STRUCTURE, MEMBRANE ASSOCIATION, AND PROCESSING OF MEPRIN SUBUNITS

By

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(ABSTRACT)

Meprins are oligomeric cell-surface metalloproteinases that are expressed at high concentrations in the renal brush border membranes of mice. Meprins consist of two types of subunits, α and β, which are the products of two different genes. The β subunit cDNA was cloned and sequenced from mouse kidneys, and the membrane association and the *in vivo* proteolytic processing of mouse meprin subunits were investigated. The primary translation product of the meprin β subunit is composed of 704 amino acids, and contains several domains, including a signal sequence at the NH_2-terminus, a prosequence, a protease domain, an adhesion domain (MAM domain), an epidermal growth factor-like domain, a potential transmembrane-spanning domain, and a short cytoplasmic tail. The β subunit is evolutionarily related to the α subunit. The α and β subunit share about 42% overall sequence identity, and have a similar arrangement of functional domains; however, a 56 amino acid segment near the COOH-terminus of α is missing in β, and the signal sequences, transmembrane and cytoplasmic domains share no significant sequence similarity. The protease domains of α and β are 55% identical. NH_2-terminal protein sequencing of detergent-solubilized meprin subunits from mouse kidneys showed that the prosequence in α is removed in the mature subunit. By contrast, only the signal sequence is removed from the mature β subunit NH_2-terminus, and the β subunit retains the prosequence. Further, the mature α subunit, but not the β subunit, is proteolyti-

cally processed at the COOH-terminus and does not contain the transmembrane and the EGF-like domains encoded by the meprin α cDNA. The β subunit is a type I integral membrane protein. By contrast, α does not transverse the membrane, and its membrane association depends on disulfide bonds. The oligomeric organization of membrane-bound meprins was analyzed by SDS-PAGE under non-reducing conditions, and by isoelectric focusing. ICR mouse kidneys express αβ heterodimers and α_2 homodimers; C3H/He mice contain β_2 dimers. Transfection of COS-1 cells with the full-length meprin α subunit cDNA resulted in the secretion of meprin dimers into the culture medium, indicating that the COOH-terminal transmembrane domain of meprin α subunits is post-translationally removed from the protein in COS-1 cells, as it is in mouse kidney cells. Replacement of the COOH-terminal 137 amino acids of α with the COOH-terminus of β, or deletion of the 56 amino acid inserted domain in α, resulted in mutant proteins that were not secreted into the medium, but rather were membrane-bound, indicating that the inserted domain of α is essential for proteolytic cleavage and secretion. Deletion of the COOH-terminal 133 residues of α did not affect meprin α dimerization or intracellular transport. The meprin α subunits secreted from transfected COS-1 cells were catalytically inactive, but could be activated by limited proteolysis with trypsin. Thus, processing at the NH_2-terminus differed in COS-1 cells and in mouse kidney. COS-1 cells did not remove the prosequence from the α subunit protein, and removal of the prosequence was essential for catalytic activity of the α subunit. These results have implications for the biosynthesis and regulation of a cell surface proteinase, and thus relate to the elucidation of the mechanisms by which biological events at the cell surface are regulated.

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bp	base pairs
BSA	bovine serum albumin
Con A	concanavalin A
DEAE	diethylaminoethyl
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
E-64	L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane
EGF	epidermal growth factor
Endo F	endoglycosidase F
Endo H	endoglycosidase H
ER	endoplasmic reticulum
IgG	immunoglobulin G
IAA	iodoacetamide
IEF	isoelectric focusing
MDCK	Madin-Darby canine kidney
OG	β-D-octylglucopyranoside
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PHA-L	Phaseolus vulgaris leukoagglutinin
pI	isoelectric point
PI-PLC	phosphatidylinositol-specifc phospholipase C
PMSF	phenylmethanesulphonyl fluoride
PVDF	polyvinylidene difluoride
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N''-Tetramethylethylenediamine
TFMS	trifluoromethanesulfonic acid
TGF	transforming growth factor
Tris	Tris[hydroxymethyl]aminomethane

I

INTRODUCTION

1.1 Proteolytic Enzymes

Proteinases mediate a wide variety of essential cellular and extracellular functions. In many instances the function is extensive digestion, the breaking down of proteins into their building blocks. Digestive proteolytic processes are often non-specific, and the action of the enzymes involved leads to the total degradation of the substrates. The digestive function of the extracellular proteases in gastric and pancreatic secretions (e.g., pepsin, trypsin, chymotrypsin), which degrade dietary proteins and peptides to amino acids and dipeptides for absorption, is well established. Similarly, through the action of renal proteases, peptides and proteins in the glomerular filtrate are broken down to amino acids that can subsequently be retrieved before excretion in the urine (Carone and Peterson, 1980). Extensive protein degradation also occurs intracellularly by lysosomal or cytosolic proteinases. In particular, cytosolic proteinases continuously degrade altered and abnormal cellular proteins as well as "normal" proteins as part of the bulk protein turnover by ubiquitin-dependent and -independent pathways (Ciechanover and Schwartz, 1989; Luscher and Eisenman, 1988). It is not well understood, however, how intracellular proteins are selected for degradation. Besides ubiquitin-conjugation, other protein modifications, such as formation of glutathione-protein mixed disulfides, oxidation, deamidation, and phosphorylation/dephosphorylation have been implicated to mark proteins for degradation (Beynon and Bond, 1986). Lysosomal proteinases are also involved in bulk protein turnover as well as specific protein degradation during conditions of starva-

tion. Lysosomal uptake of intracelluar proteins occurs by micro- and macro-autophagy. In addition there is a more selective pathway that requires a specific peptide motif (KFERQ) within the proteins to be degraded (Dice, 1990).

Many proteolytic events have a regulatory, rather than digestive, function; these processes are characterized by the cleavage of a single or a few peptide bonds, and do not lead to further proteolytic degradation of the products (Neurath, 1991). For example, several proteases with specific functions during protein maturation have been identified, e.g., signal peptidase (Evans *et al.*, 1988), mitochondrial matrix processing protease (Schneider *et al.*, 1990), and prohormone processing enzymes (reviewed by Steiner *et al.*, 1992). In addition, proteases mediate the regulation of many cellular events by the specific activation or inactivation of biologically active peptides and proteins. Examples of this category include the extracellular proteases of the blood coagulation system (reviewed by Davie *et al.*, 1991), and the proteases of the renin-angiotensin system (Ondetti and Cushman, 1982). In contrast to the digestive proteases these proteases carry out specific limited proteolysis. One of the most specific proteases known to date is renin, whose only known substrate is angiotensinogen.

The activity of a protease towards substrates is determined primarily by the accessibility of the peptide bond, and by the specificity of the enzyme for the amino acids surrounding the cleavage site. For many proteases, the amino acid on the NH_2-terminal end of the scissile bond (P1 position) is of prime importance. However, some enzymes have extended substrate binding sites, and amino acids as distant as four residues away from the scissile bond can contribute to the efficiency of binding and catalysis (Schechter and Berger, 1967). Endopeptidases are classified into four groups based on their active sites and

their catalytic mechanisms: the serine, cysteine, aspartic, and metalloproteases. Serine or cysteine proteases contain, as the names imply, an essential Ser or Cys residue in the active site, through which an acyl-enzyme intermediate is formed during catalysis. The acyl-enzyme intermediate is subsequently hydrolyzed. In contrast, the aspartic and metalloproteinases have a non-covalent mechanism of action; the catalytic mechanism of these enzymes involves general acid-base catalysis with the attack of the scissile bond by a water molecule.

Proteolysis is essentially an irreversible process, because there are no known enzymes that "repair" a broken peptide bond under normal physiological conditions; therefore it is not surprising that cells have evolved a number of regulatory mechanisms to control unwanted proteolysis (Neurath, 1991). These regulatory mechanisms include, but are not limited to, tissue- and cell-type specific expression of protease activity, expression of protease inhibitors, the controlled activation of enzymatic activity by proteolytic removal of prosequences, and the control of the subcellular location and local concentration of the protease (Bond and Butler, 1987). While proteases are rarely the primary cause of a disease, unwanted proteolytic activities characterize and contribute to the clinical symptoms of many disease states. For example, proteases can cause tissue damage during inflammation or emphysema, and are responsible for the wasting of tissue (cachexia) associated with diseases such as cancer and insulin-dependent diabetes (Bond and Beynon, 1987). In addition, transformation of cells can lead to the induction of the secretion of proteases; significantly, the secretion of proteases has been shown to correlate with the invasiveness, and thus the malignancy, of the tumor cells (Sloane *et al.*, 1990).

1.2 Cell Surface Proteinases

Cell surface proteinases mediate a variety of diverse functions. They play a role in the turnover of extracellular proteins and cell membrane proteins. In addition, membrane-bound proteinases are involved in the specific activation and inactivation of many biologically active peptides and proteins, such as growth factors, kinins, hormones (reviewed by Bond and Beynon, 1987).

The intestinal and renal brush border membranes are extraordinarily rich in cell surface peptidases, and these tissues have primarily served as sources for the identification and purification of membrane proteinases (Bond, 1991). Membrane proteinases differ in their tissue-specificity, and many brush border peptidases are also found on the surfaces of other cell types, where they presumably play a variety of roles (Kenny, 1988). While many cell surface proteinases have been well characterized on a biochemical level, the physiological functions of most of these enzymes remain unknown.

Among the mammalian cell surface proteinases that have been identified to date are angiotensin converting enzyme (E.C. 3.4.15.1), neprilysin (E.C. 3.4.24.11), endothelin-converting enzyme, miniglucagon-generating enzyme, hepsin, epididymal apical protein I, aminopeptidase P (E.C. 3.4.11.9), aminopeptidase N (E.C. 3.4.11.2), aminopeptidase A, carboxypeptidase M (E.C. 3.4.17.12), dipeptidyl peptidase IV (E.C. 3.4.14.5), and meprins (e g ., meprin A: E.C.3.4.24.18). Most cell surface proteases are zinc-metalloproteases or serine proteases.

Angiotensin converting enzyme (ACE) is an ectoenzyme found in most mammalian tissues bound to the plasma membrane of endothelial, epithelial, neural, and neuroepithelial cells (Beldent *et al.*, 1993). ACE is a zinc-metalloen-

zyme. There are two ACE isoforms derived from a single gene, by transcription from two alternate promoters (Kumar *et al.*, 1991). Somatic ACE is a monomeric 170 kDa glycoprotein containing two homologous domains, each domain bearing an active catalytic site; germinal ACE is a 110 kDa protein containing only the COOH-terminal domain of endothelial ACE (Wei *et al.*, 1991). Both are integral membrane proteins with a single COOH-terminal hydrophobic transmembrane-spanning domain. In addition to the membrane-bound forms, a soluble form of endothelial ACE exists in plasma and other body fluids, which is generated from the membrane-bound form by a post-translational proteolytic cleavage event in the COOH-terminal region (Beldent *et al.*, 1993). ACE is involved in the metabolism of the vasoactive peptides angiotensin I and bradykinin, and its physiological importance in the regulation of blood pressure is well established (Ondetti and Cushman, 1982).

Neprilysin is a monomeric metalloproteinase of approximately 93 kDa, that is present at the surface of many cell types. It has a single transmembrane spanning domain at the NH_2-terminus (Fossiez *et al.*, 1992). The enzyme has no activity against proteins, only against peptides. Neprilysin is particularly abundant in the kidney brush border membranes, where it is believed to inactivate atrial natriuretic factor (Sonnenberg *et al.*, 1988). In the brain this peptidase is involved in the degradation of various neuropeptides, including enkephalins (Schwartz *et al.*, 1981).

Endothelin-converting enzyme (ECE), a key enzyme in the production of the potent vasoconstricting peptide, endothelin, is expressed in endothelial cells and lung tissue (Takahashi *et al.*, 1993). The enzyme, purified from rat lung membranes is a monomeric protein with a molecular weight of approximately 130

kDa. ECE is a neutral metalloproteinase. Its cDNA has not been cloned and the membrane topology is not known.

Rat liver membranes contain an endopeptidase, termed miniglucagon-generating enzyme (MGE), that processes glucagon to miniglucagon (Blache *et al.*, 1993). Miniglucagon has distinct biological activities from its parent hormone; e.g., it is 1000-fold more potent than glucagon in inhibiting the plasma membrane calcium pump of hepatocytes. MGE is inhibited by metal-chelating agents and sulfhydryl-blocking reagents. The enzyme is 100 kDa in size and is present in liver, pancreas, heart, and at lower levels in skeletal muscle and intestinal mucosa. Only a partial NH_2-terminal protein sequence is available that does not correspond to the sequence of any cloned enzyme.

Hepsin is a membrane-bound serine protease that was first isolated and cloned from human liver (Leytus *et al.*, 1988). The enzyme is a monomeric protein of approximately 51 kDa with a NH_2-terminal transmembrane anchor. Hepsin is expressed in many other tissues besides liver and presumably plays an essential role in cell growth and the maintenance of cell morphology.

A metalloproteinase with significant sequence similarity to snake venom proteins has been cloned from rat and monkey epididymis (Perry *et al.*, 1992). This protein, epididymal apical protein I, is expressed exclusively in the caput region, where it localizes on the apical surface of the epithelial cells. The enzyme is androgen-regulated and implicated to play a role in sperm-egg recognition and fusion.

Dipeptidylpeptidase IV (EC 3.4.14.5) is found in a number of tissues, including liver, kidney, and small intestine. It removes the NH_2-terminal dipeptide from peptide substrates provided that the penultimate amino acid residue is pro-

line or alanine (Erickson *et al.*, 1992). In the intestine the enzyme is of particular importance for the digestion and assimilation of proline-containing peptides. Dipeptidylpeptidase IV is also thought to play a role in hormone regulation, immune response, cellular interactions with the biomatrix, and hydrolysis of renal peptides. In contrast to most other cell surface proteinases, this enzyme is a serine protease. Dipeptidylpeptidase IV is a monomeric glycoprotein of approximately 106 kDa with an NH_2-terminal membrane anchor (Erickson *et al.*, 1992).

Several membrane-bound aminopeptidases, aminopeptidase P, N, and A, are expressed on the renal and intestinal brush border membranes. All three enzymes are metalloenzymes. They presumably function in the final stage of the intestinal and renal digestion of proteins to amino acids. Aminopeptidase P is a monomeric glycoprotein of approximately 91 kDa that is expressed primarily in the lung and the kidney. The enzyme is anchored to the cell membrane through a phosphatidylinositol-glycan anchor (Simmons and Orawski, 1992). Aminopeptidase N is a homodimeric enzyme of 140-160 kDa subunits. Aminopeptidase A is also a homodimer, the subunits are approximately 125 kDa. Aminopeptidase N and aminopeptidase A have NH_2-terminal membrane anchors (Kenny and Maroux, 1982).

Carboxypeptidase M (EC 3.4.17.12) is a metalloenzyme that is expressed in kidney, intestine, lung, placenta, and endothelial cells. In kidney and intestine, the enzyme probably functions in the digestion of proteins and peptides, in other tissues it may be involved in the metabolism of biologically active peptides. The enzyme is a monomeric protein with a molecular wieght of 65,000; it is anchored to the membrane by a phosphatidylinositol anchor (Deddish *et al.*, 1990).

1.3 Meprin

Meprins are plasma membrane-bound metalloproteinases that are expressed at high concentrations in the kidney brush border membranes of mice and rats (Kounnas *et al.*, 1991; Jiang *et al.*, 1992; Kenny and Ingram, 1987), and in the intestines of mice, rats, and humans (Sterchi *et al.*, 1988; Johnson and Hersh, 1992; Gorbea *et al.*, 1993). Meprin A (E.C. 3.4.24.18) has been purified in trypsin- or papain-solubilized form from the kidneys of ICR and BALB/c mice (Beynon et al., 1981; Kounnas et al., 1991). Meprin A is responsible for the great majority (90%) of the general proteolytic activity (using azocasein as substrate) of mouse kidney brush border membranes at neutral or alkaline pH values. The enzyme has a broad substrate and peptide bond specificity; it is capable of hydrolyzing a great variety of peptides and proteins such as bradykinin, insulin B chain, glucagon, transforming growth factor-α (Butler *et al.*, 1987; Choudry and Kenny, 1991; Wolz *et al.*, 1991).

Meprin A is an oligomeric glycoprotein composed of two subunits, α and β (Gorbea *et al.*, 1991). The mouse α subunit has an apparent molecular weight, when subjected to SDS-PAGE, of approximately 90 kDa, while the β subunit is 110 kDa. The papain-purified α and β subunits are approximately 90 kDa. Experiments subjecting meprin A to gel filtration or SDS-PAGE under non-reducing conditions indicated that meprin is a disulfide-linked tetramer (Beynon *et al.*, 1981; Butler *et al.*, 1987). In addition, lectin and immunoblotting after SDS-PAGE indicated that meprins can exist as homo- and hetero-oligomers (Gorbea *et al.*, 1991). Certain inbred strains of mice, e.g., C3H/He mice, do not express the α subunit, and only homo-oligomers of β (meprin B) are found in these strains. Recent analysis of the oligomeric organization of meprin A by analytical ultracen-

trifugation indicated that, contrary to the previous interpretations from SDS gels, not all subunits in the mouse meprin complexes are linked by disulfide bridges. Meprin A was shown to be composed of disulfide-bridged dimers, which aggregate non-covalently to form higher molecular weight complexes, mainly tetramers (Marchand *et al.*, 1994). The rat and human forms of meprin have also been reported to be dimers, as judged by SDS-PAGE, and the only oligomeric form that has been detected in these species is the αβ hetero-oligomer (Sterchi *et al.*, 1988; Johnson and Hersh, 1992).

Meprin B, a homo-oligomer of β subunits, has been purified to homogeneity from the kidneys of C3H/He mice (Kounnas *et al.*, 1991). Papain-purified or membrane-associated meprin B has little or no activity against bradykinin or azocasein, and a distinct peptide bond specificity towards the insulin B chain as compared to meprin A (Kounnas *et al.*, 1991). Meprin B has latent activity toward azocasein which can be activated *in vitro* by trypsin-like enzymes; after trypsin activation meprin A and meprin B preparations have comparable specific azocaseinase activities.

Based on many structural, immunological, and enzymatic similarities between meprin A and B, and the latency of meprin B activity towards protein substrates, it was suggested that the β subunit is a polymorphic form of α that is incompletely processed to an active form *in vivo*. However, careful analysis of substrate specificities, immunological data, peptide maps of cyanogen bromide fragments, and NH_2-terminal protein sequences of the papain-purified subunits eventually proved that the α and β subunits are the products of two distinct genes (Kounnas *et al.*, 1991; Jiang *et al.*, 1992).

Cloning and sequencing of the α subunit cDNA revealed that meprins are part of the newly discovered astacin family of metalloendopeptidases (Dumermuth *et al.*, 1991; Jiang *et al.*, 1992). The astacin family of metalloendopeptidases includes a number of proteins that share a protease domain of approximately 200 amino acids that has sequence homology to the protease astacin (E.C. 3.4.24.21) from the crayfish *Astacus fluviatilis* (Titani *et al.*, 1981). While astacin is a simple digestive enzyme consisting only of the protease domain, most other members in this family are more complex molecules which contain a number of additional domains that are likely to have regulatory functions. Most of the other members of the astacin family have functions during developmental stages of the organisms in which they are expressed (Wozney *et al.*, 1988; Shimell *et al.*, 1991; Lepage *et al.*, 1992; Elaroussi *et al.*, 1994).

Sequencing of the α subunit provided important information about the primary structure of the α subunit. The α subunit possesses a number of additional domains besides its protease domain; some of these domains also occur in other members of the astacin family, while others are unique to meprins. Domains of the α subunit primary translation product include a signal sequence, a prosequence, an adhesive MAM domain, an epidermal growth factor (EGF)-like domain, and a transmembrane domain. Based on the cDNA sequence, the α subunit is predicted to be a type I integral membrane protein (Singer *et al.*, 1987); that is a protein with a single transmembrane-spanning domain near the COOH-terminus with the bulk of the protein located extracellularly. The cDNA encodes a hydrophobic stretch near the COOH-terminus, and solubilization from the plasma membrane requires either limited proteolysis with papain or toluene/trypsin, or the dissolution of the membranes with detergents (Beynon *et al.*, 1981).

Furthermore, treatment with phosphatidylinositol-specific phospholipase C does not release meprin A from membranes (Jiang *et al.*, 1992). In addition to the membrane-bound meprin, a secreted urinary form of mouse meprin has been reported, which contains α but not β subunits; it is not yet known how this soluble form is generated.

Meprin α subunits have also been cloned and sequenced from rats and humans, and were found to be more than 85% identical to the mouse α subunit (Corbeil *et al.*, 1992, Dumermuth *et al.*, 1993). The structural gene for the mouse α subunit has been localized to chromosome 17 near the major histocompatibility complex (Jiang *et al.*, 1993). The expression of the α subunit is tissue-specific and differs in mice and rats (Jiang *et al.*, 1993). Certain inbred strains of mice (e.g., C3H/He mice) are deficient in the expression of the α subunit, and thus express only the β subunit in kidney (Jiang *et al.*, 1992). The deficiency in the expression of the α subunit is inherited as an autosomal recessive trait, and a factor determining the expression of α was localized to mouse chromosome 17 (Bond *et al.*, 1984). Of those mouse strains that express α, the mRNA was only found in the kidney; no message was present in intestine, brain, heart, skeletal muscle, liver, lung, or spleen. In Sprague Dawley rats it is expressed in both kidney and intestine. In humans the message is present in intestine but not in kidney. Thus, expression of the α subunit is highly specific and differs in the species examined.

Initial cloning of the β subunit protease domain confirmed that the α and β subunits are evolutionarily related, and provided a probe to study the expression of the β subunit. The β subunit is expressed in the kidneys and the intestine of all mouse strains (Gorbea *et al.*, 1993).

1.4 Aims of These Studies

A long-range goal of our laboratory is to understand the mechanisms by which proteolytic activities are regulated *in vivo*. The present study deals with the structure, maturation, membrane association, and secretion of the major membrane-bound proteinase of mouse kidney (meprin), and thus relates to the elucidation of the mechanisms by which the expression of proteinase activity at the cell surface is regulated.

Meprins are plasma membrane homo- or hetero-oligomeric metalloendopeptidases that contain α and/or β subunits. They are expressed in high concentrations in mouse kidney brush border membranes. Cloning and sequencing of the α subunit cDNA revealed that meprins are members of the astacin family of metalloproteinases, and that meprin subunits are composed of a number of functional domains. Initial cloning of the β subunit showed that the α and β subunits are evolutionarily related. However, only the protease domain of mouse β had been sequenced, and the complete domain organization was not known for meprin β subunits from any species. The domain organization of β was of particular interest, because the β subunit in mouse kidney is significantly larger than the α subunit (110 kDa for β versus 90 kDa for α). Because both meprin subunits were estimated to have a similar carbohydrate content, differences in the domain strucure of α and β were expected. Therefore, the aim of the first part of this project was to clone and sequence the full-length meprin β subunit cDNA.

Two forms of meprin, meprin A and B, have been purified from the kidneys of different strains of mice and their physical and biochemical characteristics were studied. Virtually all previous protein characterization was performed on proteolyzed forms (papain- or trypsin-solubilized) of the enzyme, and much less

was known about the structure of the enzyme *in vivo*. Furthermore, with the cloning of the meprin cDNAs the tools were available to express meprin subunits in a eukaryotic expression system, thereby permitting the study of aspects of meprin structure that could not be determined by conventional biochemical methods. Experiments in the later part of this project were designed to provide information about the processing of the subunits and structure of meprin oligomers in the cell membrane. These aspects of meprin structure were analyzed by two approaches: (i) analysis of the membrane-bound or detergent-solubilized forms from renal brush border membrane-enriched fractions, and (ii) analysis of proteins produced using a eukaryotic expression system. The COS-1 cell line, a SV40-transformed kidney fibroblast cell line, was used in the expression studies; this cell line has been widely used for the expression of membrane-bound and secreted proteins, because COS-1 cells produce relatively high yields of recombinant protein, and carry out virtually all post-translational modifications commonly found in eukaryotic cells.

The specific aims of this project were (1) to determine the primary and domain structure of the mouse meprin β subunit, (2) to analyze the subunit organization of meprins, (3) to identify the mode of membrane association and the relationship between membrane-bound and secreted meprin subunits, and (4) to study the *in vivo* proteolytic processing of the meprin subunits. The results of these studies are presented in four sections, each of which addresses one of the specific aims. Some of the results relate to more than one aim, and are presented in the section to which they are most relevant.

II

EXPERIMENTAL PROCEDURES

2.1 Animals

Adult male C3H/He and C57/BL6 mice, and New Zealand white rabbits were purchased from Jackson Laboratories (Bar Harbor, ME). Male ICR frozen mouse kidneys were obtained from Rockland farms.

2.2 Enzyme Assays, Electrophoretic Techniques, and Protein Analyses

2.2.1 Protein determinations.- The protein content in samples was measured using bicinchoninic acid (Smith *et al.*, 1985; Pierce) with serial dilutions of bovine serum albumin (BSA) as standard, or by measuring the absorbance at 280 nm.

2.2.2 Assay for meprin activity.- Meprin activity was assayed using the general protein substrate azocasein as described by Reckelhoff *et al.* (1985). Briefly, samples containing meprin were mixed with 20 mM ethanolamine buffer, pH 9.5, containing 0.14 M NaCl to a final volume of 125 µl. The reactions were started by adding 125 µl of 22 mg/ml azocasein in the same buffer. After incubation at 37°C for 30 min the reactions were stopped by the addition of 1.0 ml of 5% trichloroacetic acid. The samples were then centrifuged for 4 min at 14,000 x g, and the absorbance at 340 nm was measured in the resulting supernatant fractions. One unit of activity is defined as an increase in absorbance at 340 nm of 0.001 per min, which is equivalent to the solubilization of 1.1 µg of azocasein per min.

2.2.3 Trypsin activation of meprin.- Samples containing meprin were treated with trypsin as described by Butler and Bond (1988). Briefly, tosyl phenylalanyl chloromethyl ketone-treated trypsin (0.5 mg/ml in 20 mM Tris-HCl buffer , pH 7.5) was added to the samples at a ratio of 10:1 (weight) of total sample protein to trypsin. The samples were incubated for 1 h at 37°C, the trypsin was then inhibited by the addition of a two-fold excess over trypsin of soybean trypsin inhibitor. Control reactions were performed with trypsin and soybean trypsin inhibitor alone to verify the effectiveness of the soybean trypsin inhibitor.

2.2.4 Purification of papain-solubilized meprin A.- Meprin A was purified from frozen ICR mouse kidneys as described by Kounnas *et al.* (1991). Briefly, 50 g of kidneys were homogenized in 20 mM Tris-HCl buffer, pH 7.5, and a membrane enriched fraction was prepared by centrifugation at 100,000 x g for 1 h. Membrane proteins were solubilized by limited proteolysis with papain (final concentration of papain of 0.1 mg/ml in 50 mM phosphate buffer, pH 6.2) as described by Butler (1989). The solubilized proteins were subjected to ammonium sulfate precipitation. The protein fraction that precipitated in the range of 40-80% ammonium sulfate saturation was desalted on Econo Pac 10 DG chromatography columns (BioRad) and subjected to anion-exchange chromatography on a Mono Q 10/10 column (Pharmacia). Proteins were eluted from the Mono Q column with a 0-1 M NaCl gradient in 20 mM Tris-HCl buffer, pH 8.5. The final purification was achieved by gel filtration chromatography on a superose 12 column (Pharmacia) using 20 mM Tris-HCl buffer, pH 7.5, containing 0.1% NaCl.

2.2.5 Purification of renal brush border membranes.- Renal brush border membrane-enriched fractions were prepared from ICR mouse, C3H/He mouse, or Sprague-Dawley rat kidneys as described by Butler and Bond (1988). Briefly, the kidneys were homogenized in 9 volumes (w/v) 2 mM Tris-HCl, pH 7.0, containing 10 mM mannitol using a polytron homogenizer (Brinkmann Instruments). $MgCl_2$ was added to a final concentration of 10 mM, and the mixture was stirred for 15 min at 4 °C. The samples were centrifuged at 1,500 x g at 4°C for 12 min, and the resulting supernatant fractions were centrifuged for 12 min at 15,000 x g at 4°C. The precipitated material from the second centrifugation step were suspended in 5 volumes of homogenization buffer, $MgCl_2$ was added to a concentration of 10 mM, and the mixtures were stirred for 15 min at 4°C. The samples were then centrifuged at 2,200 x g for 12 min, and the resulting supernatant fractions were centrifuged for 12 min at 15,000 x g at 4°C. The final sediment (brush border membranes) was resuspended in 0.2 volumes of homogenization buffer.

2.2.6 Purification of detergent-solubilized mouse meprin subunits.- Renal brush border membrane fractions were prepared from kidneys of ICR, C57BL/6 and C3H/He mice. Meprin was solubilized from the brush border membranes by incubation with 100 mM n-octyl-β-D-glucopyranoside for 2 h, shaking at 4°C, followed by centrifugation for 1 h at 100,000 x g at 4°C. The resulting supernatant fractions were applied to a 1 ml column of concanavalin A-sepharose (Pharmacia) equilibrated with Tris-buffered saline (TBS), pH 8.0, containing 100 mM n-octyl-β-D-glucopyranoside, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ (washing buffer). The column was washed with 20 column volumes of washing buffer,

before meprin subunits were eluted with elution buffer, which was washing buffer, containing 1 M α-D-methyl-mannopyranoside.

2.2.7 Partial purification of detergent-solubilized rat meprin.- Renal brush border membrane fractions were prepared from Sprague-Dawley rat kidneys. The membrane proteins were solubilized by incubation with 100 mM n-octyl-β-D-glucopyranoside for 2 h, shaking at 4°, followed by centrifugation for 1 h at 100,000 x g at 4°C. The resulting supernatant fraction was applied to a Mono Q HR 5/5 column. The column was washed with 20 mM Tris-HCl buffer, pH 8.5, containing 20 mM n-octyl-β-D-glucopyranoside, and proteins were eluted using a 0-1 M NaCl gradient. The fractions were assayed for meprin content using the azocaseinase assay. The rat meprin subunits eluted at a salt concentration of about 200 mM NaCl.

2.2.8 NH_2-terminal sequence analysis.- Detergent-solubilized, partially purified mouse or rat meprin subunits (100 μg) were boiled with 2% SDS and 5% β-mercaptoethanol for 2 min and subjected to SDS-PAGE according to the method of Laemmli and Favre (1973) on 10% polyacrylamide gels. The gels were precast one day in advance and allowed to polymerize overnight at 4°C. The gels were run for 3 h at 20 mA, before the samples were loaded. After the electrophoresis was completed, the samples were transferred to polyvinylidene difluoride (PVDF) membranes in 10 mM CAPS (3-[cyclohexylamino]-1-propane sulfonic acid) buffer, pH 11, containing 10% methanol for 2 h at a constant current of 400 mA. The PVDF membranes were stained with a 0.125% solution of Coomassie Brilliant Blue R-250 in 50% methanol/50% water, extensively rinsed with HPLC

grade water and air-dried. Portions of the Coomassie-stained membranes containing individual subunits were excised for direct NH_2-terminal sequence analyses. The NH_2-termini of meprin subunits were determined by the method of Tarr (1977). Sequence analysis was performed in the Protein Chemistry Laboratory, Department of Molecular Biology and Pharmacology, at Washington University in St. Louis.

