Molecular and Biochemical Characterization of a Serine Proteinase Predominantly Expressed in the Medulla Oblongata and Cerebellar White Matter of Mouse Brain*

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A full-length cDNA clone of a serine proteinase, mouse brain serine proteinase (mBSP), was isolated from a mouse brain cDNA library. mBSP, which has been recently reported to be expressed in the hair follicles of nude mice, is most similar (88% identical) in sequence to rat myelencephalon-specific protease. The mBSP mRNA was steadily expressed in the brain of adult mice with a transient expression in the early fetal stage during development. The genomic structure of the mouse gene for mBSP was determined. The gene, which is mapped to chromosome 7B4-B5, is about 7.4 kilobases in size and contains 7 exons. Interestingly, the 5'-untranslated region of the mBSP gene was interrupted by two introns. In situ hybridization analyses revealed that mBSP is expressed in the white matter of the cerebellum, medulla oblongata, and capsule interna and capsule interna pars retrolenticularis of mouse brain. Further, mBSP was immunolocalized to the neuroglial cells in the white matter of the cerebellum. Recombinant mBSP was produced in the bacterial expression system and activated by lysyl endopeptidase digestion, and the activated enzyme was purified for characterization. The enzyme showed amidolytic activities preferentially cleaving Arg-X bonds when 4-methylcoumaryl-7-amide-containing peptide substrates were used. Typical serine proteinase inhibitors, such as diisopropyl fluorophosphates, phenylmethylsulfon fluoride, soybean trypsin inhibitor, aprotinin, leupeptin, and benzamidine, strongly inhibited the enzyme activity. The recombinant mBSP effectively hydrolyzed fibronectin and gelatin, but not laminin, collagens I and IV, or elastin. These results suggest that mBSP plays an important role in association with the function of the adult mouse brain.

The ability of tumor cells to invade into the extracellular matrix has been linked to enzymes that are released by tumor cells or associated with the plasma membrane of tumor cells. Proteinases have been implicated in tumor cell invasion and metastasis by numerous laboratories (1–3). One such enzyme is tissue-type plasminogen activator (tPA),1 a serine proteinase widely distributed in tissues and organs (4). It has been established that tPA can be purified from the culture medium of human melanoma cell lines (Bowe) in two molecular forms of a single polypeptide chain (Mr = 64,000) and two polypeptide chains (Mr = 32,000 and 30,000), and that a proteinase(s) is involved in the proteolytic conversion of a single-chain form to the two-chain enzyme. Ichinose et al. (5) suggested that tissue kallikrein or a tissue kallikrein-like proteinase is responsible for the conversion. We recently demonstrated that, like human melanoma cells, mouse Lewis lung carcinoma and B16 melanoma cells secrete a common serine proteinase capable of rapidly converting a single-chain tPA to the two-chain enzyme (6).

In an attempt to search a mouse cancer cell cDNA library for a cDNA clone encoding the tPA-converting enzyme by means of polymerase chain reaction (PCR) using a series of degenerated oligonucleotide primers corresponding to the consensus sequences of the serine proteinase active sites, we happened to find a PCR product representing a unique proteinase that is expressed almost exclusively in the mouse brain. In the present study, we undertook molecular and biochemical analyses of this enzyme, designated mouse brain serine proteinase (mBSP), including the genomic structure and chromosomal localization, tissue distribution of the mBSP mRNA and protein product in the brain, and characterization of the recombinant enzyme. The results clearly show that mBSP is the homologue of rat myelencephalon-specific protease first reported by Scarisbrick et al. (7), and is the same enzyme very recently reported as brain and skin serine protease (BSSP) by Meier et al. (8). The current results also show that the recombinant mBSP is capable of degrading casein, fibronectin, and gelatin, suggesting a role of this enzyme in extracellular matrix protein degradation in the brain.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse B16 melanoma (RCB 0557) and Lewis lung carcinoma (RCB 0558) were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Both cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 0.3 mg/ml L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

The abbreviations used are: tPA, tissue-type plasminogen activator; PCR, polymerase chain reaction; mBSP, mouse brain serine proteinase; MCA, 4-methylcoumaryl-7-amide; Boc, t-butyloxycarbonyl; Suc, succinyl; DIG, digoxigenin; SBTI, soybean trypsin inhibitor; bp, base pair(s); kb, kilobase pairs; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BSSP, brain and skin serine protease; GFAP, glial fibrillary acidic protein.

