The Cleavage of Protein Kinase A by the Kinase-splitting Membranal Proteinase Is Reproduced by Meprin β*

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The Kinase-Splitting Membranal Proteinase (KSMSP) is a metallo-endoproteinase that clips off the carboxyl terminus tail of the catalytic (C) subunit of protein kinase A to yield a truncated, catalytically inactive protein (C'). Here we report (a) a new procedure for the purification of KSMSP, yielding a major protein band in SDS-polyacrylamide gel electrophoresis that correlates with the characteristic KSMSP activity; (b) the sequence of tryptic peptides obtained from this band, suggesting an identity between this protein and meprin β; (c) the immuno-detection by specific anti-peptide antibodies of both the α and the β subunits of meprin in KSMSP preparations; (d) the stable expression of meprin β in a mammalian cell line (293) to establish a clone that constitutively expresses the full-length precursor of meprin proteinase; and (e) the optimization of the proteolytic activation of this precursor to obtain an enzyme exhibiting the specific KSMSP cleavage, suggesting that KSMSP is either derived from, or identical with, the meprin β gene product. It is hoped that these results will shed light on the possible physiological role of KSMSP and the way it may affect protein kinase A-mediated processes.

The Kinase-Splitting Membranal Proteinase (KSMSP) is a brush-border metallo-endoproteinase originally discovered in our laboratory (1) as an enzyme that selectively clips the C subunit of protein kinase A (PKA) (40 kDa) to yield a truncated form denoted C' (34–36 kDa), which is devoid of catalytic activity (1–3). The biochemical properties of KSMSP were found to be quite intriguing: (a) it cleaves the C subunit at a distinct site in the carboxyl terminus tail of the kinase (4–8); (b) it cleaves the C subunit when it is free and active, not when inhibited by its regulatory (R) subunits, as in the R2C2 complex (2); (c) it singles out and selectively cleaves the C subunit in the presence of the large number of proteins in crude cell extracts of different tissues (3); (d) it cleaves the C subunit in its native conformation, but not if the kinase is pre-denatured (2); and (e) it distinguishes between the “open” and “closed” conformations of the C subunit (7).

In the course of these studies, it was found that KSMSP also clips off specifically the carboxyl-terminal end of the epidermal growth factor receptor and the insulin receptor (9, 10), again in a conformation-dependent manner. Somewhat unexpectedly at the time, it was found that KSMSP is an ecto-enzyme, i.e. that its active site is oriented toward the cell exterior (11), raising the question whether protein kinases, which were known to regulate biological processes within the cell, are indeed the physiological targets of KSMSP. However, studies in our laboratory which were prompted by these findings showed that protein kinase A may well have an extra-cellular regulatory role, for example in blood (12, 13).

Until recently, we could not establish unequivocally the molecular identity of KSMSP, since its purified preparations were found to vary in their specific activity, to be labile, to form aggregates, and to exhibit differences in the apparent molecular mass of the enzyme. It was difficult to establish with certainty whether the protein bands in the SDS-PAGE of such preparations is responsible for the specific KSMSP activity. Furthermore, the variable specific activity of these preparations seemed to be associated with the number of purification steps used. Therefore, we assumed that this variability might be caused by proteolytic enzymes known to be abundant in the brush-border membranes (14, 15), from which KSMSP is isolated. We reasoned that such proteolytic enzymes might not be inhibited completely by the proteinase inhibitors we added and may either activate latent form(s) of this enzyme or degrade active KSMSP in the course of its purification.

This paper describes a systematic screening of several solubilization and purification steps and the subsequent development of a rapid and efficient method for the purification of KSMSP to homogeneity. The KSMSP preparation thus obtained contains a major protein band in SDS-PAGE that correlates with the characteristic proteolytic activity of KSMSP on the C subunit of PKA. The protein in this band was fragmented, four of the resulting peptides were sequenced, and, on the basis of these sequences and a search in protein data banks, the protein was identified as the β subunit of meprin.

Meprins belong to the “astacin family” of metallo-endopeptidases (16) (based on the name of the proteinase from the crayfish Astacus astacus, which was the first to be sequenced and biochemically characterized (17)). The x-ray crystal structure of astacin was recently solved (18, 19). Mouse and rat meprins are composed of two types of subunits, referred to as α and β subunits, which are about 45% identical in their sequence (20–24). Meprin exists in homo- and heterotetrameric forms (20, 25), which can be dissociated into disulfide-linked dimers by detergent.

The full-length cDNA sequences of α and β subunits of meprin predict a similar domain structure (23, 26). Meprins are
synthesized as inactive proenzymes, and it is the removal of their prosequences that leads to their activation. There is a different proteolytic processing for each subunit in mouse kidney: the mature α subunit, but not the β subunit, undergoes this proteolytic activation (23, 27). The β subunit prosequence does not contain any known motif for precursor processing, and the enzyme is predominately delivered to the plasma membrane as azymogen. The physiological substrates of meprins are not yet established unequivocally. However, there is a substantial amount of data on the specificity of the meprin cleavage of synthetic as well as natural peptides and proteins. Among the functions that were suggested for meprins are the degradation of peptides, such as parathyroid hormone (38), α-melanocyte-stimulating hormone, bradykinin, kallikrein, substance P (29), and TGF-α (30). Meprins were proposed to degrade extracellular matrix proteins such as type IV collagen, laminin, fibronectin, and gelatin (31). They have also been implicated in the processing of urinary peptides and protein excreta and, possibly, in the release of pheromones (16, 26).

In addition to the demonstration that the KSMP activity can be reproduced by meprin β, this paper reports the preparation of specific antibodies against meprin α and against meprin β and describes expression of meprin β in a mammalian cell line (293) and establishment of a clone of these cells that constitutively expresses the full-length precursor of meprin β. Following an optimization of the proteolytic activation of the meprin β precursor, the enzyme obtained is shown to possess the specific KSMP activity, i.e., the capacity to clip off the carboxyl terminus tail of the C subunit tail of PKA.

