

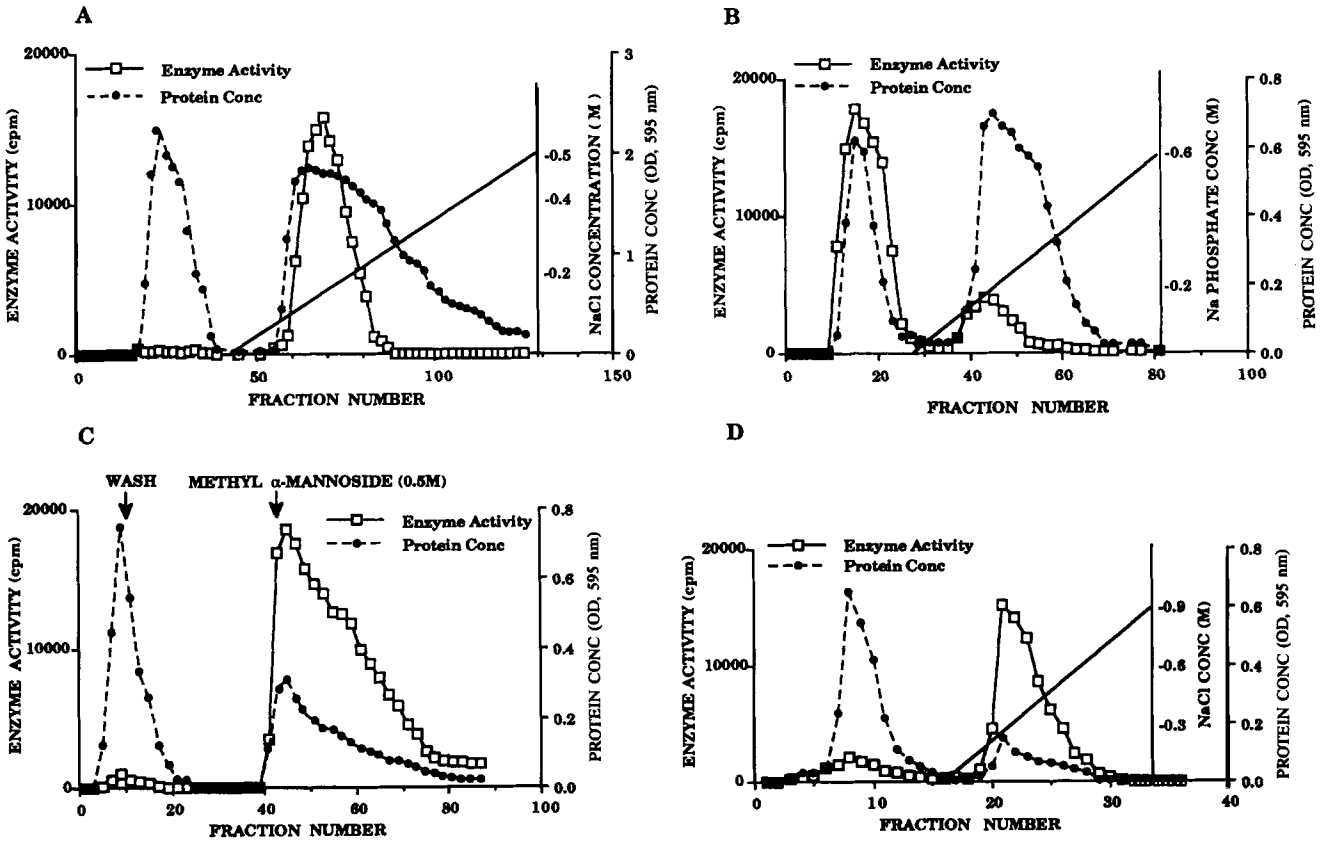


Figure 1. Chromatographic steps in purification of the metalloproteinase activity. (*A*) Purification on a DEAE-cellulose column: the solubilized enzyme was applied to a 50 × 2.5-cm DEAE-cellulose column equilibrated in buffer A and the enzyme activity retained by this column was eluted using a linear gradient of 0 to 0.4 M NaCl in buffer A. (*B*) Purification on a hydroxylapatite column: the dialyzed fractions from the DEAE-cellulose column were applied to a 2.5 × 10-cm column of hydroxylapatite that was equilibrated with buffer B. The enzyme activity was eluted in the wash whereas the bound proteins were eluted by increasing concentration of phosphate. (*C*) Purification on a concanavalin A–Sepharose column: the dialyzed and concentrated fractions from the hydroxylapatite column were applied to a 1.5 × 6-cm column of concanavalin A–Sepharose previously equilibrated in buffer C. The enzyme retained in this column was eluted with 0.5 M α-methyl mannoside as described in the text. (*D*) Purification on a gelatin-Sepharose column: the dialyzed and concentrated fractions from the concanavalin A–Sepharose column were applied to a 1 × 20-cm gelatin–Sepharose column previously equilibrated with buffer A. The enzyme activity was eluted using a linear gradient of 0 to 0.5 M NaCl in buffer A.

