Unveiling the Substrate Specificity of Meprin β on the Basis of the Site in Protein Kinase A Cleaved by the Kinase Splitting Membranal Proteinase*

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The kinetic splitting membranal proteinase (KSMP) is a metalloendopeptidase that inactivates the catalytic (C) subunit of protein kinase A (PKA) by clipping off its carboxy terminal tail. Here we show that this cleavage occurs at Glu328-Glu334, within the cluster of acidic amino acids (Asp328-Glu334) of the kinase. The $K_m$ values of KSMP and of meprin β (which reproduces KSMP activity) for the C-subunit are below 1 μM. The $K_m$ values for peptides containing a stretch of four Glu residues are in the micromolar range, illustrating the significant contribution of this cluster to the substrate recognition of meprin β. This conclusion is supported by a systematic study using a series of the C-subunit mutants with deletions and mutations in the cluster of acids. Hydrophobic amino acid vicinal to the cleavage site increase the $K_{cat}$ of the proteinase. These studies unveil a new specificity for meprin β, suggesting new substrates that are 1–2 orders of magnitude better in their $K_m$ and $K_{cat}$ than those commonly used for meprin assay. A search for substrates having such a cluster of acids and hydrophobics, which are accessible to meprin under physiological conditions, point at gastrin as a potential target. Indeed, meprin β is shown to cleave gastrin at its cluster of five glutamic acid residues and also at the M-D bond within its WMDF-NH$_2$ sequence, which is indispensable for all the known biological activities of gastrins. The latter meprin cleavage will lead to the inactivation of gastrin and thus to the control of its activity.

The presence of a kinase splitting membranal proteinase (KSMP)$^*$ in the brush-border membranes of the rat small intestine was demonstrated as early as 1979 (1). This proteinase was shown to clip the catalytic (C) subunit of PKA, yielding a distinct cleavage product (C') that was found to be devoid of the kinase activity. The biochemical characterization of KSMP as a proteinase revealed that it is an intriguing enzyme with a combination of the following unique features. (a) KSMP cleaves the C-subunit when it is free but not when inhibited by its regulatory (R) subunits, as in the R$_2$C$_n$ complex (1, 2). (b) This cleavage could not be simulated by other proteinases (trypsin, chymotrypsin, clostripain, and papain (2)), suggesting that its specificity is not due merely to an interdomain exposure in C. (c) The proteinase was found to single out and selectively cleave the C-subunit in the presence of the large number of other proteins found in crude extracts of different tissues (brain, liver, or muscle) (3). (d) KSMP was found to cleave the C-subunit in its native conformation but not if the kinase is pre-denatured (2). (e) It distinguishes between the “open” and “closed” conformations of the C-subunit (4) that were recently identified by x-ray crystallography of this kinase (5).

The cleavage of the C-subunit by KSMP leads to the removal of the carboxy terminus tail of this kinase and seemed to occur at a distinct site (6–8). Interestingly, two other kinases, the EGF- and the insulin-receptor kinases (which share certain sequence homology with the C-subunit (9)) were also shown to undergo a specific and conformation-dependent cleavage by KSMP (6, 10–12). In both receptor kinases, it was shown that the KSMP cleavage occurs at the carboxy-terminal part of the molecules. The specific and restricted character of the KSMP cleavage of C, as well as the EGF- and the insulin-receptor kinases, suggested the existence of a common structural motif that is recognized by the proteinase. Indeed, inspection of the primary sequences of the three kinases revealed that they share a stretch of acidic amino acid downstream from their common protein kinase core, raising the possibility that this stretch is an important biorecognition element for KSMP. This suggestion was supported by three additional findings. (i) The polyglutamic acid effectively inhibits the KSMP cleavage of the C-subunit ($K_i = 6 \mu M$) (7). (ii) The monoclonal antibodies against a branched polyamino acid with exposed clusters of Glu cross react with the C-subunit but not with its KSMP cleavage product (C') while a monoclonal anti-idiotype of these antibodies specifically binds to the active site of KSMP and inhibits it (7). (iii) The immunochemical mapping of the C-subunit with epitope-specific antibodies narrowed down the cleavage site location to the short region accommodating the cluster of acidic residues in C, i.e. Asp$^{328}$-Glu$^{334}$ (8).