2.2.9 Preparation of antibodies.- Polyclonal antibodies to the mouse meprin α subunit were obtained from rabbit antiserum raised against purified meprin A treated with endoglycosidase F and N-glycanase (Boehringer Mannheim). Antibodies to the β subunit COOH-terminus were prepared using the peptide RRKYRKKARANT (residues 682-693 of the β subunit), coupled to keyhole limpet hemocyanin, as antigen. The peptide was synthesized by solid phase methods at the Core Facility at Penn State University, Hershey, PA. The antigens were suspended in Freund's complete adjuvant and injected subcutaneously into male New Zealand rabbits. Three additional injections were administered to the rabbits at 14 day intervals. Fourteen days after the last injection the rabbits were bled, the blood was allowed to clot overnight, and the serum was collected. Immunoglobulin G (IgG) preparations were obtained from the sera by ammonium sulfate precipitation (50% saturation). The precipitated antibodies were desalted using gel filtration columns 10 DG (Bio Rad) and stored in phosphate buffered saline (PBS), pH 7.4 at -20°C. A monoclonal antibody to the α subunit was prepared using the hybridoma clone JHL-1; this clone was prepared at the Hybridoma facility at Virginia Commonwealth University (Hall *et al.*, 1993). Four nude mice were injected intraperitoneally with 0.5 ml pristane. Ten days later 5 x

10^6 cells of the hybridoma clone JHL-1 were injected intraperitoneally. Fourteen days after injection of the hybridoma cells, the ascites fluid was collected from the mice. The fluid was centrifuged for 10 min at 1,500 x g to remove cells, and the IgG fraction was purified from the supernatant by ammonium sulfate precipitation (50% saturation). A polyclonal antibody to a recombinant meprin β subunit protease domain was a gift from Dr. W. Jiang (Penn State University). Polyclonal antibodies to rat meprin were a gift from Dr. L. B. Hersh (University of Kentucky).

2.2.10 SDS-PAGE and immunobloting.- Proteins were subjected to SDS-PAGE in 6%, 9%, or 10% polyacrylamide gels according to the method of Laemmli and Favre (1973), and either stained with Coomassie Brilliant Blue R-250 or transferred to 0.45 mm nitrocellulose membranes. Protein transfer to nitrocellulose membranes was carried out in 50 mM Tris, 0.77 M glycine, and 40% (v/v) methanol for 2 h at a constant current of 400 mA. Most immunoblots were performed following the instructions of the Enhanced Chemoluminescence (ECL) Western blotting protocol (Amersham). Briefly, membranes were blocked overnight at 4°C with 10% dry milk protein in 20 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 0.1% Triton X-100 (TBS-T). The primary antibodies were used at a dilution of 1:10,000 in 5% milk protein in TBS-T, except for the polyclonal antibody to the deglycosylated α subunit, which was used at a dilution of 1:8,000 in TBS-T buffer containing 5% milk protein and 1% bovine serum albumin (BSA). Membranes were incubated for 1 h in the primary antibody solution, washed 3 times with TBS-T buffer, and then incubated with a 1:5,000 dilution of the secondary antibody (horseradish peroxidase-linked anti-rabbit immunoglobulin from donkey, Amersham) in TBS-T buffer containing 5% milk pro-

tein. The membranes were then washed five times with TBS-T, incubated for 1 min with ECL Western blotting detection reagents (Amersham), and exposed to X-ray film. In a few instances, e.g., analysis of deglycosylated brush border membrane proteins (Fig. 20) and isoelectric focusing (Fig. 6), a colorimetric Western blotting protocol was used. For the colorimetric procedure, the membranes were blocked in 20 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 5% milk protein (blotting solution) for 1 h at 25°C. The blocked membranes were incubated overnight with a 1:1,000 dilution of primary antibody in blotting solution, rinsed with distilled water, and washed for 15 min in blotting solution. The membranes were then incubated for 2 h with a 1:1,000 dilution of goat anti-rabbit IgG (Sigma) in blotting solution, rinsed again with distilled water, and washed for 15 min in blotting solution. Subsequently the membranes were incubated for 1.5 h with a 1:1,000 dilution of rabbit peroxidase anti-peroxidase complex (Sigma) in blotting solution, rinsed with distilled water, washed for 5 min in blotting solution, and washed for 10 min in developing buffer (20 mM Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl). The peroxidase-label was detected colorimetrically using a solution of 4-chloro-1-naphthol (3 mg/ml) and hydrogen peroxide (0.02%) in developing buffer. Prestained molecular weight markers (Sigma) were generally used for Western blots. For more accurate molecular weight determinations Combithek calibration proteins (Boehringer Mannheim) were used.

2.2.11 Lectin blotting.- Proteins were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with biotinylated lectins as described by Stroupe *et al.* (1991). Briefly, the membranes were incubated with 1 µg/ml con-

canavalin A in 10 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 3% BSA, or with 1 µg/ml *Phaseolus vulgaris* leukoagglutinin (PHA-L) in 6.7 mM potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 3% BSA for 12 h at 25°C. The membranes were washed for 15 min with a 3% solution of BSA in water, then incubated with 1 µg/ml avidin-peroxidase in 6.7 mM potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 3% BSA for 2 h at 25°C. Subsequently, the membranes were washed for 5 min with a solution of 2% BSA in water, and for 10 min with developing buffer (20 mM Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl). The peroxidase-labelled proteins were detected colorimetrically using the substrate 4-chloro-1-naphthol as described for the immunoblotting.

2.2.12 Isoelectric focusing (IEF).- Isoelectric focusing of papain-purified meprin A preparations was performed using a flatbed gel system (Hoefer). The gels were prepared with 3.75 ml bis-acrylamide (30% T, 2.7% C), 16.5 ml water, 1.125 ml glycerol, 1.125 ml ampholytes pH 4-6 (Pharmacia), 0.225 ml ampholytes pH 3-10 (Pharmacia), 158 µl ammonium persulfate, and 40 µl TEMED. The gels were run in a pH gradient from 3-10 using Anode Fluid and Cathode Fluid (Serva) as buffers. After prefocusing the gels for 1 h at 400 volts, the samples were applied to the middle of the gel. The samples were run for 2.5 h at constant power (10 watts), and the proteins were transferred to nitrocellulose membranes for Western blotting analysis. pI markers used were phycocyanine (pI 4.65), bovine carbonic anhydrase (pI 6.0), and human carbonic anhydrase (pI 6.5).

2.2.13 Two-dimensional gel electrophoresis.- Two-dimensional gel electrophoresis of ICR mouse brush border membrane proteins was performed according to the method of O'Farrell (1975). In the first dimension samples were subjected to isoelectic focusing under non-reducing conditions in 3 mm tube gels, the second dimension was SDS-PAGE under reducing conditions. The tube gels contained a pH gradient from pH 3.5 to 10, and were made with 2.75 g urea, 0.5 ml bis-acrylamide (40% T, 2% C), 1.0 ml 10% Nonidet P-40, 0.2 ml Ampholines pH 4-6 (Pharmacia), 0.05 ml Ampholines pH 3.5-10 (Pharmacia), 3.5 µl TEMED, 5 µl 10% ammonium persulfate, and 1.125 ml water. A basic (0.02 M NaOH) to acidic (0.01 M H_3PO_4) buffer chamber system was used. The samples were mixed with an equal volume of IEF sample buffer (9.5 M urea, 2% NP-40, 4% ampholines pH 4-6, and 1% ampholines pH 3.5-10 in water) prior to loading. The gels were prefocused at 400 volt-hours for 30 min, then the samples were loaded, and the gels were run for 18 h at 250 volt, followed by 1 h at 800 volt. After the isoelectric focusing was completed, the gels were removed from the tubes by air pressure, soaked for 30 min in SDS sample buffer (10% glycerol, 50 mM DTT, and 2.3% SDS in 0.0625 M Tris-HCl, pH 6.8), and placed on top of a 10% SDS-polyacrylamide gel, which had been overlaid with a melted (50°C) solution of 1% agarose in SDS sample buffer. To determine the pH gradient across the tube gels, control tube gels without samples were run at the same time as the tube gels containing samples. The control gels were cut into 5 mm thick slices, and the pH in each gel slice was measured using a pH meter (Ion Analyzer 250, Corning).

2.2.14 Phosphatidylinositol anchor determination.- Renal brush border membrane-enriched fractions were prepared from C3H/He mouse kidneys. The preparations were incubated at 37°C for 90 min in the absence or presence of 2 units of phosphatidylinositol-specific phospholipase C (isolated from *Bacillus thuringiensis*; ICN Biochemicals), centrifuged at 4°C for 1 h at 100,000 x g, and the resulting precipitate and supernatant fractions were assayed for meprin and alkaline phosphatase activity. Meprin activity was assayed using azocasein as substrate. Alkaline phosphatase activity was assayed spectrophotometrically at 37°C with 1 mM p-nitrophenylphosphate as substrate in 1 M Tris/HCl buffer, pH 9.0, containing 10 mM $MgCl_2$; the absorbance change at 410 nm was monitored. One unit of alkaline phosphatase activity is defined as µmol p-nitrophenylphosphate/min.

2.2.15 Papain-solubilization of brush border membrane proteins.- Papain (10 mg/ml stock solution) was activated by incubation with 5 mM cysteine for 15 min at 37°C in 50 mM potassium phosphate buffer, pH 6.2. Brush border membrane proteins (200 µg) from ICR mouse kidneys were suspended in 100 µl 50 mM potassium phosphate buffer, pH 6.2. Papain was added to the membranes at a concentration of 100 µg/ml, and the membranes were incubated for 1 h at 37°C. The papain was then inactivated by the addition of iodoacetamide (1 mM final concentration) and incubation at 37°C for 15 min. Solubilized proteins were separated from the membranes by centrifugation at 100,000 x g for 1 h at 4°C. This is basically the method described by Butler (1989).

2.2.16 Dithiothreitol (DTT)-treatment of brush border membranes.- Freshly prepared brush border membranes from ICR and C3H/He mouse kidneys were incubated with 50 mM DTT in 100 mM Tris-HCl, pH 8.0 for 30 min at 37°C in the presence of 10 μM L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64). E-64 was added to inhibit cysteine proteases in the sample that might become activated during the DTT treatment. To stop the reaction, iodoacetamide was added to a final concentration of 200 mM and the incubation at 37°C continued for 15 min. A portion of the samples were then stored on ice for subsequent analysis, the remaining portions were subjected to centrifugation for 1 h at 100,000 x g at 4°C. All fractions were analyzed by SDS-PAGE and immunoblotting. The relative amounts of meprin α subunits in the precipitate and supernatant fractions were estimated by densitometric comparison of the Western blot signals with those from serial dilutions of brush border membrane proteins.

2.2.17 Extraction of brush border membranes with urea or NaCl/EDTA.- Freshly prepared brush border membranes from ICR mouse kidneys were incubated for 10 min at 25°C with (i) 7 M urea in 10 mM Tris-HCl buffer, pH 7.4 in the presence of various protease inhibitors (1mM phenylmethylsulfonyl fluoride, 50 μM iodoacetamide, 10 mM EDTA, 5 μM E-64, and 100 μM 3,4-dichloroisocoumarin), or (ii) 1.5 M NaCl and 10 mM EDTA in 10 mM Tris-HCl buffer, pH 7.4. A portion of the incubation mixture was kept at 4°C for subsequent analysis by Western blotting. The remaining portions were subjected to centrifugation for 15 min at 100,000 x g at 4°C, and the resulting precipitate and supernatant fractions were analyzed by Western blotting.

2.2.18 Immunoprecipitation of meprin β subunits from brush border membrane protein containing fractions.- ICR mouse kidney brush border membranes (300 μg) were solubilized by incubation with 1% Triton-X 100 in TBS, pH 7.4 for 30 min at 4°C, and centrifugation at 100,000 x g at 4°C for 1 h. The supernatants were removed and mixed with either 10 μl of the antibody to the β subunit COOH-terminal peptide, or with 10 μl of pre-immune serum. The samples were incubated for 2 h at 4°C, mixed with 100 μl of a 50% suspension of protein A sepharose CL-4B (Pharmacia) in water, and incubated at 4°C for 2 h. The sepharose beads were precipitated in a microcentrifuge, washed 4 times with a 0.5% solution of Triton-X 100 in Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, and then washed twice with 50 mM Tris-HCl buffer, pH 6.8. The beads were boiled in 2% SDS and 5% β-mercaptoethanol for 2 min, centrifuged, and the supernatants were analyzed by SDS-PAGE.

2.2.19 Chemical deglycosylation of brush border membrane proteins.- ICR mouse brush border membrane proteins were deglycosylated with anhydrous trifluoromethanesulfonic acid (TFMS) (Sojar and Bahl, 1987) using the GlycoFree reagent system (Oxford Glycosystems). Briefly, 500 μg of a salt-free preparation of ICR mouse brush border membrane proteins was freeze-dried, and incubated for 4 h at -20°C with 50 μl anhydrous TFMS reagent. The excess TFMS was neutralized by the gradual addition of 60 μl 60% pyridine in water in an ethanol/dry ice bath. The solution was then slowly allowed to warm up to 4°C, and diluted with 400 μl 0.5% ammonium bicarbonate in water. The precipitated deglycosylated proteins were recovered from the reagent mixture by centrifugation, dissolved and analyzed by SDS-PAGE on 10% gels followed by im-

munoblotting using meprin subunit-specific antibodies for the determination of the subunit molecular weights. The completeness of the deglycosylation reaction was confirmed by performing a carbohydrate staining reaction using GlycoTrack (Oxford Glycosystems) according to the instructions of the manufacturer on a portion of the reaction mixture.

2.3. Recombinant DNA Techniques

2.3.1 Isolation of total RNA from mouse kidneys.- RNA was isolated from C3H/He mouse kidneys using the acid-phenol RNA extraction method of Chomczynski and Sacchi (1987). Briefly, 2 g fresh kidneys were homogenized in 10 ml GITC solution (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 10 mM β-mercaptoethanol). One ml 2 M NaOAc, pH 4.0, 10 ml water-saturated phenol, and 2 ml chloroform were added, the samples were vortexed, cooled on ice for 15 min, and centrifuged at 15,000 x g for 15 min at 4°C. The RNA was precipitated from the upper aquaous phase by the addition of an equal volume of isopropanol and incubation at -20°C for 1 h, followed by centrifugation at 15,000 x g for 15 min . The precipitate was dissolved in GITC solution and the isopropanol precipitation was repeated. The precipitate was washed with 75% ethanol, dried and dissolved in water.

2.3.2 Cloning of the meprin β subunit cDNA 5' and 3' ends for DNA sequencing.- An internal fragment of the meprin β subunit cDNA encoding residues 65 to 345 (nucleotides 223-1065) had been sequenced previously (Gorbea *et al.*, 1993). To obtain the sequence information of the full-length meprin β subunit cDNA, two clones containing the 5' end and 3' end of the meprin β subunit cDNA were ob-

tained by reverse transcription-polymerase chain reaction of mouse kidney RNA (Fig. 1).

The 5'-end of the meprin β subunit cDNA (283 nucleotides) was obtained by the 5' Rapid Amplification of cDNA Ends (RACE) procedure of Frohman *et al.* (1988) using the 5'-RACE System (Gibco) according to the manufacturer's instructions. Briefly, the meprin β subunit mRNA was reverse transcribed from total C3H/He mouse RNA (1 μg) by incubating the RNA with the gene-specific antisense oligonucleotide oligo-1 (Table 1), reverse transcriptase, and deoxynucleoside triphosphates (dNTPs) for 30 min at 42°C. After first strand cDNA synthesis, the RNA was degraded by incubation with RNase H for 10 min at 55°C. The first strand cDNA was purified from the excess dNTPs and the oligonucleotides on a GlassMAX DNA isolation Spin Cartridge (Gibco). A homopolymeric oligo-dC tail was added to the 3' end of the cDNA by incubation with terminal deoxynucleotide transferase (TdT) and dCTP. The tailed cDNA was amplified by the polymerase chain reaction (PCR) using gene-specific antisense oligo-2 (Table 1) and an oligo-dG anchor primer (Gibco). A portion (1:1,000) from the first round of the PCR was amplified with a "nested" gene-specific antisense primer oligo-3 (Table 1), and a "universal amplification" primer (Gibco), which hybridized to the oligo-dG anchor primer. The products from the second round of the PCR were subjected to agarose gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide, extracted from the gel using the SpinBind DNA extraction kit (FMC) and cloned into the plasmid vector pAmp 1 using the CloneAmp System (Gibco). The vector pAmp 1 was chosen, because it contains T7 and SP6 promoters on opposite ends of the multiple cloning site for direct DNA sequencing of the double-stranded plasmids using T7 and SP6 promoter primers.

Fig. 1. Cloning and sequencing strategy of the mouse meprin β subunit cDNA

Meprin β cDNA encoding residues 65-345 had been obtained previously by the polymerase chain reaction using CD-1 or C57BL/6 mouse library cDNAs as templates (Gorbea *et al.*, 1993). The 5' end of the cDNA encoding amino acids β1-76 was obtained using the 5'-RACE system; the sequence for amino acid residues β338-β704 was obtained through the 3'RACE procedure. The PCR products of the 5' RACE and 3' RACE reactions were cloned into the vector pAMP 1 (Gibco) and sequenced using the T7 and SP6 promoter primers. Deletion subclones of the 3'-RACE product were generated using *Kpn* I, *Pst* I, *Xba* I, and *Stu* I restriction sites. Arrows indicate the extent and direction of the sequencing information obtained. Arrows originating at sites other than restriction sites represent sequence obtained by extension from synthetic oligonucleotides. Abbreviations are: PCR, polymerase chain reaction; RACE, Rapid amplification of cDNA ends.

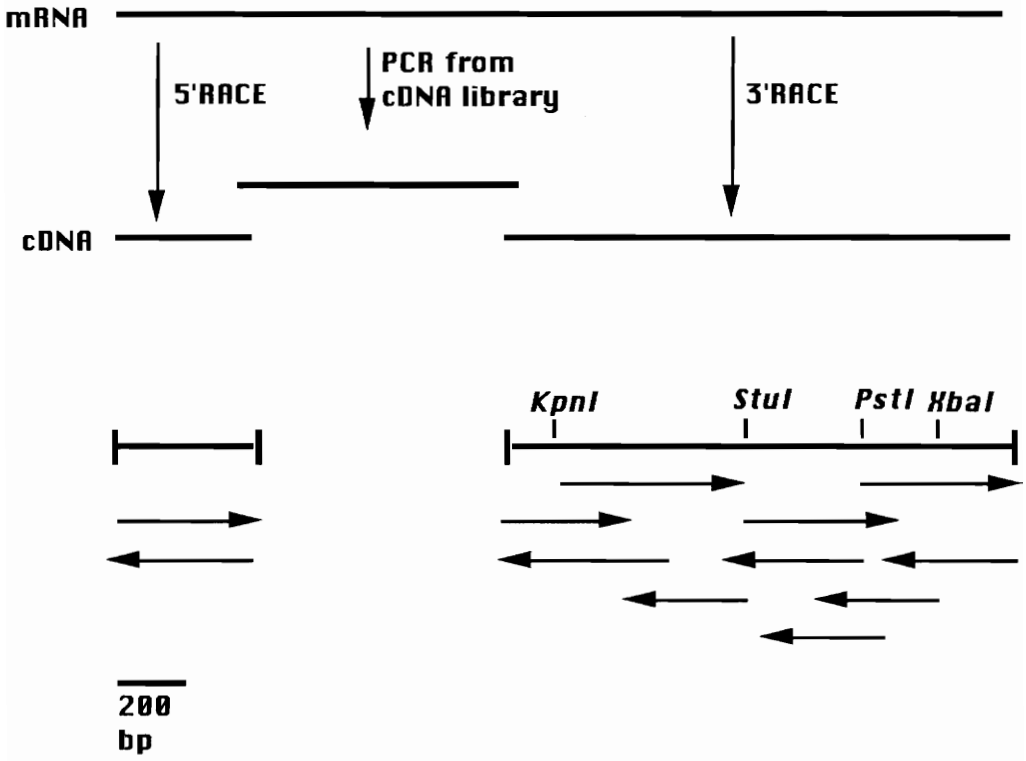

mRNA
5'RACE
PCR from
cDNA library
3'RACE
cDNA
KpnI
StuI
PstI
XbaI
200
bp

Table 1

Oligonucleotide sequences of the meprin β subunit cDNA

Oligonucleotides	Sequences	Nucleotide numbers in cDNA
A. Oligonucleotides used in 5' RACE, 3' RACE, and sequencing of the meprin β subunit cDNA		
Oligo-1 (antisense)	TCCAATGGACAAGGCTCCTGCTTC	436-456
Oligo-2 (antisense)	GCGAGATATCCCAAGGCTTGAAGTCAATGCACG	339-361
Oligo-3 (antisense)	CAUCAUCAUCAUGAATTCCAAGCTGTCCTCTAGAACATATGG	260-283
Oligo-4 (sense)	GCGCTGCAGAGCATTTTGAATGAGGGGGCCACG	983-1006
Oligo-5 (sense)	CAUCAUCAUCAUGATATCTTGTACCCCAAGAGAGGGTTTCAG	1028-1051
Oligo-6 (antisense)	CTCACACCTTTTGCCCATGTA	1952-1972
Oligo-7 (antisense)	TCCTACATTTGTTGAGTATCT	1437-1456
B. Oligonucleotides used for the generation of full-length meprin β subunit cDNA		
Oligo-8 (sense)	GCGAAGCTTGCAGCTTTCATCTGGAAGCCACAGT	1-29
Oligo-9 (antisense)	GCGGCATGCTTCTTCTATGATGGAAGGTCTCTTT	2175-2199

The oligonucleotides used in the 5' and 3' RACE procedures (oligo-1, -2, -3, -4, and -5) were designed based on the cDNA sequence of a previously cloned β subunit PCR fragment (Gorbea *et al.*, 1993). Oligo-6 and -7 were used as sequencing primers. Oligo-8 and -9 correspond to 5' and 3' untranslated regions of the meprin β subunit cDNA. The indicated nucleotide numbers refer to the published mouse meprin β subunit sequence (Gorbea *et al.*, 1993, and Fig. 2).

The 3'-end of the meprin β subunit cDNA was obtained by the 3' RACE procedure (Gibco). Briefly, cDNA was synthesized from total C3H/He mouse kidney RNA (1 μg) using an oligo-dT containing "adapter" primer (Gibco). The meprin β subunit cDNA was amplified from the reverse transcribed cDNA by the PCR using gene-specific sense oligo-4 (Table 1) and a "universal amplification" primer (Gibco), which hybridized to the adapter primer sequence. A portion (1:1,000) from the first round of the PCR was amplified with a "nested" gene-specific sense primer oligo-5 (Table 1), and the "universal amplification" primer. The product from the second round of the PCR (1290 bp) was purified and cloned into the vector pAmp 1 as described for the 5'RACE product.

2.3.3 Polymerase chain reaction (PCR).- PCR reactions were carried out on a GeneAmp PCR System 9600 (Perkin Elmer) as described by Innis and Gelfand (1990) using 5 μl reverse transcribed cDNA or 10 ng plasmid DNA as template and 5 units AmpliTac DNA polymerase in each 50 μl reaction. The following changes from the standard protocol were made regarding cycling temperatures and times: To initiate the reactions, the DNA was denatured at 94°C for 2 min. This was followed by 35 cycles of 30 sec at 94°C (denaturation), 30 sec at 55°C (annealing), and 1 min at 72°C (extension). For PCR products larger than 2 kilobases (kb), an extension time of 2 min was used instead of 1 min. The extension time in the last cycle was always 15 min. In those cases where the PCR products were subsequently expressed in COS-1 cells, the PCR reactions were carried out using Pfu DNA polymerase (Stratagene) of Ultma DNA polymerase (Perkin Elmer) instead of AmpliTac DNA polymerase to minimize the possibility of base misincorporations that might change the open reading frame.

2.3.4 Cloning of PCR products.- To facilitate cloning of the PCR products, the oligonucleotides used in the PCR amplifications had incorporated CAUCAUCAUCAU or CUACUACUACUA sequences at the 5' ends to allow directional cloning into the pAmp 1 vector (Gibco) by the Uracil DNA glycosylase method (Varshney and van de Sande, 1991) according to the instructions of the manufacturer (Gibco). Briefly, uracil DNA glycosylase and linearized plasmid pAmp 1 DNA were added to the PCR products containing uracil bases in the 5' terminal sequences. The uracil DNA glycosylase generated 3' protruding termini in the PCR products, which annealed to the linearized plasmid. The annealed chimeric molecules were used directly for the transformation of *E. coli* DH5a. For expression purposes the cloned PCR products were subsequently subcloned into the expression vector pcDNA I/Amp (Invitrogen).

2.3.5 Subcloning of the 3' RACE product.- Several deletion subclones of the 3' RACE product were constructed for DNA sequencing by digestion of the pAmp 1 vector containing the 3'RACE product insert with enzymes that cut in the multiple cloning site as well as in the meprin cDNA fragment, and subsequent self-ligation of the digested vector DNA. The original plasmid was digested with either *Kpn* I, *Pst* I, *Xba* I, *Stu* I and *SnaB* I, or *Stu* I and *Sma* I; the relative positions of the restriction sites in the 3' RACE product are shown in Fig. 1.

2.3.6 DNA sequencing.- pAmp 1 (Gibco) plasmids containing meprin β subunit cDNA fragments were sequenced using the Sanger dideoxy termination method with Sequenase (US Biochem. Corp.). The primers T7 and SP6 were used as

sequencing primers. In addition, two gene-specific primers, oligo-6 and oligo-7 (Table 1), were synthesized for use as sequencing primers. Plasmids were purified by the alkaline lysis method (Sambrook *et al.*, 1989) using the *Plasmid* Select-250 kit (5Prime 3Prime). The double-stranded plasmids were heat-denatured at 98°C for 5 min; annealing of the primers, labeling and termination reactions were done following the protocol for Sequenase (US Biochem. Corp.). Both strands were sequenced at least twice (Fig. 1, bottom).

2.3.7 Analysis of sequencing data. - DNA sequences were analyzed using the program McMolly (Soft Gene, Berlin). The deduced amino acid sequence was searched with PROSITE to find consensus sequences and patterns in the β subunit (Bairoch, 1990).

2.3.8 Generation of meprin subunit cDNA clones for expression studies.- Meprin α and β subunit cDNAs were cloned into the eukaryotic expression vector pcDNA I/Amp (Invitrogen). pcDNA I/Amp is a 5.0 kb circular plasmid vector with the following features: promoter sequences from the immediate early gene of the human cytomegalovirus (CMV) for constitutive expression of the inserted DNA sequence, transcription termination and RNA processing signals from simian virus (SV40) to enhance mRNA stability, SV40 origin of replication for episomal replication in cells expressing the SV40 large T antigen (such as COS-1 cells), T7 and SP6 promoters for *in vitro* transcription, and ColE1 origin and the ampicillin resistance gene for propagation of the plasmid in *E. coli*..

The full length β subunit cDNA was obtained from total RNA from C3H/He mouse kidney by the 3'-RACE procedure (Gibco) using an oligo-dT containing

"adapter" primer (Gibco) for the reverse transcriptase reaction and primers oligo-8 (5' sense primer; Table 1) and oligo-9 (3' antisense primer; Table 1) for the PCR amplification. The PCR product was cloned into the *Hind* III and *Sph* I sites of the expression vector pcDNA I/Amp. The resulting plasmid is referred to as pcDNA β.

Meprin α subunit cDNA encoding amino acid residues 64-760 in pBluescript SK- vector (clone B24), and meprin α subunit cDNA encoding residues 1-225 in pBluescript KS vector (clone 723S) had been isolated previously (Jiang *et al.*, 1992). The α subunit contains a unique *Nde* I site at nucleotide position 629, which is present in both clones. To obtain a full length meprin α subunit cDNA, the 5' end of the meprin α subunit cDNA (nucleotides 1-655) was amplified by PCR amplification using oligo-10 (5' sense primer; Table 2) and oligo-11 (3' antisense primer; Table 2) as primers, and clone 723S plasmid DNA as template (Table 2). Oligo-10 contained the consensus sequence for efficient eukaryotic translation initiation (C/AXXATGG; start codon underlined) surrounding the start codon to maximize the level of expression (Kozac, 1991), and an *EcoR* I site at the 5' end for cloning. To obtain the full length α subunit cDNA in pBluescript SK- vector, the PCR product was digested with *EcoR* I and *Nde* I, and cloned into the B24 plasmid that had been digested with the same enzymes. Subsequently, the full length α subunit cDNA was subcloned into the *Hind* III and *Not* I sites of the expression vector pcDNA I/Amp. This plasmid is referred to as pcDNA α.

2.3.9 Generation of the meprin α/β subunit hybrid cDNA.- This procedure took advantage of the fact that the meprin β subunit contained a *Tth* III site at position 1832, which is at the boundary of the cDNA encoding the domain with unknown

TABLE 2

Oligonucleotide sequences of the meprin α subunit cDNA

Oligonucleotides	Sequences	Nucleotide numbers in cDNA
A. Oligonucleotides used for the generation of full-length meprin α subunit cDNA		
Oligo-10 (sense)	GCGCGAATTCACCATGGCAAGGAGGCTTGGCAGATCC	16-39
Oligo-11 (antisense)	GGTCTGTGATGGTGTTGTCGTCATATGTG	627-655
B. Oligonucleotides used for the generation of C-terminal deletion mutants of the meprin α subunit		
Oligo-12 (sense)	CAUCAUCAUCAUGCCACCATGGCAAGGAGGCTTGGCAGATCC	16-39
Oligo-13 (antisense)	CUACUACUACUATCAGCCCAGCAAGCTGCCGTGCACGTG	2194-2217
Oligo-14 (antisense)	CUACUACUACUATCAGAAGTACTGTGGCCAGTTGTGGTC	2044-2067
Oligo-15 (antisense)	CUACUACUACUATCAGGGCATCGTGCTTCTGGCTGAAGC	1873-1896
Oligo-16 (antisense)	CUACUACUACUATCAATTCCGGATGGTCCAAACCCCTGC	1348-1371
Oligo-17 (antisense)	CUACUACUACUATCAATGTGTTGCGGTGCAGTTGTACAT	817-840
C. Oligonucleotides used in site-directed mutagenesis		
Oligo-18 (sense)	CACTGGCGGCCG**G**TCGA**C**CATGCATCTAGAGG	N/A
Oligo-19 (sense)	GCTTCAGCCAGAAGCACGATGCCC/TTCAGAGACCCCTGTGACCC	1873-1896/2065-2084
Oligo-20 (sense)	TACAACTGCACCGCAACACAT/GGGGTTTGGACCATCCGGAATA	819-840/1351-1372
Oligo-21 (sense)	GCCTTGGGAGAAAGCTCC**G**GA**G**GAGCCATGCTGGAGGAATCC	1930-1972
Oligo-22 (sense)	AGACATCCCAGCAGACAG**GGAG**GCTCGGTGGAGAACACTGG	1993-2033
D. Oligonucleotides used for the generation of the α/β meprin subunit hybrid		
Oligo-23 (sense)	CUACUACUACUAAAGCTTGCCACCATGGCAAGCAGGCTTGGCAG	16-35
Oligo-24 (antisense)	CAUCAUCAUCAU**GACTGGGTC**AGCTGGAACTTCAGTCCGGT	1856-1884

The indicated nucleotide numbers refer to the published mouse meprin α subunit sequence (Jiang *et al.*, 1992). Oligo-18 is part of the pcDNAI/Amp vector sequence. The mutated bases in oligo-18, -21, and -22 are shown in bold. Oligo-19 and -20 generate deletions; the position of the deleted sequence is indicated by a dash. Oligo-24 generates a TthIII site in the meprin α subunit sequence at the positions indicate in bold.

function and the cDNA stretch encoding the EGF-like domain. The 5' end of the meprin α subunit cDNA (nucleotides 16 to 1884) was amplified by PCR using oligo-12 and -13 (Table 2). The 5' terminal sense primer oligo-12 contained a *Hind*III site near the 5' end for subcloning purposes. Oligo-13 was designed to introduce a *Tth*III site into position 1880 of the meprin α subunit cDNA; this position is in the same reading frame as and corresponds to the *Tth*III site at position 1884 of the β subunit cDNA. The 5' end of the meprin β subunit cDNA was excised from the β subunit expression vector pcDNA β by digestion with *Hind*III and *Tth*III. The amplified 5' end of the meprin α subunit cDNA was digested with *Hind*III and *Tth*III, and this fragment was ligated into the digested β subunit expression vector to replace the meprin β subunit 5' fragment.