Table I. Purification of Metalloproteinase from Rat Kidneys

Fractions	Volume	Total proteins	Total activity	Specific activity	Fold purification	Yield
	ml	*mg*	*U*	*U/mg*		%
Membrane fraction	240	2524.4	21255	8.4	1	100
Solubilized enzyme	290	1158.1	28678	24.8	3	134
DEAE-cellulose	265	485.1	28573	58.9	7	134
Hydroxylapatite	16	116.4	12442	106.9	13	59
Con. A-Sepharose	130	29.1	8094	277.8	33	38
Gelatin-Sepharose I	5.5	2.7	2882	1059.6	126	14
Gelatin-Sepharose II	3.2	0.45	2621	5798.6	689	13

1 U of enzyme activity is defined as the amount of enzyme required to degrade 1 μg of gelatin per hour. Gelatin-Sepharose I and gelatin-Sepharose II refer to two successive chromatographies on gelatin-Sepharose columns.

purified by chromatography on a DEAE-cellulose column. The enzyme activity retained by this column was eluted at 150–250 mM NaCl (Fig. 1 *A*). In the next step of purification, using a hydroxylapatite column, most of the enzyme was collected in the wash whereas a small amount of the enzyme remained nonspecifically bound to the column (Fig. 1 *B*). However, this minor fraction (bound activity) could be recovered in the wash when applied to a second hydroxylapatite column. The enzyme bound quantitatively to the lectin-affinity column, concanavalin A-Sepharose, and was eluted selectively with α-methyl mannoside (Fig. 1 *C*) suggesting that the enzyme is a glycoprotein containing asparagine-linked oligosaccharide chains. The final step in the purification was another affinity chromatography using a gelatin-Sepharose column (Fig. 1 *D*). Two successive chromatographies on gelatin-Sepharose columns were required to purify the enzyme to apparent homogeneity. The gelatin-degrading activity in enzyme fractions obtained after each purification step, was inhibited by EDTA or 1,10-phenanthroline. Together, these steps of purification resulted in a homogeneous preparation of the metalloproteinase activity.

The summary of the purification is presented in Table I. These steps resulted in a specific enzyme activity of 5798 U/mg protein representing a 689-fold purification over the membrane fraction. The results of SDS-PGE under reducing conditions at each stage of purification are shown in Fig. 2. At the final stage of purification, the enzyme was homogeneous and showed a single band with a molecular mass of 85 kD on SDS-PGE. Chromatography on Sephacryl S-300 HR indicated a molecular mass of ~350 kD suggesting that the native enzyme is an oligomeric protein and most likely a tetramer (Fig. 3). The purified protein subjected to zymography using gelatin as substrate under nonreducing conditions in the presence of SDS, showed a single clear zone (from 140–180 kD) indicating that under these conditions the enzyme primarily exists in a dimer form (Fig. 4).

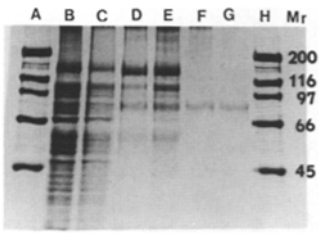


Figure 2. SDS-PAGE of the metalloproteinase fractions from various steps of purification. Aliquots of enzyme fractions (2–4 μg) obtained at various stages of purification were resolved by SDS-PAGE (8% gels) under reducing conditions and stained with Coomassie R-250 as described in the text. Lanes *A* and *H* are molecular mass standards. The enzyme fractions used in lanes *B* through *G* are as follows: *B*, solubilized enzyme; *C*, DEAE-cellulose; *D*, hydroxylapatite; *E*, concanavalin A-Sepharose; *F*, gelatin-Sepharose I; *G*, gelatin-Sepharose II.

Biochemical Characterization of the Purified Enzyme

The rate of gelatin-degrading activity was directly proportional to the time of incubation for at least 30 min and to the amount of enzyme added to the incubation up to 0.8 μg of protein. The reaction products formed at various times were resolved by size exclusion chromatography on a Sephadex G-200 column. As shown in Fig. 5, the amount of radioactivity in the slow moving peaks increased proportionately with time of incubation. Extensive hydrolysis of gelatin occurs rapidly resulting in gelatin fragments less than 14 kD in size within 30 min of incubation. The effect of pH on the gelatin-degrading activity of the purified enzyme was examined over the pH range 4 to 10 using acetate, Mes, and Caps buffers. The enzyme showed broad pH optima between pH 6 to 8 with a rapid decrease below 5 and above 9 (data not shown).