1 The authors would like to thank Drs. T. Sharp and M. Iwasa for a gift of rat myelencephalon cDNA.
penicillin, and 0.1 mg/ml streptomycin (Life Technologies, Inc.).

**Animals**—Mice (C57BL/6Ncrj strain, female) were killed by cervical dislocation, and the brains were rapidly removed, frozen in liquid N₂, and stored at −80 °C until use.

**RNA Isolation**—Total RNAs were prepared from frozen tissues or confluent monolayers of the melanoma and carcinoma cells using the guanidine isothiocyanate–cesium chloride method (9). Poly(A)⁺ RNAs were selected by oligo(dT)-cellulose column chromatography.

**Reverse Transcriptase-PCR**—The first strand cDNA was synthesized from poly(A)⁺ RNAs of mouse B16 melanoma and Lewis lung carcinoma using a SuperScript Preamplification System (Life Technologies, Inc.) according to the manufacturer’s protocol. Two degenerate oligonucleotide primers for PCR were synthesized based on the cDNA sequence for key regions of histidine and serine residues in some mouse serine proteinases (sense: 5'-GTGCGTGGTAGTATGATGGCCTATG/ATC/TCTGAGTCC/GTC/A-CTG-3' corresponding to the amino acid sequence Val-Leu-Thr-Ala-Ala-His-Cys, and antisense: 5'-AGA/CGA/GGA/CTC/CGT/ACC/CTG/ATG/ATA/GA/G-TC/GA/GCCC-3' corresponding to the amino acid sequence Gly-Asp-Ser-Gly-Gly-Pro-Leu). Conditions for PCR were 94 °C for 3 min, followed by 40 cycles of 94 °C for 15 s, 52 °C for 20 s, and 72 °C for 30 s, and then a final extension at 72 °C for 7 min. Fragments between 0.4 and 0.5 kb in size were recovered from PCR products by agarose gel electrophoresis.

The fragments were subcloned into pBluescript (II) KS⁺ (Stratagene, La Jolla, CA) cut with EcoRI. Transformation of the recombinant plasmids into *Escherichia coli* JM105 cells resulted in 105 plaques from the library were transferred to nylon membranes (Schleicher & Schuell, Dassel, Germany). The blots were hybridized with a ¹²³P-labeled probe at 42 °C in 6X SSPE, 0.5% formamide, 5X Denhardt’s solution, 1% SDS, and 100 µg/ml denatured herring sperm DNA with the ³²P-labeled full-length mBSP cDNA including exons 2–7. The membrane was washed at 50 °C in 5X SSC, 0.1% SDS, and exposed to Kodak Biomax film (Eastman Kodak Co.).

**Chromosome Localization of the mBSP Gene**—Metaphase preparations were obtained from concanavalin-stimulated splenocytes of normal male mice after bromodeoxyuridine incorporation. The mixture of two mBSP genomic DNA (α91 and α411) was labeled with biotin-16-dUTP (Roche Molecular Biochemicals) by nick-translation. After hybridization, slides were washed, blocked, and incubated with goat antibodies against biotin (Vectashield Vector Laboratory, Burlingame, CA). Slides were incubated with fluorescein isothiocyanate-conjugated rabbit antibodies against goat IgG (American Qualex, La Mirada, CA) and then with Alexa 488-conjugated rabbit antibodies against fluorescein (Molecular Probes, Eugene, OR).