2.3.10 Site-directed mutagenesis.- Site-directed mutagenesis was performed to generate a meprin α subunit deletion construct (αΔ628-683; Fig. 22, section 3.4.4), and two constructs with amino acid substitutions (constructs αRK645GG and αKR666GG; Fig. 23, section 3.4.4). The mutagenesis reactions were performed on plasmids containing meprin α subunit cDNA (pcDNA α) following the method of Deng and Nickoloff (1992) using the Transformer Site-Directed Mutagenesis Kit (Clontech). Briefly, double-stranded plasmid was denatured by heat treatment (100°C for 3 min), and two primers were simultaneously annealed to one strand of the plasmid DNA by incubation on ice for 5 min. One of the primers, referred to as the mutagenic primer, contained the desired mutation. The other primer, referred to as the selection primer, mutated a unique restriction site in the multiple cloning site of the plasmid. Both primers had been phosphorylated at the 5' end using T4 polynucleotide kinase. The sequences of the selec-

tion primer (oligo-14) and the mutagenic primers (oligos 15-17) that were used are listed in Table 2. The selection primer oligo-14 mutates the unique *Xho* I site in the multiple cloning site of pcDNA I/Amp to a unique *Sal* I site. Oligo-15 contained a deletion of the codons for Arg-628 to Tyr-683. Oligos-16 and -17 change the codons for R644/K645 or K666/R 667 to glycine codons, respectively. After annealing of the primers to the DNA strand the primers were elongated and ligated by the addition of T4 DNA polymerase and T4 DNA ligase. The resulting plasmid mixture was digested with *Xho* I to linearize wild-type plasmids, and used to transform*E.coli* BMH 71-18 *mutS,* an *E. coli* strain that is deficient in DNA mismatch repair. The next day DNA was isolated from the transformant pool, the wild-type plasmid DNA was linearized by digestion with *Xho* I, and *E.coli* DH5α was transformed with the digested DNA. Plasmids were purified from individual colonies and those that were resistant to *Xho* I digestion and could be linearized by*Sal* I were further analyzed for the desired mutations by restriction digestion (for the deletion mutant) or DNA sequencing (for the base substitutions).

2.3.11 Generation of COOH-terminal deletions of the meprin α subunit.- COOH-terminal deletion mutants for the meprin α subunit were generated by the PCR using a 5'terminal sense oligo (oligo-18) and different antisense oligos which contained stop codons at the desired positions (oligo-19 to -21; Table 2). In oligo-19 the codon encoding Leu-735 was changed to a stop codon, resulting in construct αΔ26 (Fig. 24, section 3.4.4). Oligo-20 introduced a stop codon into the position of Arg-685 (construct αΔ76, Fig. 24), oligo-21 changed the Arg-628 codon to a stop codon (construct αΔ133, Fig. 24). The oligonucleotides had CUACUACUACUA and CAUCAUCAUCAU sequences incorporated at the 5'

ends to facilitate cloning. In addition, the sense primer oligo-18 contained the consensus sequence for efficient translational initiation. The PCR products were cloned into the vector pAmp 1 by the uracil glycosylase cloning method, and directionally subcloned into the *BamH* I and *EcoR* I sites of pcDNAI/Amp.

2.4 *In vitro* Expression of Meprin Subunits

2.4.1 In vitro transcription/translation of meprin subunits.- *In vitro* translation products were obtained by *in vitro* transcription/translation of the pcDNA I/Amp plasmids containing the meprin inserts, pcDNA α or pcDNA β, using the TNT T7 Coupled Reticulocyte Lysate System (Promega). One μg of plasmid was used for each 50 μl reaction. Each reaction was carried out both in the absence and ipresence of 3.6 μl canine microsomal membranes (Promega). The translation products were separated by SDS-PAGE on 10 % polyacrylamide gels for molecular weight determinations. The gels were treated with Autofluor autoradiographic image enhancer (National Diagnostics), dried, and exposed to X-ray film at -70°C for fluorography.

2.4.2 Tissue culture.- COS-1 cells (ATCC # 1650 CRL) were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (complete DMEM) in a 37°C incubator containing 5% CO_2.

2.4.3 Transfection of COS-1 cells.- The plasmid DNA used in transfection experiments was purified using pZ523 plasmid purification columns (5 Prime 3 Prime). COS-1 cells were transiently transfected using the diethyl-aminoethyl (DEAE)-

dextran method as described by Lake and Owen (1991). Briefly, 24 h before the transfection logarithmically growing COS-1 cells were plated in 100 mm tissue culture dishes at 5 x 10^6 cells/dish. The next day the cells were incubated for 30 min at 37°C with 2 ml transfection cocktail (containing 10 μg plasmid DNA and 500 μg/ml DEAE-dextran in phosphate buffered saline, pH 7.4). The transfection cocktail was then removed by aspiration, and the cells were grown for 2.5 h in complete DMEM, supplemented with 80 μM chloroquine. After the chloroquine treatment the cells were treated for 2.0 min with 10% dimethyl sulfoxide in complete DMEM. The cells were rinsed twice with PBS (37°C), and fresh tissue culture medium was added. Cells were harvested 48 h after transfection. If the tissue culture media were to be analyzed for secreted protein, the media were replaced 32 h after transfection with serum-free medium. The serum-free medium was usually DMEM, supplemented with insulin (2.5 μg/ml), transferrin (17.5 μg/ml), 20 mM ethanolamine, bovine serum albumin (1 μg/ml), soybean trypsin inhibitor (10 μg/ml), and aprotinin (10 μg/ml). In some instances, when the media were analyzed for meprin activity, the soybean trypsin inhibitor and aprotinin were omitted from the serum-free medium to permit subsequent activation of the samples with trypsin. Omission of the protease inhibitors from the serum-free medium had no noticable effect on the stability of the secreted meprin subunits.

2.4.4 Analysis of recombinant meprin protein from transfected COS-1 cells.- Forty-eight h after transfection, the tissue culture media and the cells were harvested and analyzed for meprin protein. The media were centrifuged for 20 min at 16,000 x g to remove cell debris, and concentrated 25-fold using Centriprep-30 and Microcon-30 concentrators (Amicon). The transfected cells were harvested

by scraping in 2 ml ice-cold PBS, and centrifuging for 5 min at 300 x g at 4°C. To achieve complete lysis the cells were allowed to swell by incubation in a hypotonic buffer (10 mM Tris-HCl, pH 7.5, containing 0.1 mM iodoacetamide, 1 mM PMSF, and 10 mM EDTA) at 4°C for 5 min, before they were homogenized with a teflon-glass homogenizer. After homogenization the salt concentration of the samples was increased by the addition of 1/4 volumes of 250 mM Tris-HCl buffer, pH 7.5, containing 0.25 M NaCl. The cell homogenates were centrifuged at 4°C for 1 h at 100,000 x g, the supernatant fractions were transferred to a new tube, and the obtained precipitant fractions (membrane-enriched) were dissolved in a solution of 2% SDS in 20 mM Tris-HCl buffer, pH 7.4. The media fractions, the precipitant and the supernatant fractions from the transfected cells were analyzed for meprin protein by SDS-PAGE and Western blotting. Typically, 1/10 of the samples obtained from one 100 mm plate was loaded in each lane. The media samples were also analyzed for meprin activity before and after trypsin activation using azocasein as substrate as described in section 2.2.2, except that the samples were incubated with the substrate for 1 h at 37°C, before the reactions were stopped.

2.4.5 Endoglycosidase digestion of meprin α subunits.- Recombinant meprin samples from COS-1 cell extracts and culture media, and purified meprin A samples from mouse kidneys were enzymatically deglycosylated using either a mixture of endoglycosidase F and N-glycosidase F (endo F), or endoglycosidase H (endo H). Samples containing recombinant meprin α subunits were denatured in a solution of 0.5% SDS, 0.1% β-mercaptoethanol, and 1mM PMSF in 50 mM Tris-HCl, pH 6.8 by boiling for 5 min. The samples were then divided into three

aliquots: (i) control, (ii) endo F digestion, and (iii) endo H digestion. The endo F digestion was performed in PBS, pH 7.4, containing 0.25 mM PMSF, 10 mM EDTA, 0.1% SDS, and 0.5% n-octyl-β-D-glucopyranoside using 1.25 units of endoglycosidase F/N-glycanase F mixture (Boehringer Mannheim) per reaction. The endo H digestion was carried out in 20 mM NaOAc buffer, pH 5.2, containing 0.1% SDS, 10 mM EDTA, and 0.25 mM PMSF, using 25 milliunits of endo H (Boehringer Mannheim) per reaction. The control portion was diluted with PBS, pH 7.4 to the same volume as the other two portions. All samples were incubated at 37°C for 3 h before the reactions were stopped by boiling in SDS-PAGE sample buffer.

2.4.6 Isolation of RNA from transfected COS-1 cells.- RNA was extracted from COS-1 cells transfected with meprin β subunit cDNA according to the guanidine-HCl method (Chirgwin *et al.*, 1979). Briefly, 48 h after transfection, the tissue culture medium was removed from the cells, the cells were rinsed twice with ice-cold PBS and dissolved in a solution of 7 M guanidine-HCl, 20 mM KOAc, and 5 mM EDTA, pH 7.4. The DNA was sheared in a Dounce homogenizer, ethanol was added to a final concentration of 32%, and the RNA was precipitated overnight. The precipitate was dissolved in a solution of 6.65 M guanidine-HCl in 25 mM EDTA, pH 7.4, and a second ethanol precipitation was performed. The precipitate from the second ethanol precipitation was dissolved in a solution of 100 mM NaCl, 10 mM EDTA, and 0.5% SDS in 50 mM Tris-HCl buffer, pH 9.0, and proteins were removed by phenol-chloroform extraction. Subsequently, two more ethanol precipitations were performed. The final precipitate was dissolved in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA.

2.4.7 Northern blot analysis of transfected COS-1 cells.- Total cellular RNA (15 μg) was separated on a 1.2% agarose gel containing 1% formaldehyde as described by Sambrook *et al.* (1989), and blotted onto nylon membrane Gene Screen Plus (NEN). The membrane was analyzed under UV light to mark the positions of the 18S and 28S rRNA bands. Prehybridization was performed at 42°C in 50% (w/v) deionized formamide, 1% SDS, 10% dextran sulfate, 5x SSPE (20x: 3M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA, pH 7.4), and 5x Denhardt's solution (50x: 1 g Ficoll (type 400), 1 g polyvinylpyrrolidone, 1 g BSA, and water to 100 ml). The full-length meprin β subunit cDNA probe was labelled with α-^{32}P-dCTP using the random priming method (Prime-a-Gene, Promega). The labelled probe, purified on a Stratagene Nuc trap column, had an activity of 100,000 cpm/μl. The membrane was hybridized with $5x10^5$ cpm/ml hybridization solution at 42°C overnight. Subsequently the blots were washed twice with 1 x SSC + 0.1% SDS for 15 min at room temperature, twice with 0.1 x SSC + 0.1% SDS, once for 15 min at room temperature and once for 30 min at 50°C. The dried membrane was exposed to X-ray film at -70°C for 48 h.

III

RESULTS

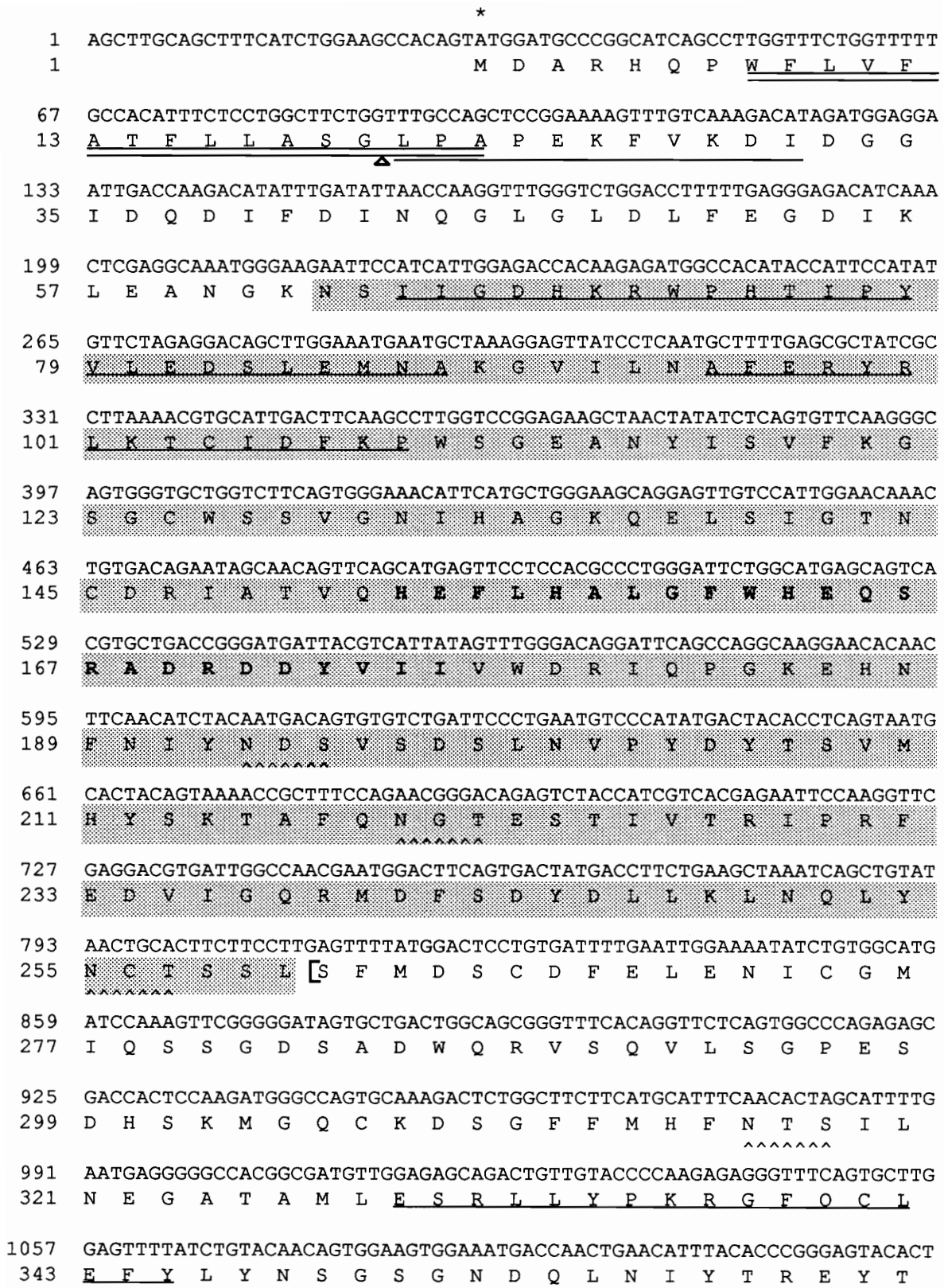
3.1 Primary Structure and Domain Structure of the Mouse Meprin β Subunit

3.1.1 Analysis of the β subunit primary sequence.- An internal fragment of the mouse meprin β subunit cDNA encoding residues 65 to 345 of the primary translation product (nucleotides 223-1065 of the full-length cDNA) had been sequenced previously (Gorbea *et al.*, 1993). To obtain the complete meprin β subunit sequence, clones containing the 5' and 3' ends of the meprin β subunit cDNA were obtained by the reverse transcriptase - polymerase chain reaction (RT-PCR) of mouse kidney RNA. The combined cDNA fragments span a region of 2306 bp in length (Fig. 2). This was consistent with results from Northern blot analysis, which indicated that the meprin β subunit mRNA is about 2.5 kb in length (Gorbea *et al.*, 1993). The cDNA contains an open reading frame of 2112 bp, a short (30 bp) 5' untranslated region, and a 3' untranslated region of about 165 bp. The 3' untranslated region of the cDNA extends into the polyadenylation tail and contains the polyadenylation signal sequence AATAAA 22 bases upstream of the polyadenylation tail. The sequence obtained by the 5' RACE procedure (nucleotides 1-283) extends 30 bases beyond the first Met codon. The sequence surrounding the first Met codon conforms to the vertebrate consensus sequence for efficient initiation of translation (A/G-X-X-A-T-G-G, initiation codon underlined); thus, this codon likely seves as the initiation codon *in vivo* (Kozak, 1987).

The open reading frame of the meprin β subunit cDNA codes for 704 amino acids (Fig. 2). The first 23 amino acids contains a hydrophobic sequence

Fig. 2. cDNA and deduced amino acid sequence of the mouse meprin β subunit

The *single-underlined* sequences were confirmed by NH_2-terminal analyses of peptides prepared from purified preparations of meprin A and B (Kounnas *et al.*, 1991). The *shaded* region is the β subunit protease domain. The 24-amino acid consensus sequence for the astacin family of metalloendopeptidases is indicated in bold. The sequences underlined by *carets* identify potential N-glycosylation sites. The *brackets* after Leu-261 and His-430 indicate the beginning and end, respectively, of the MAM domain. The *asterisk* identifies the initiation codon for translation. The *triangle* represents the potential cleavage site for signal peptidase and the beginning of the prosequence. The *dashed* underline indicates an epidermal growth factor-like domain. The *double-underlined* sequences were identified as hydrophobic regions. The stop codon and polyadenylation signal in the 3' untranslated region are indicated in bold.


*
1 AGCTTGCAGCTTTCATCTGGAAGCCACAGTATGGATGCCCGGCATCAGCCTTGGTTTCTGGTTTTT
1 M D A R H Q P W F L V F
67 GCCACATTTCTCCTGGCTTCTGGTTTGCCAGCTCCGGAAAAGTTTGTCAAAGACATAGATGGAGGA
13 A T F L L A S G L P A P E K F V K D I D G G
133 ATTGACCAAGACATATTTGATATTAACCAAGGTTTGGGTCTGGACCTTTTTGAGGGAGACATCAAA
35 I D Q D I F D I N Q G L G L D L F E G D I K
199 CTCGAGGCAAATGGGAAGAATTCCATCATTGGAGACCACAAGAGATGGCCACATACCATTCCATAT
57 L E A N G K N S I I G D H K R W P H T I P Y
265 GTTCTAGAGGACAGCTTGGAAATGAATGCTAAAGGAGTTATCCTCAATGCTTTTGAGCGCTATCGC
79 V L E D S L E M N A K G V I L N A F E R Y R
331 CTTAAAACGTGCATTGACTTCAAGCCTTGGTCCGGAGAAGCTAACTATATCTCAGTGTTCAAGGGC
101 L K T C I D F K P W S G E A N Y I S V F K G
397 AGTGGGTGCTGGTCTTCAGTGGGAAACATTCATGCTGGGAAGCAGGAGTTGTCCATTGGAACAAAC
123 S G C W S S V G N I H A G K Q E L S I G T N
463 TGTGACAGAATAGCAACAGTTCAGCATGAGTTCCTCCACGCCCTGGGATTCTGGCATGAGCAGTCA
145 C D R I A T V Q H E F L H A L G F W H E Q S
529 CGTGCTGACCGGGATGATTACGTCATTATAGTTTGGGACAGGATTCAGCCAGGCAAGGAACACAAC
167 R A D R D D Y V I I V W D R I Q P G K E H N
595 TTCAACATCTACAATGACAGTGTGTCTGATTCCCTGAATGTCCCATATGACTACACCTCAGTAATG
189 F N I Y N D S V S D S L N V P Y D Y T S V M
661 CACTACAGTAAAACCGCTTTCCAGAACGGGACAGAGTCTACCATCGTCACGAGAATTCCAAGGTTC
211 H Y S K T A F Q N G T E S T I V T R I P R F
727 GAGGACGTGATTGGCCAACGAATGGACTTCAGTGACTATGACCTTCTGAAGCTAAATCAGCTGTAT
233 E D V I G Q R M D F S D Y D L L K L N Q L Y
793 AACTGCACTTCTTCCTTGAGTTTTATGGACTCCTGTGATTTTGAATTGGAAAATATCTGTGGCATG
255 N C T S S L [S F M D S C D F E L E N I C G M
859 ATCCAAAGTTCGGGGGATAGTGCTGACTGGCAGCGGGTTTCACAGGTTCTCAGTGGCCCAGAGAGC
277 I Q S S G D S A D W Q R V S Q V L S G P E S
925 GACCACTCCAAGATGGGCCAGTGCAAAGACTCTGGCTTCTTCATGCATTTCAACACTAGCATTTTG
299 D H S K M G Q C K D S G F F M H F N T S I L
991 AATGAGGGGGCCACGGCGATGTTGGAGAGCAGACTGTTGTACCCCAAGAGAGGGTTTCAGTGCTTG
321 N E G A T A M L E S R L L Y P K R G F Q C L
1057 GAGTTTTATCTGTACAACAGTGGAAGTGGAAATGACCAACTGAACATTTACACCCGGGAGTACACT
343 E F Y L Y N S G S G N D Q L N I Y T R E Y T

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1123  ACAGGCCAGCAGGGTGGTGTTTTAACCCTTCAGAGACAAATAAAAGAGGTACCCATTGGGAGCTGG
 365  T  G  Q  Q  G  G  V  L  T  L  Q  R  Q  I  K  E  V  P  I  G  S  W

1189  CAACTTCACTATGTAACACTGCAAGTGACTAAAAAGTTTCGAGTGGTGTTTGAAGGACTCAGAGGC
 387  Q  L  H  Y  V  T  L  Q  V  T  K  K  F  R  V  V  F  E  G  L  R  G

1255  CCTGGCACGTCATCAGGTGGTCTGTCTATCGATGACATCAATCTCTCAGAAACAAGGTGTCCTCAC
 409  P  G  T  S  S  G  G  L  S  I  D  D  I  N  L  S  E  T  R  C  P  H]
                                          ^^^^^^^

1321  CATATCTGGCACATACAGAATTTCACACAGATTCTAGGCGGCCAGGACACATCTGTATACAGTCCT
 431  H  I  W  H  I  Q  N  F  T  Q  I  L  G  G  Q  D  T  S  V  Y  S  P
                     ^^^^^^^

1387  CCATTTTATTCTTCTAAAGGTTACGCTTTTCAGATCTACATGGATCTAAGATACTCAACAAATGTA
 453  P  F  Y  S  S  K  G  Y  A  F  Q  I  Y  M  D  L  R  Y  S  T  N  V

1453  GGAATTTATTTCCACCTGATCTCCGGTGCCAATGATGATCAATTACAGTGGCCATGTCCTTGGCAA
 475  G  I  Y  F  H  L  I  S  G  A  N  D  D  Q  L  Q  W  P  C  P  W  Q

1519  CAAGCTACAATGACACTCTTGGATCAAAATCCTGACATCCGACAGCGTATGTTCAACCAGCGGAGT
 497  Q  A  T  M  T  L  L  D  Q  N  P  D  I  R  Q  R  M  F  N  Q  R  S

1585  ATAACCACAGACCCAACGATGACCAGTGATAATGGAAGCTACTTTTGGGACAGGCCTTCCAAGGTG
 519  I  T  T  D  P  T  M  T  S  D  N  G  S  Y  F  W  D  R  P  S  K  V
                                    ^^^^^^^

1651  GGAGTGACGGATGTTTTCCCTAACGGAACTCAGTTTAGCAGAGGTATAGGCTATGGAACGACTGTC
 541  G  V  T  D  V  F  P  N  G  T  Q  F  S  R  G  I  G  Y  G  T  T  V
                           ^^^^^^^

1717  TTCATAACCCGAGAGAGGCTGAAGAGCAGAGAGTTCATAAAAGGAGATGACATTTACATCTTACTG
 563  F  I  T  R  E  R  L  K  S  R  E  F  I  K  G  D  D  I  Y  I  L  L

1783  ACTGTTGAAGACATATCTCACCTCAATTCTACATCAGCTGTCCCCGACCCAGTCCCCACCTTGGCT
 585  T  V  E  D  I  S  H  L  N  S  T  S  A  V  P  D  P  V  P  T  L  A
                              ^^^^^^^

1849  GTCCATAATGCCTGCTCTGAGGTTGTATGTCAGAACGGTGGCATCTGTGTTGTCCAAGATGGCAGA
 607  V  H  N  A  C  S  E  V  V  C  Q  N  G  G  I  C  V  V  Q  D  G  R

1915  GCTGAGTGCAAGTGTCCTGCAGGAGAAGACTGGTGGTACATGGGCAAAAGGTGTGAGAAGAGAGGG
 629  A  E  C  K  C  P  A  G  E  D  W  W  Y  M  G  K  R  C  E  K  R  G

1981  TCCACCCGAGACACTGTTATCATCGCTGTTTCTTCCACGGTCACCGTGTTTGCTGTGATGCTAATC
 651  S  T  R  D  T  V  I  I  A  V  S  S  T  V  T  V  F  A  V  M  L  I

2047  ATCACTCTCGTCAGTGTCTACTGCACCAGGAGGAAATATCGTAAGAAGGCTAGAGCAAATACGGCA
 673  I  T  L  V  S  V  Y  C  T  R  R  K  Y  R  K  K  A  R  A  N  T  A

2113  GCCATGACTCTAGAAAACCAACATGCGTTTTGAATATTAACTCACGCCAGCAGGTGAAATGAAAA
 695  A  M  T  L  E  N  Q  H  A  F

2178  GAGACCTTCCATCATAGAAGAATATTTTACCCAAGCCATGTGACATTTCCAACCAGATCATCTTTG

2244  TAAATAGCTGAAAGGCGTAATAAATATCCCTATATTATTTTGGTGTAAAAAAAAAAAAAAAAA
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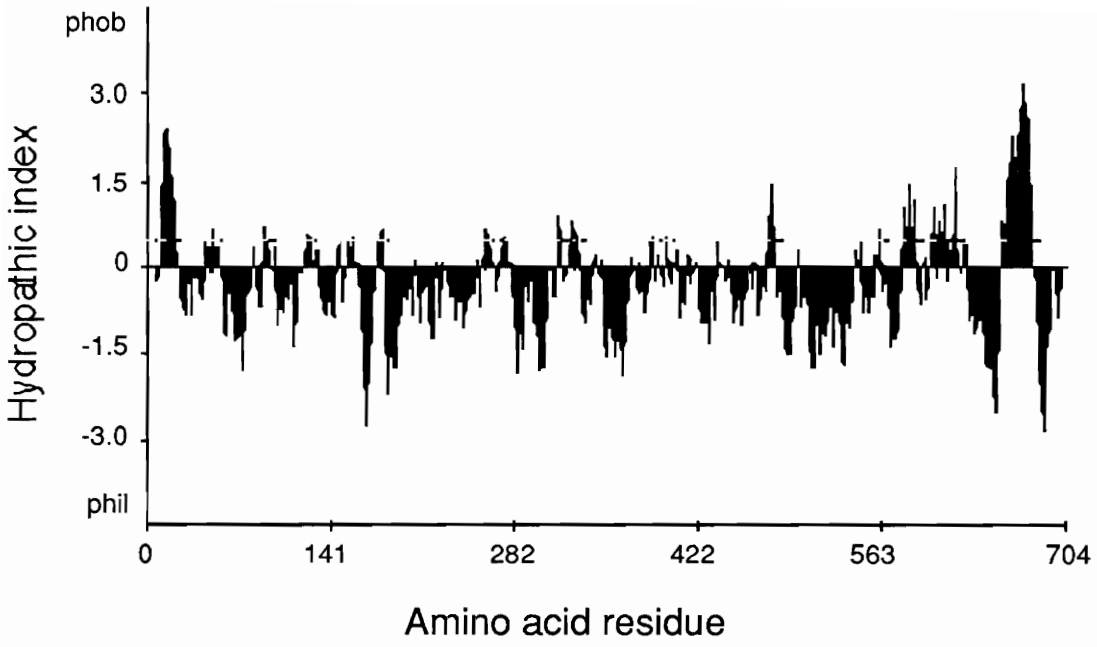
that could represent a transient signal sequence, as is present in most members of the astacin family of metalloendopeptidases (Jiang *et al.*, 1992). The protease domain of the ß subunit was assigned by alignment with other astacin family members to start at Asn-63 and end at Leu-260; this domain is composed of 198 amino acid residues. It contains the putative zinc binding pentapeptide motif, HEFLH, and the 24-amino acid consensus sequence that is characteristic of metalloendopeptidases of the "astacin family" (Dumermuth *et al.*, 1991). Immediately COOH-terminal to the protease domain, extending from Ser-261 to His-430, there is a 170 amino acid domain, the MAM domain. This domain has been described as an adhesion domain in other proteins (Beckmann and Bork, 1993). The stretch of amino acids from His-431 to Ala-610 has no identifiable function, as determined by data base searches. The COOH-terminus of the subunit contains an epidermal growth factor (EGF)-like domain (Cys-611 to Cys-646), followed by a hydrophobic sequence (Thr-655 to Val-678), a short hydrophilic sequence (Arg-682 to Arg-690), and 14 additional amino acids.

There are 9 potential N-glycosylation sites in the β subunit; four sites are present in the domain with unidentified function, two sites in the MAM domain, and three additional sites in the protease domain.

A hydropathy analysis of the mouse ß subunit (Fig. 3) revealed that the only hydrophobic regions in the meprin β subunit are the NH_2-terminal signal sequence (Trp-8 to Ala-23) and the COOH-terminal sequence (Thr-655 to Val-678). The length (24 amino acids) and hydrophobic index of the COOH-terminal region indicate that this region is a transmembrane-spanning domain. The NH_2-terminal sequence is much shorter (16 amino acids) and has features of transient signal peptides, e.g., it is preceded by a positively charged residue (Arg-4), and

Fig. 3. Hydropathy analysis of the deduced amino acid sequence of the β subunit of mouse meprin

Hydrophobic regions appear above the line, hydrophilic regions below the line. The plot was generated using a window size of 11 amino acids according to the method of Kyte and Doolittle (1982). Two segments were identified as hydrophobic regions; one is at the NH_2-terminus (residues Trp-8 to Ala-23), the other is close to the COOH-terminus (Thr-655 to Val-678).


phob
3.0
1.5
0
-1.5
-3.0
phil
Hydropathic index
0
141
282
422
563
704
Amino acid residue

contains a predicted cleavage site for signal peptidase according to the "-3,-1" rule (von Heinje, 1983).

