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Characterization of Mouse Tissue Kallikrein 5

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Mouse tissue kallikreins (*Klks*) are members of a large, multigene family consisting of 37 genes, 26 of which can code for functional proteins. Mouse tissue kallikrein 5 (*Klk5*) has long been thought to be one of these functional genes, but the gene product, mK5, has not been isolated and characterized. In the present study, we prepared active recombinant mK5 using an *Escherichia coli* expression system, followed by column chromatography. We then determined the biochemical and enzymatic properties of purified mK5. mK5 had trypsin-like activity for Arg at the P1 position, and its activity was inhibited by typical serine protease inhibitors. mK5 degraded gelatin, fibronectin, collagen type IV, high-molecular-weight kininogen, and insulin-like growth factor binding protein-3. Our data suggest that mK5 may be implicated in the process of extracellular matrix remodeling.

Key words: mouse, protease, kallikrein 5, recombinant enzyme, characterization

INTRODUCTION

Mouse tissue kallikreins (*Klks*) are members of a large, multigene family located as a gene cluster in cytogenic region B2 on mouse chromosome number 7 (Diamandis *et al*., 2004; Olsson *et al.*, 2004). Previous studies suggested that individual *Klk* gene products (mKs) are involved in many vital functions, such as kinin production, coagulation and fibrinolysis, activation and/or inactivation of peptide hormones, regulation of growth factors, and extracellular matrix protein turnover (Margolius, 1998). It is now established that the mouse has 37 *Klk* genes, 26 of which can code for functional proteins (Diamandis *et al*., 2004; Olsson *et al.*, 2004). However, only about half of the 26 genes have been characterized to date by molecular biological and biochemical approaches. Mouse tissue kallikrein 5 (*Klk5*) is one of the as-yet-uncharacterized *Klk* genes. At present, the nucleotide sequence is the only information available for this particular gene, and studies at the protein level are required to determine its biological role.

Our previous studies documented the expression of *Klk21* (Matsui and Takahashi, 2001), *Klk24* (Matsui *et al*., 2005a, b), and *Klk27* (Matsui *et al.*, 2000) in the testis and their implications in the function of this male reproductive organ. In an attempt to examine by RT-PCR whether or not the *Klk* genes described above are expressed in other mouse tissues, we happened to isolate a *Klk5* fragment from the uterus, suggesting that mK5 may play a role in the female reproductive system. To establish a basis for further physiological studies of mK5, we prepared active recombinant mK5 for biochemical characterization. The present data suggest that mK5 may play a role in various biological processes.

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MATERIALS AND METHODS

Expression vector construction

The expression vector for mK5 was prepared basically in the same way as for mK21 (Matsui and Takahashi, 2001). Briefly, a cDNA corresponding to amino acid residues 18–261 of the *Klk5* gene, including the pro-enzyme region (DDBJ/EMBL/GenBank databases NM 008456), was inserted into the *Eco*RI and *Hind*III sites of pET30a (Novagen, Madison, WI). A sense primer 5'-CCG-GAATTCGCACCTCCAGTCCAA-3', which includes the *Eco*RI site, and an antisense primer 5'-CCCAAGCTTTCAGGCATTTTTAGC-TAT-3', which includes the *Hind*III site, were synthesized. PCR using KOD plus DNA polymerase (Toyobo, Osaka, Japan) was performed using mouse skeletal muscle cDNA as the template with the above primers to create *Eco*RI sites at the 5' ends and *Hind*III sites at the 3' ends. The PCR conditions were as follows: 3 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 68°C. The reaction product was sequentially digested with *Eco*RI and *Hind*III, then gel purified and ligated in frame between the *Eco*RI and *Hind*III sites of pET30a. The orientation and sequence of the cDNA in the vector plasmid were confirmed by DNA sequencing.