EDTA and 1,10-phenanthroline markedly inhibited the purified enzyme (Table II). The inhibitory activity of EDTA

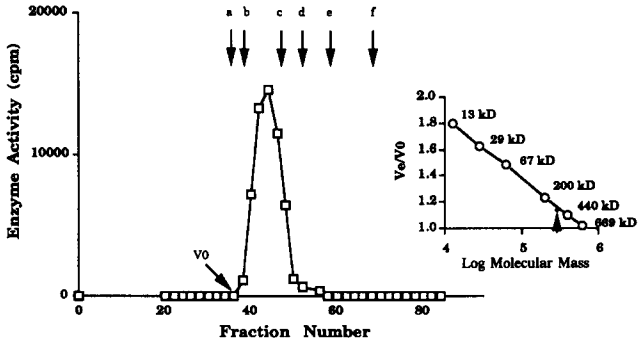


Figure 3. Determination of molecular mass of the metalloproteinase by sephacryl S-300 HR column chromatography. The purified enzyme was applied to a gel filtration column of Sephacryl S-300 HR previously equilibrated in buffer A containing 200 mM NaCl. Protein standards and their corresponding molecular masses used for calibrating the column are: (*a*) thyroglobulin (669 kD); (*b*) ferritin (440 kD); (*c*) β-amylase (200 kD); (*d*) bovine serum albumin (67 kD); (*e*) carbonic anhydrase (29 kD); and (*f*) cytochrome C oxidase (13 kD). The molecular mass of metalloproteinase was determined after comparison to the known markers.

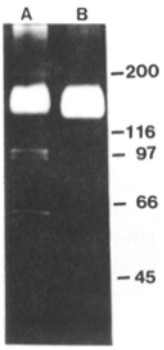


Figure 4. Gelatin-substrate zymography of the solubilized and the purified metalloproteinase. The solubilized (*A*) and the purified (*B*) enzyme was subjected to SDS-PAGE under non-reducing conditions using 8% acrylamide impregnated with 1 mg gelatin/ml. The zymogram was developed as described in the text. A clear zone indicates the presence of gelatin-degrading activity.

was overcome by zinc. DTT was also found to be a potent inhibitor of the purified enzyme (Table II). Several serine, cysteine and aspartate proteinase inhibitors did not inhibit the enzyme activity. Phosphoramidon (0.1–1 mM), an inhibitor of the neutral brush border membrane metalloendopeptidase (EC 3.4.24.11), also did not affect the enzyme activity. Various alkylating agents were also examined but neither *N*-ethylmaleimide nor iodoacetamide had any effect on the enzyme activity (Table II). This indicates that the enzyme does not require any potential free sulfhydryl group for its activity. The effect of TIMP (which inhibits the matrix-degrading metalloproteinases including the collagenases, gelatinases, stromelysins, etc.) was also examined. As shown in Table II, TIMP-2 did not inhibit the enzyme activity. We confirmed that TIMP-2 in similar concentrations was able to completely inhibit the gelatin-degrading activity of stromelysin.

Most of the matrix-degrading metalloproteinases are synthesized and secreted in their latent form as zymogens and can be activated in vitro either by trypsin or organomercurials (2, 18, 19, 25). Thus, we examined the effect of trypsin and various organomercurials on the activity of the purified enzyme. As shown in Table III neither trypsin nor organomercurials such as APMA or PCMB increased the activity of the purified enzyme.

Effect of Endoglucosaminidase H and Peptide N-Glycosidase F. As shown in Fig. 6, the enzyme is susceptible to both Endo H and peptide *N*-glycosidase F. The Endo H-treated enzyme migrated slightly faster which indicates the removal of 1 or 2 high-mannose oligosaccharide chains from the enzyme. In addition, peptide *N*-glycosidase F-treated enzyme was even smaller (by 25 kD) which indicates the removal of several complex type oligosaccharide chains from the enzyme. This data indicate the presence of asparagine-linked oligosaccharide chains and indicate the abundance of oligosaccharide chains. However, we have not examined for the presence of *O*-linked oligosaccharide chains.