**MATERIALS AND METHODS**

**Purification of KSMP—Brush border membranes (BBM) from rat kidney were prepared as described in the literature (32). All the manipulations were carried out at 4 °C, and all the buffers used during the purification of BBM contained phenylmethylsulfonyl fluoride at a final concentration of 10−4 M to reduce protein degradation. The BBM pellet obtained from 80 g of tissue (60 kidneys of 12–14-week-old male Wistar rats) was resuspended in 200 ml of a 10 mM Tris-HCl buffer (pH 7.1) containing 10 mM mannitol. This usually yielded a final protein concentration of 1 mg/ml. Octyl-β-D-glucopyranoside was added to the BBM suspension to a final concentration of 1% (w/w), bringing the protein to a detergent ratio of 1:20 (w/w). Solubilization was allowed to proceed for 30 min at 4 °C. The mixture was then centrifuged at 100,000 × g for 1 h, and the resulting octyl-β-D-glucopyranoside extract of the BBM was used for further purification. The supernatant (~200 ml) was dialyzed overnight against 1 liter of 10 mM Tris-HCl (pH 7.1) containing 1.2 M NH₄Cl and 0.5% (w/w) octyl-β-D-glucopyranoside. The turbidity was removed by centrifugation (30 min at 50,000 × g). Octyl-β-D-glucopyranoside was added to a final concentration of 1% (w/w). Chelating Sepharose (10 ml) (Pharmacia Biotech Inc.) was washed with distilled water and then loaded with Cu²⁺ ions by passing 25 ml of 50 mM CuCl₂ solution through the resin (33). The Cu²⁺-loaded resin was extensively washed with 10 mM Tris-HCl (pH 7.1), containing 1 M NH₄Cl and 1% (w/w) octyl-β-D-glucopyranoside (buffer A), and then the column material was added batchwise to the cleared BBM extract. After overnight incubation at 4 °C, the resin was mounted onto a column (1.4 × 6.5 cm) on top of 3 ml of pre-packed chelating Sepharose (free of Cu²⁺ ions) equilibrated with buffer A. The column was extensively washed with buffer A (10 column volumes) and then with 10 mM Tris-HCl (pH 7.1), containing 0.5 M NaCl and 1% (w/w) octyl-β-D-glucopyranoside (buffer B). The column was then eluted stepwise with 50 mM histidine in buffer B, and the fractions containing KSMP activity emerged in about one column volume. The pooled fractions were extensively dialyzed against 10 mM Tris-HCl buffer (pH 7.1), containing 1.5 mM MgCl₂ and 1% (w/w) octyl-β-D-glucopyranoside (buffer C). The dialyzed KSMP preparation was applied batchwise onto 5 ml of a DEAE-Sepharose column (Pharmacia) pre-equilibrated with buffer C. After a 30-min incubation at 4 °C, the column was washed with 75 ml of buffer C, and the enzyme was eluted by an NaCl gradient (150 mM, 0–500 mM) in buffer C. The emerging fractions were assayed for KSMP activity and then pooled for further studies.

**Assay of KSMP Activity—**The C subunit of PKA purified from bovine heart was described earlier (34) and was used as a substrate (1). The standard reaction mixture (20 μl) contained 20 μg/ml of the C subunit and 3–50 μg/ml of the KSMP preparation (depending on the purity of the enzyme) in 20 mM Tris-HCl buffer (pH 7.1), containing 1.5 mM MgCl₂ and 1% (w/w) octyl-β-D-glucopyranoside. KSMP preparations from different purification steps were extensively dialyzed against the reaction buffer prior to assaying the activity. The KSMP cleavage was allowed to proceed for 10 min at 22 °C and then arrested by adding the SDS-PAGE loading buffer (35). The samples were then subjected to SDS-PAGE, and the components in the reaction mixture were visualized by Coomassie Blue staining. Quantitatively, the KSMP activity was determined by densitometric scanning of the C to C′ conversion using the equation % Activity = C/[(C + C′)]. One unit of the KSMP activity is defined as the amount of enzyme converting 1 μg of C to C′ per min at 22 °C.

**Sequence Analysis of KSMP—**The KSMP preparation eluted from the DEAE-Sephabel column was further purified by SDS-PAGE (5–15% acrylamide gradient). The protein bands were visualized by zin-cimidazole staining (36), and those having an apparent molecular mass of 85 and 75 kDa were excised from the gel for analysis. The proteins in these gel slices were eluted with 10 mM Tris-HCl (pH 7.1) containing 0.5% (w/v) octyl-β-D-glucopyranoside. Trypsin (sequencing grade, Boehringer Mannheim) was added to these extracts (protein to trypsin ratio, 50:1), and trypsinolysis was allowed to proceed for 2 h at 37 °C. Trifluoroacetic acid was added to a final concentration of 0.5% (v/v), and the peptide mixture was applied on an Ultraphas ODS reversed phase HPLC column (150 × 4.6 mm, Beckman). The column was developed with a linear gradient of acetonitrile (0–40%, within 100 min) in 0.1% (v/v) trifluoroacetic acid, and the absorbance of the effluent was monitored at 210 nm (Hewlett Packard 1040A diode array detector). The chosen peaks were collected and then rechromatographed on the same column. Sequence analysis was performed with a gas-phase sequencing apparatus (Applied Biosystems). The peptide sequences obtained were used for a search in GenBank®/EMBL Data Bank using the program Findpatterns (Genetic Computer Group, Wisconsin).

**Specific Anti-peptide Antibodies against the “Head” and the “Tail” of the Catalytic Subunit of PKA—**The antibodies were raised against synthetic peptides at the amino terminus and at the carboxyl terminus of the C subunit. The peptides chosen were P-1, AKKGSEQVESKEFLARK; P-2, KGPGDTSNFDDYEEE; and P-3, SINERCGKESFEP, the numbers indicating their positions in the sequence of the C subunit (cf. Fig. 6B). The preparation of the antibodies and the characterization of their specificity is described elsewhere (8).