**In Situ Detection of the mBSP mRNA**—Antisense and sense RNA probes were prepared by *in vitro* transcription of a reverse transcriptase-PCR-amplified fragment of mBSP cDNA with T3 or T7 RNA polymerase using a DIG RNA labeling kit (Roche Molecular Biochemicals). Mouse brain sections (10 µm) were cut on cryostat and thinned onto slides coated with silan. Sections on slides were fixed in 4% paraformaldehyde (Wako) in phosphate-buffered saline (PBS) for 15 min. After washing with PBS, the sections were treated with 20 µg/ml proteinase K (Wako) in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA for 10 min at 37 °C, postfixed in the same fixative, permeabilized in 0.2 µM HCl for 10 min, acetylated with 0.25% acetic anhydride in 0.1% triethanolamine (Merck, and dehydrated through an ascending alcohol series. The DIG-labeled RNA probe (antisense or sense) in hybridization buffer containing 50% formamide, 0.5 µl NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10% dextran sulfate (Wako), 1X Denhardt’s solution, 0.25% SDS, and 0.2 mM MgCl₂ tRNA (Roche Molecular Biochemicals) were placed on the sections, and then incubated at 50 °C for 18 h. The sections were washed at 50 °C in 50% formamide, 2X SSC for 30 min. Sections were then treated with 40 µg/ml RNase A in RNase buffer (0.5 µl NaCl, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA) for 30 min at 37 °C. Subsequently, sections were washed in 0.1X SSC for 20 min at 50 °C, and then in 0.2X SSC for 20 min at 50 °C twice. The hybridized probes were detected using a DIG nucleic acid detection kit (Roche Molecular Biologicals). Sections were treated with 1% blocking reagent in DIG buffer 1 (100 µM Tris-HCl (pH 8.5), 150 µM NaCl) for 30 min, and incubated in 500X diluted anti-DIG antibody in DIG buffer 1 for 30 min. Sections were washed with DIG buffer 1, 0.1X SSC, 0.1% SDS, 0.1X SSC for 30 min at 37 °C, and then washed in 0.1% SDS, 0.1X SSC for 30 min at 37 °C.

**Immunohistochemical Detection of the mBSP**—Whole brains dissected from mouse (8 weeks old) were fixed in 4% paraformaldehyde/PBS (pH 7.0). After dehydration in serial concentrations of ethanol and xylene, they were embedded in paraffin and sectioned at a 10 µm thickness. The sections were mounted on a coverslip, and dried overnight at 45 °C. Following deparaffinization and hydration, the sections were immersed in PBS-BT buffer (0.1 g of bovine serum albumin, 50 µl of Tween 20, 0.1 g of NaF in 100 ml of PBS), and incubated in normal goat serum (Sigma) for 30 min at room temperature. Anti-mBSP antiserum/PBS-BT (1:100 dilution) or rabbit polyclonal antibody against gial fibrillary acidic protein (GFAP, Sanbio, Netherlands/PBS-BT (1:300 dilution) was applied on the coverslip for 2 h at room temperature. After washing three times with PBS-BT, the sections were treated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Cappel Products, West Chester, PA/PBS-BT (1:100 dilution) containing propidium iodide (Sigma) for 1 h at room temperature. The coverslip was mounted on a slide with 70% glycerin containing 5% n-propyl gallate/1X PBS-saline (pH 7.4). The presence of antibodies in the tissues was analyzed under a confocal laser scanning microscope (Fluoview, Olympus, Tokyo, Japan).

**Preparation of Antisera**—The antigen used was the recombinant mBSP eluted from an Ni²⁺ column as described in the following section. Specific antisera was raised by injecting female rabbits (2 months old)
with 200 μg each of the above protein emulsified with Freund's complete adjuvant. Boosting was started 2 weeks later by injecting each at 2-week intervals with 200 μg of the antigen emulsified with Freund's incomplete adjuvant. The antisera were obtained from the blood collected after three booster injections.

Preparation of Recombinant mBSP—The expression vector for mBSP was constructed by inserting its cDNA (amino acids 14–246) including the complete pro-enzyme region of the mBSP gene into EcoRI site of pET30a (Novagen, Madison, WI). PCR was performed with oligonucleotide primers pro-mBSP (5'-GGAGATCTGCGTGGCGAAGAACGGAGA-3') and mBSP-AS (5'-GGAGATCTGCGTGGCGAAGAACGGAGA-3') to create EcoRI sites at the 5' and 3' ends. The reaction product was sequentially digested with EcoRI, gel purified, and ligated in-frame in the EcoRI site of expression vector pET30a. The orientation and sequence of the BSP cDNA in the pET3 plasmid were confirmed by DNA sequencing. The ligated vector was transformed to E. coli strain BL21 (DE3) pLysS (Novagen), and the cells were grown at 37 °C in 500 ml of

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mBSP activity was determined with MCA.

Zymography—Casein and gelatin zymography were performed according to the methods previously described (12). Briefly, mBSP was electrophoresed on 12% SDS-PAGE gels containing 0.1% casein (Wako) or 1 mg/ml type A porcine skin gelatin (Sigma) under nonreducing conditions. After electrophoresis, gels were washed twice in 2.5% Triton X-100 for 30 min, and then incubated with shaking in 0.1 M glycine-NaOH (pH 8.0) for 18 hr at 37 °C. The gels were stained with 0.25% Coomassie Brilliant Blue to visualize zones of lysis.