We have recently shown that C-degrading activity of KSMP can be reproduced by the β-subunit of rat meprin (13). Meprin is a membranal metalloendoproteinase found in the intestinal and renal brush-border membranes of the mouse (14, 15), rat (16–18), and man (19, 20). Meprins belong to the astacin family of endopeptidases (21–24) and are usually composed of two types of subunits, α and β, that exist as homo- and heterotetramers bound to each other through disulfide bridges (25, 26). In spite of a large number of studies on meprins, the substrate specificity and the physiological assignment are not established yet even though several suggestions have been set forth.
The demonstration that meprin β possesses a KSMP activity (13) raised the possibility that the specificity of this enzyme might be different from the one currently accepted. Furthermore, the protein and peptide substrates commonly used for meprins (27–30) may mislead the search for its physiological substrate. More, the protein and peptide substrates commonly used for meprin cleavage do not allow unbiased conclusions to be drawn concerning the specificity of this enzyme. The KSMP cleavage site in the C-subunit is an example of a peptide substrate cleavage that may not be physiological and may thus to the control of its activity.

MATERIALS AND METHODS

Purification and Assay of KSMP and of the Catalytic Subunit of PKA—The purification and assay of KSMP (from rat kidney) (13) and of the catalytic subunit of PKA (35) (from bovine heart) were carried out as described earlier.

Purification of Meprin β Precursor—The meprin β precursor was expressed in the 293 human embryo kidney cell line as reported earlier (13). The KSMP purification procedure described before (13) was applied for the purification of the expressed meprin β precursor, with some modifications. The meprin β expressing clone was grown on the line, fitted to the experimental values by the least square method, and \[ \frac{1}{v} = \frac{K_m}{V_{max}} + \frac{1}{S} \frac{1}{V_{max}} \]

where \( v \) is the rate of cleavage, \( K_m \) and \( V_{max} \) are Michaelis constants, and \( [S] \) is the initial concentration of the substrate. Briefly, the rate of cleavage was measured in each case by monitoring the formation of the cleavage products and then plotting the activity versus the initial substrate concentration in a double reciprocal plot. The line described the experimentally determined kinetic parameters by the least square method.

Cleavage of Synthetic Peptides—The kinetic constants \( K_m \) and \( V_{max} \) were measured as described by Cleland (38). A single substrate mechanism for the reaction, the relationship between the velocity of the reaction and the substrate concentration is given by the equation

\[ \frac{1}{v} = \frac{K_m}{V_{max}} + \frac{1}{S} \frac{1}{V_{max}} \]

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Cleavage of Synthetic Peptides—Cleavage of synthetic peptide substrates was performed in a 20 mM Tris-HCl buffer, pH 7.1, with an addition of 1.5 mM MgCl₂ and 0.1% octyl-D-glucopyranoside. Analytical scale reaction mixtures were 30 μl final volume, preparative scale reaction mixtures were 200 μl, and the minimal amount of a peptide in the reaction mixture was not below 0.5 nmol. The peptide concentrations in the reaction mixtures ranged from 1 to 200 μM, and the reaction was allowed to proceed for a time interval within the linear region of dependence on time. The cleavage was arrested by adding trifluoroacetic acid to the reaction mixture to a final concentration of 0.5%, and then its volume was adjusted to 500 μl by trifluoroacetic acid and injected to a reverse-phase HPLC column (LiChroCART 125–4 LiChroSphere RP-18, Merck, Germany). A flow rate of 1 ml/min was used, and elution of the bound material was achieved using a linear gradient of acetonitrile. The effluent was monitored simultaneously at 210 and 280 nm with a diode-array detector (Hewlett-Packard). A quantitative version of the peak area into the amount of peptide in it was calculated from a calibration curve. A quantitative amino acid analysis of the eluted peaks was run along with measuring the area of the peaks. The enzyme activity was defined as micromole of product released by 1 mg of enzyme/1 min.