**Specific Anti-peptide Antibodies against the α and β Subunits of Meprin—**Regions that share no sequence homology in the α subunit (5′-KIQTFQGDSDHN-3′), and the β subunit (5′-YMTREY TAGHGDQVY-3′) of rat meprin were selected as identity for preparing specific peptide-antibody antibodies against each of the subunits (8).

The peptides were synthesized with a solid phase synthesizer, purified by reversed phase HPLC, and analyzed by amino acid composition or sequence to confirm their structure. The peptides were cross-linked with Keyhole Limpet Hemocyanin (Pierce) as a carrier, and the resulting conjugates were used for the immunization of rabbits, as described earlier (37). The immune sera obtained were fractionated by an ammion sulfate precipitation (repeated twice), and the resulting IgG fractions were used in this study.

**Cloning of the β Subunit of Meprin—**A total RNA pool isolated from rat kidney by the thiocyanate method described in the literature (38) was used as a template for cDNA synthesis from a d(T)₁₇ primer by the avian myeloblastosis virus reverse transcriptase (Boehringer). The DNA of interest was then amplified by PCR, using specific oligonucleotide primers designed on the basis of the reported sequence of the meprin β subunit DNA (EMBL data base access no. M88601). These oligonucleotides, which contained restriction sites convenient for further manipulations, were annealed with the 5′- and 3′-non-coding regions of the gene in question. The primers had the following sequences: 5′-primer with a KpnI restriction site, 5′-CTAGGGTACCAGGCTG CGACCTTAAAAGGAC-3′; 3′-primer carrying a BamHI restriction site, 5′-GTCGGGATCCAATTTAGTTTATGAGCCACT-3′. The KpnI-BamHI restriction fragment of the PCR product was cloned into the pcDNA-3 vector (Invitrogen) for a transient and stable expression in mammalian cells. The general integrity of the gene was ascertained by its translation in a rabbit reticulocyte lysate system (TNT™-coupled transcription/translation kit) in the presence or in the absence of canine microsomal membranes (CMM) (Promega).
Expression of the Meprin β Subunit in Mammalian Cells—A human embryonic kidney (HEK) cell line (denoted 293) was used for the stable expression of the meprin β subunit. A pcDNA-3 vector carrying the β subunit gene was introduced into the cells by electroporation (38). The clones were selected by growth on the Dulbecco's modified Eagle's medium containing 10% fetal calf serum, a standard mixture of combined antibiotics (BioLab, Israel), and 0.4–0.6 mg/ml of geneticin sulfate (G-418, Life Technologies, Inc.). Geneticin-resistant clones were picked up and grown separately on the selective medium (in the presence of 0.4–0.6 mg/ml of geneticin) until they formed a confluent monolayer. The cells were then collected with a rubber scraper, washed by phosphate-buffered saline three times, and resuspended in a 20 mM Tris-HCl buffer (pH 7.1), containing 1.5 mM MgCl₂ and 1% (w/v) octyl-β-D-glucopyranoside. The insoluble particles were removed by centrifugation at 14,000 × g for 15 min, and the supernatants were diluted with a 20 mM Tris-HCl buffer (pH 7.1) containing 1.5 mM MgCl₂ and 0.1% (w/v) octyl-β-D-glucopyranoside, to a final protein concentration of 0.2 mg/ml. They were then assayed for KSMP activity.

Assay of the KSMP Activity of the Expressed Meprin β Subunit—The octyl-β-D-glucopyranoside extracts of the clones (final volume, 100 μl; protein concentration, 0.2 mg/ml) were incubated with 100 ng of the C subunit of PKA. The reaction was allowed to proceed for 3–20 h at 22 °C and then arrested by adding the SDS-PAGE loading buffer (35). Aliquots (20 μl) of each reaction mixture were subjected to SDS-PAGE, transferred onto a nitrocellulose membrane, and stained with the antibodies against different epitopes of the catalytic subunit of PKA (8). Cross-reactive material was visualized by staining with anti-rabbit antibodies conjugated with peroxidase (Sigma) using an ECL detection system (Amersham). As a control, we used octyl-β-D-glucopyranoside extracts of geneticin-resistant clones obtained by transfecting 293 cells with the pcDNA3 v vector in vitro.

Activation of the Meprin β Subunit by Trypsin—Trypsin digestion of the crude membranal preparation of the meprin-expressing cells and rat kidney BBM was carried out as described earlier (20). The trypsin to protein ratio was kept at 1 to 30. The reaction was allowed to proceed for 1 h at 37 °C and arrested by adding a 10-fold excess (w/v) of soybean trypsin inhibitor. Completion of trypsin inhibition was verified in every experiment by running an appropriate control, which included (in addition to the substrates) trypsin and its inhibitor but no meprin. The proteolytic action of meprins on azocasein was performed according to the method described earlier (39). The reaction mixture (1 ml) contained 11 mg/ml azocasein (Sigma), 10 mM ethanolamine (pH 9.5), 70 mM NaCl, and 75–100 μg of the meprin preparation (membranes or an octyl-β-D-glucopyranoside extract of the membranes). The cleavage of the azocasein was allowed to proceed at 37 °C. Aliquots (250 μl) were withdrawn at different times, and the reaction was arrested by adding 1 ml of 5% trichloroacetic acid. The resulting precipitate was removed by centrifugation in a table-top centrifuge for 10 min at 15,000 × g. The absorbance of the digested azocasein fragments in the supernatant was measured at 540 nm. The change of absorbance with time was monitored and compared to a control reaction with no added enzyme.