3.1.2 Comparison of the meprin α and β subunit.- Alignment of the mouse ß subunit with the α subunit reveals an overall identity in the amino acid sequences of 42% (Fig. 4). The protease domains are 55% identical. There is, however, very little homology in the NH_2-terminus (first 37 amino acids of ß), or in the sequences COOH-terminal to the EGF-like domain (after Glu-647 of ß), although both subunits contain hydrobic sequences in this region. Interestingly, a segment spanning from Arg-628 to Tyr-683 in the α subunit is missing in the ß subunit. Fifteen of the seventeen cysteine residues in the β subunit are conserved between the α and β subunit primary translation products. These cysteine residues may be important for the overall structure of meprin subunits, presumably by forming intra- or intermolecular disulfide bridges.

The domain structures of α and ß are compared diagramatically in Fig. 5. The overall domain structure of α and β is very similar. The signal sequence in β is slightly smaller than in α, and the COOH-terminal segment after the hydrophobic sequence (predicted to be cytoplasmic) is slightly larger. The α subunit has an additional domain inserted between the domain with unidentified function and the EGF-like domain that is completely missing in the β subunit.

3.2 Oligomeric Organization of Mouse Renal Meprins

A significant discovery was that meprins can exist as different isoforms, and that the expression of one or more of these isoforms differed in different mouse strains (Gorbea *et al.*, 1991). Based on results using electrophoretic techniques,

Fig. 4. Alignment of the deduced amino acid sequences of the mouse α and β subunit

The upper sequence is the β subunit, the lower sequence is the α subunit. Identical residues are indicated by *lines*; chemical similarities between two residues are indicated by *asterisks*. For optimal alignment, several gaps were inserted into the sequences. The brackets < and > indicate the beginning and end, respectively, of a domain. Alignment was aided by the use of the program Mc Molly (Soft Gene, Berlin).


beta MDARHQPWFLVFATFLLASGLPAPEKFVKDIDGGIDQDIFDINQ 44
alpha MARRLGRSSSFAIMLWIQPACLLSLIFSAHIAAVSIKHLLNGSDHDTDVGEQKDIFEINL 60
<protease
beta GLGLDLFEGDIKLEANGKNSIIGDHKRWPHTIPYVLEDSLEMNAKGVILNAFERYRLKTC 104
alpha AAGLNLFQGDILL_PRTRNAMRDPSSRWKLPIPYILADNLELNAKGAILHAFEMFRLKSC 119
beta IDFKPWSGEANYISVFKGSGCWSSVGNIHAGKQELSIGTNCDRIATVQHEFLHALGFWHE 164
alpha VDFKPYEGESSYIIFQKLSGCWSMIGDQQVG_QNISIGEGCDFKATIEHEILHALGFFHE 178
beta QSRADRDDYVIIVWDRIQPGKEHNFNIYND_SVSDSLNVPYDYTSVMHYSKTAFQ_NGTE 222
alpha QSRTDRDDYVNIWWDQIITDYEHNFNTYDDNTITD_LNTPYDYESLMHYGPFSFNKNESI 237
protease><MAM
beta STIVTRIPRFEDVIGQRMDFSDYDLLKLNQLYNCTSSLSFMDSCDFELENICGMIQSSGD 282
alpha PTITTKIPEFNTIIGQLPDFSAIDLIRLNRMYNCTATHTLLDHCDFEKTNVCGMIQGTRD 297
beta SADWQRVSQVLSGPESDHSKMGQCKDSGFFMHFNTSILNEGATAMLESRLLYPKRGFQCL 342
alpha DADWAHGDSSQPE_QVDHTLVEQCKGAGYFMFFNTSLGARGEAALLESRILYPKRKQQCL 356
beta EFYLYNSGSGNDQLNIYTREYTTGQQGGVLTLQRQIKEVPIGSWQLHYVTLQVTKKFRVV 402
alpha QFFYKMTGSPADRFEVWVRRDDNAGKVRQLAKIQTFQGDSDHNWKIAHVTLNEEKKFRYV 416
MAM>
beta FEGLRG_PGTSSGGLSIDDINLSETRCPHHIWHIQNFTQILG___GQDTSVYSPPFYSSK 458
alpha FLGTKGDPGNSSGGIYLDDITLTETPCPAGVWTIRNISQILENTVKGDKLV_SPRFYNSE 476
beta GYAFQI__YMDLRYSTN___VGIYFHLISGANDDQLQWPCPWQQATMTLLDQNPDIRQRM 513
alpha GYGVGVTLYPNGRITSNSGLLGLTFHLYSGDNDAILEWPVENRQAIMTILDQEADTRNRM 535
beta FNQRSITT__DPTMTSDNGSYFWDRPSKVGVTDVFPNGTQFSRGIGYGTTVFITRERLKS 571
alpha SLTLMFTTSKNQTSSAINGSVIWDRPSKVGVYDKDCDC__F_RSLDWGWGQAISHQLLKR 592
beta REFIKGDDIYILLTVEDISHLNSTSAVPDPVPTLA_____ 616
alpha RNFLKGDSLIIFVDFKDLTHLNRTEVPASARSTMPRGLLLQGQESPALGESSRKAMLEES 652
<EGF
beta _____VHNACSEVVCQNGGICVVQDGRAECKCPA 635
alpha LPSSLGQRHPSRQKRSVENTGPMEDHNWPQYFRDPCDPNPCQNEGTCVNVKGMASCRCVS 712
EGF> <TM TM>
beta GEDWWYMGKRCEKRGSTRDTVIIAVSSTVTVFAVMLIITLVSVYCTRRKYRKKARANTAA 695
alpha GHAFFYAGERCQAM_HVHGSLLGLL___IGCIAGLIFLTFVTFSTTNGKLRQ 760
beta MTLENQHAF 704

Fig. 5. Domain structures of mouse meprin α and β subunit deduced amino acid sequences

The domains are aligned relative to the protease domain. Both mouse subunits have a signal sequence (S) at the N-terminus, followed by a potential prosequence (Pro). Close to the COOH-terminus there is an epidermal growth factor-like domain (EGF) followed by a transmembrane spanning domain (TM) and a short cytoplasmic domain (C). Between the protease domain and the EGF-like domain is an adhesion domain (MAM) and a domain of unknown function (X). In addition, the α subunit has an inserted domain (I) comprised of 56 amino acids which is not present in the β subunit.

Meprin α

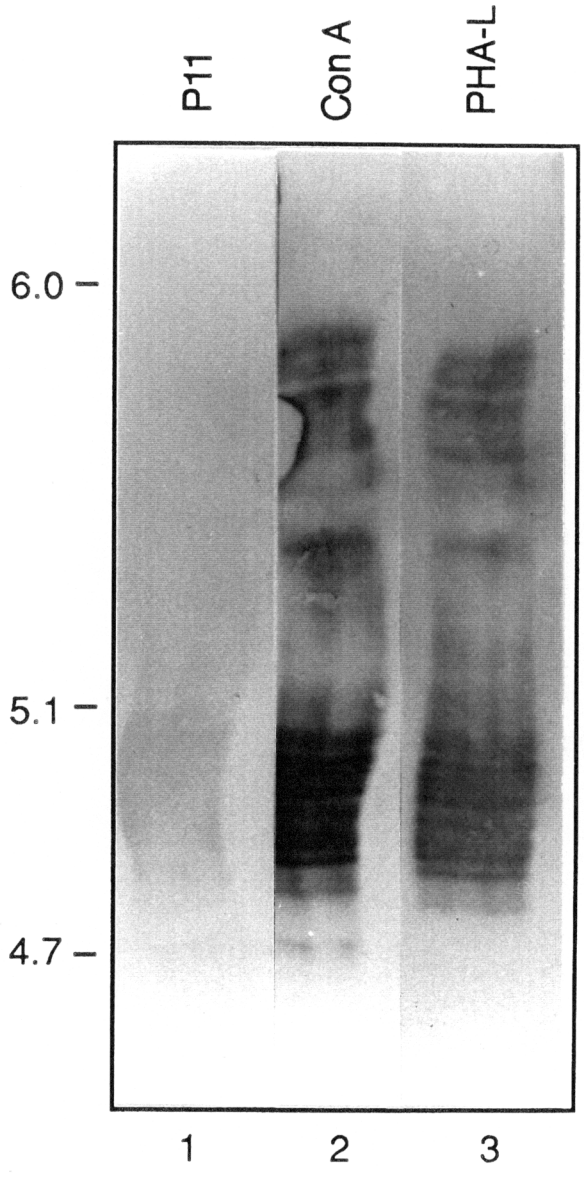
Meprin β

it was suggested that three isoforms of meprin exist in ICR mouse kidneys with the subunit compositions α_4, $\alpha_2\beta_2$, and β_4, and that all subunits in these complexes are linked by disulfide bonds. In contrast, the kidneys of some mouse strains with low meprin activity, e.g., C3H/He mice, were reported to contain only β_4 tetramers. More recent analyses of the oligomeric structure of meprin A by analytical ultracentrifugation indicated however, that the basic disulfide-linked unit of meprin was a dimer, and that under native conditions two sulfhydryl-linked dimers associate non-covalently to form tetramers (Marchand *et al.*, 1994).

To further analyze the oligomeric organization of meprin subunits, the isolation of individual meprin isoforms on the basis of charge differences was attempted. Papain-purified meprin A from ICR mouse kidneys was separated by analytical isoelectric focusing (IEF), and the subunit compositions of the isoelectric forms were analyzed by lectin blotting and immunoblotting (Fig. 6). A large number of isoelectric forms (about 15), ranging in pI from 4.7 to 6.0, were detected by blotting with the lectin concanavalin A (con A), a lectin that recognizes carbohydrate moieties on both α and β subunits (Stroupe *et al.*, 1991; Fig. 6, lane 2). The α subunit-specific lectin PHA-L (Stroupe *et al.*, 1991) recognized the same number of isoelectric forms as con A (Fig. 6, lane 3), indicating that every isoelectric form of meprin contains α subunits. Lane 1 was probed with the antiserum P11; this antiserum recognizes mainly meprin β subunits, but shows cross-reactivity with meprin α subunits. The P11 antibody reacted mainly with a subset of the isoelectric forms, in a pI range of 4.7 to 5.1. Thus, purified meprin A consisted of many more isoelectric forms than could be accounted for by differences in subunit composition; other factors must contribute to charge heterogeneity. One subgroup of isoelectric forms (pI range 5.1 o 6.0) contained mainly

Fig. 6. Isoelectric focusing of papain-purified meprin A

Papain-purified meprin A from ICR mouse kidneys was subjected to isoelectric focusing, transferred to nitrocellulose, and analyzed by lectin blotting and immunoblotting as described in "Experimental Procedures". Ten μg of meprin A protein was applied to each lane. Lane 1 was probed with an antiserum to the meprin β subunit (P11); lanes 2 and 3 were probed with the biotinylated lectins concanavalin A and PHA-L, respectively. Concanavalin A reacts with meprin α and β subunits, PHA-L is specific for meprin α subunits (Stroupe *et al.*, 1991). The positions of the pI markers are indicated on the left. Abbreviations: con A, concanavalin A; PHA-L, *Phaseolus vulgaris* leukoagglutinin.


P11
Con A
PHA-L
6.0
5.1
4.7
1
2
3

or only α subunits, the other subgroup (pI range 4.7 to 5.1) contained α and β subunits.

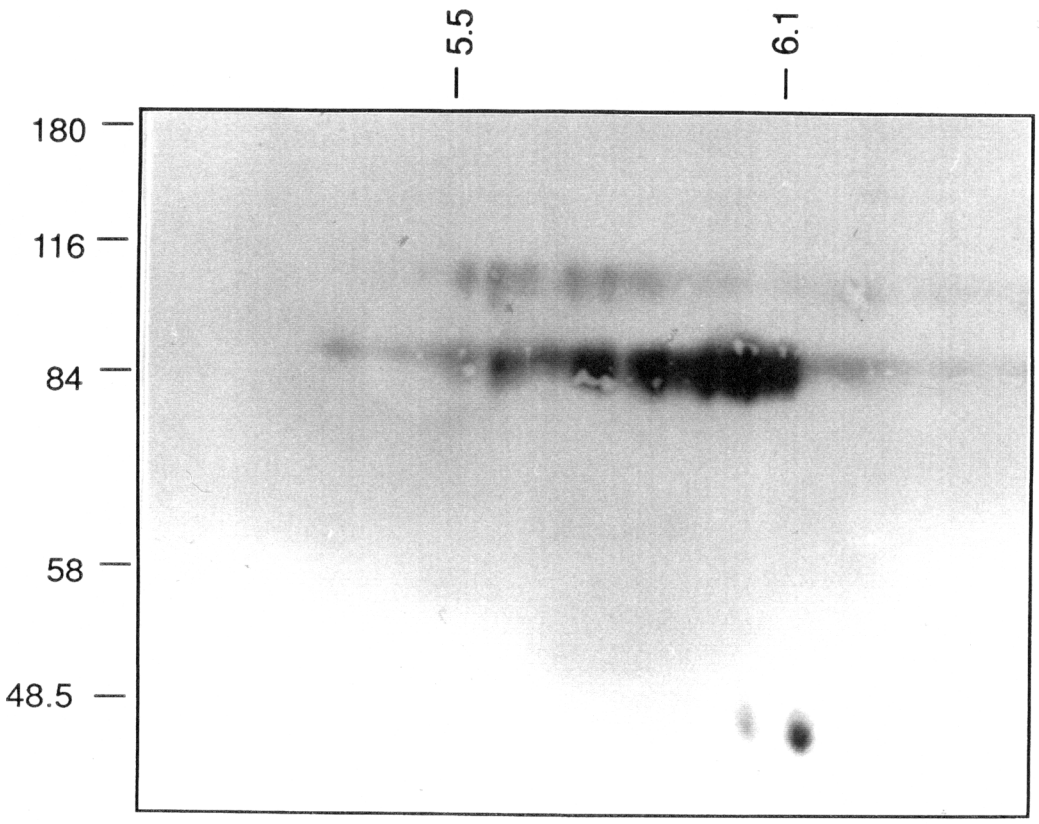
To determine whether the charge heterogeneity of meprin was present in membrane-bound subunits or was introduced into the protein as an artefact of protein purification, two-dimensional gel electrophoresis of brush border membrane proteins was performed (Fig. 7). ICR mouse brush border membrane proteins were solubilized by incubation with octylglucoside, and the solubilized proteins were subjected to IEF under non-reducing conditions, followed by SDS-PAGE under reducing conditions in the second dimension. The meprin subunits were detected by lectin blotting with biotinylated concanavalin A. Previous findings had indicated that at the con A concentrations used the meprin α and β subunits were the predominant brush border membrane proteins detected (Gorbea *et al.*, 1991). The detergent-solubilized meprins consisted of a series of isoelectric forms, ranging in pI from 5.5 to 6.1. A subset of isoelectric forms (pI 5.5 to 5.8) clearly contained α and β subunits (approximately 90 and 110 kDa, respectively); the forms in the pI range 5.8 to 6.1 contained mainly α subunits. Thus, native forms of meprin also consist of a series of isoelectric forms; the molecular basis for this charge heterogeneity remains unknown.

The results from the analytical IEF and the two-dimensional gel electrophoresis both indicated that ICR mice express homo-oligomers of only α subunits and hetero-oligomers containing α and β subunits. They provide no evidence for β homo-oligomers in ICR mice; however, it is possible that β homo-oligomers comigrated with α subunit-containing isoforms.

The oligomeric organization of membrane-bound mouse meprins was also analyzed by SDS-PAGE of mouse kidney brush border membranes under

Fig. 7. Two-dimensional electrophoresis of membrane-bound meprins

Brush border membrane proteins from ICR mouse kidneys (100 μg) were solubilized by incubation with β-D-octylglucopyranoside, subjected to 2 dimensional electrophoresis, transferred to nitrocellulose, and the meprin subunits were visualized by lectin blotting using biotinylated concanavalin A as described in "Experimental Procedures". The first dimension was isoelectric focusing under non-reducing conditions. The isoelectric focusing was carried out in tube gels containing 6 M urea, 1% NP-40, and a basic (pH 10) to acidic (pH 3) pH gradient. The second dimension was SDS-PAGE under reducing conditions. pI values are shown on the top of the gel; the positions of the SDS-PAGE molecular weight markers are indicated on the left.


5.5
6.1
180
116
84
58
48.5

non-reducing conditions followed by immunoblotting (Fig. 8). Of great importance for these experiments was the availability of antibodies that are completely subunit-specific. Notably, the injection of deglycosylated meprin A, which contained α and β subunits, into rabbits yielded an antibody preparation that was completely specific for the α subunit; this antibody was used in most subsequent experiments. A specific antiserum to the β subunit was obtained by injecting rabbits with a peptide present in the COOH-terminal region of β (residues 682-693 of the β subunit primary translation product), a region that shows no homology to α.

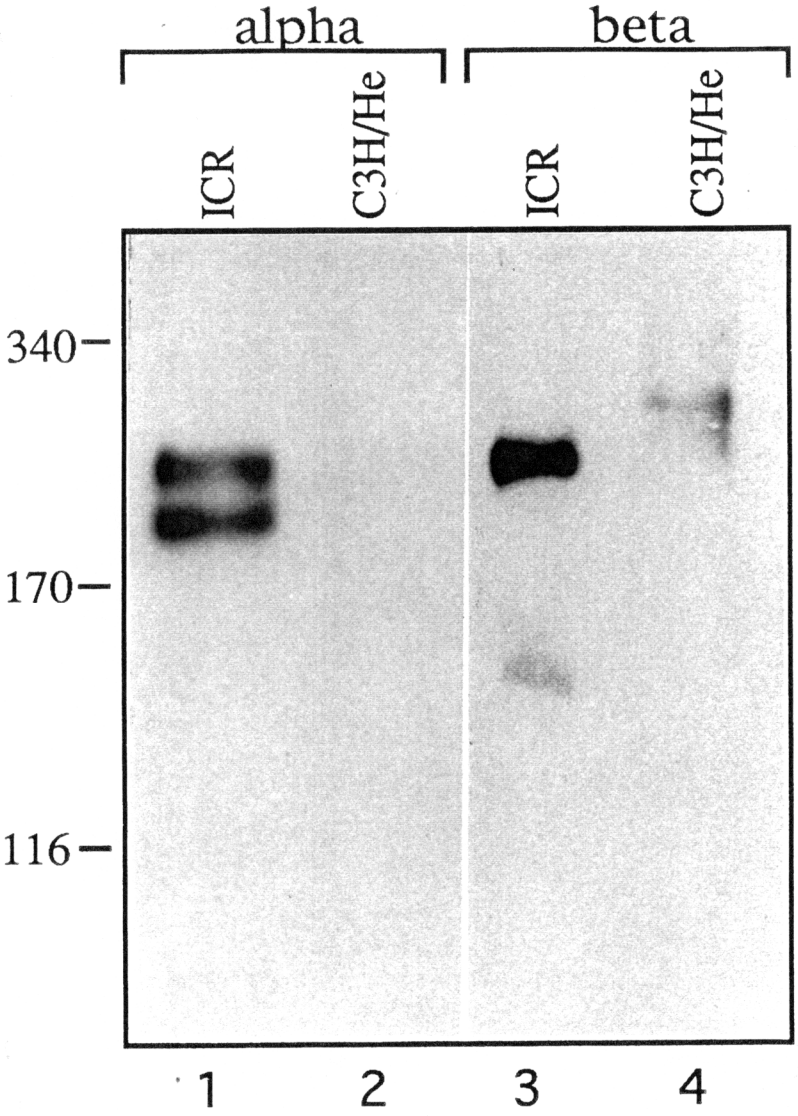
In preparations from ICR mice, two high molecular weight complexes of 204 and 224 kDa were identified after SDS-PAGE in 6% gels under non-reducing conditions (Fig. 8). These molecular weight estimates indicated that the basic oligomeric unit of membrane-bound mouse meprins is a disulfide-linked dimer, rather than a tetramer as previously reported. The 204 kDa protein reacted only with the antibody raised to the α subunit, and appears to be a α_2 homodimer; the 224 kDa protein is a heterodimeric protein, which reacted with both the anti-α as well as the anti-β subunit antibody. C3H mouse kidney preparations contained only the β_2 homodimer with an apparent molecular weight of approximately 240 kDa in this gel system. Thus, ICR mouse kidneys contain disulfide-linked α_2 dimers and $\alpha\beta$ dimers, but no β_2 dimers; C3H/He mice contain only β_2 dimers.

3.3 Membrane Association of Meprin Subunits

All data previous to these studies indicated that both meprin subunits are type I integral membrane proteins, that is proteins with a single COOH-terminal membrane-spanning domain. The evidence for this hypothesis was that solubilization

Fig. 8. Immunoblots of non-reduced kidney brush border membrane proteins with meprin subunit specific antisera

Brush border membrane proteins (25 μg) were subjected to SDS-PAGE (6% gels) under non-reducing conditions, the proteins were transferred to nitrocellulose, and probed with antibodies to the deglycosylated meprin α subunit (lanes 1 and 2) or anti-β subunit peptide antibodies (lanes 3 and 4). Lanes 1 and 3 contained membrane fractions from ICR mice, lanes 2 and 4 contained membrane fractions from C3H/He mice.


alpha
beta
ICR
C3H/He
ICR
C3H/He
340
170
116
1
2
3
4

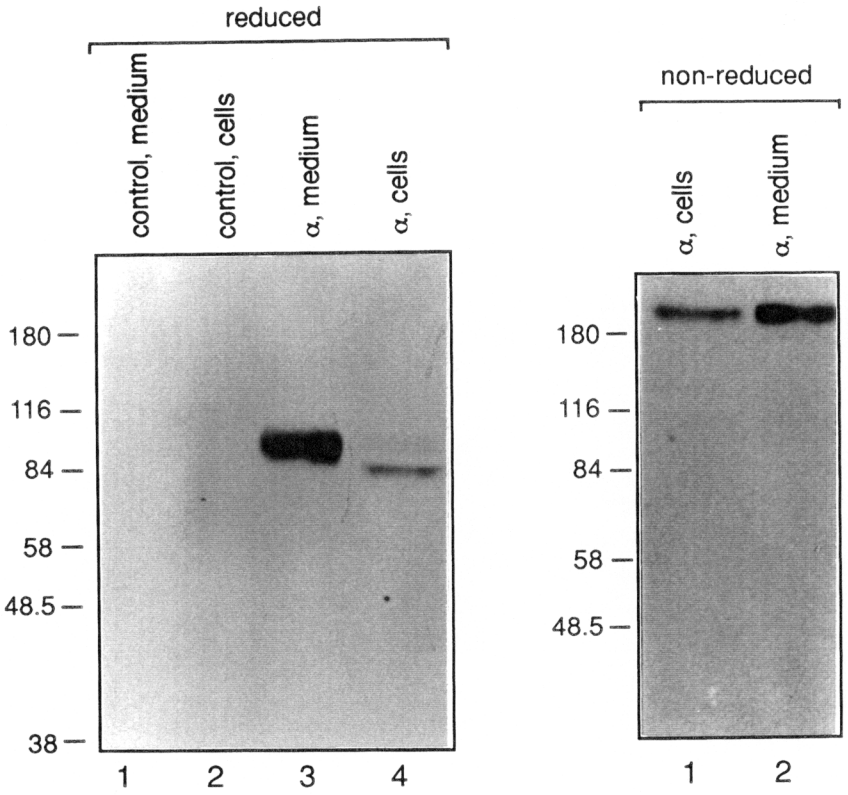
of meprins from renal brush border membrane proteins required limited proteolysis with papain or trypsin/toluene (Kounnas *et al.*, 1991). In addition, treatment of brush border membranes from ICR mice with phospholipase C does not solubilize meprin A (Jiang *et al.*, 1992). Finally, the cDNA sequences encode hydrophobic potentially transmembrane-spanning domains near the COOH-termini.

The membrane association of meprin subunits was analyzed using two experimental systems: (1) COS-1 cells transfected with meprin cDNA; this system was used, because it allowed analysis of each subunit individually, and (2) renal brush border membrane enriched fractions; the membranes in these preparations retain the structural organization of the apical membranes, where meprins are found *in vivo*.

3.3.1 Analysis of the membrane association in transfected COS-1 cells.- The experimental design was to transfect COS-1 cells with meprin α or β subunit cDNA, and to identify domains involved in membrane association by deletion analysis. COS-1 cells were transfected with the wild-type meprin α subunit cDNA, and the culture medium and the cell fractions were analyzed for meprin protein by SDS-PAGE and immunoblotting (Fig. 9). Prior to SDS-PAGE the transfected cells were homogenized and high speed (100,000 x g) precipitate fractions (membrane-enriched) and supernatant fractions were prepared. Most of the immunoreactive material was found in the tissue culture medium (Fig. 9, lane 3). The secreted α subunit had a molecular mass of about 95 kDa. A small fraction of the expressed meprin subunits was also found in the cell precipitate fraction (Fig. 9, lane 4); the cell-associated meprin consisted of a series of bands with slightly different mobilities on SDS-PAGE; the apparent molecular masses

Fig. 9 Immunoblot analysis of the mouse meprin α subunit expressed in COS-1 cells

COS-1 cells were transfected with meprin α subunit cDNA as described in "Experimental Procedures". Concentrated tissue culture media (indicated as "medium") and cell membrane fractions (indicated as "cells") were subjected to SDS-PAGE and immunoblotting using anti meprin α subunit antibodies. The left gel was run under reducing conditions, the right gel was run under non-reducing conditions. Left gel, lanes 1 and 2 contained control samples transfected with vector DNA only; left gel, lanes 3 and 4, and right gel, lanes 1 and 2 contained samples from transfection with meprin α subunit cDNA. Each lane contained 10% of the sample obtained from one 100 mm dish. The positions of molecular mass markers (in kDa) are shown on the left.

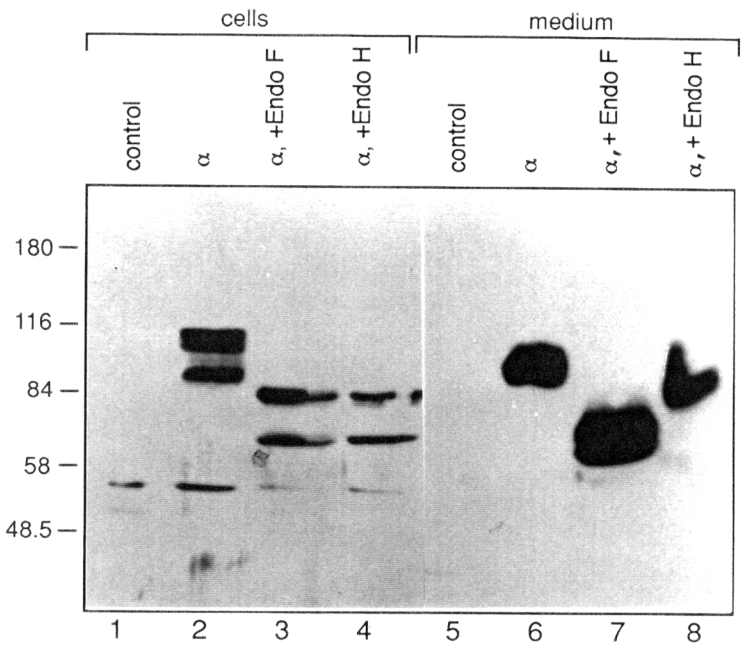

reduced
control, medium
control, cells
α, medium
α, cells
180
116
84
58
48.5
38
1
2
3
4
non-reduced
α, cells
α, medium
180
116
84
58
48.5
1
2

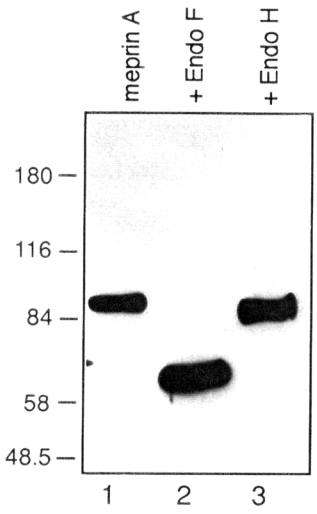
ranged from 86 kDa to 102 kDa. The supernatant fractions of the cell homogenate did not contain any immunoreactive material (data not shown). Both the secreted and the cell-associated meprin subunits were oligomeric, as judged by SDS-PAGE under non-reducing conditions (Fig. 9, right panel). Thus, contrary to the prediction from the cDNA information most of the wild-type α subunit was expressed as a secreted soluble protein rather than a membrane-bound protein.

To determine whether the cell-associated meprin subunits are expressed on the cell surface, the recombinant meprin subunits were subjected to endoglycosidase-H (endo-H) or endoglycosidase-F (endo-F)/N-glycanase treatment (Fig. 10). Endo-F will cleave N-linked high-mannose, biantennary hybrid, and biantennary complex oligosaccharides from glycoproteins. In contrast, endo-H will cleave only high mannose, but not complex N-linked oligosaccharides from glycoproteins. Since N-linked carbohydrates are initially synthesized in the ER as high mannose chains, and maturation of some of these high mannose chains to complex carbohydrate chains occurs in the Golgi apparatus, resistance to digestion by endo-H indicates that the protein has transited the Golgi apparatus. Sensitivity to endo-H digestion indicates localization of the glycoprotein in the ER. Fig. 10, top panel shows the immunoblot analysis of untreated, endo-F and endo-H treated meprin subunits from the medium and cell fraction of transfected COS-1 cells. Untreated cell-associated meprin migrated as a series of bands with apparent molecular masses ranging from 86 to 102 kDa (Fig. 10, lane 2). Endo-F treatment produced two bands with molecular masses of approximately 81 and 65 kDa (Fig. 10, lane 3). Treatment with endo-H produced the same result as endo-F treatment (Fig. 10, lane 4), indicating that the cell-associated meprin does

Fig. 10. Effect of endoglycosidase treatment on the meprin α subunit

Protein samples were treated with either a mixture of endoglycosidase F/N-glycanase (indicated as Endo F) or with endoglycosidase H (indicated as Endo H) as described in "Experimental procedures", and subjected to SDS-PAGE (10%) under reducing conditions, followed by immunoblotting using anti meprin α subunit antibodies. Top, samples from transfected COS-1 cells. Lanes 1 to 4 contain cell membrane fractions, lanes 5 to 8 contain tissue culture medium samples. Lanes 1 and 5 contained samples from cells transfected with vector DNA only (control transfection), lanes 2 to 4 and lanes 6 to 8 contained samples from cells transfected with the meprin α subunit cDNA. Each lane contained 10% of the sample obtained from one 100 mm dish. Bottom, papain-purified meprin A samples (1 μg) from ICR mouse kidneys. The samples were either untreated (lane 1), treated with endo F (lane 2) or treated with endo H (lane 3). The positions of molecular mass markers (in kDa) are shown on the left.


cells
medium
control
α
α, +Endo F
α, +Endo H
control
α
α, + Endo F
α, + Endo H
180
116
84
58
48.5
1
2
3
4
5
6
7
8

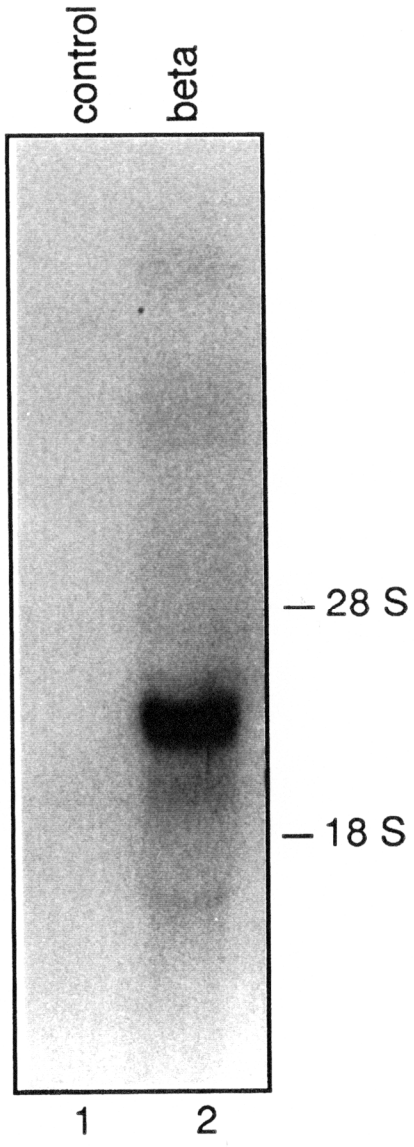

meprin A
+ Endo F
+ Endo H
180
116
84
58
48.5
1
2
3

not contain complex carbohydrate chains. In contrast, the secreted meprin subunits in the culture medium contained carbohydrate chains that are resistant to endo-H treatment (Fig. 10, lanes 5 to 8). Endo-F treatment reduced the molecular mass of the secreted meprin α subunit from approximately 92 kDa to a broad band, possibly two bands with a molecular mass range of 56 to 74 kDa; endo-H treatment reduced the molecular mass to approximately 79 kDa. The bottom panel shows the migration of untreated, endo-F treated, and endo-H treated purified meprin A from ICR mouse kidneys. Endo-F treatment reduced the molecular weight from 88 kDa to 67 kDa; endo-H treatment resulted in only a slight reduction of the molecular mass from approximately 88 kDa to 85 kDa. Thus, meprin subunits secreted from COS-1 cells and purified meprin subunits from mouse kidney are mature proteins containing some complex N-linked carboydrate chains. The mouse kidney meprin subunits have a larger fraction of complex carbohydrates than the COS-1 cell expressed secreted meprin subunits, which might reflect cell-type dependent differences in the expression of glycosyltransferases. In contrast, the COS-1 cell-associated meprin subunits are immature forms that are retained in a pre-Golgi compartment, rather than mature surface expressed proteins. Together, these experiments indicated that in transfected COS-1 cells the mature α subunit is a secreted protein, not an integral membrane protein.