Production and purification of active mK5

The ligated vector (pET30a-mK5) was transformed to *E. coli* strain BL21. The cells were grown at 37°C, induced with isopropyl-1-thio-β-D-galactoside, harvested by centrifugation, and lysed by freeze-thawing. The samples were washed twice with 0.5% Triton X-100, solubilized in 50 mM Tris-HCl (pH 7.8) containing 6 M urea and 0.5 M NaCl, and incubated for 12 h at room temperature in the same buffer containing 6 M urea and 0.5 M NaCl. The solubilized

samples were fractionated on a Ni^{2+}-chelate column (5-ml volume) (Novagen) previously equilibrated with 50 mM Tris-HCl (pH 7.8) containing 0.5 M NaCl and 6 M urea (Matsui and Takahashi, 2001). Elution was conducted with 50 mM histidine in the same buffer containing 0.5 M NaCl and 6 M urea. Eluted protein was extensively dialyzed against 50 mM Tris-HCl (pH 8.0). The protein thus prepared was a fusion protein containing at the N-terminus 51 extra amino acids, all of which originated from the plasmid sequence, in addition to the complete pro-mK5 sequence. This fusion protein was then incubated in 50 mM Tris-HCl (pH 8.0) with trypsin-Sepharose 4B, and the resulting active mK5 enzyme preparation was further purified by column chromatography on a Resource Q column (1-ml bed volume) (AKTA FPLC system) previously equilibrated with 50 mM Tris-HCl (pH 8.0). The materials retained were eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. One-milliliter fractions were collected and the enzyme activity of mK5 was assayed with a synthetic substrate, Pro-Phe-Arg-MCA, containing 4-methylcoumaryl-7-amide (MCA) (Peptide Institute, Osaka, Japan).

Enzyme activity

Recombinant mK5 activity was determined according to the method of Barrett (1980) with a slight modification. Briefly, the reaction solution contained 0.1 M Tris-HCl buffer (pH 8.0), 0.1 mM MCA substrate, and the enzyme sample in a final volume of 0.5 ml. The reaction was initiated by the addition of the substrate and stopped by the addition of 2.5 ml of 30 mM sodium acetate buffer (pH 4.3) containing 100 mM monochloroacetic acid. The release of fluorophore 7-amino-4-methylcoumarin (AMC) was measured by spectrofluorometry at an excitation wavelength of 370 nm and an emission wavelength of 460 nm.

Kinetic parameters

Kinetic parameters were determined for various MCA substrates. Initial velocities, extrapolated from plots of product versus time, were transformed into double-reciprocal plots. Maximum velocities (V_{max}), K_m, and k_{cat} values were obtained from the intercepts of these plots. The active mK5 concentration was determined using the active-site titrant *p*- nitrophenyl-*p'*-guanidinobenzoate HCl according to the method of Chase and Shaw (1967).

Inhibition of mK5 by protease inhibitors

Active mK5 (0.1 μg) was pre-incubated with various concentrations of protease inhibitors at 37°C for 15 min in 0.1 M Tris-HCl buffer (pH 8.0). After addition of 0.1 mM Pro-Phe-Arg-MCA, the residual enzyme activity was measured.

Degradation of protein substrates by mK5

Gelatin zymography was performed according to the method previously described by Heussen and Dowdle (1980). Briefly, mK5 was electrophoresed on 12% SDS-PAGE gels containing 1 mg/ml type A porcine skin gelatin (Sigma, St. Louis, MO) under non-reducing 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 8.3) for 18 h at 37°C. The gels were stained with 0.25% Coomassie Brilliant Blue to visualize zones of lysis.

Casein (Wako Pure Chemical Industries Ltd., Osaka, Japan) was added to mK5 at a ratio of 10:1 (w/w) in 50 mM Tris-HCl buffer (pH 8.0), and the mixtures were incubated at 37°C. Aliquots removed at different time points were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining.

Degradation of fibronectin and laminin was performed as previously described by Rajapakse *et al*. (2005). Briefly, 4 μg of human plasma fibronectin (Chemicon, Temecula, CA), and 4 μg mouse laminin (Biomedical Technologies Inc., Stoughton, MA) were each incubated for 18 h at 37°C in 20 μl of 50 mM Tris-HCl buffer (pH 8.0) with 400 ng of mK5. The reactions were stopped by the addi-