Degradation of Collagen Type IV, Laminin, and Fibronectin. We examined the ability of the purified enzyme to degrade major basement membrane components. [^{3}H]-collagen type IV was incubated with the purified enzyme for various times and the hydrolyzed products were analyzed in

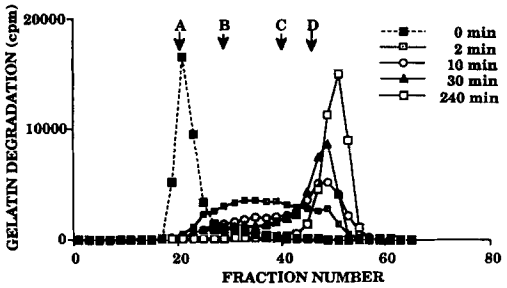


Figure 5. Time course of gelatin degradation by the purified matrix-degrading metalloproteinase. The assay mixtures containing 1 μg of the purified enzyme and 60,000 cpm of [^{3}H]-gelatin was prepared as described in the text. The assay mixtures were incubated for various times as indicated in the figure and the reaction products were resolved by gel filtration on a calibrated column of Sephadex G-200. (*A*) blue dextran; (*B*) albumin (67 kD); (*C*) carbonic anhydrase (29 kD); (*D*) cytochrome C (13 kD).

Table II. Effect of Selected Proteinase Inhibitors on the Purified Enzyme

Inhibitor	Concentration	Enzyme activity
		% control
1,10-Phenanthroline	2.5 mM	33 ± 3
	5.0 mM	18 ± 2
EDTA	1.0 mM	40 ± 1
	5.0 mM	20 ± 3
EDTA + 10 mM Zn^{2+}	5.0 mM	107 ± 2
DTT	0.5 mM	20 ± 1
	1.0 mM	3 ± 1
E-64	10.0 μM	98 ± 2
	20.0 μM	103 ± 3
Leupeptin	200.0 μM	99 ± 2
Pepstatin A	200.0 μM	88 ± 4
PMSF	1.0 mM	99 ± 1
SBTI	1.0 mg/ml	89 ± 4
Aprotinin	140.0 k/U	100 ± 4
Phosphoramidon	5.0 μM	97 ± 1
	10.0 μM	96 ± 2
NEM	1.0 mM	96 ± 1
	5.0 mM	109 ± 1
Iodoacetamide	1.0 mM	98 ± 1
TIMP-2	10.0 μg/ml	103 ± 3
	20.0 μg/ml	98 ± 2

The purified enzyme (~1 μg) was preincubated for 10 min with varius amounts of inhibitors as indicated. The substrate ([^{3}H]gelatin, 30,000 cpm) was then added and incubations continued for another 30 min at 37°C. The products were determined in supernatants after TCA precipitation. Values are mean ± SE.

Table III. Effect of Organomercurials and Trypsin

Agents	Concentration	Enzyme activity
		% control
APMA	0.5 mM	101 ± 2
	1.0 mM	98 ± 2
PCMB	0.5 mM	101 ± 2
	1.0 mM	88 ± 1
Trypsin	10.0 μg/ml	105 ± 2
	20.0 μg/ml	113 ± 3

The purified enzyme (~1 μg) was preincubated for 30 min with various concentrations of organomercurials as indicated. The substrate ([^{3}H]gelatin, 30,000 cpm) was then added and incubations were continued for another 30 min at 37°C. The reaction products were determined in the supernatants after TCA precipitation. Trypsin was preincubated with the purified enzyme for 20 min at 25°C followed by 20-fold excess of SBTI. The substrate was then added and incubations carried out as described above for organomercurials. Values are mean ± SE.

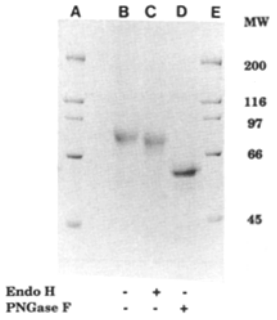
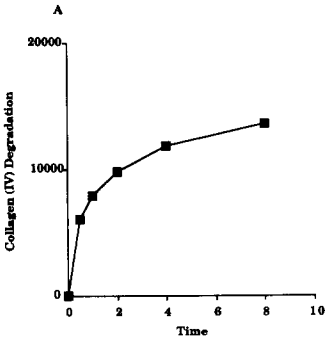
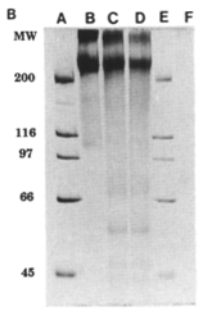


Figure 6. Effect of deglycosylation of the matrix degrading metalloproteinase on its migration on SDS-polyacrylamide gels. The purified enzyme was treated with Endo H and peptide *N*-glycosidase F as described in the text. The control and treated enzyme proteins were resolved by SDS-PAGE and the proteins were detected with Coomassie blue.

the supernatants after TCA precipitation. As shown in Fig. 7 *A*, the enzyme is capable of hydrolyzing [^{3}H]collagen type IV. In further experiments, the reaction products formed on incubation of the purified enzyme with mouse EHS laminin were resolved by SDS-gel electrophoresis (Fig. 7 *B*). As can be seen both light and heavy chains of laminin (7) are susceptible to degradation and the proteolytic fragments of 68, 57, 47, and 44 kD are produced. Similarly, the purified enzyme was capable of degrading fibronectin resulting in proteolytic fragments of 112, 88, and 44 kD as shown in Fig. 7 *C*.