Construction of Mutants and Their Translation in a Cell-free System—The wild-type murine Cβ-subunit gene cloned into the pcDNA3 vector (Invitrogen) under the control of the T7 promoter was a generous gift from Dr. S. S. Taylor (University of California, San Diego). Site-directed mutations were introduced by oligonucleotide-directed mutagenesis of a uracil-containing single-stranded Kunkel template (37). The translation of the coding sequences was carried out in the TNT-coupled transcription/translation rabbit reticulocyte expression system (Promega), as recommended by the manufacturer, and per-
amino acids of this kinase, formed in the presence of \(^{35}\text{S}\) methionine (Amersham, UK). KSMP (7, 10, 11) and with our more recent immunological agreement with earlier experiments in our laboratory, which implicated the involvement of this cluster of acidic amino acids that was not positively identified (cysteine), whereas the two amino acids (Glu and Arg) that are not in bold were identified, but their yield was too low to be determined unequivocally (see also Table I for quantitative results of the sequence analysis).

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**RESULTS AND DISCUSSION**

**KSMP Cleaves the C-subunit at the E\(^{322}\)-E\(^{333}\) Bond within Its Cluster of Acidic Amino Acids**—To get an insight into the molecular basis of the specific cleavage of the C-subunit by KSMP, it was essential to determine exactly the bonds cleaved in the kinase by this proteinase. This recently became possible upon achieving a purification of KSMP to homogeneity (13).

To identify the exact sites of the KSMP cleavage in the C-subunit, we carried out this cleavage on a preparative scale, and then separated the cleavage products by reverse phase HPLC and sequenced them. Two new peaks were shown to be formed, a major and a minor peak (HPLC data not shown). In quantitative terms, the major peak was about 20-fold more abundant than the minor one. As seen in Table I, the major peak had the sequence EEIRVSINEK\(^*\)GKEFS resulting from a cleavage of the E\(^{322}\)-E\(^{333}\) bond within the cluster of acidic amino acids of this kinase, i.e. residues D\(^{328}\)-E\(^{334}\). This is in agreement with earlier experiments in our laboratory, which implicated the involvement of this cluster of acidic amino acids in the recognition of the C-subunit and of other kinases by KSMP (7, 10, 11) and with our more recent immunological mapping of the cleavage site with specific anti-peptide antibodies (8). The minor peak had the sequence DFLKK (Table I), which fits the stretch DFLKK in the amino-terminal region of the C-subunit (Fig. 1).

**KSMP and Meprin \(\beta\) Have Identical \(K_m\) and \(V_{max}\) values for the C-subunit**—In an attempt to gain further support to our finding that the KSMP cleavage of the C-subunit can be reproduced by meprin \(\beta\), we compared the \(K_m\) and \(V_{max}\) values of KSMP with meprin \(\beta\) in the cleavage of this kinase. A preparation of KSMP obtained from rat kidney and recombinant meprin \(\beta\) purified from transfected 293 cells (13) were used in this comparison. The cleavage was performed at different concentrations of the C-subunit as described under “Materials and Methods,” securing a linear dependence of the proteolysis with time and with the concentration of the proteinase. Each of the reaction mixtures was subjected to SDS-PAGE followed by Coomassie Blue staining. The extent of proteolysis was determined by computing densitometry of these gels, monitoring the amount of the clipped C-subunit (i.e. C’) formed. Plotting the KSMP (C-subunit-degrading) activity versus the concentration of the C-subunit in double reciprocal coordinates gave straight lines (Fig. 2) from which the \(K_m\) and \(V_{max}\) values were calculated. Average values for these \(K_m\) and \(V_{max}\) values were calculated from three independent experiments and were found to be \(K_m = 0.44 \pm 0.03 \mu\text{M}\) and \(V_{max} = 19 \pm 9 \text{nmol/min} \times \text{mg}\) for KSMP and were \(K_m = 0.58 \pm 0.07 \mu\text{M}\) and \(V_{max} = 22.8 \pm 2.7 \text{nmol/min} \times \text{mg}\) for the expressed meprin \(\beta\).