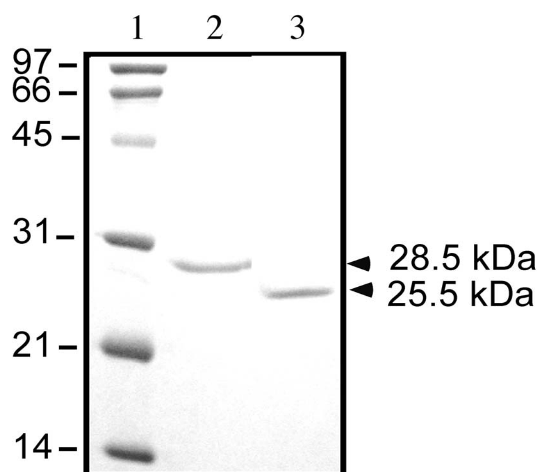

Fig. 1. Purity of purified recombinant mK5. Purified active mK5 was subjected to SDS-PAGE analysis under reducing (lane 2) and non-reducing (lane 3) conditions and visualized by Coomassie Brilliant Blue R-250 staining. Lane 1, molecular mass standards (kDa).

Table 1. Kinetic parameters of mK5 as measured on MCA-containing substrates

Substrates	V_{max}	K_m	k_{cat}	k_{cat}/K_m
	μmol/min/mg	mM	min^{-1}	$mM^{-1}\cdot min^{-1}$
Pro-Phe-Arg-MCA	1.5	0.03	39	1300
Z-Phe-Arg-MCA	0.3	0.13	7.6	58
Z-Leu-Arg-MCA	0.1	0.05	2.7	54
Pyr-Gly-Arg-MCA	0.1	0.03	1.6	53
Z-Val-Val-Arg-MCA	0.2	0.11	4.7	43
Boc-Val-Leu-Lys-MCA	ND			
Boc-Glu-Lys-Lys-MCA	ND			
Suc-Leu-Leu-Val-Tyr-MCA	ND			
Ac-Ile-Glu-Thr-Asp-MCA	ND			
Suc-Ala-Ala-Pro-Phe-MCA	ND			

Z, benzyloxycarbonyl; Pyr, pyroglutamyl; Boc, tert-butyloxycarbonyl; Suc, succinyl; Ac, acetyl; ND, not determined because of no significant activity.

Table 2. Effects of protease inhibitors on mK5 activity

Inhibitor	Concentration	Inhibition (%)
DFP	2.0 mM	87
Leupeptin	0.1 mM	70
Aprotinin	0.1 mg/ml	34
Antipain	0.2 mM	13
SBTI	0.1 mg/ml	0
Benzamidine	1.0 mM	0
E-64	0.5 mM	12
o-Phenanthroline	2.0 mM	10
Pepstatin	0.1 mM	0
TPCK	0.2 mM	14
TLCK	0.2 mM	7
EDTA	0.1 mM	0
Chymostatin	0.2 mM	0

DFP, diisopropyl fluorophosphate; SBTI, soybean trypsin inhibitor; TPCK, N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone; EDTA, ethylenediamine tetraacetic acid

tion of SDS sample buffer, and the reaction mixtures were boiled and subjected to SDS-PAGE. After electrophoresis, gels were stained with 0.25% Coomassie Brilliant Blue.

The effect of mK5 on collagen types I and IV was examined according to the method described previously by Ogiwara *et al*. (2005). Briefly, 25 µg of FITC-conjugated bovine collagen types I and IV (Yagai Corporation, Yamagata, Japan) were incubated with 500 ng of mK5 for 16 h at 33°C in 100 µl of 50 mM Tris-HCl buffer (pH 8.0). After incubation, the reactions were terminated by adding 300 µl of stopping reagent (42% ethanol, 50 mM Tris-HCl (pH 9.5), and 0.2 M NaCl). Samples were incubated on ice for 10 min, and then centrifuged at 10,000×*g* for 10 min. Aliquots of the resulting supernatants were diluted to a volume of 3.0 ml with distilled water, and the fluorescence was measured at an excitation wavelength of 459 nm and an emission wavelength of 520 nm.