RESULTS

Molecular Characterization of KSMP—In an attempt to establish unequivocally the molecular identity of KSMP, we screened several solubilization and purification steps and developed a rapid and efficient method for the purification of KSMP to homogeneity. In this screening, the KSMP activity was monitored by the specific and limited degradation of the catalytic subunit (C) of PKA (1). BBM from rat kidney were purified, using the procedure of Evers et al. (32), based on a precipitation of the membranes by Cu²⁺ ions. This procedure includes seven consecutive centrifugation steps and yields a brush-border membrane preparation enriched with KSMP (data not shown). Solubilization of the purified membranes with 1% octyl-β-D-glucopyranoside (w/v) was found to bring most of the KSMP activity into solution, together with some other membrane proteins. In an attempt to establish optimal conditions for the preservation of the KSMP activity in solution, we compared several detergents in their ability to do so. These included octyl-β-D-glucopyranoside, Nonidet P-40, Triton X-100, Lubrol WX, sodium deoxycholate, Tween 20, Tween 80, and CHAPS. A crude octyl-β-D-glucopyranoside extract of rat renal BBM was dialyzed against a buffer containing no detergent; then one of the detergents mentioned above was added, to a final concentration of 0.1% (w/v), and the KSMP activity was measured as indicated under “Materials and Methods.” Removal of the 1% (w/v) octyl-β-D-glucopyranoside by dialysis against a buffer that contains no detergent leads to a reduction in the KSMP activity, which is optimally restored if the buffer is supplemented with 0.1% (w/v) octyl-β-D-glucopyranoside (data not shown). Therefore, we used it routinely for the preservation and assay of KSMP.

Purification of KSMP—Following the solubilization of KSMP, the purification procedure consisted of two chromatographic steps: metal affinity chromatography on chelating Sepharose with immobilized Cu²⁺ ions (Fig. 1A) and chromatography on a DEAE-Sepharose anion exchange column (Fig.
KSMP activity were pooled and further purified by chromatography on a DEAE-Sephacel column (Fig. 1, lane 6). The crude octyl-β-D-glucopyranoside extract of the membranes; lane 3, wash-through from the chelating Sepharose column; lane 4, material eluted by histidine from the chelating Sepharose column; lane 5, Pool I from the DEAE-Sephacel column; lane 6, Pool II from the DEAE-Sepahce column. The lane marked C in panel B shows the position of the intact C subunit (no cleavage).

The crude octyl-β-D-glucopyranoside extract obtained from the brush-border membranes was applied on the chelating Sepharose column. The majority of the loaded proteins were tightly bound to the resin even after extensive washing. It was subsequently eluted with 20 mM histidine (Fig. 1A). Gradient elution with histidine did not result in any better resolution of the 85-kDa band and the presence of the specific KSMP activity was observed in all the KSMP purifications carried out by the method described above. As seen in Table I, the overall purification factor obtained was only 176-fold (6.8% yield) when measured by the increase in KSMP specific activity. It should be noted, however, that the purification factor of the KSMP protein (measured with specific anti-KSMP antibodies raised against distinct sequences in KSMP) is significantly higher (data not shown), suggesting that while the processes of solubilization and chromatography were systematically optimized, they still damaged somewhat the catalytic efficacy of KSMP.

Sequence Analysis of the Protein Bands Present in the KSMP Preparation—To determine the sequence of the protein(s) responsible for the KSMP activity, we excised the 85- and 75-kDa protein bands from the SDS-PAGE (Fig. 2, lane 6), eluted them, and subjected them to extensive proteolysis with trypsin. The resulting tryptic peptides were subjected to reversed phase HPLC (data not shown). Several peptides were obtained from the 85-kDa protein band, and four of them were rechromatographed and then subjected to sequence analysis. The following results were obtained (yield in picomoles is given in parentheses): Peptide 1, Met (48) Asp (48) Phe (23) Ser (10) Asp (15) Tyr (4); Peptide 2, Glu (93) Tyr (76) Thr (13) Ala (45) Gly (28) His (19) Gln (21) Asp (21) Gly (12) Val (13) Leu (10) Thr (12) Gln (7) Arg (3); Peptide 3, Val (65) Leu (47) Tyr (27) Pro (25) Lys (4); and Peptide 4, Val (28) Gly (117) Val (118) Gln (81) Ala (66) Phe (71) Phe (75) Pro (53) Asn (41) Gly (17) Thr (54) Gln (23) Ser (5) Phe (17) Arg (17). On the basis of these results and a database search, an unambiguous identity between the sequence of these peptides and stretches in the β subunit of rat meprin (25) (access number M88601 in GenBank™/EMBL Data Bank) was established (Fig. 3). It should be noted that the amino acid residue in meprin β preceding each of the sequenced peptides was either an arginine or a lysine residue, as expected for tryptic cleavage sites. In view of these results, we concluded that the 85-kDa protein band is, most likely, the β subunit of rat meprin.

A similar tryptic cleavage was carried out on the 75-kDa band. Again, four peptides were sequenced, and a data base search was carried out. The major peptide obtained (GDPGNSTDGILYLDI) was identical with a stretch (positions 409–423) in the α subunit of rat meprin (21) (EMBL data base accession number S43408). The second peptide (MTDDNYSYLDPRSK) matched with the primary structure of meprin β subunit at positions 524–538, suggesting that the β subunit of meprin may undergo proteolysis either in vivo or in the course of purification. The third and fourth peptides (YSDELHDPPT and TMFGYGLSFIQEDF) were identified as positions 345–354 and 395–409, respectively, in the rat L-type neutral amino acid transporter (access numbers A41785 and A45263 in the NBRF Data Bank). The presence of this protein in the KSMP preparation is probably due to a nonspecific copurification, although a possible meaningful interaction between this protein and meprin β cannot be completely ruled out. Since the 85-kDa band was present in all the KSMP preparations we obtained so far, we assumed that the β subunit of meprin could be responsible for the KSMP activity. However, the possible

**Fig. 2.** SDS-PAGE analysis of the protein content (panel A) and of the C-degradation activity (panel B) obtained at the various steps of purification of KSMP. Monitoring of protein content and of C-degradation were carried out by Coomassie Blue staining. Lane 1, brush-border membranes; lane 2, 1% (w/w) octyl-β-D-glucopyranoside extract of the membranes; lane 3, wash-through from the chelating Sepharose column; lane 4, material eluted by histidine from the chelating Sepharose column; lane 5, Pool I from the DEAE-Sephacel column; lane 6, Pool II from the DEAE-Sepahce column. The lane marked C in panel B shows the position of the intact C subunit (no cleavage).