Several attempts were made to express the mouse meprin β subunit in COS-1 cells. However, no protein could be detected after transfection of the meprin β subunit cDNA into COS-1 cells. It is not clear why this was the case. Northern blot analysis showed that COS-1 cells transfected with β subunit cDNA produce the β subunit message (Fig. 11). Also, *in vitro* transcription/translation of

Fig. 11. Northern blot of total RNA isolated from COS-1 cells transfected with meprin β subunit cDNA

Total RNA was isolated as described in "Experimental Procedures". RNA samples (20 μg) were subjected to formaldehyde-gel electrophoresis in the presence of ethidium bromide (0.5 μg/ml), transferred to Gene Screen membrane, and hybridized to a full-length ^{32}P-labeled mouse meprin β subunit cDNA probe (nucleotides 1-2199, see Fig. 2). Lane 1 contained RNA from COS-1 cells transfected with the pcDNAI/Amp expression vector (control), lane 2 contained RNA isolated from cells transfected with the pcDNAI/Amp expression vector containing the meprin β subunit insert. The positions of the ribosomal RNA markers are indicated on the right.

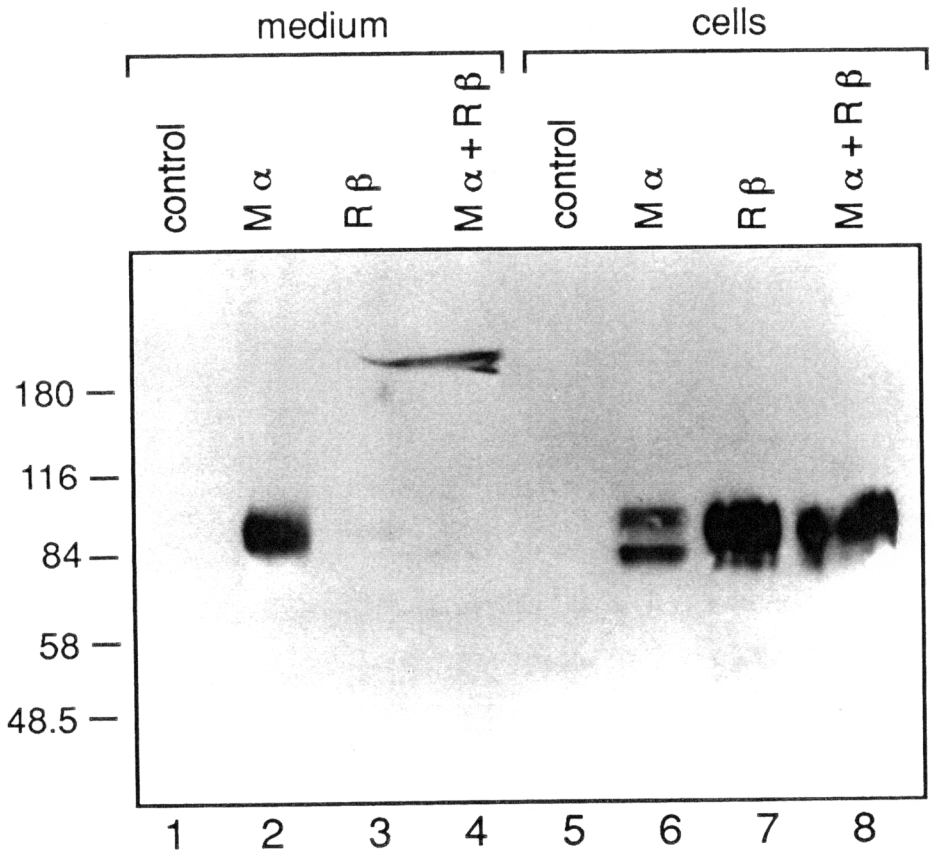

control
beta
— 28 S
— 18 S
1
2

the meprin subunit cDNAs resulted in the production of meprin β protein of the expected molecular weight (Fig. 21; section 3.4.3). It is possible that the available antibodies to the β subunit are not sensitive enough to detect the β subunit protein in the transfection system; the antibodies to the β subunit have detection limits that are at least one order of magnitude higher than the detection limits of the α subunit-specific antibodies. Also, the expression level might be lower for the mouse β subunit than for the mouse α subunit.

Rat meprin α subunits, when expressed in human 293 cells, were also secreted into the culture medium; by contrast, rat β subunits were expressed on the cell surface of transfected 293 cells (Johnson and Hersh, 1994). However, if the rat α subunits were cotransfected with rat β subunits, the α subunits localized to the membrane, indicating that membrane association of α subunits depended on the presence of β subunits. To test whether the mouse α subunit transfected in COS-1 cells can also associate with the cell membrane through meprin β subunits, COS-1 cells were transfected with the mouse α subunit cDNA alone or in combination with the rat β subunit cDNA (a gift from Dr. L. Hersh). Since the rat and mouse β subunits are 90% identical, it was expected that the rat subunit could substitute for the mouse subunit in this experiment. The samples were analyzed by immunoblotting with a polyclonal antiserum to rat meprin which recognized both subunits (Fig. 12). When the α subunit was expressed alone, the majority of the α subunit protein was detected in the culture medium (Fig. 12, lanes 2 and 6). Like in the 293 cell system, the β subunit was found only in the cell membrane fraction, but not in the medium, indicating that the β subunit is a transmembrane protein (Fig. 12, lanes 3 and 7). When the α and β subunit were cotransfected (Fig. 12, lanes 4 and 8), the α subunit was not secreted into the

Fig. 12. Transfection of COS-1 cells with mouse meprin α and rat meprin β subunit cDNA alone and in combination

Ten μg of the expression vector pcDNAI/Amp harboring the wild-type or mutant meprin α subunit cDNA were transfected into COS-1 cells grown on 100 mm plates. Concentrated tissue culture media (indicated as "medium") and cell membrane fractions (indicated as "cells") from the transfected cells were prepared as described in "Experimental Procedures". Samples transfected with either vector DNA (lanes 1 and 5), mouse meprin α subunit cDNA (lanes 2 and 6), rat β subunit cDNA (lanes 3 and 7), or mouse α and rat β cDNA (lanes 4 and 8) were subjected to SDS-PAGE (10% gel) under reducing conditions, followed by immunoblot analysis using anti rat meprin antibodies. Lanes 1 to 4 contained medium samples, lanes 5 to 8 contained cell membrane samples. Each lane contained 10% of the sample obtained from one 100 mm dish. The positions of molecular mass markers (in kDa) are shown on the left.


medium
cells
control
M α
R β
M α + R β
control
M α
R β
M α + R β
180
116
84
58
48.5
1
2
3
4
5
6
7
8

medium, but localized only to the cell membrane fraction. Thus, in COS-1 cells, β is essential for the association of the mouse α subunit with the cell membrane.

3.3.2 Membrane association of meprin subunits in mouse kidney membranes.-

The results obtained from transfection of COS-1 cells with full-length meprin subunit cDNAs indicated that α is not an integral membrane protein, whereas β is, and that α is membrane-associated in the presence of β. The experiments in this section were designed to determine whether this was also the case for the mouse kidney subunits.

To determine whether the meprin ß subunit is attached to kidney brush border membranes via a phosphatidylinositol anchor, membranes were prepared from C3H/He kidneys (a mouse strain that contains only β subunits) and treated with phosphatidylinositol-specific phospholipase C (Table 3). The results indicated that meprin was not released from the membranes with the phospholipase treatment whereas alkaline phosphatase, which is attached by the phosphatidylinositol anchor, was released under the same conditions. Thus, there is no evidence for a phosphoinositol linkage for the ß subunit or meprin B.

To analyze the type of membrane association of the meprin subunits, meprin subunits were solubilized from ICR mouse kidney brush border membranes by limited proteolysis with papain, and the membrane-associated and papain-solubilized subunits were compared by probing with subunit-specific antibodies, some of which recognize specific domains (Fig. 13). Three antibodies were used: a polyclonal antibody raised to the deglycosylated meprin α subunit , an antibody raised to a β subunit peptide (amino acids 682-693) COOH-terminal of the hydrophobic domain, and an antibody to a recombinant meprin β subunit

Table 3

Effect of phospholipase C treatment on solubilization of meprin B activity

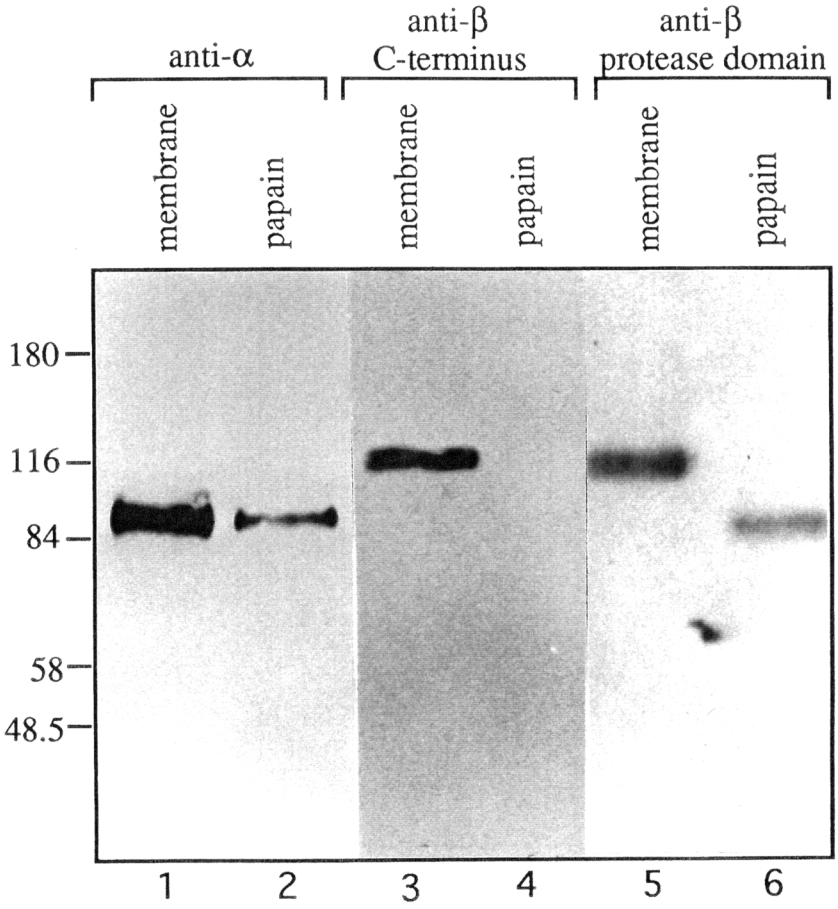
PLC units added	Meprin activity		
	In precipitated fraction	In supernatant fraction	% activity in precipitate
0	6.33	0.73	90
2	6.00	0.06	99

PLC units added	Alkaline phosphatase activity		
	In precipitated fraction	In supernatant fraction	% activity in precipitate
0	978	26.5	94
2	62.3	920	8

Brush border membrane enriched fractions were prepared from C3H/He mouse kidneys. The membrane fractions were incubated at 37°C for 90 min in the presence or absence of phosphatidylinositol-specific phospholipase C (PLC), centrifuged at 4°C for 1 h at 100,000 x g, and the enzyme activities were measured in the resulting precipitated and supernatant fractions. Units for meprin are expressed as increase in absorbance at 340 nm of 0.001/min; for alkaline phosphatase, 1 μmol 4-nitrophenylphosphate/min.

Fig. 13. Western Blotting of membrane-bound and papain-solubilized meprin with domain-specific antibodies.

Total brush border membranes (25 μg, lanes 1, 3, and 5) and papain-solubilized fractions from 25 μg brush border membranes (lanes 2, 4, and 6) were subjected to SDS-PAGE (10 % gel) under reducing conditions, transferred to nitrocellulose membranes, and probed with antibodies directed against different domains. Lanes 1 and 2 were probed with an antibody to the deglycosylated α subunit, lanes 3 and 4 with a COOH-terminal β subunit peptide antiserum, and lanes 5 and 6 with an antibody raised against the β subunit protease domain. Positions of the molecular mass markers (in kDa) are shown on the left.


anti-α
anti-β
C-terminus
anti-β
protease domain
membrane
papain
membrane
papain
membrane
papain
180
116
84
58
48.5
1
2
3
4
5
6

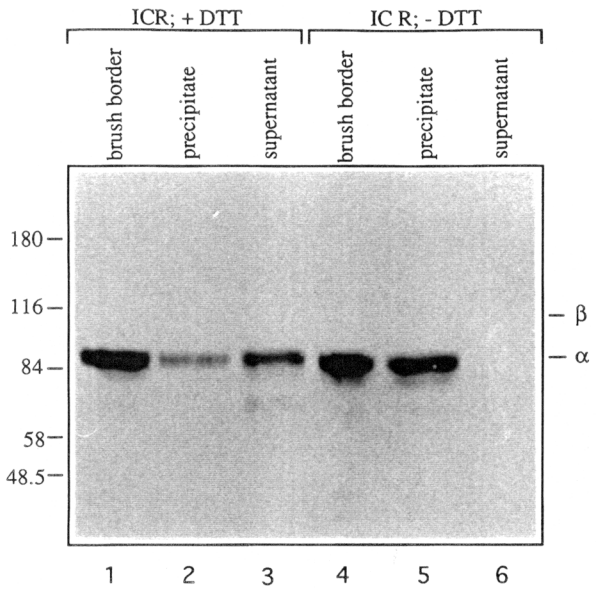
protease domain. When meprin subunits were solubilized from brush border membranes by papain treatment, the size of the α subunit did not change detectably (lanes 1 and 2); thus, it is unlikely that papain removes a transmembrane-spanning anchor from α during the solubilization process. The membrane-bound β subunit reacted with the antibody to the COOH-terminal peptide (lane 3). When β was solubilized with papain, it decreased in size from 110 kDa to 90 kDa (lanes 5 and 6) with concomittant loss of the COOH-terminal epitope (lanes 3 and 4).

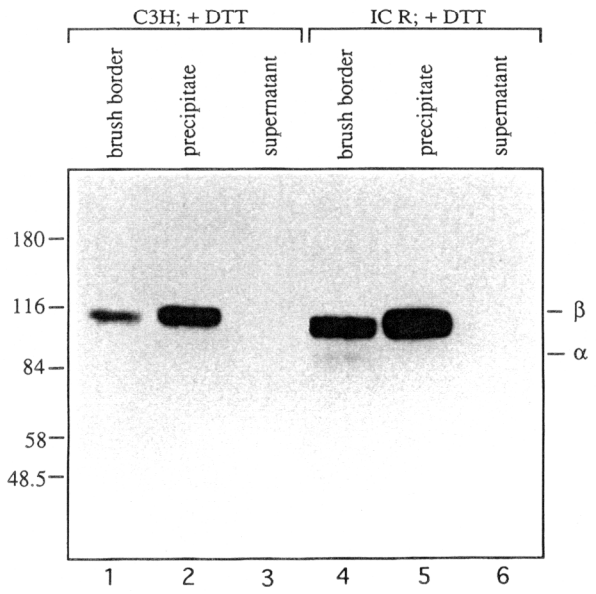
In order to determine whether some or all mouse meprin α subunits can be solubilized by treatment with reducing agents, brush border membranes were treated with the reducing agent DTT in the presence of the cysteine protease inhibitor E-64 (Fig. 14). E-64 was added in order to inhibit cysteine proteases in the sample that might become activated by reducing agents. The majority of α subunit protein was solubilized from the ICR mouse membrane fractions by this treatment (panel A); in contrast no meprin β subunit protein became soluble under the reaction conditions from either ICR or C3H mouse kidney membranes (panel B). The extent to which the α subunit was solubilized was estimated to be about 90% by densitometric analysis of the Western blotting signals using known amounts of brush border membranes as standards.

Together, the data indicated that the mouse α subunit is not an integral membrane protein. Rather than interacting directly with the lipid bilayer, the α subunit is linked to the membrane by a mechanism that requires the presence of disulfide bonds. In contrast, the β subunit interacts with the membrane through its hydrophobic membrane-spanning domain near the COOH-terminus.

Fig. 14. Release of the α subunit, but not the β subunit, from renal brush border membranes by treatment with dithiothreitol (DTT)

Renal brush border membranes were treated with 50 mM DTT as described in "Experimental Procedures", and centrifuged at 100,000 x g for 1 h at 4°C. The obtained precipitated and supernatant fractions were analyzed by immunoblotting. Top gel, immunoblotting of samples from ICR mouse kidneys with an antibody to the deglycosylated α subunit. Lanes 1 and 4 contained ICR brush border membrane fractions (5 μg); lanes 2 and 5 contained fractions that precipitated from 5 μg ICR mouse renal brush border membranes, and lanes 3 and 6 contained supernatant fractions from 5 μg brush border membrane proteins. Lanes 1 to 3 contained DTT-treated samples; lanes 4 to 6 control samples that were treated the same way as the samples in lanes 1 to 3, except that the DTT was omitted from the incubation buffer. Bottom gel, immunoblotting of DTT-treated fractions from C3H/He (lanes 1 to 3) and ICR (lanes 4 to 6) mouse renal brush border membranes with anti-β subunit peptide antibodies. Lanes 1 and 4 contained brush border membrane fractions (25 μg); lanes 2 and 5 contained precipitant fractions from 25 μg brush border membranes; lanes 3 and 6 contained supernatant fractions from 25 μg brush border membranes. Positions of the molecular mass markers (in kDa) are shown on the left.


ICR; + DTT
IC R; - DTT
brush border
precipitate
supernatant
brush border
precipitate
supernatant
180
116
84
58
48.5
β
α
1
2
3
4
5
6


C3H; + DTT
IC R; + DTT
brush border
precipitate
supernatant
brush border
precipitate
supernatant
180
116
84
58
48.5
β
α
1
2
3
4
5
6

To test whether α_2 dimers associate with the membrane through non-covalent interactions with $\alpha\beta$ heterodimers or other proteins, brush border membranes were treated with high concentrations of salt and EDTA or urea (Fig. 15). Treatment of brush border membranes with 1.5 M NaCl/10 mM EDTA did not solubilize meprin protein (Fig. 15, panel A); however, treatment with 7 M urea solubilized a significant fraction of meprin α subunits. The β subunit remained completely retained in the membrane fraction (Fig. 15, panel B), indicating that the urea treatment did not disrupt the membrane nonspecifically. SDS-PAGE under non-reducing conditions showed that the urea-solubilized meprin was exclusively α_2 homodimeric (Fig. 15, panel C). Thus, meprin α_2 homodimers associate with the membrane through noncovalent interactions with other membrane components.

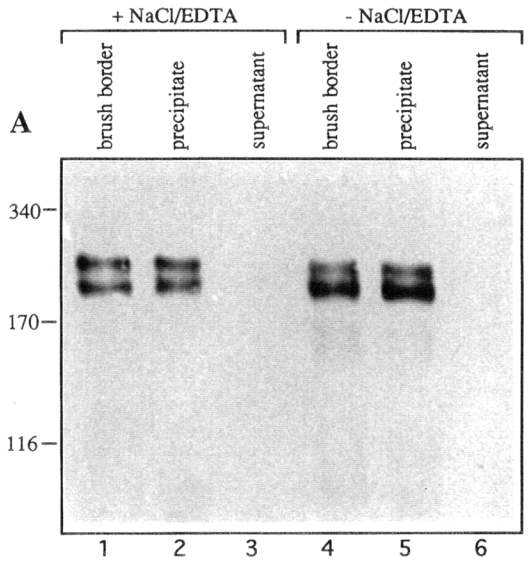
Rat kidney brush border membranes also express meprin α and β subunits. The oligomeric organization of rat meprin, analyzed by two-dimensional gel electrophoresis (SDS-PAGE under non-reducing conditions in the first dimension; SDS-PAGE under reducing conditions in the second dimension), was $\alpha\beta$ heterodimeric; no other isoforms were detected using this gel system (Johnson and Hersh, 1992). To test whether rat and mouse meprins have a similar oligomeric organization, brush border membranes from Sprague-Dawley rats were analyzed by immunoblotting before and after urea treatment. Fig. 16, top panel shows a comparison of the mouse and rat meprin subunits by Western blotting using antibodies raised to the mouse subunits. Both mouse meprin antisera cross-reacted with the rat meprin subunits. The mouse α subunit had an apparent molecular weight of approximately 90 kDa, the rat α subunit of 77 kDa. The mouse β subunit migrated as a 110 kDa protein, the rat β subunit as a 90

Fig. 15. Immunoblots of ICR mouse renal brush border membrane fractions treated with NaCl/EDTA or urea

A, brush border membranes (200 μg) were treated with 1.5M NaCl/10 mM EDTA as described in "Experimental Procedures", and centrifuged for 15 min at 100,000 x g at 4°C. 25 μg brush border membrane proteins (lanes 1 and 4), precipitate fractions (lanes 2 and 5), and supernatant fractions (lanes 3 and 6) derived from 25 μg protein were subjected to SDS-PAGE (6% gel) under non-reducing conditions, and probed with anti-deglycosylated α subunit antiserum. Lanes 1 to 3 contain NaCl/EDTA treated samples; lanes 4 to 6 contain control samples that had been incubated under the same conditions except that NaCl and EDTA were omitted.

B, brush border membranes (200 μg) were treated with 7 M urea as described in "Experimental Procedures", centrifuged for 15 min at 100,000 x g at 4°C, and the resulting fractions subjected to SDS-PAGE (10% gel) under reducing conditions. Lanes 1 and 4 contained 25 μg brush border membrane proteins, lanes 2 and 5 contained precipitate fractions obtained from 25 μg brush border membrane proteins, and lanes 3 and 6 contained supernatant fractions obtained from 25 μg brush border membrane proteins. Lanes 1 to 3 were probed with anti-deglycosylated α subunit antiserum, lanes 4 to 6 were probed with anti-β subunit peptide antibodies.

C, 25 μg urea-treated brush border membranes (lane 1) and the supernatant fraction obtained from 25 μg brush border membrane proteins after urea treatment were subjected to SDS-PAGE (6% gel) under non-reducing conditions, and probed with anti-deglycosylated α subunit antiserum. The positions of the molecular mass markers (in kDa) are shown on the left.


+ NaCl/EDTA
- NaCl/EDTA
A
brush border
precipitate
supernatant
brush border
precipitate
supernatant
340
170
116
1
2
3
4
5
6

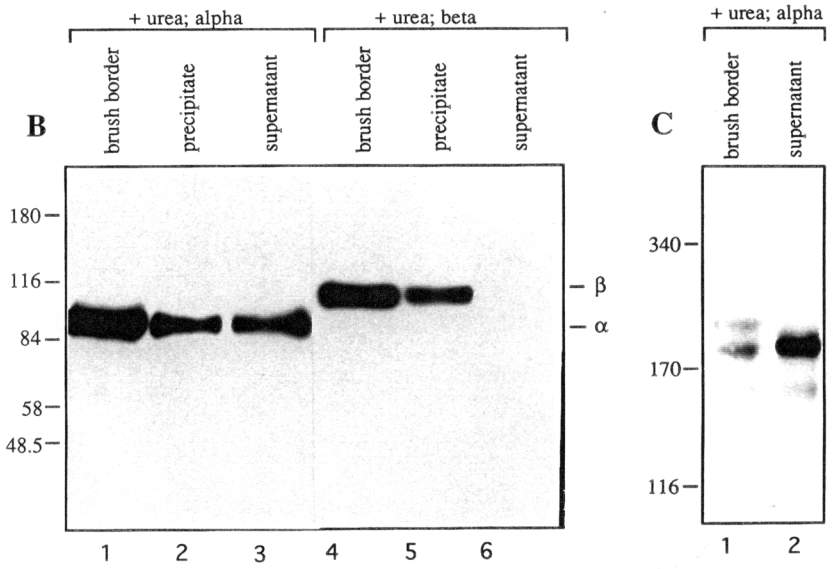
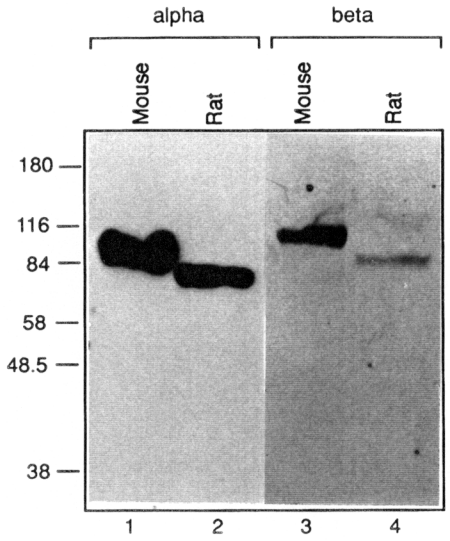
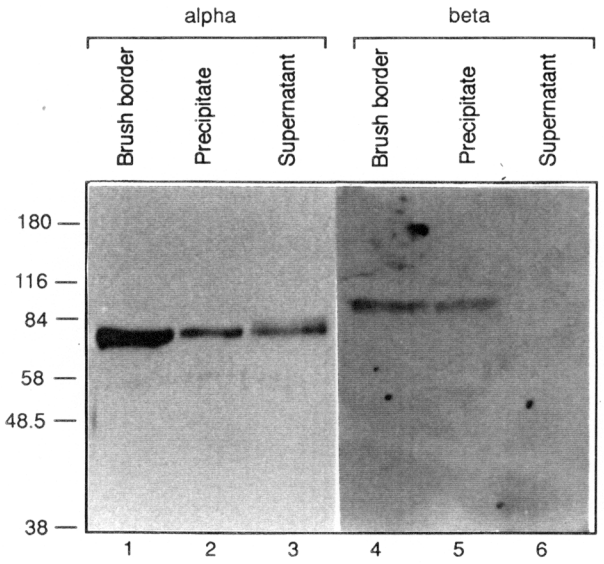

+ urea; alpha
+ urea; beta
B
brush border
precipitate
supernatant
brush border
precipitate
supernatant
180
116
84
58
48.5
β
α
1
2
3
4
5
6
+ urea; alpha
C
brush border
supernatant
340
170
116
1
2

Fig.16. Western blot analysis of rat renal brush border membrane proteins before and after treatment with urea

Top, comparison of mouse and rat meprin subunits by immunoblotting. Brush border membrane proteins from ICR mouse kidneys (25 μg) or Sprague-Dawley rat kidneys (60 μg) were subjected to SDS-PAGE (10% gels) under reducing conditions and probed with polyclonal antibodies to the deglycosylated mouse meprin α subunit (lanes 1 and 2) or to the β-subunit protease domain (lanes 3 and 4).

Bottom, immunoblot of rat renal brush border membrane fractions treated with 7 M urea. The brush border membranes were treated with 7 M urea as described in "Experimental Procedures", centrifuged for 15 min at 100,000 x g at 4°C, and the resulting fractions were subjected to SDS-PAGE (10% gels) under reducing conditions. Lanes 1 and 4 contained 75 μg brush border membrane proteins, lanes 2 and 5 contained precipitate fractions obtained from 75 μg brush border membrane proteins, and lanes 3 and 6 contained supernatant fractions obtained from 75 μg brush border membrane proteins. Lanes 1 to 3 were probed with antibodies to the deglycosylated mouse meprin α subunit, lanes 4 to 6 were probed with antibodies to the mouse meprin β-subunit protease domain. The positions of the molecular mass markers (in kDa) are shown on the left.


alpha
beta
Mouse
Rat
Mouse
Rat
180
116
84
58
48.5
38
1
2
3
4


alpha
beta
Brush border
Precipitate
Supernatant
Brush border
Precipitate
Supernatant
180
116
84
58
48.5
38
1
2
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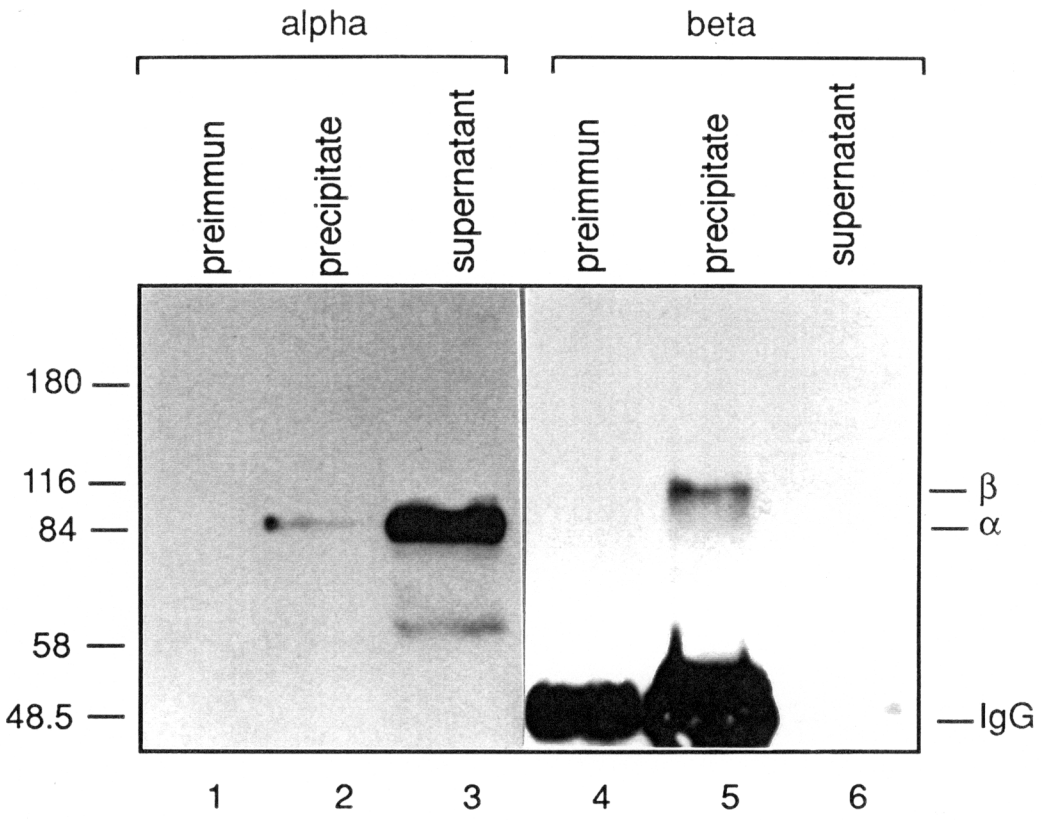
5
6

kDa protein. The smaller molecular weights of the rat subunits compared to the mouse subunits may be caused by differences in glycosylation. When renal brush border membranes from Sprague-Dawley rats were treated with 7 M urea, about 50% of the meprin α subunits were solubilized, while no rat β subunits became soluble (Fig 16, bottom panel). This indicates that rat and mouse meprins have a similar oligomeric organization; in rats, as in mice, α_2 homodimers exist in addition to αβ heterodimers, and a fraction of rat meprin α subunits is not covalently attached to β subunits.