Five micrograms of purified high-molecular-weight kininogen (Calbiochem, La Jolla, CA) were incubated at 37°C with 500 ng of active mK5 in 0.1 M Tris-HCl buffer, pH 8.0. Aliquots were removed

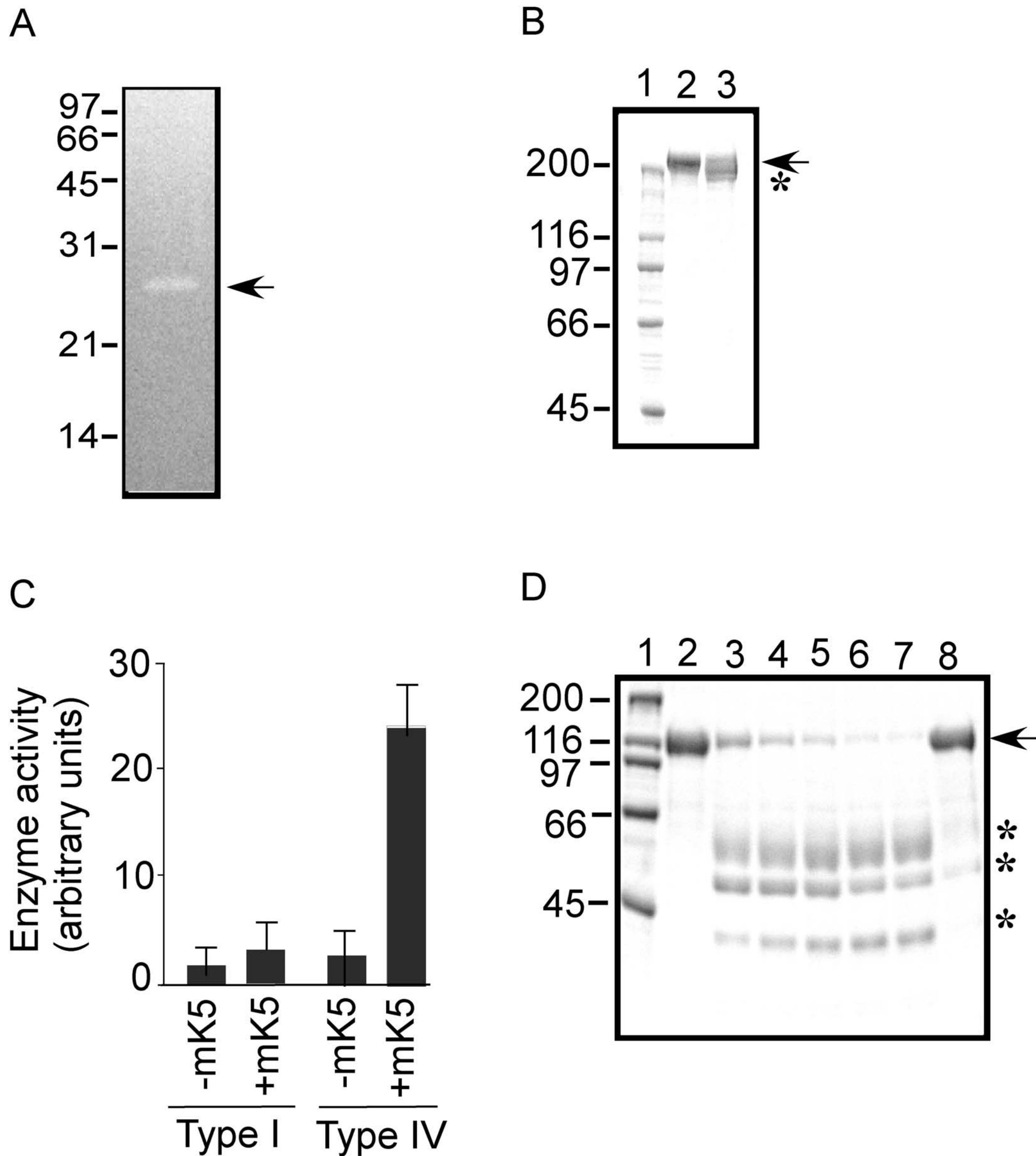

Fig. 2. Degradation by mK5 of extracellular matrix and plasma proteins. **(A)** Gelatin zymography of mK5. Purified mK5 (0.5 µg) was loaded on SDS-PAGE gels containing 1 mg/ml gelatin. Electrophoresis was conducted under non-reducing conditions. The arrow indicates the band produced by gelatinolytic activity of mK5. Positions of protein molecular-mass standards (kDa) are indicated to the left. **(B)** Effect of mK5 on fibronectin. Human fibronectin was separately incubated without (lane 2) or with (lane 3) mK5 for 18 h at 37°C and was subjected to SDS-PAGE under reducing conditions followed by Coomassie Brilliant Blue R-250 staining. Lane 1, molecular-mass standards (kDa). The arrow and asterisk indicate the intact and degraded forms of fibronectin, respectively. **(C)** Effect of mK5 on collagens. FITC-conjugated collagens type I and type IV were incubated without mK5 (-mK5) and with mK5 (+mK5) for 18 h at 33°C, and the fluorescence intensities of the supernatants were measured. Values of means±SEM from three separate experiments are shown. **(D)** Effect of mK5 on high-molecular-weight kininogen. The kininogen was incubated with mK5 at 37°C for 0 h (lane 2), 0.5 h (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), or 6 h (lane 7) and was subjected to SDS-PAGE under reducing conditions followed by Coomassie Brilliant Blue R-250 staining. Lane 8, incubation for 6 h without mK5. Lane 1, molecular-mass standards (kDa). The arrow and asterisks indicate the intact and degraded forms of kininogen, respectively.

at various time intervals, the reactions were stopped by the addition of SDS sample buffer, and the samples were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining.

Twenty-five nanograms of human insulin-like growth factor binding protein-3 (IGFBP-3) (Genzyme, Cambridge, MA) were incubated with 10–80 ng of recombinant mK5 at 37°C in 20 μl of 0.1 M Tris-HCl (pH 8.0). The reaction was stopped by the addition of SDS sample buffer. The reaction mixtures were boiled, separated on 12% SDS-PAGE, and transferred to a polyvinylidene difluoride transfer (PVDF) membrane (Millipore). The blotted membrane was incubated with goat anti-human IGFBP-3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a 1:1000 dilution and subsequently with donkey anti-sheep/goat IgG (Amersham Pharmacia, Biotech, Tokyo, Japan). Immunoreactive signals were detected using an ECL Western Blot Detection Kit (Amersham Biosciences, Buckinghamshire, England) according to the protocol provided by the manufacturer.