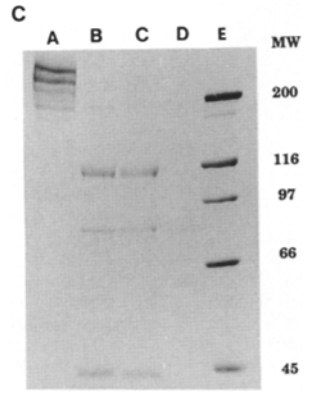


Figure 7. Degradation of type IV collagen, laminin, and fibronectin by the purified metalloproteinase. (*A*) [^{3}H]-collagen was incubated for various times in 100 mM Tris–HCl, pH 7.5, with 1 μg of the purified enzyme. At the end of incubations, the radioactivity was assayed in the supernatants after TCA precipitation. (*B*) Mouse EHS laminin (20 μg) was incubated with the purified enzyme (1 μg) for various times and reaction products were resolved by SDS-PAGE. Lane *A*, molecular weight markers; lane *B*, laminin without enzyme; lane *C*), 15 h incubation; lane *D*, 30 h incubation; lane *E*, molecular weights; lane *F*, purified enzyme. (*C*) Fibronectin (20 μg) incubation products were resolved by SDS-PAGE. Lane *A*, fibronectin without enzyme; lane *B*, 15-h incubation; lane *C*, 30-h incubation; lane *D*, purified enzyme; lane *E*, molecular weight markers.

Evidence That the Matrix Degrading Enzyme Is Meprin

Amino Acid Sequence of the Purified Enzyme. The NH_2-terminal peptide and two internal peptides selected from the HPLC profile obtained on digestion of the purified enzyme with lys C endopeptidase were used for amino acid sequence. Unexpectedly, the amino acid sequence of these peptides showed complete homology to the previously reported amino acid sequence of the α subunit of rat meprin from 68–77, 379–391, and 392–401 (5, 10) (Fig. 8). It is important to note that there is essentially no homology to the β subunit of meprin (Fig. 8).

Comparison of the Purified Enzyme with Meprin. Meprin has not been previously reported to degrade gelatin or ECM components. Thus we carried out additional studies to provide further evidence that the matrix-degrading enzyme is indeed meprin. Meprin has been previously purified after papain treatment of cortical membranes monitoring azocasein degrading activity (10, 12). We, therefore, purified gelatin-degrading activity as well as azocasein-degrading activity from cortical membranes after treatment with papain. Enzymes purified by both these methods yielded two polypeptide chains of ~84 and 72 kD by SDS-PAGE (Fig. 9), indicating that when the gelatin-degrading activity is purified after papain digestion, it also yields two bands similar to the previous descriptions of meprin purification (10, 12). In addition, the enzyme, purified after detergent solubilization of the membranes, yields a single band of 85 kD corresponding to the larger 84-kD subunit of the purified papain-solubilized enzyme. Moreover, the enzyme, purified either after papain treatment or after detergent solubilization, migrated to similar positions on a gelatin substrate gel (data not shown).

Meprin was able to degrade collagen type IV, laminin, and fibronectin resulting in proteolytic fragments similar to those produced with the purified detergent-solubilized enzyme (Fig. 10). In addition, the detergent-solubilized enzyme was able to degrade azocasein and insulin, known substrates for meprin (data not shown).

Immuno-crossreactivity of Matrix-degrading Enzyme and Meprin. Antiserum raised against the purified detergent-solubilized enzyme or meprin (papain-solubilized enzyme) was incubated with the solubilized membrane fraction of the renal cortex as described in Materials and Methods. As shown in Fig. 11 (*A* and *B*), both of the antibodies immunoprecipitated the gelatin-degrading activity whereas no effect was observed when preimmune serum was used. These data demonstrate that the purified enzyme accounts for most of the gelatin-degrading activity of the renal cortex. In addition, these results show that the antibody against meprin is able to immunoprecipitate the gelatin-degrading activity in the renal cortex. Western blot analyses reveals that the antibody raised against detergent-solubilized purified matrix-degrading enzyme or to papain-solubilized azocasein-degrading activity (meprin) cross-reacted with both the enzymes (Fig. 11, *C* and *D*).