Degradation of Extracellular Matrix Components by mBSP—4 μg of human plasma fibronectin (Chemicon, Temecula, CA), 4 μg of mouse laminin (Biomedical Technologies Inc., Stoughton, MA), 20 μg of acid-soluble type I collagen from calf skin (Sigma), and 20 μg of acid-soluble type IV collagen from human placenta (Sigma) were each incubated at 37 °C for 18 hr in 50 μl Tris-HCl buffer (pH 8.0), with mBSP (500 ng) in a final volume of 20 μl. Reactions were stopped with SDS sample buffer, and the reaction mixtures were boiled and subjected to SDS-PAGE using a 6% gel. After electrophoresis, gels were stained with 0.25% Coomassie Brilliant Blue.

The activity of mBSP on elastin was assessed using elastin-orecin (Elastin Products Co., Inc., Owensville, MO) as a substrate according to the method of Apple (13).

Western Blotting Analysis—Samples were subjected to SDS-PAGE (14) under reducing and nonreducing conditions, and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) by the method of Towbin et al. (15). The blotted membrane was incubated with rabbit anti-mBSP antiserum at 1:3000 dilution and subsequently with goat anti-rabbit IgG antibody (Amersham Pharmacia Biotech). Immunoreactive signals were detected using an ECL Western blot detection kit (Amersham Pharmacia Biotech) according to the protocol provided by the manufacturer.

RESULTS

Molecular Cloning of mBSP—mRNAs prepared from mouse Lewis lung carcinoma cells and B16 melanoma cells were used...
for reverse transcriptase-PCR with two degenerated oligonucleotide primers as described under “Experimental Procedures.” Among the 160 PCR products analyzed, one with a length of 434 bp was found to have a nucleotide sequence highly homologous to that of the serine protease, rat myelencephalon-specific protease, which has been reported by Scarisbrick et al. (7). This 434-bp cDNA fragment was then used as a probe for Northern blot analysis conducted with mRNAs isolated from several mouse tissues. As shown in Fig. 1A, an intense band of 1.3 kb was detected only in the brain. Based on this observation, we screened a mouse brain cDNA library using the same cDNA fragment as a probe, and isolated a full-length cDNA clone (the nucleotide sequence has been deposited in the DDBJ/EMBL/GenBank™ Data Bank under accession number AB015206). This clone was 1,115 bp long, including the 5' -noncoding region (213 bp), the coding nucleotide sequence (738 bp), and the 3' -noncoding region (164 bp). There are 5 ATG codons between nucleotide positions 193 and 222. Since the codon at positions 214–216 best meets the criteria for the initiation site of the translation (16), we tentatively assume that translation starts at this ATG codon. As a result, the open reading frame codes for a protein of 246 amino acids with a molecular weight of 26,664.

During the course of this study, Meier et al. (8) reported a cDNA clone encoding a novel serine protease designated BSSP. The authors demonstrated a predominant expression of this gene in the hair follicles of nude mice. mBSP is undoubtedly the same molecule as BSSP, and is highly homologous to rat myelencephalon-specific protease (88% identical in amino acids, Ref. 7). mBSP also exhibited 67% identity to human zyme (17), the same molecule as BSSP, and is highly homologous to rat myelencephalon-specific protease, which has been reported by Scarisbrick et al. (7). The active site residues, His 62, Asp 106, and Ser 197 (numbered from the putative translation initiation Met), of the catalytic triad are encoded by exons 4, 5, and 7, respectively.

**Expression of mBSP—** In adult mice, the mBSP mRNA was exclusively detected in the brain among the tissues examined (Fig. 1A), confirming the result of Meier et al. (8). To determine the changes of mBSP gene expression during development, a time-course experiment was conducted by Northern blot analysis using RNAs obtained from various stages of developing mice. In the prenatal periods, the mRNA was transiently decreased during development, a computer search for consensus binding sites of transcription factors indicated the presence of motifs for Sp1, AP1, and C/EBP.