1B). The crude octyl-β-D-glucopyranoside extract obtained from the brush-border membranes was applied on the chelating Sepharose column. The majority of the loaded proteins were excluded (Figs. 1A and 2A, lane 3). However, KSMP remained tightly bound to the resin even after extensive washing. It was subsequently eluted with 20 mM histidine (Fig. 1A). Gradient elution with histidine did not result in any better resolution of the KSMP activity from the other proteins (data not shown). The eluted fractions possessing KSMP activity (Fig. 2) were pooled and further purified by chromatography on a DEAE-Sephacel column (Fig. 1B). Application of an NaCl gradient to this column yielded two protein peaks, one of which possessed KSMP activity (Fig. 1B) and contained two protein bands (75 and 85 kDa). Measuring the KSMP activity in the emerging fractions along this peak suggested that the C-degrading activity correlates with the presence of the 85-kDa band, since pool II (Fig. 1B), which contained more of this band, had a higher KSMP activity (Fig. 2, A and B, compare lanes 5 and 6). In more quantitative terms, pool II had a higher specific activity (Table I). It should be mentioned that the pool II preparation did not possess either amino- or carboxy-peptidase activities described in the literature (14, 40) (data not shown). The KSMP preparation comprising pool II was used for further structural analysis of the enzyme. Although the relative amounts of the 75- and 85-kDa bands varied in different preparations of KSMP, the correlation between the occurrence of the 85-kDa band and the presence of the specific KSMP activity was observed in all the KSMP purifications carried out by the method described above. As seen in Table I, the overall purification factor obtained was only 176-fold (6.8% yield) when measured by the increase in KSMP specific activity. It should be noted, however, that the purification factor of the KSMP protein (measured with specific anti-KSMP antibodies raised against distinct sequences in KSMP) is significantly higher (data not shown), suggesting that while the processes of solubilization and chromatography were systematically optimized, they still damaged somewhat the catalytic efficacy of KSMP.

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The cleavage of C by KSMP or by meprin β can be made to proceed to completion. In most experiments, we monitor initial rates to secure linearity (cf. Fig. 2B). It should be noted, however, that since the assay is carried out in the presence of a detergent (to keep KSMP soluble) and since denatured C is not cleaved by KSMP, an incomplete cleavage is sometimes observed in assays involving a prolonged incubation as in Fig. 6A.
anti-peptide antibodies designed to be specific to each of these subunits. To tackle this question, we prepared the preparation of subunit-specific anti-peptide antibodies. This approach allowed us to identify stretches in their primary structure that share no sequence homology. These stretches (Fig. 4) indicate amino acid residues that within the sequence of peptide 4 indicates amino acid residues that were identified but their yield could not be determined quantitatively.

Involvement of the α subunit of this enzyme could not be excluded at this stage. To tackle this question, we prepared anti-peptide antibodies designed to be specific to each of these subunits.

**Immunoclonal Identification of the α and β Subunits of Meprin in KSMP Preparations**—A comparison of the sequence of the α and β subunits of meprin enabled us to identify stretches in their primary structure that share no sequence homology. These stretches (Fig. 4C), which were distant from their pro-sequences (and thus would not be removed upon maturation of the enzymes (41)) were chosen as idiotypes for the preparation of subunit-specific anti-peptide antibodies. Both the crude extract of rat brush-border membranes and purified KSMP preparations were found to contain proteins that are recognized by these anti-meprin antibodies; the anti-α subunit antibodies cross-reacted with the 75-kDa band (Fig. 4A, lanes 1–3), whereas the anti-β subunit antibodies detected the 85-kDa band (Fig. 4B, lanes 1–3). It should be noted that (a) the prominence of the 85-kDa band in these preparations was roughly proportional to their relative KSMP activity, further implicating the β subunit of meprin in this activity, and (b) the results in panels A and B suggest that during the purification of KSMP both the α and the β subunits of meprin presumptively undergo proteolytic degradation, as indicated by the presence of protein bands of lower molecular mass, which cross-react with the specific anti-α and the anti-β subunit antibodies (Fig. 4A, lane 2, and B, lanes 2 and 3). It should be noted that the specificity of the anti-meprin antibodies toward α and β subunits is quite sharp, as illustrated by the immunostaining of a crude extract from cells expressing the β subunit of meprin (see below). Thus, anti-β subunit antibodies specifically recognize the 105-kDa band of the meprin β subunit precursor in a crude membrane preparation of meprin β expressing cells (Fig. 4B, lane 4), whereas anti-α subunit failed to detect any cross-reactive material in this preparation (Fig. 4A, lane 4).

**Cloning of Meprin β**—To demonstrate unequivocally that the involvement of the α subunit of this enzyme could not be excluded at this stage. To tackle this question, we prepared anti-peptide antibodies designed to be specific to each of these subunits.

**Kinase A Cleavage by KSMP Reproduced by Meprin β**

| Purification step | Protein | Total activity* | Specific activity | Purification fold | Yield of activityb |
|-------------------|---------|----------------|------------------|------------------|------------------|
|                   | mg      | units          | units/mg         |                  |                  |
| Crude homogenate  | 1600    | 5100           | 2.6              | 1                | 100              |
| Brush-border membranes | 102 | 3998           | 39               | 15               | 78               |
| 1% Octyl-glucoside extract | 46 | 5607           | 122              | 47               | 110              |
| Chelating agarose column | 3.2 | 744            | 233              | 90               | 14.6             |
| DEAE-Sephacel column |        |                |                  |                  |                  |
| Pool I            | 1.8     | 98             | 54               | 176              | 6.8              |
| Pool II           | 0.8     | 345            | 455              |                  |                  |

* 1 unit of the KSMP activity was defined as amount of the enzyme degrading 1 μg of the catalytic subunit of PKA per min at 22 °C under the standard assay conditions.

b KSMP activity in the 1% octyl-β-D-glucoside extract was taken as 100%.