The Substrate Recognition of KSMP Analyzed by the Cleavability of the C-subunit Mutants—In order to evaluate the structural requirements of KSMP for its substrate recognition, we constructed a series of the C-subunit mutants, with deletions or substitutions in the cluster of acidic amino acids of the C-subunit encompassing its KSMP cleavage site. The first step toward this analysis was to set up an adequate expression system for monitoring the cleavage of the C-subunit and its mutants since we found out that the wild-type C-subunit itself, though fully active as a protein kinase (38), is not cleaved if expressed in bacteria (data not shown). The rabbit reticulocyte lysate translation system using \(^{35}\text{S}\) methionine was found to be appropriate for that purpose. The wild-type C-subunit produced in this system was cleaved

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**TABLE I**

Sequence of peptides released from the C-subunit by KSMP

The C-subunit was incubated with a KSMP preparation, and the resulted products were separated by reverse-phase HPLC.

| Cycle | Amino acid residue | Yield pmol |
|-------|-------------------|------------|
| Peptide 1 | E | 678.7 |
| 1 | E | 579.5 |
| 2 | R | 345.8 |
| 3 | S | 13.4 |
| 4 | I | 273 |
| 5 | N | 189.3 |
| 6 | E | 212.7 |
| 7 | E | 116.7 |
| 8 | I | 116.7 |
| 9 | K | 107.0 |
| 10 | K | 149.4 |
| 11 | K | 22.6 |
| 12 | F | 28.2 |
| 13 | S | 2.3 |

| Peptide 2 | D | 175.8 |
| 1 | F | 215.3 |
| 2 | L | 181.4 |
| 3 | K | 179.4 |
| 4 | K | 192.9 |

\(^a\) Amino acid residue that was identified, but the yield could not be determined quantitatively.

\(^b\) Amino acid residue that was not positively identified.
Fig. 2. Determination of kinetic parameters (K_m and V_max) of the cleavage of the C-subunit by KSMP and by meprin β. The cleavage was carried out at various concentrations of the C-subunit (from 0.33 to 4 μM) and monitored by the formation of the C’ (in nmol) per min by 1 mg of enzyme. The formation of the C’ was calculated from densitometric scans of the corresponding band on SDS-PAGE stained with Coomassie Blue. The activity versus initial concentration of the C-subunit was plotted in a double reciprocal plot, and the K_m and V_max values were calculated as described under “Materials and Methods.”

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The C-subunit expressed in E. coli is not myristylated and does not undergo the same post-translational modifications that occur in the mammalian enzyme. The lack of cleavage in spite of full catalytic activity is intriguing and is currently being studied in our laboratory.

Fig. 3. The effect of deletions and of single, double, and triple site mutations within the cluster of acidic amino acids in the C-subunit (see also Scheme 1) on their cleavability by KSMP. A, cleavage of the C-subunit mutants carrying deletions at the cluster of acidic amino acids. The C-subunit and the indicated mutants were translated in a rabbit reticulocyte lysate translation system in the presence of [35S]methionine. The cleavage was allowed to proceed for the indicated time periods as described under “Materials and Methods,” and the products (if any) were separated by SDS-PAGE. The protein bands were then visualized by autoradiography, and the resulting autoradiographs were subjected to densitometric scanning for quantitation. The initial rates of cleavage (measured in arbitrary units) were compared as a percentage of the initial rate of cleavage of the wild-type C-subunit (taken as 100%). B, the effect of substitutions by alanine of the various acidic amino acids in the cluster of acids on the cleavage of the the C-subunit mutant by KSMP. The cleavage and its quantitation were carried out as described in panel A.

Use of Synthetic Peptides Derived from the C-subunit to Establish the Substrate Specificity of KSMP—To complement our results on the specificity of KSMP that were based on the sequence of the cleavage products and on mutations of the C-subunit, we attempted to elucidate the recognition of KSMP by the use of synthetic peptides derived from the segment in the C-subunit that encompasses the KSMP cleavage site. We prepared four synthetic peptides whose sequences were derived from the following segments in the C-subunit: 1) K^319–Y^335, 2) P^327–I^335, 3) S^325–Y^330, and 4) Y^330–P^333 (Fig. 4). Each of these peptides was cleaved by a pure KSMP preparation, and the cleavage products were resolved by reverse-phase HPLC and analyzed by a determination of their amino acid composition or sequence. As seen in Fig. 4, KSMP cleaves the peptides between acidic amino acids in positions that correspond either to the E^332–E^333 bond, or to the D^328–D^329 bond in C. The cleavage
site of the peptides in the middle of the cluster of glutamic acid residues is identical to the KSMP cleavage site detected in the C-subunit and described above (E332-E333). The lack of cleavage of the D328-D329 bond may be due to steric hindrance, or an unfavorable juxtaposition of this D-D bond of the C-subunit and the KSMP active site. Interestingly, the three-dimensional structure of the C-subunit established by x-ray crystallography showed that, while the backbone of the cluster of acidic amino acids in the C-subunit exhibits relatively high B factors and thus a greater flexibility and availability (5), the D328-D329 bond may be less available for interaction with KSMP since D329 forms a salt bridge with K47 (39) that would neutralize the negative charge of D329 and also restrict its flexibility.