**Chromosome Localization of the mBSP Gene—** We performed Southern blot hybridization using the cDNA encompassing exons 2–7 as a probe. When mouse genomic DNA was digested

| Exon no. | Exon size | Sequence at exon/intron junction | Phase | Intron size |
|----------|-----------|---------------------------------|-------|-------------|
| 1        | 174       | GAC TTA CAGtgtaggtagg             | ttcatgcagGTC GGA TCA | 917 |
| 2        | 156       | CAC CAG CAGtggtagtga             | cctctgaagATG CCC ATG | 391 |
| 3        | 61        | AAA TCA Gtggattaa                | cttcccagCC TGG TCG | 1 |
| 4        | 157       | Lys Ser A                       | 1a Trp Ser | 681 |
| 5        | 248       | Lys Lys e                       | Asn Leu | 2 |
| 6        | 137       | GAA AAT Gtggtagg                | acctcttagGT | 1 |
| 7        | 296       | Glu Asn G                        | ly Asp Phe | 464 |

![DNA sequences of the 5'-flanking region of the mBSP gene](http://www.jbc.org/figure1.png)
with EcoRI and BamHI, two hybridization-positive bands (5.0 and 6.5 kb for EcoRI; 1.2 and 6.8 kb for BamHI) were detected. On the other hand, digestion with HindIII and XbaI produced three bands (1.8, 2.8, and 10.0 kb for HindIII; 2.0, 7.3, and 8.5 kb for XbaI) (data not shown). The patterns were consistent with the numbers of the respective restriction enzyme sites present in the genomic sequence.

We next attempted to determine the chromosomal localization of the mBSP gene by in situ hybridization with the mBSP clone on R-banded metaphase chromosomes that had been prepared from concanavalin-stimulated normal male mouse spleenocytes. As shown in Fig. 4, fluorescence signals were detected on chromosome 7B4-B5 and this result was reproducible. These results indicate that mBSP is encoded by a single, unique gene that resides on chromosome 7B4-B5.

Histological Studies—Sections of adult mouse brains were analyzed for in situ detection of the mBSP mRNA using DIG-labeled RNA probes. The antisense probe detected specific signals in the white matter of the cerebellum (Fig. 5, C and D). The molecular layer, Purkinje cell layer, and granular layer did not show any positive signals. Clear signals were also observed in the medulla oblongata (Fig. 5, E and F), and capsula interna (CAI) and capsula interna pars retrolenticularis (CAIR) (Fig. 5, G and H). No significant expression of mBSP mRNA was detected in any other part of the brain.

To obtain information on the identity of mBSP-producing cells, immunohistochemical analyses were conducted with the cerebellum. Two adjacent sagittal sections were stained with specific antibody against mBSP or antibody against GFAP, a well known marker protein for astrocytes. As shown in Fig. 6A, the mBSP antibody produced strong fluorescent signals almost exclusively in the cerebellar white matter. This is in good agreement with the result obtained by in situ hybridization analysis as described above. mBSP is apparently distributed in areas surrounding the nuclei of mBSP-producing cells. As expected, GFAP-immunoreactive astrocytes were found only in the white matter of the cerebellum (Fig. 6B). The distribution of GFAP-immunoreactive astrocytes corresponds in part to that of the mBSP-immunoreactive cells, suggesting that astrocytes are at least one kind of cells producing mBSP in vivo. However, the current immunohistochemical data also indicate that other type(s) of neuroglial cells may produce mBSP in the mouse cerebellum.

Characterization of the Recombinant mBSP—Since the peptide bond Ser13-Ala14 is presumed to be the putative cleavage site by a signal peptidase (23), the nucleotide sequence, which starts with the Ala14 codon and ends with the stop codon, was subcloned into the EcoRI site of the E. coli expression vector pET30. Transformation of this construct into E. coli resulted in production of a 38-kDa protein (data not shown). Considering that the recombinant protein should contain 51 extra amino acid residues, which originate from the plasmid sequence, at the NH2 terminus, in addition to 233 residues of its own pro-mBSP, the size was somewhat greater than expected. This fusion protein, which had been obtained by fractionating on an Ni2+-chelate column, was digested with an immobilized lysyl endopeptidase, and the activated enzyme was purified using a SBTI-Sepharose 4B column (Fig. 7). The direct Edman degradation of the purified sample gave a single NH2-terminal amino acid sequence of Val-Val-His-Gly-Gly-Pro-X-Leu (- the 7th residue was not clearly identified). However, two polypeptides with a close molecular weight were separated at around 22,000 in the SDS-PAGE (Fig. 7). These polypeptides were reactive with the rabbit anti-mBSP antibody as examined by Western blot analysis (data not shown). Since the polypeptides were both retained on and recovered from the affinity column of SBTI-Sepharose, they are presumed to be enzymatically active. Based on these results, we concluded that activation with lysyl endopeptidase occurred by cleaving at the peptide bond of Lys27-Val28. A plausible explanation for the presence of two active enzymic forms in the recombinant enzyme preparation is that part of the activated mBSP might have undergone an additional hydrolysis by lysyl endopeptidase at a peptide bond near the COOH terminus.