The data presented clearly show that the membrane association of α_2 dimers depends on non-covalent interactions with other membrane proteins. These other membrane proteins are most likely αβ heterodimers, because (i) the native form of meprin *in vitro* is a tetramer, and (ii) in the COS-1 cell expression system the presence of β subunits was sufficient to anchor α subunits to the cell membrane. To demonstrate the proposed interaction of α_2 dimers with αβ heterodimers, experiments were designed to determine whether α_2 dimers coprecipitate when β subunits are precipitated with β subunit-specific antibodies and protein A-sepharose. All attempts to precipitate β using the antibody to the β subunit protease domain failed, presumably because the antibody did not recognize the native conformation of β. To test whether α_2 homodimers interact with αβ heterodimers in the presence of detergents, meprins were solubilized from brush border membranes using Triton-X 100, and the β subunits were precipitated by incubation with the antibody to the β subunit COOH-terminal peptide. The resulting precipitate and supernatant fractions were analyzed by Western blotting for α and β subunit content (Fig. 17). When probed with the antibody to β, immunoreactive material was found in the precipitate, but not in the super

Fig. 17. Immunoprecipitation of meprin β subunits from ICR mouse kidney brush border membranes

Brush border membrane proteins (300 μg) were solubilized by incubation with Triton-X 100 and divided in two portions. One half of the sample was subjected to immunoprecipitation with the polyclonal antibody to the β subunit COOH-terminal peptide and protein A sepharose beads as described in "Experimental Procedures"; the other half was immunoprecipitated using preimmune serum. The precipitate and supernatant fractions were analyzed by immunoblotting. Lanes 1 to 3 were probed with a monoclonal antibody to the meprin α subunit; lanes 4 to 6 were probed with a polyclonal antibody to the β subunit COOH-terminal peptide. Lanes 1 and 4 contained precipitate samples from incubation with preimmun serum. Lanes 2 and 5 contained samples that precipitated with the β subunit-specific antibody. Lanes 3 and 6 contained the supernatant fraction from the immunoprecipitation with the β subunit-specific antibody. In each lane, one tenth of the total precipitate or supernatant fraction was added to the gels; thus, the amount of sample in each lane corresponded to the precipitate or supernatant derived from 15 μg brush border membrane protein.


alpha
beta
preimmun
precipitate
supernatant
preimmun
precipitate
supernatant
180
116
84
58
48.5
β
α
IgG
1
2
3
4
5
6

natant, indicating that the antibody had precipitated the β subunits quantitatively (Fig. 17, lanes 5 and 6). In contrast, when probed with a monoclonal antibody to the α subunit, most immunoreactive material was found in the supernatant, indicating that only a fraction of α subunits was bound to β subunits. Thus, in the presence of detergent α_2 dimers do not interact with αβ heterodimers.

3.4. Proteolytic Processing of the Meprin Subunits

3.4.1. NH_2-terminal processing of meprin subunits in mouse kidneys.- A hydropathy analysis of the mouse meprin subunits identified a NH_2-terminal hydrophobic signal sequence in the meprin subunits. Between the signal sequence and the protease domain is a short prosequence (42 amino acids for β, 44 amino acids for α). NH_2-terminal sequencing of papain-purified meprin A indicated that the prosequence of α is not present on the purified form of the enzyme, however, it was not known whether papain treatment removes the prosequence from the NH_2-terminus. To determine whether the hydrophobic NH_2-terminal sequence and/or the following prosequence is present on the mature meprin subunits, detergent-solubilized meprin was purified from C3H/He, ICR, and C57BL/6 mice for NH2-terminal sequence analysis.

Since the enzyme had been purified only in proteolyzed form after trypsin or papain treatment, and the detergent-solubilized forms showed significantly different behavior on purification columns, it was necessary to develop a new purification procedure. First, various detergents were analyzed for their effectiveness in solubilizing meprin activity and their compatibility with the meprin activity assay. It was found that meprin subunits were best solubilized by treatment with 100 mM octylglucoside. At this concentration about 82% of meprin activity

was released from brush border membranes, while only 40% of the total protein was solubilized, resulting in a two-fold purification (Table 4). Raising the octylglucoside concentration above 100 mM increased the solubility of total protein, but not of meprin activity. The octylglucoside-extracted brush border membranes were subjected to affinity chromatography on concanavalin A-sepharose, a lectin to which meprin subunits were known to bind with high affinity (Gorbea *et al.*, 1991). As expected, meprin subunits bound with high affinity to the column, and a portion of the meprin activity could be eluted with buffer containing 1 M α-methylmannoside (Fig. 18). The eluted fractions had a specific activity that was about 110-fold higher than the homogenate (Table 5), and only one major contaminating protein with a molecular mass of approximately 25 kDa was present (Fig. 18, bottom panel). Fractions containing meprin subunits were separated by SDS-PAGE, transferred to PVDF membranes, and the individual subunits were excised for direct NH_2-terminal sequencing.

The NH_2-terminus of the β subunit from all three mouse strains was found to start at Leu-21 (Table 6). Thus, the first 20 amino acid residues (Met-1 to Gly-20) correspond to a transient NH_2-terminal signal peptide, and there is no difference in processing between β subunits in the β_2 homodimers of C3H/He mice and the αβ heterodimers of C57BL/6 and ICR mice. Leu-21 was also the NH_2-terminus of papain-solubilized preparations of the β subunit (Kounnas *et al.*, 1991). When the ß subunit was treated with trypsin, the new NH_2-terminus became Asn-63 which is the beginning of the protease domain (Jiang *et al.*, 1992). Trypsin also activated meprin B against azocasein, indicating that removal of the 42 amino acid segment (Leu-21 to Lys-62) is critical for the proteolytic activity of the β subunit against large substrates (Kounnas *et al.*, 1991). The NH_2-terminal

Table 4

Solubilization of meprin activity from brush border membranes by β-D-octylglucopyranoside (OG)

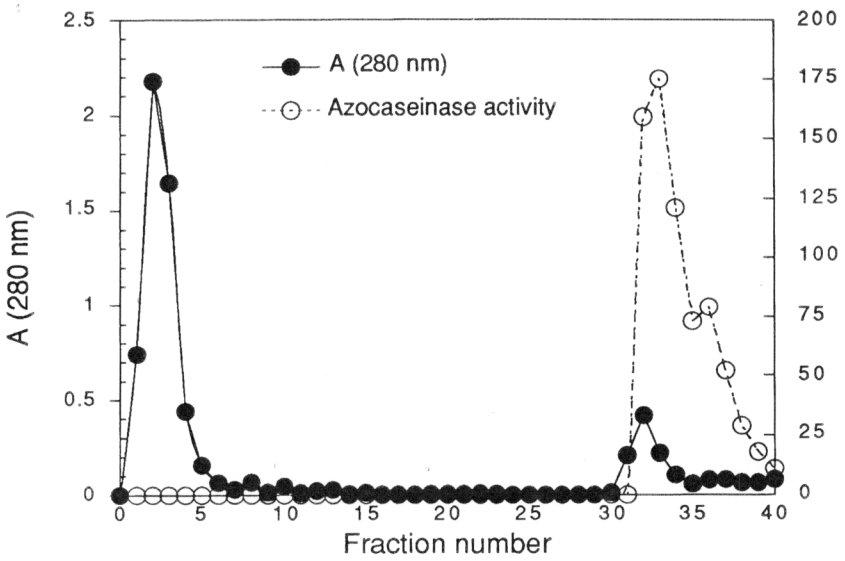
concentration of OG (mM)	% protein solubilized	% meprin activity solubilized
17.5	14.4	22.7
25.0	29.1	59.0
35.0	31.4	63.2
70.0	35.4	81.3
100.0	39.1	81.6

ICR mouse renal brush border membrane fractions (4 mg protein/ml) were incubated for 2 h at 4°C with the indicated concentrations of the detergent β-D-octylglucopyranoside, centrifuged for 1 h at 100,000 x g at 4°C, and the protein content and meprin acitivy were measured in the resulting precipitate and supernatant fractions. Meprin activity was measured using azocasein as substrate. One unit is defined as increase in absorbance at 340 nm of 0.001/min, which is equivalent to the solubilization of 1.1 μg of azocasein per min.

Fig. 18. Partial purification of mouse meprin subunits by concanavalin A affinity chromatography

Top, Concanavalin A chromatography profile of brush border membrane proteins from ICR mouse kidneys. Meprin subunits were solubilized from brush border membrane fractions by extraction with β-D-octylglucopyranoside, subjected to concanavalin A-sepharose affinity chromatography as described in "Experimental Procedures", and the resulting fractions (1 ml) were assayed for protein content and azocasein-degrading activity. After 30 fractions were collected, 1 M α-methylmannoside was added to the elution buffer.

Bottom, SDS-PAGE of detergent-solubilized purified meprin samples after concanavalin A affinity chromatography. Ten μg protein was subjected to SDS-PAGE (10 % gel) and stained with Coomassie-Brilliant Blue R-250. The positions of the molecular mass markers are indicated on the left; the positions of the meprin α and β subunits, determined by immunoblotting, are shown on the right.


A (280 nm)
Azocaseinase activity
A (280 nm)
Fraction number
2.5
2
1.5
1
0.5
0
200
175
150
125
100
75
50
25
0
0
5
10
15
20
25
30
35
40

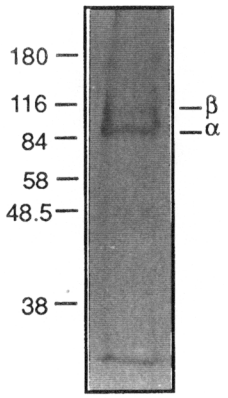

180
116
84
58
48.5
38
β
α

Table 5

Purification of detergent-solubilized mouse meprin oligomers from ICR mouse kidney

Sample	Proteinase Activity	Protein	Specific Activity	Yield	-fold purification
	total units	*mg*	*units/mg*	%	
Homogenate	12,363	972	13	100	1
Solubilized brush border membranes	4,990	18	285	40	22
Concanavalin A	1,132	0.8	1415	9	109

Mouse kidneys (5g) were homogenized in 9 volumes of 2 mM Tris-HCl buffer, pH 7.0, containing 10 mM mannitol. Brush border membrane enriched fractions were prepared, the membrane proteins were extracted with β-D-octylglucopyranoside, and subjected to Concanavalin A affinity chromatography as described in "Experimental Procedures". Proteinase activity was assayed with azocasein.

Table 6

NH_2-terminal sequences of detergent-solubilized renal meprin subunits

Mouse α subunit	
subunit purified from ICR mice	NAMRDPSSR
subunit purified from C57BL/6 mice	NAMRDPSSR
deduced from cDNA sequence (residues 78-86)	NAMRDPSSR
Rat α subunit	
subunit purified from Sprague-Dawley rats	XXLXDPSXXXKPPIPYILA
deduced from cDNA sequence (residues 67-85)	NALRDPSSRWKPPIPYILA
Mouse β subunit	
subunit purified from ICR mice	XPAPEKFVKD
subunit purified from C57BL/6 mice	LPAPEKFVKD
subunit purified from C3H/He mice	XXXPEKFVKDI
deduced from cDNA sequence (residues 21-31)	LPAPEKFVKDI

Detergent-solubilized meprin subunits from mouse and rat kidney were purified as described in "Experimental Procedures". The proteins were applied to SDS-PAGE (10 % gels) under reducing conditions, transferred to PVDF membranes, and individual bands were excised for direct NH_2-terminal protein sequencing. The mouse α subunit cDNA sequence was determined by Jiang *et al.* (1991), the rat α subunit cDNA sequence was determined by Corbeil *et al.* (1993). The mouse β subunit cDNA sequence is shown in Fig. 2.

amino acid of the mouse α subunit was found to be Asn-78, which is the beginning of the protease domain (Table 6). This was also the NH_2-terminus of papain- or trypsin-purified meprin α subunits. Thus, in contrast to the β subunit the α subunit has the prosequence removed *in vivo*. NH_2-terminal sequencing was also performed on partially purified rat meprin subunits (Table 6). The NH_2-terminal amino acid of the rat α subunit was Asn-67, which is the NH_2-terminal amino acid of the rat α protease domain. Thus, the NH_2-termini of the α subunits are processed similarly in mouse and in rat kidneys. No sequence was obtained for the rat β subunits.

3.4.2 NH_2-terminal processing of the meprin α subunit in transfected COS-1 cells.- The meprin α subunit secreted from transfected COS-1 cells was analyzed for azocaseinase activity before and after trypsin treatment (Table 7). No azocaseinase activity was detected in the control media (media harvested from cells transfected with vector DNA only) before or after trypsin treatment (data not shown). Untreated media, containing the secreted recombinant meprin α subunits, also contained no detectable activity; however, after trypsin treatment of the media samples, the specific activity of α subunits was comparable to the activity of purified kidney meprin A. In addition, immunoblotting of the recombinant secreted meprin α subunit before and after trypsin treatment showed that the α subunit molecular weight decreased from 95 to 90 kDa upon trypsin treatment (Fig. 19), which is consistent with the removal of the prosequence by trypsin. These data indicate that, in contrast to the mouse kidneys, COS-1 cells do not remove the prosequence from α. It is not known why processing of the NH_2-terminus of α differs in COS-1 cells and mouse kidney cells, but a working hy

Table 7

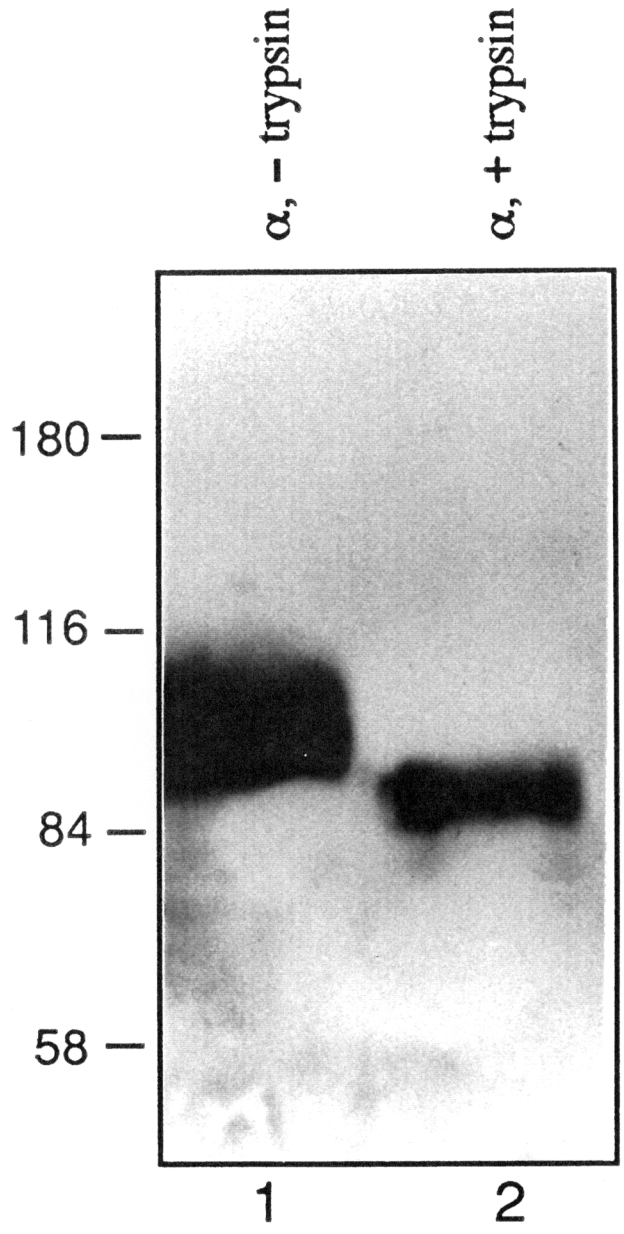
Azocaseinase activity of recombinant meprin α subunits secreted from transfected COS-1 cells and papain-purified meprin-A before and after trypsin treatment

Treatment	Azocaseinase activity	
	recombinant α subunit (units/mg meprin)	meprin-A (units/mg)
untreated	n.d. (<200)	5,000
trypsin treated	10,900	9,000

Papain-purified meprin-A was prepared as described in "Experimental Procedures". The activity of the recombinant meprin α subunit was determined in the tissue culture medium taken from cells transfected with meprin α subunit cDNA. The amount of meprin protein in the culture medium was determined by densitometric comparison of the Western blotting signals with signals from known amounts of meprin A. Azocasein activity was measured over a time course of 1 h as described in "Experimental Procedures" using 20 µl medium sample (corresponding to 15% of the total medium obtained from one 100 mm plate). Abbreviations: n.d., not detectable.

Fig. 19. Immunoblot analysis of secreted meprin α subunits before and after activation with trypsin

COS-1 cells were transfected with cDNA encoding the wild-type meprin α subunit. The tissue culture medium from the transfected cells was harvested, concentrated, and a portion was treated with trypsin as described in "Experimental Procedures". The untreated sample (lane 1) and the trypsin-treated sample (lane 2) were subjected to SDS-PAGE (10% gel) under reducing conditions, followed by immunoblot analysis using anti meprin α subunit antibodies. The protein in each lane corresponded to 10% of the medium obtained from one 100 mm dish. The positions of molecular mass markers (in kDa) are shown on the left.


α, - trypsin
α, + trypsin
180
116
84
58
1
2

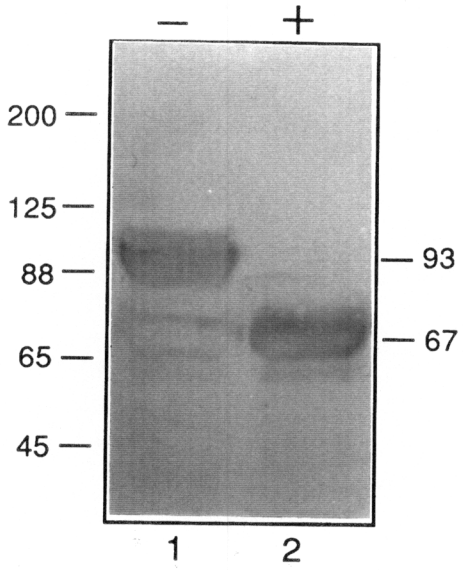
pothesis is that the proprotein processing enzyme for α is not expressed in COS-1 cells.

3.4.3 COOH-terminal processing of meprin subunits in mouse kidneys.- Data presented in the previous sections indicated clearly that the mouse β subunit is a transmembrane protein, anchored to the membrane through its COOH-terminal region. By contrast, α is not a transmembrane protein, although the cDNA predicts that α has a transmembrane helix near the COOH-terminus. The experiments described in this section were performed to determine whether α undergoes COOH-terminal processing in mouse kidneys *in vivo*.

In order to determine the protein molecular weights of mouse kidney meprin subunits, brush border membrane preparations from ICR mouse kidneys were chemically deglycosylated. The deglycosylated proteins were separated by SDS-PAGE, and the meprin subunits were visualized by Western Blotting using two different antibodies (Fig. 20). Both subunits were estimated to contain about 30% carbohydrate. The deglycosylated α subunit had a molecular mass of about 67 kDa, β was approximately 79 kDa. These molecular mass estimates were compared with molecular masses calculated from the cDNA-derived amino acid sequences (Table 8). Alpha and β differ in NH_2-terminal processing (removal of the prosequence in α, retained prosequence in β), making Asn-78 the first residue of the α subunit, and Leu-21 the NH_2-terminal amino acid of the β subunit (see section 3.4.1). When this difference is taken into account, the cDNAs of α and β predict nearly identical molecular weights for the mature proteins, if it is assumed that no additional post-translational modification occurs (77,200 and 77,300 for α and β, respectively). The apparent molecular mass of the deg-

Fig. 20. Immunoblot analysis of chemically deglycosylated ICR mouse brush border membrane proteins

Brush border membrane proteins (500 μg) were chemically deglycosylated using anhydrous trifluoromethanesulfonic acid (TFMS) as described in "Experimental Procedures", and subjected to SDS-PAGE and immunoblotting. Fifty μg of protein were applied to each lane. Lane 2 in the top gel and lane 1 in the bottom gel contained TFMS-treated brush border membrane proteins (+), lane 1 in the top gel and lane 2 in the bottom gel contained untreated brush border membrane proteins (-). The top gel was probed with an antibody to the deglycosylated meprin α subunit, the bottom gel was probed with antiserum P11, a peptide antibody which recognized both meprin subunits. The positions of the molecular mass markers (in kDa) are indicated on the left; molecular mass estimates for the meprin subunits are indicated on the right.


−
+
200
125
88
65
45
93
67
1
2

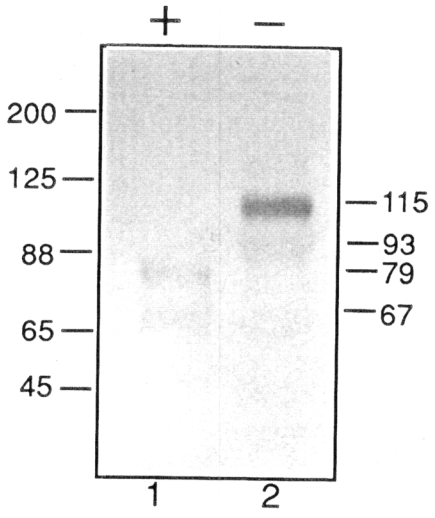

+
−
200
125
88
65
45
115
93
79
67
1
2

Table 8

Comparison of experimentally determined and cDNA inferred molecular weights (MW) of mouse meprin subunits

Meprin subunit	Experimentally determined MW	MW predicted from cDNA(amino acids)
NH_2-terminally processed subunits found in mouse kidney membranes		
α subunit; untreated	93,000	-
α subunit; chem. deglycosylation	67,000	77,200 (78-760)
β subunit; untreated	115,000	-
β subunit; chem. deglycosylation	79,000	77,300 (21-704)
In vitro translation products		
α subunit; - microsomes	83,000	85,700 (1-760)
α subunit; + microsomes	108,000	-
β subunit; - microsomes	76,000	79,600 (1-704)
β subunit; + microsomes	98,000	-

The molecular weights of the *in vitro* translated mouse meprin subunits and the mouse kidney subunits before and after deglycosylation were determined by SDS-PAGE (Figs. 16 and 17). Each experimental value is the average of at least two determinations. Molecular weights were calculated from the cDNA information using the program McMolly (Soft Gene, Berlin). The NH_2-termini of the mature subunits were determined as described in Table 6.

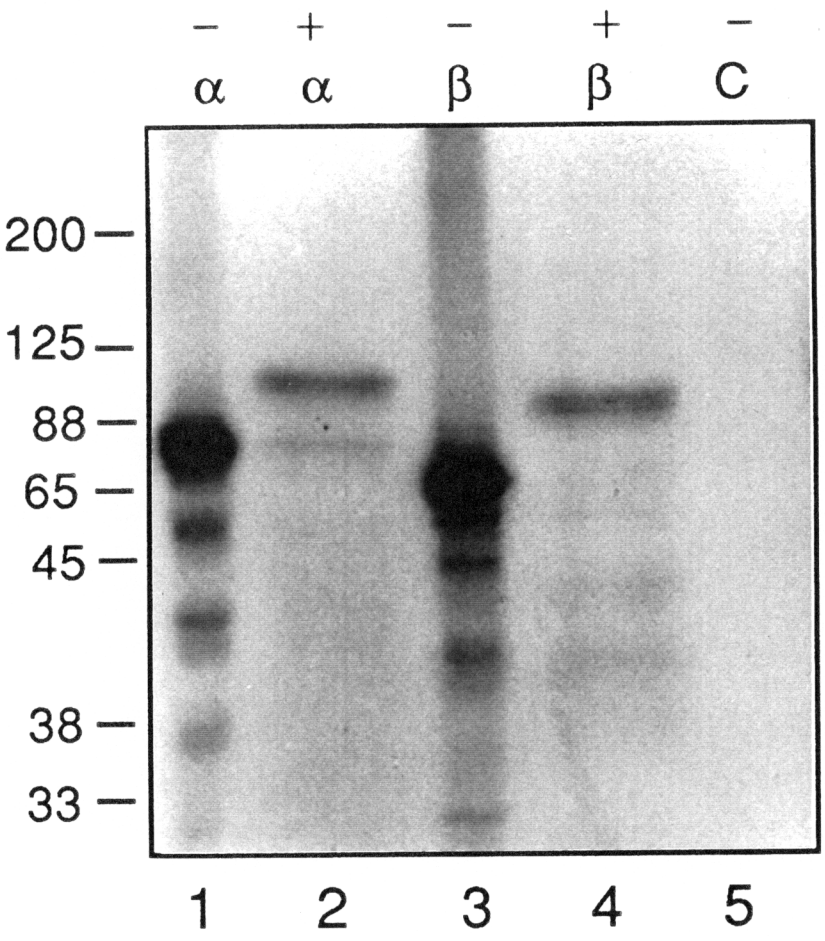
lycosylated β subunit was in agreement with this predicted molecular mass; however, the apparent molecular mass of the deglycosylated α subunit was much smaller than predicted.

The mobility on SDS-gels of *in vitro* translated meprin subunits was also analyzed (Fig. 21; Table 8). Membrane proteins or secreted proteins synthesized in a reticulocyte lysate system in the absence of microsomal membranes are primary translation products devoid of the post-translational modifications that they undergo during transit through the secretory pathway. In the presence of microsomal membranes, modifications that are localized in the ER, e.g., signal peptide cleavage and N-linked core glycosylation, occur. In the absence of microsomal membranes α was synthesized mainly as a protein of 83 kDa (Fig. 21, lane 1), while β was approximately 76 kDa (Fig 21, lane 3). A few smaller proteins were also detected, these are most likely translation products that were initiated at internal Met codons, a phenomenon that is commonly observed in *in vitro* translation systems. Both estimates agreed with those calculated for the primary translation products from the cDNA (85.7 kDa for α, 79.6 kDa for β). In the presence of microsomes, the masses of both subunits increased due to the addition of N-linked core carbohydrate chains.

Since the *in vitro* translated meprin subunits showed no abnormal mobilities on SDS gels, the fast migration of the deglycosylated α subunit of mouse kidney membranes indicates that this form is a smaller, proteolytically processed form, rather than an abnormally fast migrating form. From the molecular mass determinations it was estimated that in the mature mouse kidney α subunit a fragment of about 10 kDa is removed from the COOH-terminus. This estimate indicates that processing of α occurs in or near the inserted domain of α (Fig. 5,

Fig. 21. *In vitro* transcription/translation of meprin subunit cDNAs in the absence and presence of microsomal membranes

pcDNAI/Amp plasminds containing full-length meprin subunit cDNAs were *in vitro* transcribed/translated using the TNT T7 Coupled Reticulocyte system (Promega) in the presence (indicated as +) or absence (indicated as -) of canine microsomal membranes. One μg of plasmid was used for each 50 μl reaction. The [^{35}S] methionine-labeled proteins (5 μl) were subjected to SDS-PAGE, followed by fluorography. Lanes 1 and 2 contained samples translated using meprin α cDNA, lanes 3 and 4 contained meprin β cDNA translation products. The control sample (lane 5) was transcribed/translated in the absence of plasmid DNA. The positions of molecular mass markers (in kDa) are shown on the left.


–
+
–
+
–
α
α
β
β
C
200
125
88
65
45
38
33
1
2
3
4
5

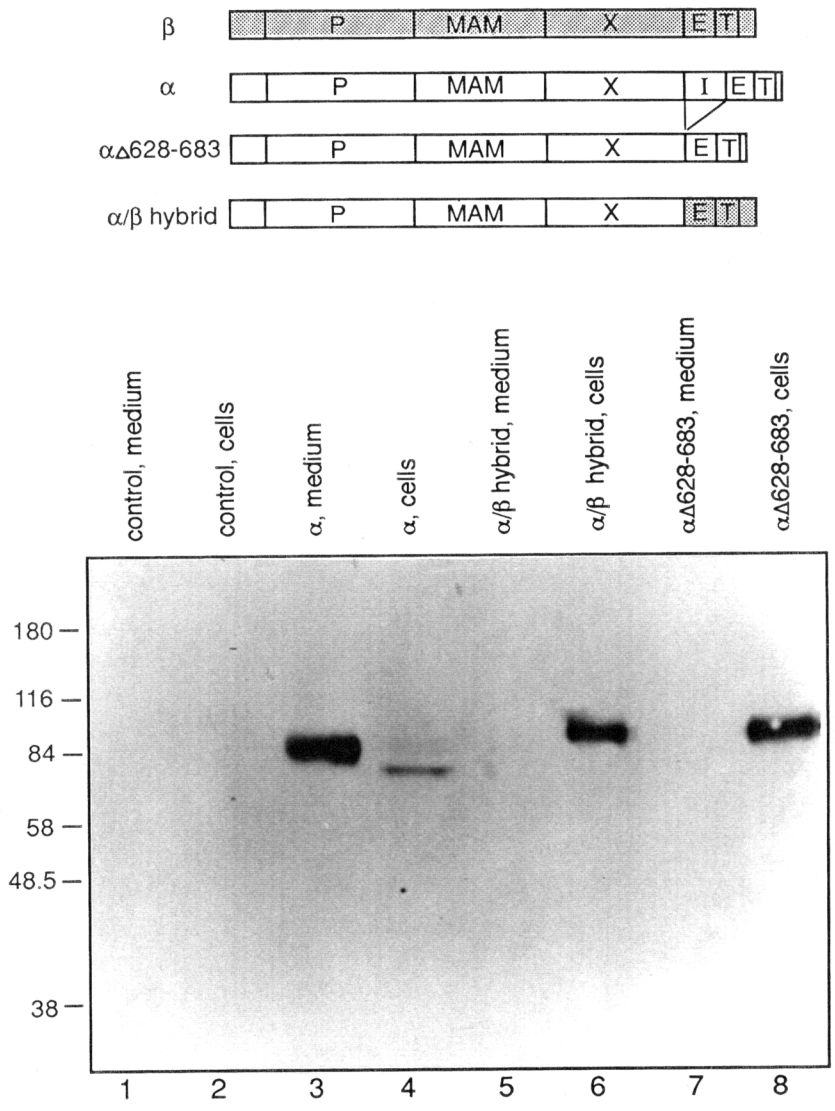
section 3.1.2), which is the only region where the α and β meprin subunits are completely different. Further supprt for the conclusion that the α subunit is cleaved in the inserted domain of α comes from immunological evidence indicating that the α subunit EGF-like domain is absent from the mature subunit (Marchand *et al.*, 1994).