The action of mK5 on human single-chain tissue-type plasminogen activator (American Diagnostica Inc., Greenwich, CT) was examined according to the method described previously by Ohnishi *et al.* (2004).

RESULTS

Preparation of mK5

In order to obtain a *Klk5* cDNA encoding mK5, we conducted RT-PCR using the primer set described in Materials and Methods. Total RNAs isolated from salivary glands and skeletal muscles of adult mice were used as templates. PCR products amplified from the RNAs were found by sequence analyses to contain two mouse *Klk* species, *Klk1* and *Klk5*. In the present study, a full-length cDNA isolated from mouse skeletal muscle was used for subsequent experiments.

The recombinant protein was expressed in *E. coli* (BL21) transformed with the vector pET30a-mK5, and the enzyme was purified as described in Materials and Methods. SDS-PAGE analysis of the active enzyme sample eluted from a Resource Q column gave a single polypeptide band. The apparent molecular mass was estimated to be 28.5 kDa under reducing conditions and 25.5 kDa under non-reducing conditions (Fig. 1).

Enzymatic properties of mK5

The enzyme showed no detectable activity loss for at least 2 months when stored at –20°C. The electrophoretic pattern did not change under such storage conditions.

The enzyme activity of the recombinant mK5 was measured at various pHs using Pro-Phe-Arg-MCA as the substrate. Activity was detected over a pH range of 7–10, with the optimum pH at 9.5

Fluorogenic peptide substrates with the AMC cleavage group were tested with mK5 to determine its kinetic constants (Table 1). Pro-Phe-Arg-MCA was very rapidly hydrolyzed. Other substrates, *i.e.*, Z-Phe-Arg-MCA, Z-Leu-Arg-MCA, Pyr-Gly-Arg-MCA, and Z-Val-Val-Arg-MCA, were hydrolyzed by mK5 to a lesser extent. Among the substrates hydrolyzed, the highest k_{cat} value and lowest K_m value were obtained with Pro-Phe-Arg-MCA. Substrates containing Lys at the P1 position were not hydrolyzed. The enzyme showed no activity for the synthetic substrates tested that contained tetrapeptides.

The enzyme activity of mK5 was strongly inhibited by diisopropyl fluorophosphate (DFP) and leupeptin (Table 2). A slight inhibition was observed with aprotinin. None of the other inhibitors tested showed significant inhibition of mK5 activity. Assays of two different batches of enzyme preparation gave the same results.