When the purified, active mBSP was kept at 4 °C or frozen at −20 °C in 50 mM Tris-HCl buffer (pH 8.5) for 2 weeks, the enzyme activity was reduced by 67% and 45%, respectively. Electrophoretic analysis of the samples stored as above revealed that this loss of enzyme activity was accompanied by degradation of the polypeptide due to autolysis.

Enzyme activity of the recombinant mBSP was measured at various pH levels using Boc-Pro-Phe-Arg-MCA as substrate. The activity was detected at pH range 6–10 in a typical bell shape, and the optimum pH was 8.5.

Substrate specificity of mBSP was examined using various MCA-containing peptide substrates, and the results are shown in Table II. The recombinant pro-mBSP showed little or no enzyme activity toward any substrate, but was dramatically activated by lysyl endopeptidase treatment. Among substrates tested, the best was Boc-Pro-Phe-Arg-MCA. Boc-Phe-Ser-Arg-MCA was the second best substrate, and Boc-Glu-Ala-Arg-MCA was also a good substrate. BenzylArg-MCA and Lys-containing substrate Boc-Val-Leu-Lys-MCA were hydrolyzed very little by the enzyme. The chymotrypsin substrates, Suc-Ala-Ala-Pro-Phe-MCA and Suc-Leu-Leu-Val-Tyr-MCA, were resistant to the enzyme.

Table III shows the effects of proteinase inhibitors on the enzyme activity. D i ssopropyl fluorophosphate, phenylmethylene sulfonyl fluoride, SBTI, aprotinin, leupeptin, antipain, and benzamidine strongly inhibited the activity. The results are consistent with the idea that mBSP is a serine proteinase.

Examination of Enzyme Action on Some Extracellular Matrix
Proteins—To gain some insight into the biological role of mBSP, its action on protein substrates, including several extracellular matrix proteins, was examined. Zymographic analysis revealed that heat-denatured casein and gelatin were degraded by mBSP (Fig. 8A). Five extracellular matrix proteins were tested for the enzyme. Incubation of the enzyme with fibronectin apparently produced several degraded polypeptides (Fig. 8B), whereas laminin, collagen I, collagen IV, and elastin were resistant to the enzyme action (data not shown). Finally, we examined the effect of mBSP on human tPA. The recombinant enzyme was unable to convert single-chain tPA to its two-chain form at a significant rate. These results suggest that fibronectin and gelatin could be candidate protein substrates of mBSP in vivo.

DISCUSSION

Our attempt to search for the serine proteinase responsible for proteolytic conversion of single-chain tPA to two-chain tPA using mouse cancer cells eventually led to identification of mBSP. The mRNA of this proteinase was expressed exclusively in the mouse brain under normal physiological conditions. Very recently, however, Meier et al. (8) isolated from nude mouse skin a cDNA clone encoding BSSP and having the same amino acid sequence as mBSP. Further comparison of the mBSP protein sequence with those of other related proteins reported to date indicated that rat myelencephalon-specific protease (7) shows the highest homology (88% identity) to the current proteinase. Humanzyme (17), which is also known as neurosin (18) and protease M (19), is in the next place in homology with 67% identity. This value is significantly low in comparison with that of rat myelencephalon-specific protease. We believe that mBSP is a mouse homologue of rat myelencephalon-specific protease. This idea is supported by following the similarities in the regiospecificity of gene expression of these two serine proteases. 1) Both genes are specifically expressed in the central nervous system. 2) Within the central nervous system, rat myelencephalon-specific protease is expressed at the highest level in the medulla oblongata and spinal cord. Similarly, the region exhibiting predominant expression of the mBSP gene in...
A Mouse Brain Serine Proteinase

FIG. 7. Purification of active recombinant mBSP by SBTI-Sepharose 4B column chromatography. The recombinant fusion protein treated with immobilized lysyl endopeptidase was applied to a column of SBTI-Sepharose 4B in 50 mM Tris-HCl buffer (pH 8.0), containing 0.2 mM NaCl. The retained enzyme was eluted by applying 0.1 M glycine-HCl buffer (pH 3.0), at the position indicated by an arrowhead. Fractions of 1 ml were collected and were monitored for protein concentration and enzyme activity. The protein concentration was determined by measuring the optical density of the fractions at 280 nm, and enzyme activity was determined using Boc-Val-Pro-Arg-MCA as substrate. Fractions 25–27 were pooled, and 10 μl of aliquots was subjected to SDS-PAGE analysis under nonreducing conditions (see inset). Two separate polypeptides having an apparent molecular mass of 21.5 and 22.5 kDa are indicated.