3.4.4 COOH-terminal processing of the meprin α subunit in transfected COS-1 cells.- Because meprin α subunits undergo COOH-terminal processing in transfected COS-1 cells, just as in mouse kidney cells, this expression system is a potentially useful system to identify the proteolytic processing site in the α subunit, as well as the identity and subcellular location of the processing enzyme. To obtain more information about the COOH-terminal proteolytic processing site in COS-1 cells, recombinant mutant meprin subunits were constructed and expressed in COS-1 cells (Fig. 22). One of the constructs tested was a chimeric meprin subunit, in which the α subunit region COOH-terminal of the unknown domain (residues 624-760 in α) was replaced with the corresponding COOH-terminal portions of the β subunit (residues 613-704 in β) (construct α/β, Fig. 22, top panel); this construct was designed to determine whether the COOH-terminal 137 residues of the primary translation product of α are required for processing. A second construct, a deletion mutant of the α subunit, in which the inserted domain (residues 628-683 of α) was deleted by site-directed mutagenesis (construct αΔ628-683; Fig. 22, top panel), tested more specifically the role of the inserted domain in processing. Analysis of the culture media and the cell precipitate fractions by immunoblotting showed that, in contrast to the wild-type α subunit, none of the mutant meprin subunits were secreted into the culture medium,

Fig. 22. Cell-associated expression of a meprin α subunit deletion mutant and a meprin α/β hybrid subunit

Top, schematic diagrams of the wild-type meprin subunits, the α subunit deletion mutant, and the chimeric meprin subunit. The individual domains are indicated by the abbreviations: P, protease domain; MAM, adhesion domain; X, unknown domain; I, inserted domain; E, epidermal growth factor-like domain; T, transmembrane domain. The α subunit domains are shown in white, shaded areas represent β subunit sequence.

Bottom, expression of the wild-type and mutant α subunits in COS-1 cells. Ten μg of the expression vector pcDNAI/Amp harboring the wild-type or mutant meprin α subunit cDNA were transfected into COS-1 cells grown on 100 mm plates. Concentrated tissue culture media (indicated as "medium") and cell membrane fractions (indicated as "cells") from the transfected cells were prepared as described in "Experimental Procedures". Samples from cells transfected with either vector DNA (lanes 1 and 2), wild-type meprin α subunit cDNA (lanes 3 and 4), α subunit deletion mutant cDNA (lanes 5 and 6), or α/β subunit hybrid cDNA (lanes 7 and 8) were subjected to SDS-PAGE (10% gel) under reducing conditions, followed by immunoblot analysis using anti meprin α subunit antibodies. Each lane contained 10% of the sample obtained from one 100 mm dish. The positions of molecular mass markers (in kDa) are shown on the left.


β
α
αΔ628-683
α/β hybrid
P
MAM
X
I
E
T
control, medium
control, cells
α, medium
α, cells
α/β hybrid, medium
α/β hybrid, cells
αΔ628-683, medium
αΔ628-683, cells
180
116
84
58
48.5
38
1
2
3
4
5
6
7
8

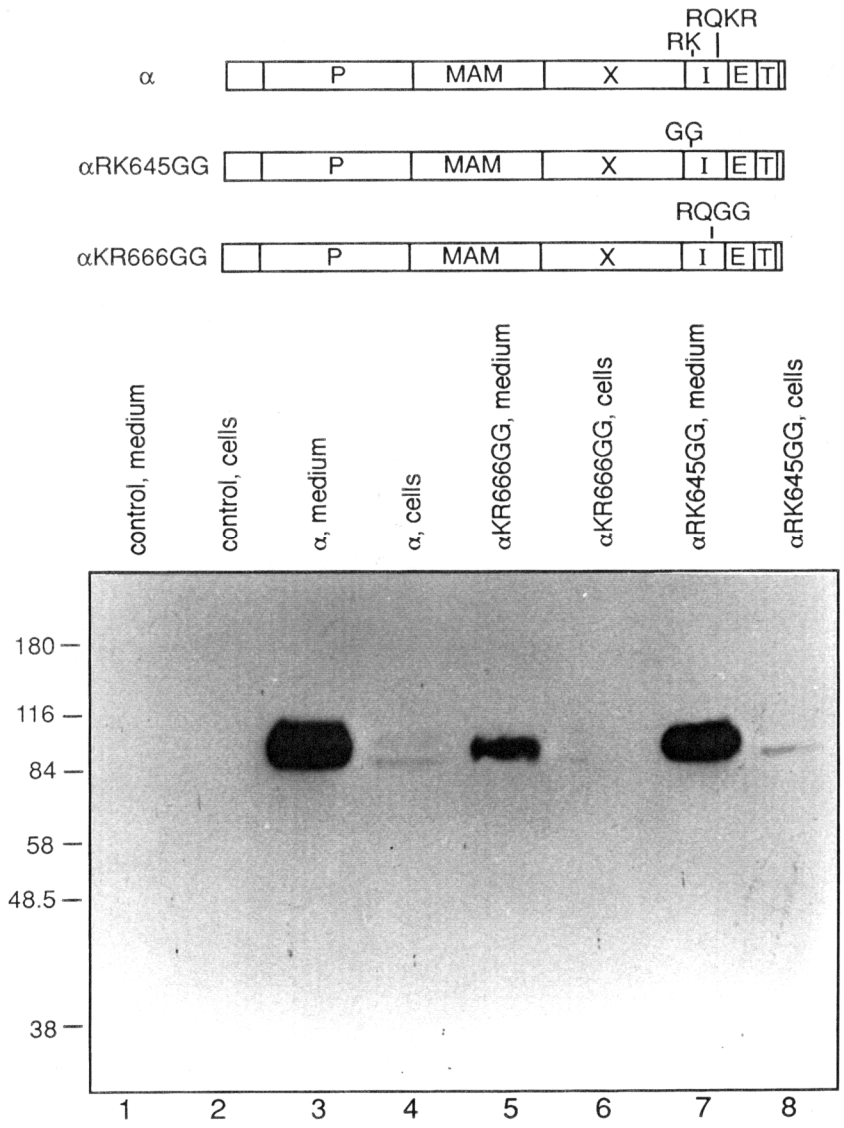
they were found exclusively in the cell membrane fraction (Fig. 22, bottom panel). In addition, the molecular masses of the cell-associated mutant subunits were slightly larger than the molecular masses of the secreted wild-type α subunit (approximately 105 kDa for the α/β chimeric subunit, 102 for the αΔ628-683 mutant, compared to 95 kDa for the wild-type secreted α subunit). The larger molecular sizes of the mutant subunits compared to the wild-type subunit provide further evidence that these mutants are not processed at the COOH-terminus, but retain the EGF-like and transmembrane domain. It was concluded that the inserted domain in α is essential for processing.

One possible explanation for the finding that the inserted domain of α is essential for COOH-terminal processing is that the inserted domain contains the cleavage site that is recognized by the processing enzyme. Thus, the amino acid sequence of the inserted domain was searched for putative processing sites. It is well established that post-translational processing of proteins frequently occurs at dibasic sites; this reflects the requirement of many processing proteases for basic residues in the P1 and P2 positions of the substrates (Van de Ven et al., 1993). The inserted domain of α contains two such dibasic sites, amino acids R645/K646 and K666/R667. Amino acids K666/R667 are also part of the consensus sequence for cleavage by furin-like processing enzymes (R-X-K/R-R). To test whether the furin-like consensus sequence or the other dibasic site are the cleavage site in α, the dibasic sites were replaced by glycine residues (Fig. 23, top panel) by site-directed mutagenesis, and the resulting mutant proteins were expressed in COS-1 cells and analyzed for secretion and cellular localization (Fig. 23, bottom panel). Eliminating either of the two dibasic sites did not prevent

Fig. 23. Expression of mutant meprin α subunits lacking dibasic sites

Top, schematic diagrams of the wild-type and mutant meprin subunits. The α subunit domains are indicated by the abbreviations: P, protease domain; MAM, adhesion domain; X, unknown domain; I, inserted domain; E, epidermal growth factor-like domain; T, transmembrane domain. The wild-type α subunit contains two dibasic sites within the inserted domain at positions 666/667 and 645/646. In the mutant α subunits one of the dibasic sites is replaced with two glycine residues as indicated.

Bottom, expression of the wild-type and mutant α subunits in COS-1 cells. Ten μg of the expression vector pcDNAI/Amp harboring the wild-type or mutant meprin α subunit cDNA were transfected into COS-1 cells grown on 100 mm plates. Concentrated tissue culture media (indicated as "medium") and cell membrane fractions (indicated as "cells") from the transfected cells were prepared as described in "Experimental Procedures". Samples from cells transfected with either vector DNA (lanes 1 and 2), wild-type meprin α subunit cDNA (lanes 3 and 4), αKR666GG DNA (lanes 5 and 6), or αRK645GG DNA (lanes 7 and 8) were subjected to SDS-PAGE (10% gel) under reducing conditions, followed by immunoblot analysis using anti meprin α subunit antibodies. Each lane contained 10% of the sample obtained from one 100 mm dish. The positions of molecular mass markers (in kDa) are shown on the left.


RQKR
RK
α
P
MAM
X
I
E
T
GG
αRK645GG
RQGG
αKR666GG
control, medium
control, cells
α, medium
α, cells
αKR666GG, medium
αKR666GG, cells
αRK645GG, medium
αRK645GG, cells
180
116
84
58
48.5
38
1
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4
5
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7
8

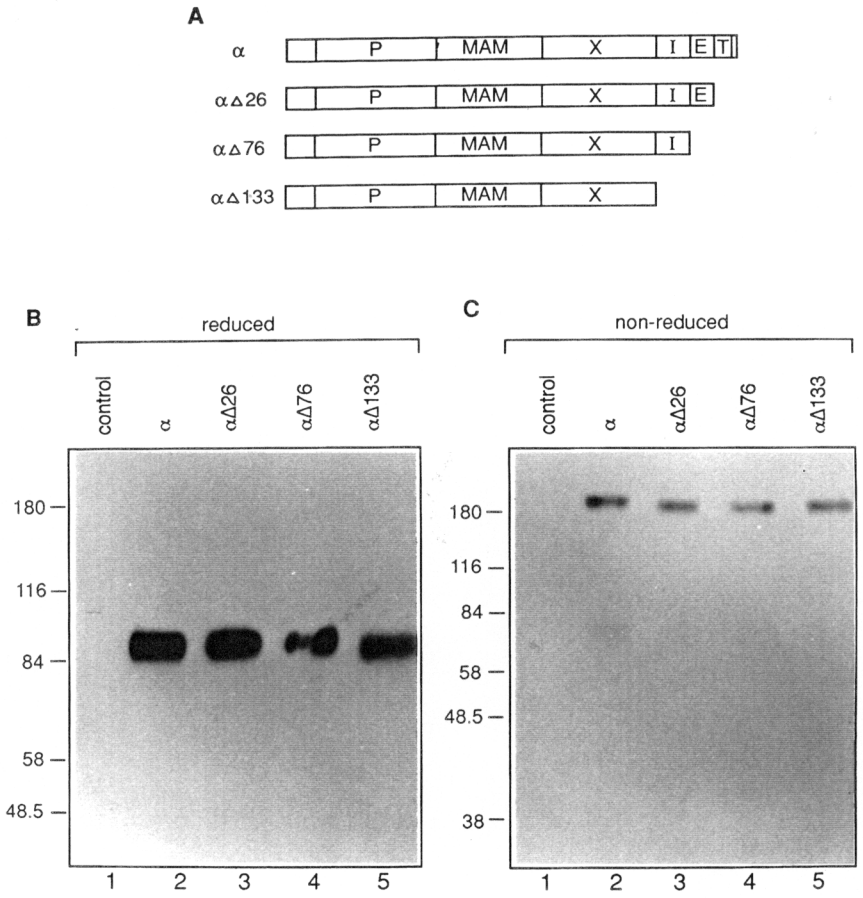
secretion of the meprin α subunit into the culture medium, indicating that none of these sites is essential for processing.

It is not known why meprin α subunits are initially synthesized with a COOH-terminal extension. One hypothesis that was tested here is that the COOH-terminal extension may be important during early stages of meprin biosynthesis. To determine whether the transmembrane domain or other portions of the COOH-terminal extension in α are initially important during meprin biosynthesis for either dimerization or transport of meprins through the secretory pathway, COOH-terminal deletions were constructed and expressed in COS-1 cells (Fig. 24). Deletion of up to 133 amino acids from the COOH-terminus of α did not have any detectable effect on the ability of the meprin α subunits to dimerize or to be secreted (Fig. 24, bottom panel). Thus, the COOH-terminal extension of α is not essential for dimerization of α subunits or intracellular transport.

Fig. 24. Expression of COOH-terminal deletion mutants of the meprin α subunit in COS-1 cells

Panel A, schematic diagrams of the wild-type and mutant meprin subunits. The individual domains are indicated by the abbreviations: P, protease domain; MAM, adhesion domain; X, unknown domain; I, inserted domain; E, epidermal growth factor-like domain; T, transmembrane domain.

Panels B and C , immunoblot analysis of the meprin α subunit COOH-terminal deletion mutants. COS-1 cells were transfected with pcDNAI/Amp vector DNA (lane 1), cDNA encoding wild-type meprin α subunit (lane 2), or cDNA encoding α subunit deletion mutants (lanes 3 to 5). The tissue culture media from the transfected cells were harvested, concentrated, and subjected to SDS-PAGE (9% gel) under reducing (panel B) or non-reducing conditions (panel C), followed by immunoblot analysis using anti meprin α subunit antibodies. Each lane contained 10% of the medium obtained from one 100 mm dish. The positions of molecular mass markers (in kDa) are shown on the left.


A
α
P
MAM
X
I
E
T
αΔ26
αΔ76
αΔ133
B
reduced
control
α
αΔ26
αΔ76
αΔ133
180
116
84
58
48.5
1
2
3
4
5
C
non-reduced
control
α
αΔ26
αΔ76
αΔ133
180
116
84
58
48.5
38
1
2
3
4
5

IV

DISCUSSION

The meprin β subunit and the astacin family.- One of the first goals of this thesis research was to determine the complete primary sequence of the mouse meprin β subunit and thereby to obtain information about the structure of meprin β subunits and their relationship to meprin α subunits and to other members of the "astacin family" of metalloendopeptidases. The primary sequence of β was obtained by generating meprin β subunit cDNA by reverse transcription-polymerase chain reactions, and cloning and sequencing of the PCR products. The ß subunit contains many of the features of other members of the astacin family. Like the other members of this family the β subunit contains a catalytic domain that is composed of about 200 amino acids and is approximately 30% identical to the crayfish protease astacin. The catalytic domain of β contains the zinc binding motif HEXXH, which is present in most zinc metalloproteases known to date; in addition it contains the 24 amino acid consensus sequence that is unique to this family (Jiang and Bond, 1992). The astacin family is an old one in that it is predicted that the protease domain existed before vertebrates and invertebrates diverged (Dumermuth *et al.*, 1991). Twenty members of this family have been identified to date by protein or DNA sequence analyses. Members of the astacin family include proteins from nematode, hydra, frog (Maéno *et al.*, 1993), fish (Yasamasu *et al.*, 1992), quail (Elaroussi and DeLuca, 1994), and sea urchin (Lepage *et al.*, 1992; Reynolds *et al.*, 1992; Hwang *et al.*, 1994), the Drosophila developmental protein *tolloid*, (Shimell *et al.*, 1991), human bone morphogenetic protein-1 (Wozney *et al.*, 1988), and the subunits of mouse, rat, and human

meprins (Jiang and Bond, 1992). These metalloendoproteinases are expressed in developing as well as mature organisms and appear to have diverse functions, including digestion, processing of extracellular proteins, degradation of egg envelope, pattern formation, activation of growth factors, and cartilage and bone formation. All members are believed to have protease activity *in vivo*, but in most cases the physiological substrates are not known. Like most proteins of the astacin family the meprin β subunit is much larger than astacin and contains additional domains that may serve to regulate its function and/or location. The meprins are the only members of the family that contain COOH-terminal hydrophobic sequences that appear to be responsible for anchoring the protein to membranes. All other members of the family described thus far are secreted from cells.

The meprin α and β subunits share significant sequence homology throughout every region that is present in the mature proteins; there is less homology in the regions that are not present in the mature protein of one or both subunits, e.g., in the signal sequence, prosequence, COOH-terminal region. The high degree of homology between the mouse α and β subunits indicates that they were derived from a common ancestral gene that was duplicated and subsequently diverged to give rise to two distinct proteins. It is likely that the ancestral meprin subunit gene originated by the fusion of various modular elements encoding the individual domains found in meprin subunits, as has been proposed for the origin of multidomain proteins in general (Bork, 1992). Notably, the α subunit contains an inserted domain that is not present in β. It is possible that the DNA encoding this inserted domain is a separate module, possibly encoded by a single exon, that was either lost from the β subunit gene or that is spliced differently.

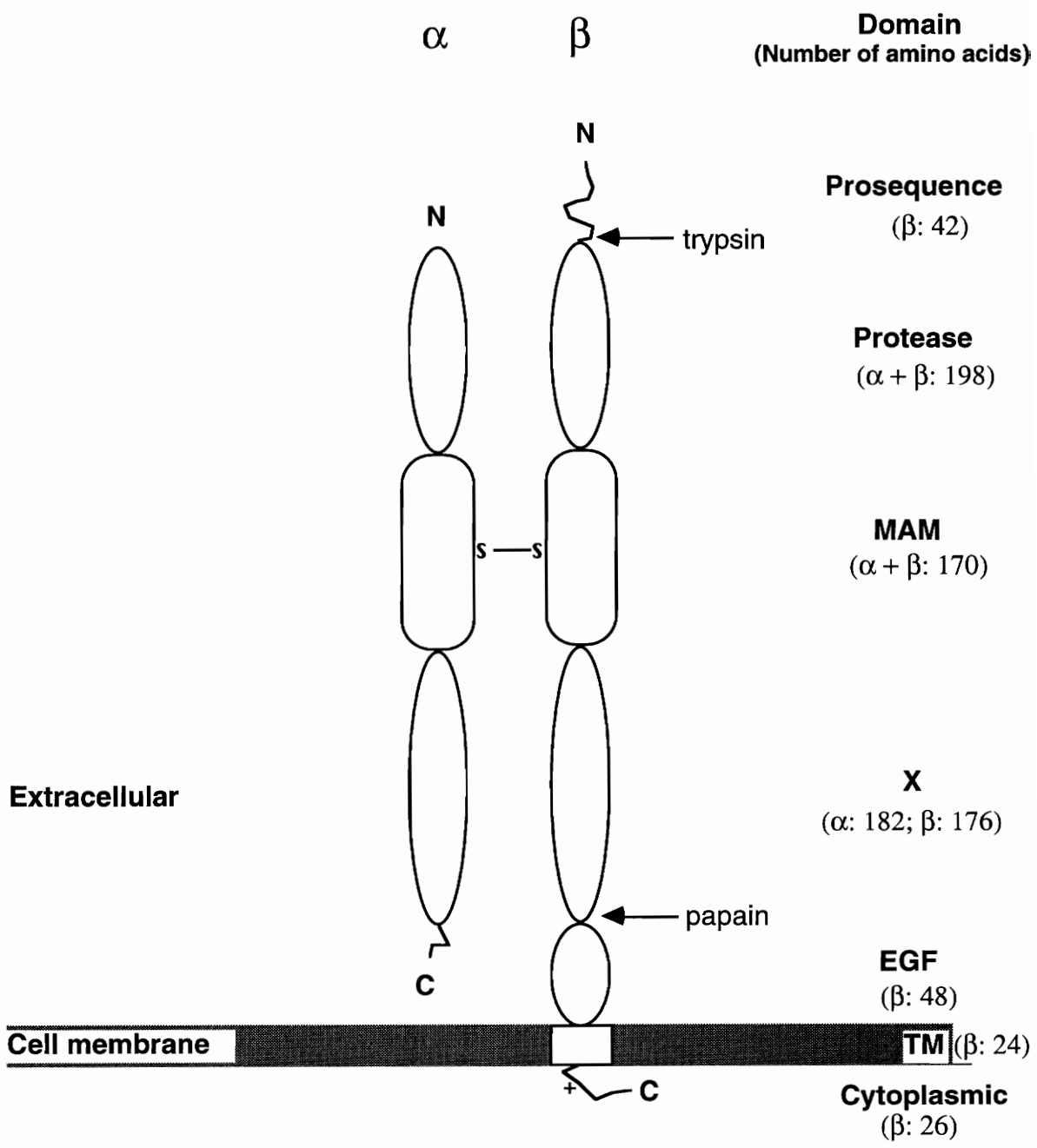
Today, the genes for the α and β subunits are on different chromosomes (Gorbea *et al.*, 1993; Jiang *et al.*, 1992), and the tissue-specific differences in the expression of α and β subunits indicate that the genes have evolved to incorporate different regulatory elements. Analysis of the genomic organization of the meprin genes will provide further information relating to the evolutionary relationship of α and β.

Structure of the meprin α and β subunit.- The cloning of the complete meprin β subunit and the determination of the *in vivo* proteolytic processing of meprin α and β subunits provides new information about the structure of the mature mouse kidney meprin subunits. Fig. 25 shows a schematic diagram of a meprin αβ heterodimer in mouse kidney membranes. Both mature meprin subunits contain three major domains: an astacin-like protease domain, an adhesion domain (MAM), and an additional domain of as yet unknown function. In both subunits the signal sequences are removed. The NH_2-termini of α and ß are shown to differ as determined experimentally by NH_2-terminal protein sequencing: for α the prosequence is removed, while ß retains the prosequence. The prosequence in β can be removed *in vitro* by treatment with trypsin-like proteases (Fig. 25, arrow; Kounnas *et al.*, 1991).

The mouse β subunit associates with cell membranes through the hydrophobic domain located near the COOH-terminus; this gives it the topology of a type I integral membrane protein (Singer *et al.*, 1987). On the extracellular side near the membrane, the β subunit has an EGF-like domain. The ß subunit contains a cluster of positively charged amino acids on the cytoplasmic side of the membrane, which may function as a stop-transfer sequence to anchor the

Fig. 25. Model of the αβ meprin dimer from ICR mouse kidney membranes

The proposed domain structure of the mature subunits is drawn schematically. The domains and the number of amino acids present in each domain are indicated on the right. The α subunit is located extracellularly, as is the bulk of the β subunit. The β subunit has a transmembrane anchor. The + on the COOH-terminal tail denotes a cluster of positively charged residues in the cytoplasmic domain of β. An intersubunit disulfide bond is predicted to be formed between cysteine residues in the MAM domains. The letters N and C indicate the NH_2- and COOH-termini of the subunits, respectively.The arrows point to the approximate positions of peptide bonds that are susceptible to proteolytic cleavage. Abbreviations: X, domain of unknown function; TM, transmembrane domain.


α
β
Domain
(Number of amino acids)
N
N
Prosequence
(β: 42)
trypsin
Protease
(α + β: 198)
S—S
MAM
(α + β: 170)
Extracellular
X
(α: 182; β: 176)
papain
C
EGF
(β: 48)
Cell membrane
TM
(β: 24)
+
C
Cytoplasmic
(β: 26)
Intracellular

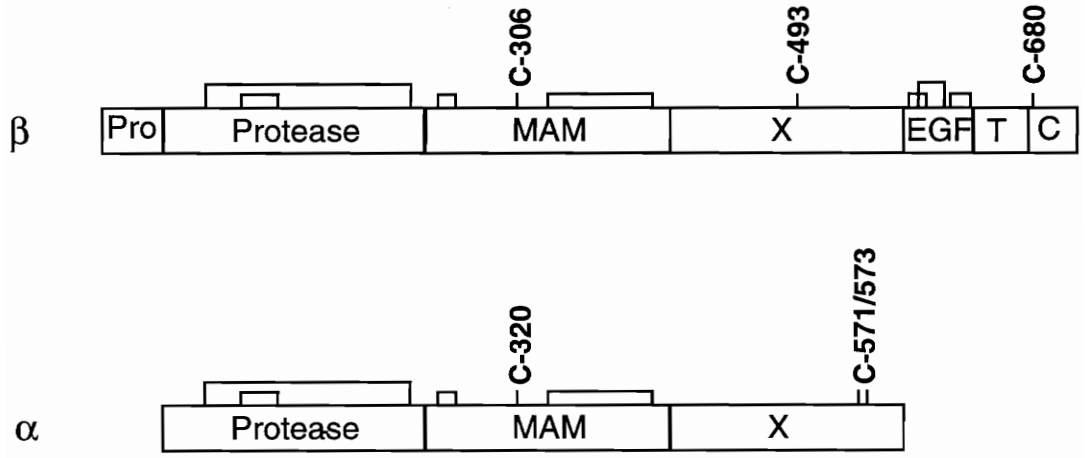
molecule at the cell surface (Mauxion *et al.*, 1989). In addition, the cytoplasmic domain contains two potential phosphorylation sites; one is a potential protein kinase C phosphorylation site at Thr-681 (in the consensus sequence TRR), the other is Thr-693 (in the consensus sequence RXXT), a potential site for calmodulin kinase II phosphorylation (Kennelly and Krebs, 1991). It remains to be established whether one or both of these sites are phosphorylated *in vivo*. The cytoplasmic domain of β has the potential to interact with intracellular proteins, and the positively charged residues and as well as phosphorylated residues could be important for such interactions. In contrast to β, the mature α subunit is proteolytically processed at the COOH-terminus and contains neither the COOH-terminal hydrophobic membrane-anchoring domain nor the EGF-like domain encoded by the message. This is supported by substantial experimental evidence: first, COS-1 cells, 293 cells, or MDCK cells transfected with full-length mouse, rat, or human meprin α cDNA secrete a soluble protein into the culture medium, indicating that, at least in these cell systems, α does not have a membrane anchor (this thesis; Johnson and Hersh, 1994; Grünberg *et al.*, 1993). Second, significant amounts of α subunits can be solubilized from mouse kidney membranes by reducing agents or urea, indicating that the mouse kidney form of α does not have an intrinsic membrane anchor. Third, molecular weight estimates of the polypeptide portion of mature mouse kidney α subunits indicate that at least 10 kDa are removed from the COOH-terminus. Fourth, an antibody raised to a peptide located in the EGF-like domain of α (residues 688-699 of the α subunit) did not react with the mature form of α; it did however react with the primary translation product of α synthesized in a rabbit reticulocyte lysate system (Marchand *et al.*, 1994). Taken together, the data indicate that the mature α subunit in mouse kid-

ney, as well as in an experimental COS-1 cell expression system, is a COOH-terminally truncated extracellular protein. There is no evidence for RNA processing or alternative splicing for meprin subunits. Only one message has been identified for each α and β subunit in mouse kidney tissue, and those are full-length transcripts (Jiang et al., 1992; Gorbea et al., 1993). Therefore, it is likely that COOH-terminal processing occurs as a proteolytic event.

There is at least one intersubunit disulfide bond in the αβ meprin dimers, as determined by SDS-PAGE of brush border membrane proteins under non-reducing conditions. As a working hypothesis it is proposed that a conserved Cys residue in the MAM domain (Cys-320 of α and Cys-306 of β) forms an intersubunit disulfide bond between meprin subunits. The mature α subunit contains 11 cysteine residues; nine of them are conserved in α and β. Cys-320 is one of the conserved residues, and it is the only conserved Cys residue not implicated in the formation of intramolecular disulfide bonds (Fig. 26). Four Cys residues in the astacin-like protease domain are predicted to form intramolecular disulfide bonds by analogy with astacin for which the disulfide bonding pattern has been determined by protein sequencing (Titani *et al.*, 1987). Similarly, four of the five Cys residues in the MAM domain are conserved in all MAM domains identified to date and have been proposed to be important for the overall structure of the adhesion domain by forming intramolecular disulfide bonds (Beckmann and Bork, 1993). The fifth cysteine residue in the meprin MAM domain is conserved in meprins, but not in MAM domains of other proteins. There are two additional cysteine residues in the unknown domain of α, and one in the unknown domain of β; these are not conserved between α and β (Fig. 26). Cys-680 of β is in the cytoplasmic domain and likely to be in the reduced state.

Fig. 26. Cysteine residues in the mature mouse meprin α and β subunits and proposed arrangement of intramolecular disulfide bonds

The figure shows a diagram of the domains that are present in the mature mouse meprin subunits. The domains are : Pro, prosequence; protease, astacin-like protease domain; MAM, adhesion domain; X, domain of unknown function; EGF, epidermal growth factor-like domain; T, transmembrane domain; C, cytoplasmic domain. Proposed intrasubunit disulfide bonds within the meprin subunits are indicated by brackets. The disulfide bridges in the protease domain are proposed based on the arrangement of disulfide bonds in astacin (Titani *et al.*, 1987). The disulfide bonding pattern in the EGF-like domain has been established for murine EGF (Savage *et al.*, 1973). Four of the five cysteine residues in the MAM domain are conserved among all known MAM domains and are predicted to form intrachain disulfide bonds; the arrangement shown is hypothetical. A fifth cysteine residue in the MAM domain is conserved in meprins, but not in other MAM domains. Cysteine residues that are not predicted to form intrachain disulfide bonds are indicated by vertical bars and their residue numbers. The amino acid numbers were assigned as in Fig. 4 (section 3.1.2).


β
Pro
Protease
MAM
X
EGF
T
C
C-306
C-493
C-680
α
Protease
MAM
X
C-320
C-571/573

The α subunit, with the prosequence removed, is in a conformation that is fully active against peptide and protein substrates. Meprin B, which contains only β subunits, has poor activity against peptides, and is known to have latent activity towards larger protein substrates, such as azocasein (Kounnas *et al.,* 1991). When the prosequence is removed by trypsin-like enzymes, meprin B exhibits comparable azocaseinase activity to that seen with meprin A (Kounnas *et al.*, 1991), thus latency towards proteins is clearly associated with the prosequence that is present on the mature form of the subunit in the membrane. Proprotein processing and activation is a common feature of proteolytic enzymes, as well as hormones and neuropeptides (Neurath, 1991). These molecules are synthesized as inactive precursors, the activation of which involves limited proteolysis at a specific cleavage site, followed by a conformational change in the protein. The specific mechanism of activation depends on the geometry of the active site and its structural relation to the activation peptide in the precursor molecule. Because the proform of β is fully active against one small substrate, YLVC(SO_3^-)GERG, but latent towards larger protein substrates, it has been proposed that the prosequence inhibits activity by sterically limiting the access of larger protein substrates, but not smaller peptide substrates, to the active site. Recently, a new model for the active conformation of the meprin protease domains has been proposed based on the crystal structure of astacin, the prototype of the "astacin family" enzymes (Bode *et al.*, 1992). The X-ray structure of astacin showed that the NH_2-terminus of the protease is buried forming an internal water-linked salt-bridge with a conserved Glu residue (Glu-103 in astacin) near the active site. Computer-assisted modelling indicated that NH_2-terminally extended proforms cannot assume such a conformation. It has been proposed that the activating

cleavage event liberates the mature NH_2-terminus, allowing the new NH_2-terminal residue to fold into the active site, thus triggering the conformational transition from a looser proform to a more compact active state. This activation mechanism is reminiscent of the trypsin-related proteases (Huber and Bode, 1978). By contrast, it differs from the "cysteine switch" activation mechanism suggested for the vertebrate collagenases where the prosegment folds across the active site, with a conserved cysteine in the propeptide coordinating to zinc (Springman *et al.*, 1990) . The proform of α, as expressed in COS-1 or 293 cells, is completely inactive towards azocasein and small peptide substrates, unless the prosequence is removed by trypsin *in vitro* (this thesis; Stambolieva and Bond, unpublished results; Johnson and Hersh, 1994). The trypsin-treated recombinant α subunits have proteinase and peptidase activity comparable to meprin A purified from mouse kidney. The complete lack of activity of pro-α against substrates of any size is consistent with the model of activation proposed by Bode *et al.*, 1992, which indicated that the conformation of the active site itself, and not just the access to the active site, changes upon activation.