Discussion

In the present study, we have purified to homogeneity the

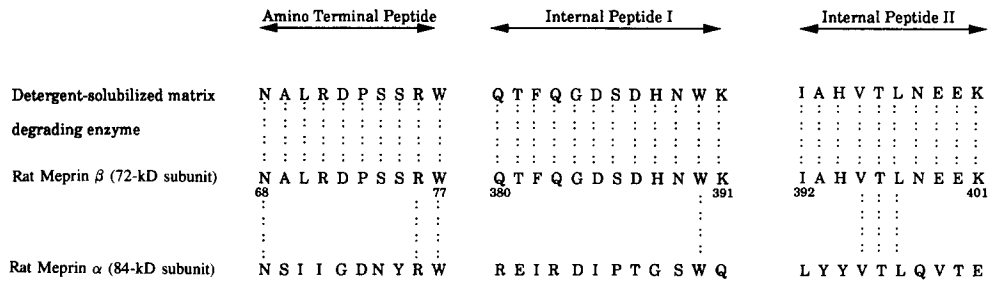


Figure 8. Comparison of the amino acid sequence of amino terminal and two internal peptides of detergent solubilized matrix degrading enzyme to those of rat meprin α (84-kD subunit) and rat meprin β (72-kD subunit). The amino acid sequence of rat meprin α is described by Corbeil, et al. (20) and that of rat meprin β by Johnson and Hersh (21).

proteinase responsible for the vast majority of matrix-degrading activity in the kidney cortex at neutral pH. This matrix-degrading enzyme is an oligomeric protein of ~340–360 kD as indicated by size exclusion chromatography and is apparently composed of four subunits of 85 kD based on SDS-PAGE under reducing conditions. Under nonreducing conditions in the presence of SDS, the enzyme primarily exists in dimer form as is evident from the gelatin zymogram. This enzyme has the capacity to degrade type IV collagen, laminin, and fibronectin, all important components of the extracellular matrix.

Unexpectedly, the amino acid sequence of NH_2-terminal and two internal peptides of the purified enzyme showed complete homology to the primary amino acid sequence of the α subunit of rat meprin. Meprin (EC.3.4.24.18), a plasma membrane-associated metalloendopeptidase present in the proximal tubule of the kidney (3), has not been previously reported to degrade ECM components. The enzyme is a major constituent of the membranes and comprises ~5% of the total microvillar proteins. The enzyme has been previously purified from mouse (13) and rat (12) kidneys as azocasein- or insulin B-chain–degrading activity following its release from the brush border membranes by treatment with papain (3). The purified papain-solubilized meprin from rat kidney yields two polypeptides of ~72 and 84 kD (10, 12). We, therefore, purified gelatin-degrading activity as well as azocasein-degrading activity from cortical membranes after treatment with papain. Our data demonstrate that when the enzyme is purified after detergent solubilization, it yields a single band on SDS-PAGE which corresponds to meprin A (8). In contrast, when the enzyme is prepared after papain solubilization, it yields two bands, the 84-kD band which corresponds to meprin A and the 72-kD band which corresponds to meprin B (5, 8, 10). Further evidence that the detergent-solubilized enzyme is meprin A, are the data with Western blots in which the antibody prepared against the detergent-solubilized enzyme has only minimal cross-reactivity with the 72 kD band. This is in keeping with the complete homology of the NH_2-terminal and the two internal peptides of the purified matrix-degrading enzyme with meprin A and the marked mismatch with meprin B.

In addition to demonstrating that the biochemical properties of the matrix-degrading enzyme purified from detergent-

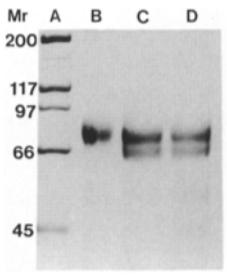


Figure 9. Comparison of the purified enzymes from detergent-solubilized and papain solubilized membranes by SDS-PAGE. Lanes *A*, molecular mass standards; lane *B*, detergent-solubilized gelatin-degrading enzyme; lane *C*, papain-solubilized gelatin-degrading enzyme; lane *D*, papain-solubilized azocasein-degrading enzyme (meprin).