Our results described above suggest that mK5 is a serine protease with strict trypsin-like cleavage specificity.

Hydrolysis of protein substrates by mK5

Zymographic analysis using gelatin was conducted for mK5. A single lytic band was seen at a position correspond-

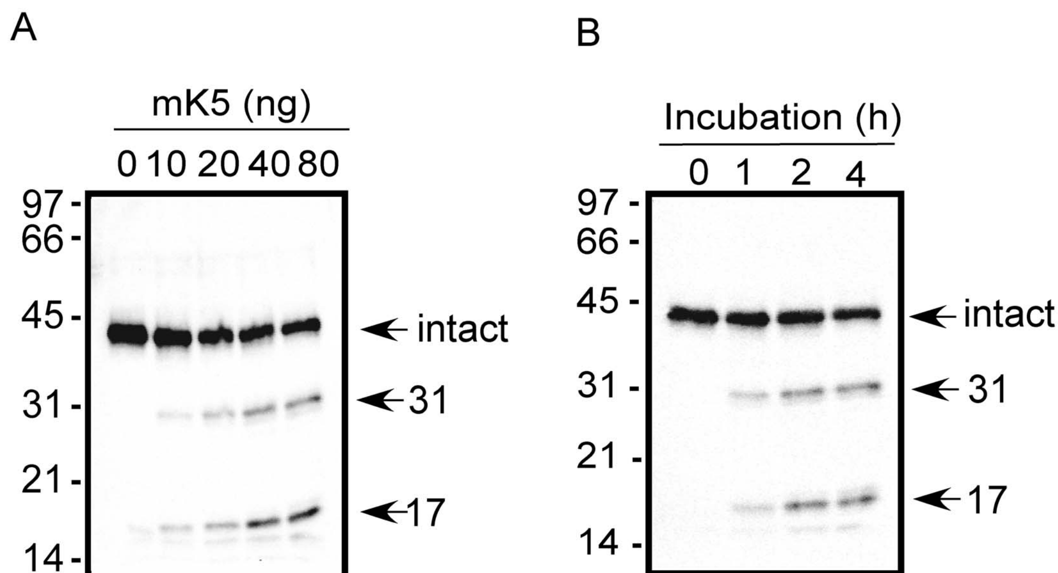

Fig. 3. Action of mK5 on human recombinant IGFBP-3. **(A)** Human IGFBP-3 (25 ng) was incubated for 2 h at 37°C with the indicated amounts of mK5 in 20 μl of 100 mM Tris-HCl (pH 8.0) and was subjected to SDS-PAGE followed by Western blot analysis using anti IGFBP-3 antibody. **(B)** IGFBP-3 (25 ng) was incubated with mK5 (40 ng) at 37°C for the periods indicated at the top of the figure and was subjected to SDS-PAGE/Western blot analysis as described in (A). In both panels, the positions of molecular-mass protein standards (kDa) are indicated to the left, and intact IGFBP-3 and its degraded products (31 and 17 kDa) to the right.

ing to Mr=25.5 kDa (Fig. 2A). Incubation of intact fibronectin (Mr=220 kDa) with mK5 generated two degradation products (Mr=200 and 190 kDa) (Fig. 2B). mK5 cleaved type IV but not type I collagen (Fig. 2C). High-molecular-weight kininogen was a substrate for mK5 (Fig. 2D). Casein and laminin were resistant to mK5 (data not shown). mK5 did not convert the single-chain tissue-type plasminogen activator to the two-chain form of the enzyme (data not shown).

The effect of mK5 on IGFBP-3 was examined. The enzyme degraded the intact protein (Mr=41 kDa) in a time-dependent as well as in a dose-dependent manner, with the concomitant production of 31 and 17 kDa fragments (Fig. 3A and 3B).