**Fig. 3. Alignment of the tryptic peptides obtained from the 85-kDa band of the purified KSMP preparation (Fig. 2A, lane 6) with amino acid sequences in the β subunit of meprin. Numbers indicate positions of the amino acid residues in the sequence of meprin. The question mark in Peptide 2 indicates a sequence cycle where the amino acid residue could not be determined unequivocally. The asterisk within the sequence of peptide 4 indicates amino acid residues that were identified but their yield could not be determined quantitatively.**

**Fig. 4. Western blot analysis of different KSMP preparations with the anti-peptide antibodies against the α (panel A) or the β subunit (panel B) of meprin. Panel C describes the sequences of the synthetic peptides that were chosen for raising specific anti-meprin α and anti-meprin β antibodies, since they are characteristic of each subunit (no homologous sequences in the companion subunit). Hatched and gray boxes indicate (respectively) the positions of the signal peptides and pro-sequences. Lane 1, 1% (w/w) octyl-β-D-glucopyranoside extract of a membrane preparation from the mammalian cells expressing the meprin β subunit.**
Kinase A Cleavage by KSMP Reproduced by Meprin β

In Vitro Translation of the Meprin β Subunit—In an attempt to establish an expression system in which we could check the functional relationship between KSMP and meprin β, we translated the meprin coding sequence in a rabbit reticulocyte lysate in the presence of CCM. The translation mixture was subjected to centrifugation, and the translated meprin was found in the membranal fraction only (Fig. 5, lane 4) and not in the supernatant (Fig. 5, lane 3). The rabbit reticulocyte lysate translation system, supplemented with CMM, apparently provides essential post-translational modifications and a correct targeting of meprin to the membrane. However, the meprin β obtained in this system still failed to display the characteristic KSMP activity, i.e. the specific cleavage of the C subunit of PKA (data not shown).

A Stable Expression of the Meprin β Subunit—In an attempt to find out whether meprin β is responsible for the KSMP activity originally observed in BBM (1–3), we undertook to express meprin β in mammalian cells and to find out whether it possesses KSMP activity. A pcDNA-3/meprin β construct carrying a geneticin resistance as a selective marker was introduced into the human embryo kidney cell line called 293. Transfected cells were selected by growing them on a selective medium containing geneticin sulfate (G418). As many as 50 of the geneticin-resistant clones were assayed for the specific KSMP activity using the catalytic subunit of PKA as a substrate (1, 8), and the degradation of C into clipped C′ was monitored with a specific set of anti-C antibodies described elsewhere (8). The clone with the highest C-degrading activity was selected (further referred to as the β subunit-expressing clone) and used in this study. Three types of antibodies were used for monitoring the clipping of C. They are referred to as anti-C and used for monitoring the clipping of C. They are referred to as anti-C-terminus (C-terminus) and anti-C-terminus (C-terminus) (8). The anti-peptide antibodies raised against the P-1 and P-2 peptides recognize both the intact C and the clipped C′ (Fig. 6A, Panel A, lanes denoted Control), or with the same cells after transfection with the meprin β subunit/pcDNA 3 construct, and thus expressing meprin β (lanes denoted Express.). The cleavage (if any) was allowed to proceed for 0 or 18 h at 22°C. The samples were subjected to SDS-PAGE, transferred to nitrocellulose paper, and stained with the indicated anti-peptide antibodies (αP-1, αP-2, and αP-3 panels). Panel B, location of the epitope-specific anti-C subunit antibodies used here for the detection of KSMP-like activity. Gray rectangles indicate the positions of the peptide epitopes P-1, P-2, and P-3 (see also Ref. 8 and “Materials and Methods” for further details). The position of the KSMP cleavage site in the C subunit sequence is marked by an arrow.

A Stabilization of the Meprin β Subunit—In an attempt to find out whether meprin β is responsible for the KSMP activity originally observed in BBM (1–3), we undertook to express meprin β in mammalian cells and to find out whether it possesses KSMP activity. A pcDNA-3/meprin β construct carrying a geneticin resistance as a selective marker was introduced into the human embryo kidney cell line called 293. Transfected cells were selected by growing them on a selective medium containing geneticin sulfate (G418). As many as 50 of the geneticin-resistant clones were assayed for the specific KSMP activity using the catalytic subunit of PKA as a substrate (1, 8), and the degradation of C into clipped C′ was monitored with a specific set of anti-C antibodies described elsewhere (8). The clone with the highest C-degrading activity was selected (further referred to as the β subunit-expressing clone) and used in this study. Three types of antibodies were used for monitoring the clipping of C. They are referred to as anti-C and anti-C-terminus (C-terminus) and anti-C-terminus (C-terminus) (8). The anti-peptide antibodies raised against the P-1 and P-2 peptides recognize both the intact C and the clipped C′ (Fig. 6A, Panel A, lanes denoted Control), or with the same cells after transfection with the meprin β subunit/pcDNA 3 construct, and thus expressing meprin β (lanes denoted Express.). The cleavage (if any) was allowed to proceed for 0 or 18 h at 22°C. The samples were subjected to SDS-PAGE, transferred to nitrocellulose paper, and stained with the indicated anti-peptide antibodies (αP-1, αP-2, and αP-3 panels). Panel B, location of the epitope-specific anti-C subunit antibodies used here for the detection of KSMP-like activity. Gray rectangles indicate the positions of the peptide epitopes P-1, P-2, and P-3 (see also Ref. 8 and “Materials and Methods” for further details). The position of the KSMP cleavage site in the C subunit sequence is marked by an arrow.