It should be mentioned that the peptides 319KGPGDTSNFPDDYEEEEEI335 and 325SNFDDY330, but not the peptide 327FDYEEEEEI335, were found to have an additional (minor) cleavage site between F327 and D328 (Fig. 4). The rate of this minor cleavage is at least ten-fold slower than the rate of cleavage between the two aspartic acid residues.

Cleavage of PKA by KSMP Unveils the Meprin β Specificity

For a more quantitative comparison between KSMP and meprin β, the kinetic parameters ($K_m$ and $K_{cat}$ values) of these two enzyme preparations were measured with a synthetic peptide (YEEEEI) containing a single cleavage site to simplify the measurements and their processing. As seen in Table II, the $K_m$ and $K_{cat}$ values of KSMP and meprin β with this substrate were found to be quite similar, further supporting our conclusion (13) that meprin β and KSMP are closely related, if not identical.

The Kinetic Parameters of Meprin β Obtained with Peptide Substrates Point to a New Specificity Profile for this Metalloendopeptidase—As a member of the astacin family of metalloendopeptidases, meprin β should have an arylamidase activity (24) and have a preference for bonds flanked by neutral or positive residues. However, the kinetic measurements on the synthetic peptides revealed a remarkable specificity in the meprin β for bonds adjacent to charged residues, suggesting a role in the processing of negatively charged peptides.
Comparison of kinetic parameters of different peptide substrates for meprin β and KSMP

| Peptide substrate | $K_m$ ($\mu$M) | $K_{cat}$ (mol/min/mg) | $K_{cat}/K_m$ | $V_{max}$ (mol/min/mg) |
|-------------------|----------------|------------------------|---------------|------------------------|
| LHRH$^{a,b}$     | 270            | 11.2                   | 4.1 x 10$^{-4}$ | 0.45 ± 0.05             |
| Substance P$^c$  | 86             | 5.0                    | 3.5 x 10$^{-4}$ | 1.20 ± 0.08             |
| Bradykinin$^c$   | 325            | 1.5                    | 0.5 x 10$^{-4}$ | 2.53 ± 0.10             |
| YEEIE (KSMP)     | 2 ± 0.2        | 0.6                    | 3.0 x 10$^{-4}$ | 0.45 ± 0.05             |
| YEEIE            | 4 ± 1.2        | 2.1                    | 5.2 x 10$^{-4}$ | 1.20 ± 0.08             |
| YEEIA            | 10 ± 2.1       | 4.3                    | 4.3 x 10$^{-4}$ | 2.53 ± 0.10             |
| AEEIE            | 7 ± 2.8        | 1.4                    | 20.4 x 10$^{-4}$ | 0.83 ± 0.05             |
| SNFDDY           | 56 ± 3.2       | 64.6                   | 115.0 x 10$^{-4}$ | 37.35 ± 9.50             |
| MEEIE (ME)       | 4 ± 1.6        | 0.4                    | 10.0 x 10$^{-4}$ | 0.28 ± 0.08             |
| EEEAY            | 75 ± 4.1       | 9.9                    | 13.2 x 10$^{-4}$ | 5.71 ± 1.85             |

$^a$ $V_{max}$ is expressed in mol of formed products in 1 min by 1 mg of enzyme.

$^b$ Calculated from Stephenson and Kenny (27).

$^c$ Luliberin (luteinizing hormone-releasing hormone).

The kinetic parameters data from different sources were reduced to the comparable values. Arrows in the sequences of peptides indicate the cleavage sites that were cleaved by KSMP from rat kidney (indicated in brackets), or by purified recombinant meprin β-subunit.

Meprin β for these acid peptides is significantly higher (12–100-fold, as reflected in its $K_{cat}/K_m$ values) than the comparative values obtained for the hormones LHRH, substance P, and bradykinin (Table II).