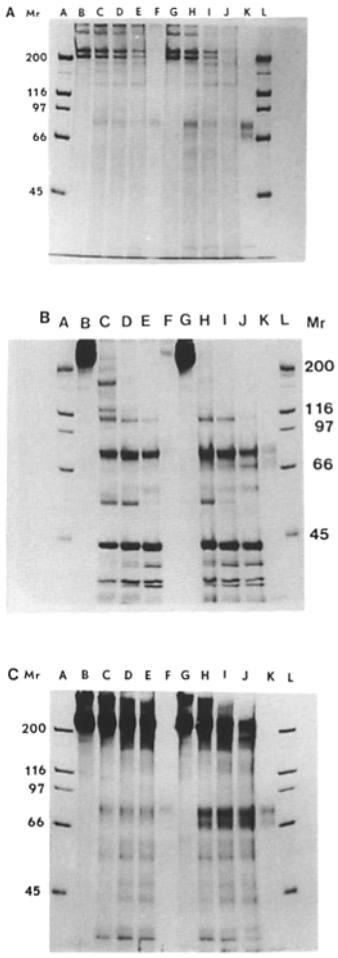


Figure 10. Comparison of matrix-degrading activity of the purified metalloproteinase to that of purified meprin. (*A*) Collagen type IV (30 μg) was incubated with 1 μg of purified cortical metalloproteinase (lanes *C–F*) or with 2 μg of purified meprin (lanes *H–K*) for various times and reaction products were analyzed by SDS-PAGE. Lanes *A* and *L*, molecular mass standards; lanes *B* and *G*, collagen type IV controls; lanes *C* and *H*, 1-h incubation; lanes *D* and *I*, 2-h incubation; lanes *E* and *J*, 4-h incubation; lanes *F* and *K*, purified metalloproteinase and purified meprin, respectively. (*B*) Fibronectin (50 μg) was incubated with 1 μg of purified cortical metalloproteinase (lanes *C–F*) or with 1 μg of pure meprin (lanes *H–K*) for various times and reaction products were resolved by SDS-PAGE. Lanes *A* and *L*, molecular mass standards; lanes *B* and *G*, fibronectin controls; lanes *C* and *H*, 1-h incubation; lanes *D* and *I*, 4-h incubation; lanes *E* and *J*, 8-h incubation; lanes *F* and *K* are purified metalloproteinase and purified meprin respectively. (*C*) Laminin (50 μg) was incubated with 1 μg of purified cortical metalloproteinase (lanes *C–F*) or with 2 μg of purified meprin (lanes *H–K*) for various times and products were resolved by SDS-PAGE. Lanes *A* and *L*, molecular mass standards; lanes *B* and *G*, laminin controls; lanes *C* and *H*, 4-h incubation; lanes *D* and *I*, 12-h incubation; lanes *E* and *J*, 24-h incubation; lanes *F* and *K* are purified metalloproteinase and purified meprin, respectively.