Structure of meprin isoforms in mouse kidney.- The results presented herein have lead to a new model for the oligomeric organization of meprin subunits. Previous results had indicated that mouse kidneys express several isoforms of meprin oligomers, and that the expression of isoforms differed in the mouse strains examined (Gorbea *et al.*, 1991). ICR mice were reported to express membrane-bound α_4, $\alpha_2\beta_2$, and β_4 tetramers, while in C3H/He mice only the β_4 tetramers were detected. All subunits in the tetrameric complexes were believed to be linked by disulfide bridges. The present studies indicate that the oligomeric

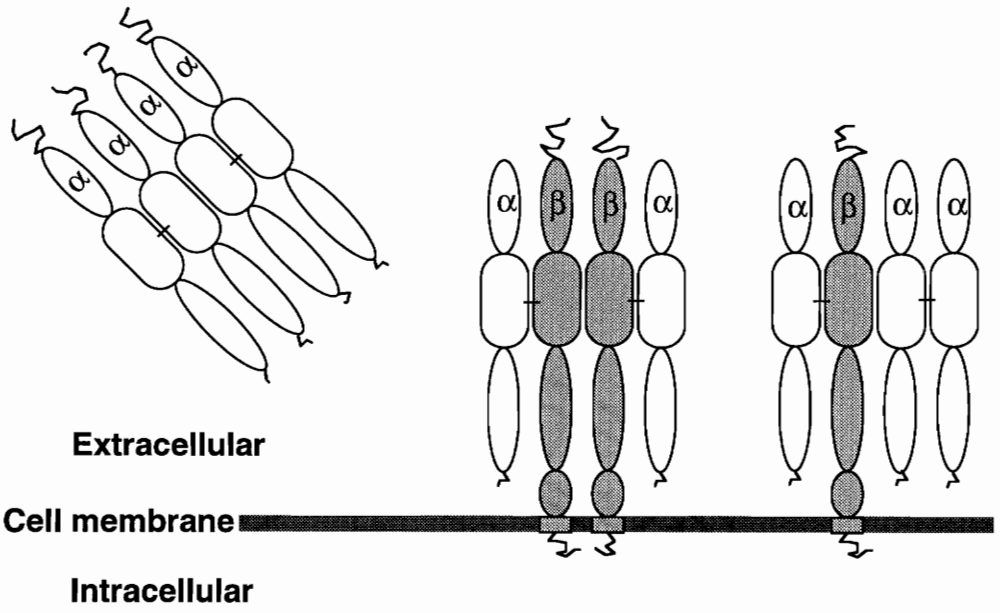
organization is somewhat different. A modified model for the isoforms of meprin A and meprin B in mouse kidney is shown in Fig. 27. The basic disulfide-linked unit of meprins is a dimer, rather than a tetramer as was previously reported. Meprin A is defined as any form of meprin that contains an α subunit. Meprin A from ICR mice contains αβ heterodimers and α_2 homodimers, as determined by SDS-PAGE under non-reducing conditions (Fig. 8, section 3.2). Meprin B, a homo-oligomer of ß subunits, is expressed in C3H/He mouse kidney. Meprin B is composed of β_2 dimers, as determined by SDS-PAGE. In the experiments herein, the β_2 dimer was not observed in ICR mouse brush border membranes; it was only observed in C3H/He mouse membranes. The lack of β_2 in ICR mouse kidneys may be related to the finding that α is synthesized at twice the rate of β, and is more abundant in ICR mouse membranes than β (Hall *et al.*, 1993). In addition, it is possible that the αβ heterodimer is a preferred or more stable form than the β_2 homodimer. There is some evidence suggesting that ICR mouse kidney membranes contain more β subunits than C3H/He mouse membranes, which would be consistent with the hypothesis that α subunits stabilize β in the membranes. Immunoblots using antibodies to the β subunit detected more β in ICR mouse kidney membranes than in C3H/He mouse membranes (e.g., Fig. 8, section 3.2). Also, trypsin-activation of brush border membrane samples indicated that ICR mouse membranes contain more trypsin-activatable activity than C3H/He mouse membranes (Stroupe *et al.*, 1989).

It is now clear that α subunits do not interact directly with the lipid bilayer, but associate with cell membranes through at least two mechanisms: (i) covalent disulfide linkages to meprin β subunits, and (ii) non-covalent interactions of α_2 homodimers with membrane proteins. Purified preparations of the enzyme exist

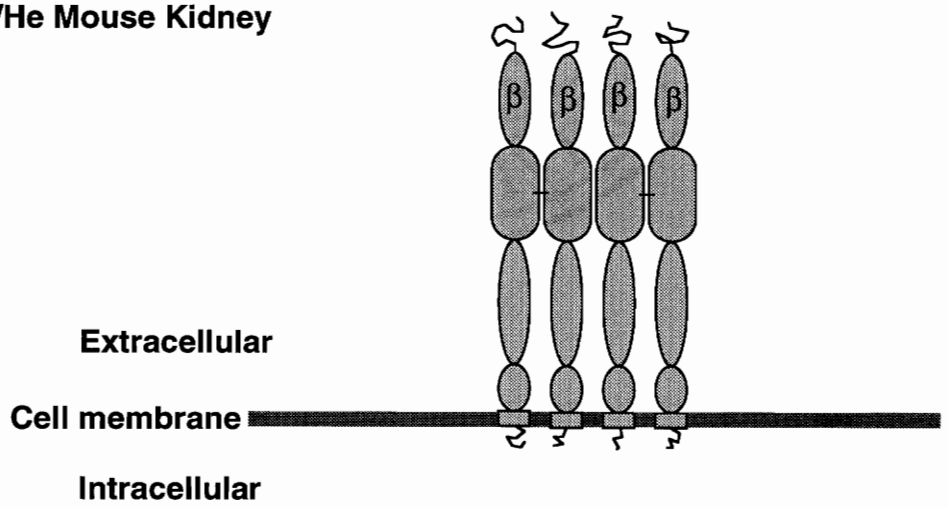
Fig. 27. Model for the meprin isoforms expressed in mouse kidney

The top panel shows ICR mouse kidney membranes, the bottom panel shows C3H/He mouse kidney membranes. The meprin subunits are shown schematically, α subunits in white and β subunits in grey. The meprin β subunits and the secreted meprin α subunits are shown with the prosequences retained; the membrane-associated α subunits do not contain the prosequences. Intersubunit disulfide bonds are depicted by horizontal bars. Each isoform consists of two disulfide linked dimers which associate non-covalently to form tetramers. ICR mouse membranes contain disulfide-linked $\alpha\beta$ dimers and α_2 dimers; membrane-bound tetramers are formed by the association of two $\alpha\beta$ dimers, or one $\alpha\beta$ dimer and one α_2 dimer. In either instance, the β subunit anchors all subunits to the membrane. Homotetramers of α subunits are secreted from the cells into the tubule lumen. C3H/He mouse kidney contain β_4 homotetramers formed by the association of two disulfide-linked β_2 dimers.

A. ICR Mouse Kidney



B. C3H/He Mouse Kidney



as higher oligomers, mainly tetramers, *in vitro* (Marchand *et al.*, 1994). On the basis of the *in vitro* oligomeric structure, it is proposed that there is a non-covalent association of meprin dimers to form tetramers *in vivo*. The tetrameric forms of meprins that are predicted to be present in ICR and C3H/He mouse kidney are shown in Fig. 27. In C3H/He mouse kidney, the predominant form is proposed to be a β_4 tetramer formed by the non-covalent association of two β_2 dimers. In ICR mouse kidney, membrane-bound $\alpha\beta$ dimers associate noncovalently with $\alpha\beta$ dimers or α_2 dimers. Thus, the meprin β subunit anchors both heteromeric and homomeric α subunits to the membrane. It is possible that membrane proteins other than the $\alpha\beta$ meprin dimer are also involved in binding α_2 homo-oligomers to the membrane. However, the fact that purified meprins are mainly tetrameric, and that α_2 is secreted from transfected cells if β is not cotransfected with α, strongly indicates a direct role for the β subunit in the association of all forms of α with the membrane (this thesis; Johnson and Hersh, 1994). ICR mouse brush border membranes contain significantly more α subunits than β subunits, thus it appears that the predominant form in the membrane is the tetramer composed of α_2 and $\alpha\beta$ dimers. The model in Fig. 27 shows the β subunit, rather than the α subunit, of the heterodimer interacting non-covalently with the other $\alpha\beta$ or α_2 dimers; this was an arbitrary choice made because of the two-dimensional nature of the model. In reality it is likely that both α and β subunits contribute to the interactions of the dimers in the membrane-bound complexes. It should be noted that he tetrameric organization has been demonstrated experimentally only for meprin A, not for meprin B. The domain(s) in α and/or β that mediate the non-covalent interactions between meprin dimers have not yet been identified.

As mentioned previously, 90% of α subunits become soluble by treatment with reducing agents. Meprin subunits contain a 170 amino acid domain, the MAM domain, which is also present in the cell surface receptors A5 protein and protein tyrosine phosphatase μ(Beckmann and Bork, 1993). It has been proposed that MAM domains have an adhesive function in membrane proteins. This domain may mediate noncovalent interactions between meprin subunits and meprin dimers. Four conserved cysteine residues in the MAM domains are likely to form intramolecular disulfide bonds and are presumably important for the overall structure of these domains. Disruption of these disulfide bridges is likely to destroy the adhesion properties of the domains. It is, therefore, reasonable to propose that the efficient solubilization of meprin α subunits by reducing agents was brought about not only by the reduction of covalent disulfide bonds linking α and β subunits, but also by the disruption of the noncovalent interactions of meprin α_2 dimers with αβ heterodimers.

The analysis of rat brush border membranes by immunoblot analysis indicated that mouse and rat meprins may be more similar in structure than previously thought. Both rat α and β subunits were found to be slightly smaller than the mouse α and β subunits, respectively. This may be caused by species-dependent differences in glycosylation, because the predicted primary amino acid structures are similar. The rat brush border membranes clearly contained α_2 dimers that could be solubilized by urea treatment. Purified rat meprins are tetrameric, as judged by their retention time on gel filtration columns (Kenny and Ingram, 1987), thus it is likely that homo- and heterodimers of rat meprin are organized into tetramers in rat kidney membranes in a way analogous to the ICR mouse enzyme.

The model in Fig. 27 shows further that homo-oligomers of α are secreted from the cells into the urine in ICR mouse kidney. The secreted forms of α are shown with the prosequences retained, as was determined experimentally by NH_2-terminal sequencing of secreted urinary α subunits (Gorbea *et al.*, 1994). The proform of mouse, rat, or human α is also the form that is secreted from transfected COS-1 cells, 293 cells, or Madin Darby canine kidney (MDCK) cells (this thesis; Johnson and Hersh, 1994; Grünberg *et al.*, 1993). There are a large number of other membrane-bound proteins that have soluble counterparts; examples include receptors, cell adhesion molecules, leukocyte antigens, and ectoenzymes (reviewed by Ehlers, and Riordan, 1991). Angiotensin converting enzyme (ACE) is an example of another brush border membrane proteinase that has a soluble isoform produced by proteolytic processing of the membrane-bound form. Cleavage and release of soluble ACE occurs at a monobasic residue in the COOH-terminal domain of membrane-bound ACE after the enzyme has reached the cell surface (Ramachandran *et al.*, 1994). The membrane-bound form of ACE retains the COOH-terminal hydrophobic domain, whereas both the membrane-bound and the secreted forms of α are extracellular components. It is reasonable to suggest that the membrane association and secretion of meprin α subunits is regulated by protein interactions, e.g., by the ability of α to associate with β–anchored proteins. Alpha subunits that fail to associate with β–anchored proteins are secreted.

Known brush border peptidases are anchored to the plasma membrane either by a COOH-terminal hydrophobic domain, e.g., ACE (Wei *et al.*, 1991), by hydrophobic peptide sequences near the NH_2-termini, e.g., neprilysin (Devault *et al.*, 1987) and aminopeptidase N (Olsen *et al.*, 1988), or via a COOH-terminal

phosphatidylinositol anchor, e.g., aminopeptidase P (Hooper *et al.*, 1987), carboxypeptidase M (Deddish *et al.*, 1990). The proposed membrane association of the meprin α subunit through protein interactions is unique among the known brush border peptidases. However, it may serve as a model for soluble proteins that associate with membrane proteins in a specific way to function at the cell surface. e.g., to generate high local concentrations of effectors at the membrane. Other proteins that fall into this category are complement proteins and plasminogen activators (Sim *et al.*, 1993; Nykjaer *et al.*, 1994).

Biosynthesis of meprin subunits.- The biosynthesis of membrane proteins involves a complex sequence of co-and post-translational modifications. Common modifications include signal peptide cleavage and other forms of proteolytic processing, disulfide bond formation, oligomerization, and N-linked and O-linked glycosylation. While the analysis of meprin biosynthesis was not the main focus of these studies, the results form the basis for several conclusions and hypotheses relating to meprin biosynthesis.

With the cloning and NH_2-terminal sequencing of the mature subunits it is clear that the signal sequences of both subunits are removed during biosynthesis. This presumably occurs cotranslationally or very shortly after translation in the endoplasmic reticulum, where the signal peptidase activity is located (Evans *et al.*, 1988).

Dimerization and intersubunit disulfide bond formation of the mouse meprin subunits occur very early during biosynthesis in the endoplasmic reticulum. This is apparent from the analysis of wild-type meprin α subunits transfected into COS-1 cells (Fig. 9 and Fig. 10, section 3.3.1). While most of the α

subunits are secreted into the culture medium, a small fraction of α subunits are retained in the endoplasmic reticulum, as determined by their sensitivity to endo H digestion. SDS-PAGE under non-reducing conditions showed that the ER-retained α subunits are dimeric, indicating that the ER is the site of intersubunit disulfide bond formation. This is also the site where the human form of meprin aquires intersubunit disulfide bonds (Sterchi *et al.*, 1988). This may be the case for membrane-bound and secreted proteins in general, because protein disulfide isomerase, a protein that is implicated for catalysis of disulfide bond formation, is present in the lumen of the endoplasmic reticulum (Noiva and Lennarz, 1992). It is possible that tetramer formation of meprin subunits also occurs in the ER, although no experiments were done that specifically addressed this point. Recently it has been recognized that the localization of protein oligomerization in the ER provides the basis for an important regulatory mechanism of expression. In several instances it has been established that assembly of multisubunit complexes in the ER is a prerequisite for the transport of the newly synthesized proteins through the secretory pathway (e.g., Rutledge *et al.*, 1992; Hennecke and Cosson, 1993). This presumably prevents the expression of incompletely assembled non-functional protein complexes on the cell surfaces, which could be potentially detrimental to the organism.

The results further show definitively that the biosynthesis of α , but not β, in mouse kidney involves the removal of the prosequence. As in most other proenzymes, cleavage occurs at a basic residue. A survey of the processing sites in 154 proproteins showed that almost 50% of proproteins are cleaved after Lys-Arg, 30% at other dibasic motifs, and 14% at a monobasic residue (Hutton, 1990). The enzyme that processes α apparently does not recognize β. The pro-

protein cleavage in α occurs following the residues R-T-R (residues 75-77 in α, Fig. 4), the corresponding sequence in the β subunit is N-G-K (residues 60-62 in β, Fig. 4). It is thus possible that prosequence cleavage depends on a basic residue in position P3, or that the processing enzyme requires Arg in the P1 position. It would be informative to analyze the requirements for prosequence cleavage in α by site-directed mutagenesis; however, no cell expression system has been identified to date that will process the wild-type α subunit prosequence. The R-T-R cleavage motif in α is somewhat similar to, but distinct from, the motifs recognized by members of the recently discovered family of processing enzymes of the subtilisin family (reviewed by Steiner *et al.*, 1992). This family includes the proteases furin, PC2, PC1/PC3, PC4, and PACE. All members of this family were identified by PCR methods searching for homologues to the yeast gene product kexin. PC1/PC3 and PC2 cleave at K-R and R-R dibasic sites, while furin cleaves at R-X-K/R-R sites. Furin is located in the trans-Golgi network, as determined by immunofluorescence staining; PC1/PC3 and PC2 are present in transport vesicles (Bresnahan *et al.*, 1990; Smeekens *et al.*, 1991). The endoproteolytic activities and subcellular locations of PACE and PC4 have not been determined. While furin and PC1/PC3 cannot be the NH_2-terminal processing enzyme for α, it is likely that there are more members of this family yet to be identified, one of which might have the specificity to cleave at R-X-R sites or at monobasic sites. Notably, the amino acid sequence preceding the cleavage site in α, R-T-R, closely resembles the cleavage site in the proform of another brush border enzyme, lactase-phlorizin hydrolase (Oberholzer *et al.*, 1993). In this protein, cleavage occurs at the sequence K-T-R. Proprotein processing of lactase phlorizin hydrolase is a post-Golgi event, occuring either in the trans-Golgi

network, transport vesicles, or at the cell surface, by an unidentified protease (Lottaz *et al.*, 1993). The subcellular location of the meprin α prosequence processing enzyme remains to be established. Interestingly, secreted forms of α, either from mouse urine samples or from COS-1 cells, retain the prosequence. One possible interpretation of this finding is that the NH_2-terminal processing occurs at the cell surface, and those subunits that have only transient interactions with the cell membrane leave as unprocessed zymogens.

The results in this thesis establish that the biosynthesis of α involves COOH-terminal processing, and provide preliminary information about the COOH-terminal processing site in α. Western blotting of brush border membranes with anti-α subunit antibodies detected only one electrophoretic form, indicating that the processing of the α subunit COOH-terminus (as well as the NH_2-terminus) occurs quantitatively. The calculated molecular size of the mature α subunit polypeptide indicates that COOH-terminal processing of the primary translation product occurs in a region immediately NH_2-terminal to the EGF-like domain. This region contains a 56 amino acid inserted domain that is not present in the β subunit. It is the only region that is totally unique to α. Several attempts by our laboratory to identify the processing site by COOH-terminal protein sequencing have failed. Because COOH-terminal processing of the α subunit in transfected COS-1 cells closely resembled the processing in mouse kidney cells, it is likely that the protein processing machinery that is responsible for the COOH-terminal processing is the same in both systems. Therefore experiments were designed to define the processing site by site-directed mutagenesis. The results further substantiated the hypothesis that the inserted domain is a crucial factor determining whether a meprin subunit will be processed. First, replacement of

the COOH-terminal 137 residues of α (including the inserted domain) with the COOH-terminus of β prevented processing, resulting in a protein that retained the membrane-spanning sequence; and second, deletion of the inserted domain from the α subunit also prevented COOH-terminal processing of α (Fig. 22, section 3.3.4). Therefore, the 56 amino acid inserted domain is clearly essential for processing. The simplest interpretation of these results is that the processing site is located within the 56 amino acids of the inserted domain. It cannot be completely ruled out, however, that the absence of the inserted domain in the mutant proteins changed their conformations in such a way that a processing site located elsewhere in the protein is no longer exposed on the protein surface.

Analysis of the sequence of the inserted domain showed no significant sequence homology to any other protein sequences in the data bases. The inserted domain, however, contains several sites that commonly serve as processing sites in other proteins, notably a RQKR sequence that conforms to the consensus sequence (RXK/RR) for furin processing enzymes (Van de Ven *et al.*, 1993). There are many membrane and secreted proteins that are processed at these sites, e.g., the precursors of von Willebrand factor (Van de Ven *et al.*, 1990) and of β-nerve growth factor (Bresnahan *et al.*, 1990). There is also a dibasic site in the inserted domain, that could function as processing site. Deletion of the furin recognition site or the dibasic site by site-directed mutagenesis did not prevent the secretion of α (Fig. 23, section 3.4.4); thus, these sites are not essential for processing. It is either possible that the dibasic sites are not used as processing sites, or that in the absence of these processing site an alternative cleavage site can be used. In any case the result demonstrated that furin-like enzymes cannot be the only processing enzymes of the α subunit.

Finally, it is possible that the proteolytic system that cleaves α has a broad specificity, as is seen for growth factor processing enzymes (reviewed by Massagué and Pandiella, 1993). Growth factor precursors resemble meprin α subunits in that they are synthesized as membrane-bound precursors with COOH-terminal membrane anchors, and are processed to mature, soluble proteins by NH_2-and COOH-terminal cleavage. The COOH-terminal processing and solubilization of membrane-bound growth factor precursors has been studied in great detail, yet only in a few instances are the identities of the cleaved peptide bonds known. One example is the precursor of transforming growth factor (TGF)-α, which is cleaved at an Ala-Val peptide bond. Various non-conservative amino acid substitutions in the cleavage site had no detectable effect on cleavage efficiency, and deletion of a 12 amino acid sequence surrounding the cleavage site was necessary to block pro-TGF-α cleavage, whereas more discreet mutations were insufficient to prevent cleavage (Perez *et al.*, 1990). It is possible that the processing sites in growth factors, as in meprin α, are not specific recognition sites, but rather are composed of an exposed region that is vulnerable to the attack by a variety of proteases. In addition, it is possible that the processing enzymes themselves are non-specific.

The subcellular location of the COOH-terminal processing enzyme remains to be established. Processing enzymes are generally thought to be membrane-bound and could reside in compartments such as the Golgi apparatus (where furin-like enzymes reside), or at the cell surface (where the processing enzymes for ACE and growth factors are believed to reside) (Massagué and Pandiella, 1993; Ramachandran *et al.*, 1994). The results from the analysis of COS-1 cells transfected with wild-type α subunit cDNA provide some preliminary

evidence that COOH-terminal processing occurs in the endoplasmic reticulum. Deglycosylation of the recombinant α subunits retained in the endoplasmic reticulum produced two polypeptides with molecular weights of 81,000 and 65,000 (Fig. 10, section 3.3.1). The 65 kDa protein is most likely a proteolytically processed form of α. While no work has been done to determine the character of proteolytic modification that this protein has undergone (NH_2- or COOH-terminal), its size is similar to that of mature α subunits in mouse kidney (67 kDa). Thus, it is possible that this is the COOH-terminally processed form, while the 81 kDa polypeptide is a precursor. The presence of both precursor and processed form in the ER indicates that processing may occur in the endoplasmic reticulum.

The forces that drive meprin oligomerization remain to be determined. In many instances, the oligomerization of multisubunit membrane proteins is mediated by specific interactions between the transmembrane domains (reviewed by Cramer *et al.*, 1992). Examples of proteins in this category are the integrins, the T cell receptor, glycophorin A, and the CD8 molecule (Briesewitz *et al.*, 1993; Rutledge *et al.*, 1992; Lemmon *et al.*, 1992; Hennecke and Cosson, 1993). EGF-like domains have also been reported to be important participants in subunit protein-protein interactions (Baron *et al.*, 1991). The meprin α subunits are initially synthesized with a COOH-terminal extension that contains a transmembrane domain and an EGF-like domain; this raised the hypothesis that the COOH-terminal extension might be important during biosynthesis and oligomerization. The hypothesis that α subunit dimerization is mediated by interactions of transmembrane helices is further supported by the finding that the α transmembrane domain contains two motifs that have been identified as crucial for interactions in the transmembrane domains of glycophorin A and the EGF-receptor

(Lemmon *et al.*, 1992; Ullrich and Schlessinger, 1990). The present experiments with COOH-terminal deletion mutants indicated that neither the meprin α transmembrane domain nor its EGF-like domain are essential for dimerization of α subunits or their transport through the secretory pathway. It is still possible, however, that the COOH-terminal region of α mediates interactions with β during αβ heterodimer synthesis.

Several other possible functions for the COOH-terminal region of the α subunit primary translation product can be proposed. An important aspect of the biosynthesis of any brush border membrane protein that has not been addressed in this thesis is the sorting of the proteins to the apical, versus the basolateral, membrane domain. Many researchers interested in protein targeting have postulated that the sorting of brush border membrane proteins to the apical domain is mediated by signals within the proteins to be sorted; however, the molecular structures of the putative apical targeting signals have remained elusive (Simons and Wandinger-Ness, 1990). It is thus possible that the COOH-terminus of α contains information that is relevant for sorting. This could be tested by expressing COOH-terminal deletions in a polarized cell line, such as CaCo-2, rather than a non-polarized cell line, such as COS-1. It would also be of interest to determine if the α subunit EGF-like domain has growth factor-like activity. If the COOH-terminal processing of α occurs at a single site, a membrane-bound EGF-like domain will be produced. If more than one cleavage is involved, it is possible that EGF-like domains with potential biological activity are secreted into the proximal tubules. If COOH-terminal processing occurs in the ER, as suggested, it is also possible that the COOH-terminal fragment is degraded by the ER-asso-

ciated proteolytic system. Ongoing studies in our lab are aimed at determining the fate of the COOH-terminal fragment after cleavage.

Physiological role and regulation of meprin subunits.- One of the most challenging tasks for researchers interested in any cell surface protease remains the unambigous identification of those substrates that are physiologically relevant *in vivo*, and meprins are no exception in this regard. Most of the data regarding substrates come from *in vitro* studies, and the contributions that meprins make to any process *in vivo* are unknown. This is true for proteases in general; for most proteases, membrane-bound or intracellular, the physiological functions have yet to be identified. Most inhibitors of proteases are not enzyme-specific, but inhibit a number of proteases of the same mechanistic class. The identification of specific processes, in which a particular protease is involved, is further complicated by the observation that proteolytic activities are often redundant. If a particular protease is inhibited, another protease may be able to fulfill its function.

Meprins have been implicated to be involved in a number of processes. They are expressed in a tissue-specific manner on the brush border membranes of mouse, rat, and human intestine, and of mouse and rat kidneys. Meprins have the potential to act on other membrane-bound brush border proteins or on soluble peptides and proteins in the tubule or intestinal lumen. The tissue-specific expression of meprin subunits is species-dependent, indicating that meprins may have different functions in the species in which they are expressed. In mice, the α subunit is expressed at very high levels in kidney, but not in the intestine. It has been estimated that meprin accounts for approximately 5% of the total brush border membrane proteins in mouse renal brush border membranes. The α sub-

unit is active against a broad range of peptides and protein substrates and is likely to affect the protein and peptide composition in the urine. Meprin possibly inactivates biologically active peptides in the glomerular filtrate. Together with other renal brush border proteinases, meprins might be involved in the digestion of peptides and proteins in the glomerular filtrate to permit their retrieval before excretion in the urine. Because meprin α subunits are not expressed in human kidney, it is possible that the α subunit has a rodent-specific function in kidney. In this context it has been proposed that meprins affect olfactory responses of rodents and thereby influence mouse behavior, e.g., mate selection and recognition of offspring (Flannery *et al.*, 1991). The urine of rodents, unlike the urine of humans, contains a high concentration of proteins, e.g., soluble proteins that are synthesized in the liver and subsequently filtered across the glomerulus (mouse urinary proteins, MUPS), and soluble class I histocompatibility antigens. Both classes of proteins have been found to bind olfactants and to transport them to the urine. Thus, a rodent-specific function for meprin might be the processing or degradation of olfactory binding proteins. Alpha subunits are secreted in rodent kidney; thus α subunits have the potential to function at a site that is distinct from the site of biosynthesis, e.g., in the lumen of the distal tubules. Beta subunits are integral membrane proteins that can only act at the surface of the proximal tubule cells, where they are synthesized. The β subunit has very little peptidase activity and a low protease activity unless activated. In the kidneys, where β is not activated, β may have a function that is unrelated to proteolysis. Beta may function as a receptor for peptides or other proteins. It is possible that the main function of β in rodent kidney is to anchor α to the membrane.

Beta is also expressed in the intestine of every species in which meprin subunits have been identified. In the mouse intestine, the β subunit is activated, probably due to the high concentration of trypsin in the gastrointestinal tract (Gorbea *et al.*, 1994). It is likely that the human and rat meprin β subunits in the intestine also are activated. The activated meprin β subunit has very little peptidase activity *in vitro*, thus it is unlikely that the function of β is to degrade peptides. The trypsin-activated β subunit, however, has considerable proteinase activity. Rats and humans, but not mice, also express α subunits in the intestine. Given the abundance of the soluble proteases chymotrypsin and trypsin in the intestine, it is unlikely that meprin subunits contribute significantly to the bulk degradation of dietary proteins. This suggests that meprin β subunits have a specific function in the intestine that requires proteinase activity, but is separate from the digestion of dietary proteins for nutrient acquisition.

Certain mouse strains are completely deficient in the expression of the α subunit, yet no abnormal phenotype is associated with this deficiency. The ß subunit, by contrast, is present in the kidneys of all mouse strains tested, and in intestine as well. It is therefore possible that the α subunit has a very specialized function that can be compensated for, and that the ß subunit performs essential activities at the membrane. A long-range project that has the potential to give new insights into meprin function would be the development of a transgenic mouse model in which the gene for the β subunit has been knocked out. This would provide information as to whether the function of the β subunit is essential and hopefully give some information relating to its function.

Conclusions.- This thesis project addressed aspects of the structure, processing, membrane-association and secretion of meprin, a major brush border membrane protease from rodent kidney, and rodent and human intestine. It was shown that meprin α and β subunits have similar deduced primary sequences. The meprin α and β subunits are processed differently, resulting in mature proteins that are much more distinct than would have been predicted from the primary sequences alone. Beta, with the prosequence retained, is a latent proteinase in mouse kidney, while α, with the prosequence removed, is active at the cell surface. The oligomeric organization of meprins is that of disulfide-linked dimers, which associate non-covalently to form tetramers. The expression of various isoforms of meprin oligomers differs in the mouse species examined. The β subunit is an integral membrane protein. In contrast, α subunits are extracellular proteins, which can be secreted or remain membrane-associated through covalent and non-covalent interactions with β-containing proteins. The proposed membrane association of the meprin α subunit through protein interactions is unique for the known brush border peptidases. However, it may serve as a model for soluble proteins that associate with membrane proteins in a specific way in order to function at the cell surface.

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PUBLICATIONS:

Kennelly, P.J., Leng, J., and Marchand, P. (1992). The MgATP-binding site on chicken gizzard myosin light chain kinase remains open and functionally competent during the calmodulin-dependent activation-inactivation cycle of the enzyme. *Biochemistry* **31**, 5394-5399.

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ABSTRACTS:

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