It should be noted that substitution of either the Tyr or the Ile residues by alanine in the peptide YE...E was observed with the C-subunit-derived peptides (Table II). The kinetic parameters of meprin β for the YEEEI and SNFDDY peptides are considerably distinct. The SNFDDY peptide was found to have a substantially reduced affinity ($K_m$ = 80 $\mu$M), which we believe is associated with a reduction of the overall charge of the cluster of acidic amino acids. However, due to an increasingly high rate of cleavage, the $K_{cat}/K_m$ ratio for SNFDDY is within the range determined for the high affinity substrates (Table II).

Is Gastrin a Physiological Substrate of Meprin β?—In view of the finding reported here that meprin β has a distinct preference for substrates containing a cluster of negatively charged amino acids, we carried out a search in the data base for peptides and proteins containing stretches of at least four acidic amino acid residues. This search revealed quite a few peptides and proteins with such stretches and, consequently, candidate substrates for meprin β. Among these, gastrin seemed to be of particular interest since it is found in the gastrointestinal tract and in the kidney where it can be exposed to meprin. Discovered in 1905 as an acid-stimulating factor (41), gastrin is now regarded also as an important growth-stimulating hormone (42). Gastrin occurs in multiple hormonal forms that are produced as a result of proteolytic processing and may contain from 71 to 6 amino acid residues (32, 43). Its most abundant forms (G-34 and G-17) contain a stretch of five glutamic acid residues (Fig. 7) (34). All known biological effects of gastrin reside in the conserved carboxyl-terminal tetrapeptide amide WDMD-NH$_2$, which is common to all gastrins and also to the cholecystokinins (Scheme 2B).

Analysis of the fragments resulting from gastrin cleavage by either KSMP or meprin β revealed proteolysis at three distinct sites (Fig. 7). Two adjacent cleavage sites were found within the cluster of glutamic acid residues. For the sake of simplicity, the kinetic parameters of the cleavages were measured on the gastrin fragments rather than on the whole molecule (Fig. 7). The first fragment, MEE...EAY, accommodated the two alternative cleavage sites identified in gastrin 17. The affinity of meprin β for this peptide was found to be very similar to that observed with the C-subunit-derived peptides (Table II). The
of the mouse prosequence of gastrin. The cleavage at the Met-Asp bond (which will inactivate gastrin) occurs at E332-E333, within the cluster of acidic amino acids (D328-E334) of this kinase. The Km for cleavage of the carboxyl-terminal fragment of gastrin, EEAYGWMD, resulted in clipping off its last two residues (cleavage at the Met-Asp bond). Since any modification in the carboxyl-terminal tetrapeptide amide WMDF-NH₂ grossly reduces or abolishes all its known biological effects (33, 34, 44), this cleavage will inactivate gastrins. Notably the Km for the cleavage at this site was found to be substantially higher than the Km for the cleavage at the Met-Asp bond within its WMDF-NH₂ sequence. However, the Km for this cleavage was much higher, and thus, it had a comparable Km/Km value (Table II). The decreased affinity for the EEAYGWMD peptide compared with the site in the cluster of glutamic residues was actually expected in view of the findings reported here regarding the important contribution of clustered acidic to the affinity of meprin β for its substrates.

It should be emphasized, however, that in spite of the fact that clusters of acidic are recognized and cleaved by meprin β with a significantly lower Km, hydrophobic amino acid residues most likely play an important role in the cleavage of physiological substrates by this proteinase. They do so with a high Km but also with a high Km and thus with a high catalytic efficacy. The evidence supporting the importance of hydrophobic amino acid residues is summarized in the following. (i) The protein substrate (C-subunit) with which KSMP was originally discovered (1–3), as well as the EGF- and insulin-receptor kinases (7, 10, 11), contain hydrophobic amino acid residues within or adjacent to their cluster of acidic amino acids. (ii) The dye 1-anilino-8-naphthalenesulfonate, which is known to bind to hydrophobic sites in proteins, inhibits the cleavage of the C-subunit by KSMP (45). (iii) While a copolymer composed of Glu and Tyr is cleaved by KSMP, a polymer of Glu amino acid residues alone is not cleaved by it and acts as a competitive inhibitor of this proteinase (7, 45). (iv) The hydrophobic proteinase inhibitor chymostatin inhibits the cleavage of the C-subunit by KSMP though at relatively high concentrations (≥ 10⁻³ M) (3). (v) In general, members of the astacin family of peptidases have been shown to cleave peptides containing hydrophobic amino acid residues and to possess an arylamidase activity (26).