FIG. 8. Activities of purified recombinant mBSP on protein substrates. A, for casein and gelatin zymography, 0.5 μg of purified recombinant mBSP was loaded on 12% SDS-PAGE gels containing 0.1% casein or 1 mg/ml gelatin. Molecular masses (kDa) of standard proteins are indicated at left. B, human fibronectin (450 kDa) was separately incubated with (+) or without (−) mBSP (0.5 μg) for 18 h at 37 °C, and was subjected to SDS-PAGE. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250. Molecular masses (kDa) of standard proteins are indicated at left.

TABLE II
Enzyme activities of the recombinant mBSP toward various MCA substrates

| Substrate                | Specific activity (nmol/min/μg protein) |
|--------------------------|----------------------------------------|
| Boc-Val-Pro-Arg-MCA      | 74.9 (100)                              |
| Boc-Phe-Ser-Arg-MCA      | 71.2 (95)                               |
| Boc-Gln-Ala-Arg-MCA      | 44.3 (59)                               |
| Boc-Gln-Gly-Arg-MCA      | 10.7 (14)                               |
| Boc-Leu-Lys-Arg-MCA      | 9.1 (12)                                |
| Pro-Phe-Arg-MCA          | 8.0 (11)                                |
| Z-Phe-Arg-MCA            | 7.2 (10)                                |
| Boc-Gln-Arg-MCA          | 6.8 (9)                                 |
| Boc-Leu-Lys-MCA          | 3.1 (4)                                 |
| Bz-Arg-MCA               | 2.4 (3)                                 |
| Suc-Ala-Ala-Pro-Phe-MCA  | ND                                      |
| Suc-Leu-Leu-Val-Tyr-MCA  | ND                                      |

TABLE III
Effects of inhibitors on enzyme activity

| Inhibitor | Concentration | Inhibition |
|-----------|---------------|------------|
| DFP       | 0.5 mM        | 65         |
| PMSF      | 0.5 mM        | 96         |
| SBTI      | 0.1 mg/ml     | 97         |
| TLCK      | 0.1 mM        | 11         |
| TPCK      | 0.1 mM        | 7          |
| Bestatin  | 0.02 mM       | 9          |
| Pepstatin | 0.01 mM       | 19         |

the mouse brain is the medulla oblongata. 3) Like rat myelencelphal-specific protease, mBSP is expressed in the cortex of the cerebellum.

Although the mBSP mRNA is transiently detected with poly(A)⁺ RNA isolated from developing mouse embryos, the proteinase is a brain-specific protein. The expression of mBSP in the brain was found to initiate at 28 days after birth. This observation suggests the presence of factors and mechanisms that strictly control mBSP gene expression. To establish a basis for further investigation into the regulatory mechanism of the gene expression, the structure, including its 5'-untranslated region, of the mBSP gene was determined in this study. The gene spans 7.4 kb in total, and contains 7 exons. The exon/intron organization in the coding region was basically the same as those of trypsin-family genes such as trypsin (AB017032), cathepsin G (24), and granzymes (25). Interestingly, the 5'-untranslated region of this gene was interrupted by two introns. Obviously this is a unique feature of the mBSP gene, inasmuch as no intron has been reported at this position for trypsin-type serine proteinase genes, with the exception of the neprospine gene, which contains an intron in the 5'-untranslated region (26). Comparison of our genomic sequence (DDBJ/EMBL/GenBank™ AB032402) against data base entries revealed that, like the mBSP gene, the human zyme (GenBank™ AF149289) exhibits a similar exon/intron organization, having two intervening sequences in its 5'-untranslated region. These findings may indicate that the mBSP gene is derived from an ancestor gene common to the trypsin family proteinases, and that the current gene, perhaps together with the human zyme gene, subsequently evolved in a manner different from that of trypsin (5 exons) or neprospine (6 exons). In this context, we should note the further similarity between the mBSP and human zyme genes with respect to their chromosome localizations. In the present study, the mBSP gene was localized to mouse chromosome 7B4-B5, while Little et al. (17) mapped the human zyme gene to chromosome 19q13.3. Interestingly, this region of human chromosome 19 is known to share a region of synteny with mouse chromosome 7. Despite such common features between these two genes, we tentatively assume that they are not homologous genes because of the noticeable sequence differences described above. In order to validate this assumption, however, it will be necessary to identify the human homologue of mBSP.
Meier et al. (8) have isolated a full-length cDNA from a dE18 mouse embryonic skin library exhibiting a different 5′-end distinct from that of our cDNA. The present data on the genomic structure clearly demonstrate that such a difference is due to an alternative splicing event occurring at the 5′-untranslated region of the mBSP gene. The 5′-end guanine of the isolated skin cDNA corresponded to the guanine (2655) of the gene (AB032402), which was found in intron-1 of the mBSP primary transcript in mouse brain and 60 nucleotides upstream of the intron-1/exon-2 boundary. The exact role of the primary transcript in mouse brain and 60 nucleotides upgene (AB032402), which was found in intron-1 of the mBSP isolated skin cDNA corresponded to the guanine (2655) of the gene (AB032402). This information includes the mouse gene structure, chromosome localization, and histological localization of the mRNA and protein product in the brain. In addition, this paper describes for the first time the enzymic function of mBSP using a recombinant protein, and demonstrates its action on the extracellular matrix proteins, fibronectin and gelatin. The present data should help elucidate the biological role of the enzyme not only in the central nervous system but also in the skin.