DISCUSSION

The mouse *Klk5* gene was previously identified as a member of the tissue kallikrein multigene family located on chromosome 7 (Mason *et al.*, 1983). Based on the nucleotide sequence of *Klk5*, the gene product mK5 was postulated to be a functional protease. However, little information on this protein was currently available. To our knowledge, there had been only one study of this protein, which dealt with the mRNA expression of the kallikrein in the mouse uterus and decidua (Chan *et al.*, 1999). Since our laboratory is interested in the roles of proteases, including tissue kallikreins, in uterine function, we decided to characterize mK5 biochemically.

mK5 hydrolyzed the synthetic substrate Pro-Phe-Arg-MCA with a k_{cat}/K_m value ($mM^{-1}\cdot min^{-1}$) of 1300, which was approximately 25 times greater than the values for the other substrates tested, indicating that Pro-Phe-Arg-MCA was the best substrate. mK21 (Matsui and Takahashi, 2001) and mK24 (Matsui *et al*., 2005a, b), both of which have been recently characterized in our laboratory, also showed strong activity for this substrate. Like mK21 and mK24, mK5 does not cleave Lys-X bonds at all. Table 1 indicates that mK5 is highly specific for the hydrolysis of peptide bonds at the carboxyl side of arginine residues.

The serine protease nature of mK5 was evident from strong inhibition by the protease inhibitors DFP and leupeptin. However, some other typical serine protease inhibitors, such as SBTI and benzamidine, did not affect mK5 activity. The resistance of mK5 to these latter two inhibitors was rather surprising, because they substantially inhibited the previously characterized mK21 and mK24 (Matsui and Takahashi, 2001; Matsui *et al*., 2005a, b). In this regard, we should note our recent observation that mK1 (true mouse tissue kallikrein) is also resistant to SBTI and benzamidine (our unpublished data). The fact that mK5 resembles mK1 in its behaviors toward protease inhibitors may indicate that mK5 and mK1 have highly similar protein structures. Indeed, recent studies by Olsson and Lundwall (2002) on the organization and evolution of mouse *Klk* genes have established that *Klk1* (coding mK1) and *Klk5* (coding mK5) are located alongside each other, and that they are structurally closest among the mouse *Klk* genes.

The present study clearly demonstrates that mK5 hydrolyzes various proteins, including ECM proteins (gelatin, fibronectin, and collagen type IV), high-molecular-weight kininogen, and IGFBP-3. These facts suggest that mK5 may play roles in a variety of biological processes.

Fibronectin is an adhesive cell-surface protein important for the interaction of cells with adjacent cells or ECM components. Degradation of this protein causes the disintegration of local ECM environments, leading to increased mobility of the cells residing in the ECM. Such an event is particularly important in the process of ECM remodeling. The present finding that mK5 can degrade fibronectin indicates that the protease may be involved in the ECM remodeling process. The finding that mK5 shows collagenolytic activity for collagen type IV is consistent with this idea.

Another interesting finding is that mK5 cleaves IGFBP-3. IGFBP-3 is a member of a family of peptides that have a strong affinity with IGF-I and IGF-II, and it plays an inhibitory role in the activity of IGF. It is generally believed that IGFBP proteolysis can reverse the inhibition due to the increased concentration of free IGFs near their receptor. The involvement of many proteases in IGFBP proteolysis has been reported (Firth and Baxter, 2002). Our data suggest that mK5 is another candidate protease for the degradation of IGFBP-3. IGFBP-3 hydrolysis by mK5 produced two major polypeptides (31 and 17 kDa), pointing to a cleavage at a single peptide bond by mK5. We previously reported that IGFBP-3 is cleaved by mK21 (Matsui and Takahashi, 2001). Interestingly, the hydrolysis patterns of IGFBP-3 appear to be the same between mK21 and mK5. The human IGFBP-3 preparation used in this study contains various sites susceptible to proteases. Because mK5 specifically cleaved Arg-X bonds and because IGFBP-3 is reported to be hydrolyzed by many trypsin-like proteases at Arg^{97}-Ala^{98}, we tentatively suggest this peptide bond may be the site of cleavage. However, the validity of this assumption needs to be established by future experiments. In short, mK5 could be a protease regulating the activity of IGFs through the degradation of IGFBP-3.

In this study, we observed that mK5 hydrolyzes high-molecular-weight kininogen. However, the biological significance of this activity is unknown at present.

In summary, this report describes the first characterization of recombinant mK5, which is encoded by an as-yet-unexamined mouse tissue kallikrein gene (*Klk5*). Our study demonstrates the serine protease nature of mK5 with cleavage specificity for Arg-X bonds and suggests that mK5 may play a role in the ECM remodeling process. Our data should be helpful for future physiological studies of mK5.

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