In Vitro Translation of the Meprin β Subunit—In an attempt to establish an expression system in which we could check the functional relationship between KSMP and meprin β, we translated the meprin coding sequence in a rabbit reticulocyte lysate in the presence of CCM (Fig. 5, lane 2), which provide the components necessary for post-translational modifications such as glycosylation. Indeed, under these conditions the translated protein band exhibited a shift in its molecular mass (from 80 to 105 kDa), in accordance with the previously observed increase in molecular mass of meprin subunits following glycosylation (26, 43). Concurrent with this assumption, the meprin β translated in the presence of CCM was found now to translocate to the membranes; when the CMM fraction from the translation mixture was subjected to centrifugation, the translated meprin was found in the membranal fraction only (Fig. 5, lane 4) and not in the supernatant (Fig. 5, lane 3). The rabbit reticulocyte lysate translation system, supplemented with CMM, apparently provides essential post-translational modifications and a correct targeting of meprin to the membrane. However, the meprin β obtained in this system still

KSMP activity can be attributed to meprin β, we cloned and expressed this protein. Total RNA was isolated from rat kidney and used for cDNA synthesis. The cDNA obtained as a result of a reversed transcription was used as a template for PCR amplification of the coding sequence of meprin β. A PCR was run, using primers specific to the 5′- and 3′-non-coding regions of the β subunit gene, and the product was cloned into a pcDNA-3 expression vector. The general integrity of the cloned meprin β gene was ascertained by its in vitro translation in a rabbit reticulocyte lysate system (Fig. 5, lane 1), where one major band with an apparent molecular mass of 80 kDa was detected. This band had a somewhat lower molecular mass compared with the molecular mass of KSMP (85 kDa) when purified from rat kidney (see above). Therefore, we assumed that the lower molecular mass of the meprin β subunit band may be due to the fact that in this expression system, post-translational modification(s) such as glycosylation, which are known to occur with meprins (42), do not take place. The exact nucleotide sequence of the β subunit cloned gene was confirmed by direct sequencing. No mismatches that could have been introduced by PCR were found.

FIG. 5. Autoradiogram of an SDS-PAGE analysis of the products formed upon in vitro translation of the meprin β subunit gene in a rabbit reticulocyte lysate in the presence of [35S]methionine. The translation was performed either in the absence (lane 1) or in the presence of CMM. The reaction mixture translated in the presence of CMM was applied on the gel as such (lane 2), or after centrifugation to separate the supernatant (lane 3) from the pellet, which contained the CMM (lane 4). The position of molecular weight markers are shown alongside the gel. Note that (a) after translation in the lysate alone (no post-translational modifications such as glycosylation), the products remain in solution, and the major protein band has an apparent molecular mass of ~80 kDa; (b) if the translation is carried out in the presence of CCM (which allows glycosylation), the product has an apparent molecular mass of ~105 kDa; and (c) the glycosylated product is now in the pellet, presumably as a result of its targeting to the membrane.

FIG. 6. Immuno-identification of a KSMP-like C subunit-degrading activity in the lysate of the cells expressing the rat meprin β subunit, using anti-peptide antibodies that specifically recognize the amino terminus (αP-1) and the carboxyl terminus (αP-2 and αP-3) parts of the C subunit. Panel A, immunostaining of the C subunit preparation incubated with KSMP purified from rat kidney (lanes denoted KSMP), with the pcDNA 3 transfected cell line (lanes denoted Control), or with the same cells after transfection with the meprin β subunit/pcDNA 3 construct, and thus expressing meprin β (lanes denoted Express.). The cleavage (if any) was allowed to proceed for 0 or 18 h at 22°C. The samples were subjected to SDS-PAGE, transferred to nitrocellulose paper, and stained with the indicated anti-peptide antibodies (αP-1, αP-2, and αP-3 panels). Panel B, location of the epitope-specific anti-C subunit antibodies used here for the detection of KSMP-like activity. Gray rectangles indicate the positions of the peptide epitopes P-1, P-2, and P-3 (see also Ref. 8 and “Materials and Methods” for further details). The position of the KSMP cleavage site in the C subunit sequence is marked by an arrow.
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Fig. 7. Effect of trypsin on the proteolytic activity of crude preparations of the meprin β expressed in the 293 cell line. The meprin sources used were either membranes from the transfected cell line (denoted Membranes) or an extract of these membranes obtained by solubilization with 1% (w/w) octyl-β-D-glucopyranoside (denoted O.G.Extract). Each of these preparations was treated with trypsin (+) for 60 min at 37 °C or incubated for the same time without trypsin (−) as a control. The proteolytic activity of these preparations was measured on two substrates: azocasein (panel A) or the C subunit (panel B). The activation ratio represents the ratio between the activity obtained after trypsin treatment and the activity obtained in the corresponding control (no trypsin), which was taken as 1.

18 h is immunochemically identical to the C'-form produced upon cleavage of C by KSMP purified from rat kidney (Fig. 6, panel A, lanes containing KSMP).

**Meprin β Expressed in Mammalian Cells Requires a Different Tryptic Activation to Reveal Its KSMP and Its Azocaseinolytic Activities—**The KSMP (C-degrading) activity found in the clone that expresses the meprin β subunit was extremely low compared with that of KSMP isolated from rat kidney. Since it was shown before that both the α and β subunits of meprin are synthesized as inactive precursors when transiently expressed in either 293 (44) or COS-7 (45) cell lines, we studied the effect of trypsin on the activation of KSMP preparations obtained from rat kidney (data not shown), and on preparations of meprin β expressed in the 293 cell line. Both the azocaseinolytic and the C subunit-degrading activities were significantly enhanced upon trypsinolysis of the meprin β expressed in the 293 cell line (Fig. 7, panels A and B). However, essentially no enhancement in either the azocaseinolytic or the C subunit-degrading activity was observed in the rat kidney BBM or in the octyl-β-D-glucopyranoside extract of BBM (data not shown). Interestingly, the trypsinolysis affected differently the C subunit-degrading activity and the azocaseinolytic activity of meprin β, depending on whether meprin β was membrane-bound or solubilized. The activation of the solubilized meprin β toward azocasein was about 6-fold higher than the activation of the membrane-bound meprin β (Fig. 7A). However, the enhancement of the KSMP activity of membrane-bound meprin β by trypsinolysis was 16-fold higher than the enhancement in activity of its solubilized form (Fig. 7B). This major difference in activation of the membrane-bound (immobile) versus the detergent-solubilized (mobile) enzyme might be due to a difference in accessibility, and thus in the susceptibility of these two forms of meprin β to proteolysis by trypsin, and to a difference in the distinct proteolytic processing of meprin β, which is required for optimal cleavage of each one of these two substrates. In other words, these results suggest that differently clipped forms of meprin β may be responsible for the cleavage of C and for the cleavage of azocasein.