The recombinant mBSP shows trypsin-like enzyme activity toward MCA-containing peptide substrates. The inhibitor profiles of mBSP are compatible with this idea, except that 1-chloro-3-tosylamido-7-amino-2-heptanone is not a potent inhibitor of the enzyme. At present, it is unknown why the inhibitor does not show a significant inhibition for mBSP. The enzyme may have a unique structure distinct from other trypsin-like proteinases in its active site. mBSP preferentially hydrolyzes substrates containing Arg in the P1 position. In a broad sense, the substrate specificity of mBSP apparently resembles that of mouse neurepsin (30), although only 45% identity is found in the amino acid sequences of these proteins. More importantly, mBSP was demonstrated to exhibit a hydrolytic activity toward protein substrates. Fibronectin, gelatin, and casein were found to be degraded effectively by the enzyme. The ability of mBSP to degrade the former two extracellular matrix proteins (fibronectin and gelatin) is of particular interest from a biological point of view, since the enzyme is thought to function in the extracellular space. If mBSP is spatially localized with these extracellular matrix proteins in the same tissues, the proteinase could hydrolyze them in vivo. Existing evidence indicates that fibronectin is a major extracellular matrix protein expressed in the nervous system (31). Alternatively, mBSP may be involved in the neural injury leading to neuronal and glial cell death, phagocytosis, glial cell proliferation, and migration. Extensive degenerative changes associated with this process definitely require the degradation of a variety of proteins by many extracellular proteinases. mBSP could be a proteinase for this process. Indeed, the involvement of rat myelencephalon-specific protease, here demonstrated to be a mBSP homologue, in the process of spinal cord injury was suggested by Scarsbrick et al. (7). In this connection, it would be interesting to examine whether mBSP mRNA would be induced after brain injury. It should be noted, however, that mBSP plays a fundamental role in normal brain homeostasis, since the mBSP gene is steadily expressed in adult mice.

We initially isolated a PCR fragment of the mBSP clone from mRNAs of mouse cancer cells, such as Lewis lung carcinoma and B16 melanoma cells. Since these cancer cells commonly secrete a serine proteinase capable of converting human single-chain tPA to its two-chain enzyme (6), we were interested in whether or not mBSP is the enzyme in question. However, no such converting activity was demonstrated for the current enzyme, indicating that the putative tPA-converting enzyme is distinct from mBSP. Nevertheless, mBSP may play a role in the invasive and metastatic process of cancer cells, given that it effectively degrades fibronectin and gelatin.

In conclusion, the present report provides new information on mBSP that has not been described in the two previous related papers (7, 8). This information includes the mouse gene structure, chromosome localization, and histological localization of the mRNA and protein product in the brain. In addition, this paper describes for the first time the enzymic function of mBSP using a recombinant protein, and demonstrates its action on the extracellular matrix proteins, fibronectin and gelatin. The present data should help elucidate the biological role of the enzyme not only in the central nervous system but also in the skin.

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