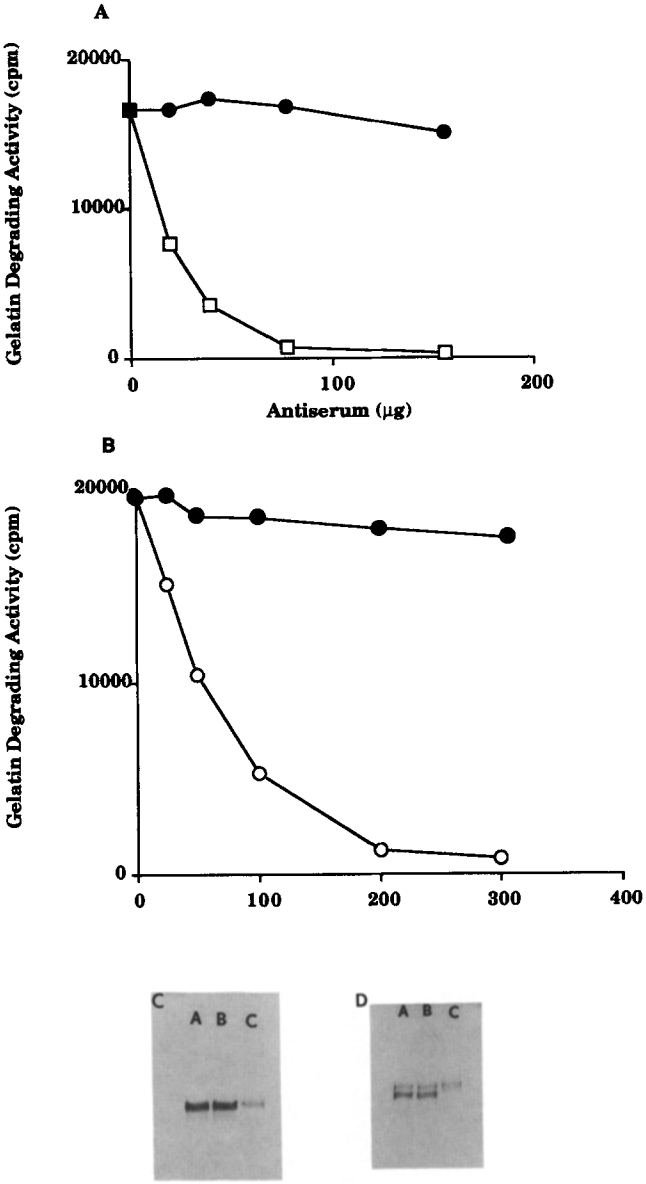


Figure 11. Immunoprecipitation studies and western blot analyses with polyclonal antibodies. (*A*) Immunoprecipitation of the gelatin-degrading activity by antibody to the purified detergent-solubilized enzyme. The solubilized enzyme from the renal cortex membrane was incubated with various amounts of the polyclonal antibody raised against the purified metalloproteinase. After 4 h of incubation a suspension of washed *Staphylococcus aureus* was added and after 2 h the suspension was centrifuged. The enzyme activity in the supernatant was measured. □—□ antibody; ●—●, preimmune serum. (*B*) Immunoprecipitation of the gelatin-degrading activity by antibody to the purified meprin (papain-solubilized azocasein-degrading enzyme). The solubilized enzyme from the renal cortex membrane was incubated with the polyclonal antibody raised against meprin and the gelatin-degrading activity was immunoprecipitated as described in *A*. ○—○ antibody; ●—●, preimmune serum. (*C*) Western blot analysis using polyclonal antibody to the purified detergent-solubilized enzyme. Lanes *A*, papain-solubilized gelatin-degrading enzyme; lane *B*, meprin (papain-solubilized azocasein-degrading enzyme); lane *C*, detergent-solubilized gelatin-degrading enzyme. (*D*) Western blot analysis using polyclonal antibody to meprin. Lanes *A*, papain-solubilized gelatin-degrading enzyme; lane *B*, meprin (papain-solubilized azocasein-degrading enzyme); lane *C*, detergent-solubilized gelatin-degrading enzyme.

solubilized membranes are similar to those reported for meprin, we also showed that meprin was able to degrade gelatin and type IV collagen. Meprin also degraded laminin and fibronectin resulting in proteolytic fragments similar to those produced with the detergent-solubilized enzyme. It should be noted that comparison of the specific activities of meprin and the well known matrix-degrading metalloproteinases indicate that meprin is as effective as gelatinase A in degrading gelatin and collagen type IV, but less effective than gelatinase B. However, as compared to stromelysin, gelatin is a better substrate for meprin (21, 22). The detergent-solubilized enzyme was able to degrade azocasein and insulin, known substrates for meprin. Moreover, antibodies raised against meprin were able to immunoprecipitate the gelatin-degrading activity in the renal cortex and Western blot analysis revealed cross-reactivity between the respective antibodies and the purified enzymes. Taken together, these data provide compelling evidence that the detergent-solubilized matrix-degrading enzyme is meprin A. The immunoprecipitation data as well as our previous studies (27) demonstrate that this enzyme accounts for most of the matrix-degrading activity of the renal cortex. Our preliminary data with immunohistochemical staining utilizing polyclonal antibody to the purified cortical metalloproteinase also demonstrated staining in the tubules but not in the glomeruli. This is in keeping with the study reported by Bond et al. (6) in which they have shown that meprin is localized in tubules but not in glomeruli. This indicates that despite similar biochemical characteristics, the cortical (tubular) and glomerular enzymes appear to be distinct from each other.

The renal tubular epithelium is the target in many forms of ischemic and toxic renal injury resulting in cell death and acute renal failure (15). The tubular epithelium is supported by a tubular basement membrane, a specialized form of extracellular matrix composed primarily of type IV collagen, laminin, and proteoglycans (1). Recently, it has become apparent that, in addition to providing structural support, the extracellular matrix may also promote cell proliferation, cell migration, cell differentiation, and cell attachment to basement membrane, all potentially important factors in regeneration. During injury, the exposure of the functional sites of extracellular matrix may produce effects critical to regeneration. Indeed, in a recent study, we demonstrated marked alterations in renal tubular extracellular matrix components following ischemia-reperfusion injury to the kidney (26). Thus it is conceivable that meprin, when released from injured tubules due to ischemic or toxic renal injury, may play an important role in regeneration by exposure of residual epithelial cells to active sites of laminin or other components of extracellular matrix important for regeneration. Since this is the major matrix-degrading enzyme in the kidney, it may play a role either in injury and regeneration or in other cellular functions.

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