**DISCUSSION**

Since the various protein kinases share a homologous catalytic core (residues 40–300 in PKA) (46–49) but differ in their individual segments located outside this core, it seems reasonable to assume that the function of these end segments is associated with the individual regulation or substrate recognition of each kinase. Therefore, a proteolytic enzyme such as KSMP, which specifically inactivates PKA by clipping off its tail segment, may play a regulatory role in systems controlled by this kinase.

For several years now, we have been studying the role of such an end segment in PKA. KSMP was discovered in the course of these studies (1, 2). It attracted our attention not merely because it selectively inactivates PKA but also because of the specificity with which it clips off the carboxyl terminus tail of the C subunit of PKA. In addition, KSMP was found to clip off the carboxyl terminus tail of two other important kinases: the epidermal growth factor receptor kinase (9) and the insulin receptor kinase (10). In all three kinases, the cleavage was shown to be conformation-dependent (2, 7, 9, 10).

The KSMP activity (defined as the ability to clip off the carboxyl-terminal tail of the C subunit of PKA) was biochemically characterized and functionally identified in brush-border membranes of the small intestine of the rat, mouse, rabbit, and guinea pig and in the kidney of the rat, mouse, and guinea pig (3). Assayed by the above mentioned criterion, KSMP was previously shown to be a neutral endopeptidase, with a pH optimum between 6.8 and 7.6, and to be inhibited by chelating agents such as EDTA and EGTA. Upon removal of metal ions with EDTA and reconstitution with various ions, Zn²⁺ was shown to optimally restore the proteolytic activity of the enzyme, suggesting that KSMP is a metalloenzyme and that Zn²⁺ is most likely the native cofactor of the enzyme (3). Interestingly, KSMP was not inhibited by phosphoramidon, a very efficient competitive inhibitor for the neutral metallo-endopeptidase 24.11 (EC 3.4.24.11) found in brush-border membranes of the small intestine and of the kidney (14), excluding the possibility that KSMP is identical with this endopeptidase.

With the subsequent discovery in brush-border membranes of a phosphoramidon-insensitive neutral endopeptidase which, in different laboratories was named meprin (50, 51), endopeptidase-2 (39, 52), or PABA peptide hydrolase (53, 54), we suspected that KSMP might be identical with this peptidase, as they had some biochemical properties in common. To test this possibility, we obtained a sample of mouse meprin-a (27) (which is now known to be a heterotetramer composed of both α and β subunits of meprin (20, 26)) and some anti-meprin-a antibodies (kindly provided by Dr. Judith S. Bond, then at the Virginia Commonwealth University). We found that while the meprin-a sample we obtained did act on α-casein, it had no KSMP (C subunit-cleaving) activity, and while the anti-meprin-a antibodies stained very well this meprin-a preparation on a Western blot, we could not establish a correlation between...
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KSMP activity and the extent of staining by these antibodies. To get a deeper insight into the possibility that a KSMP-like activity may be involved in the regulation of PKA (and possibly other protein kinases), we undertook (a) to elucidate the functional role of the carboxyl-terminal tail of the C subunit; (b) to prepare specific tools for the detection of a KSMP-like activity in various tissues and subcellular organelles; (c) to determine the sequence of KSMP and use it for the preparation of anti-peptide antibodies against specific segments of this enzyme associated with its C-degrading activity; and (d) to clone and express this proteinase and thus to set the stage for the preparation of mutants with deletions, truncations, and site-directed mutations to assess the relationship between the structure of such mutants and their function as specific kinase-splitting proteinases.

Along these lines, we have prepared a set of antibodies against the head and the tail end segments of the C subunit, with which it is possible to tell apart C from C′, and thus to monitor a KSMP activity (8). One of the antibodies was shown to impede the KSMP cleavage of C to C′ (8). These antibodies will hopefully enable us to identify KSMP activity in different tissues. Most importantly, we devised a rapid and efficient method for purifying and sequencing KSMP to establish unequivocally its molecular identity. This paper provides evidence suggesting that the sequence of the protein associated with KSMP activity is identical with that of the β subunit of meprin. It reports the preparation of specific anti-peptide antibodies against the α and the β subunits of meprin and describes the stable expression of the β subunit of meprin in a mammalian cell line (293) and the establishment of a clone of these cells that constitutively expresses the full-length precursor of meprin β. It also describes the proteolytic activation of this precursor leading to a significant enhancement of caseinolytic and of KSMP (C subunit-clipping) activities. Interestingly, we show that these two activities of the precursor are distinctly different, namely that the conditions that bring about an optimal activation ratio monitored by caseinolytic activity yield a very low activation ratio monitored by KSMP activity and vice versa. This last finding might explain why we could not reproduce at the time the KSMP activity with the meprin-a preparation obtained by the method described by Macadam et al. (15), which used papain for the solubilization of the enzyme from the membrane, since (as shown here) the precise conditions used for the activation of the meprin β precursor determine whether it will yield an optimal KSMP or an optimal caseinolytic activity. In addition, this finding emphasizes the importance of using the appropriate substrate when establishing a procedure for precursor activation.

It is hoped that the sequence-specific anti-KSMP antibodies described here will allow a direct localization of KSMP-like enzymes, where and when KSMP is present in an inactive form. The ability to detect inactive yet activable species of an enzyme is of particular importance since, by nature of their physiological assignment, regulatory enzymes have to be present in a latent form until called into action. The combination of detection by activity and detection by physical presence is also useful for the identification of natural inhibitors of KSMP or for the characterization of the physiological circumstances under which KSMP may be turned on.
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