It is, therefore, possible that the degradation of gastrin by meprin β may draw its affinity from the interaction of meprin β with the cluster of acidic (Kₚ = 1.5 µM) while the fast cleavage at the Met-Asp bond (which will inactivate gastrin)

may originate from the high Kₚ value (63.4 s⁻¹) for this cleavage. It is also possible that the cleavage at the cluster of acidic facilitates the subsequent evacuation of the active site.

The Prosequence of Meprin β Contains Seven Acidic and Seven Hydrophobic Amino Acid Residues within a Stretch of Twenty Acidic Amines and Differ from Meprin α in This Respect—Meprins are synthesized as inactive proenzymes whose prosequences are removed when they are called upon to act. One of the plausible mechanisms for an auto-inhibition of enzymes is the blocking of the active site by a substrate-like prosequence, which can then be removed for the purpose of activation by the enzyme itself or by another enzyme, in response to an appropriate regulatory stimulus. In view of the specificity profile described above for meprin β, we looked into its prosequence, searching for features related to the specificity ensuing from this study and for a difference between meprin β and meprin α that might reflect the known difference in specificity between these two subunits (24). At the same time, we attempted to find out whether, in view of the specificity implicated in this study, there may be evidence supporting or disproving an autoinhibition mechanism of the type described above. Scheme 2 illustrates such a comparison for the rat, mouse, and human prosequences of meprins α and β. From this comparison, it is evident that (i) the prosequence of meprin β possesses a stretch of acidic and hydrophobic amino acids (in line with the specificity profile described above). This is especially prominent in the stretch D⁵⁸-E¹⁷⁴, say in the rat, in which out of 20 amino acids, seven are acidic (mostly Asp), seven are hydrophobic (mostly Ile and Leu), and not one is a basic amino acid. (ii) The parallel stretch in promeprin α has six hydrophobics but only three acidics and one basic amino acid. (iii) Five of the acidic and hydrophobic residues in the β prosequence come in pairs of DI or DL, raising the possibility that this repeated motif may have a distinct inhibitory significance. These suggestions regarding the difference in specificity between the β and the α subunits of meprin, and regarding the molecular basis of their autoinhibition, will hopefully shed light from a new angle on the biorecognition of these metalloendopeptidases. However, their ultimate proof will have to rely on molecular modeling studies (based on the astacin structure (46)) and possibly to await the determination of the three-dimensional structures of these precursors by x-ray crystallography.

CONCLUSION

This paper provides evidence to show that the cleavage of KSMP in the C-subunit occurs at E¹⁸¹-E¹⁸⁲, within the cluster of acidic amino acids (D¹⁸⁸-E¹⁸⁹) of this kinase. The Km values of KSMP and of meprin β (which we recently showed to reproduce KSMP activity) for the C-subunit are below 1 µM. The Km for peptides containing a stretch of four Glu residues are in the micromolar range, suggesting a significant contribution of this cluster of acidics to the biorecognition of meprin β. This conclusion is supported by experiments with a series of C-subunit mutants with deletions and mutations in the cluster of acidides. In addition, hydrophobic amino acids vicinal to the cleavage site seem to play an important role in the function of this proteinase, increasing its Km.

These studies unveil a new specificity profile for meprin β, suggesting new candidate substrates that are 1–2 orders of magnitude better (lower in their Km and higher in their Km) than substrates commonly used for meprin β. Specifically, the search for substrates that have such a cluster of acidides and hydrophobics and are accessible to meprin β under physiological conditions, pointed at the hormone gastrin as a potential target. Indeed, we show here that, at least in vitro, meprin β cleaves gastrin at its cluster of five glutamic acid residues and also at the Met-Asp bond within its WMDF-NH₂ sequence...
whose unmodified structure has been claimed to be indispensable for all the known biological activities of gastrins (33, 34). The latter meprin cleavage will lead to the inactivation of gastrin and thus to the control of its activity. In view of the fact that meprins have been implicated in key biological processes such as growth, development, and tissue remodeling, the search for such target substrates, and the demonstration that they occur in vivo, is quite an important task.

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