Plasminogen-Independent Initiation of the Pro-urokinase Activation Cascade in Vivo. Activation of Pro-urokinase by Glandular Kallikrein (mGK-6) in Plasminogen-Deficient Mice†

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ABSTRACT: The plasminogen activation (PA) system is involved in the degradation of fibrin and various extracellular matrix proteins, taking part in a number of physiological and pathological tissue remodeling processes including cancer invasion. This system is organized as a classical proteolytic cascade, and as for other cascade systems, understanding the physiological initiation mechanism is of central importance. The attempts to identify initiation routes for activation of the proform of the key enzyme urokinase-type plasminogen activator (pro-uPA) in vivo have been hampered by the strong activator potency of the plasmin, that is generated during the progress of the cascade. Using gene-targeted mice deficient in plasminogen (Plg −/− mice) [Bugge, T. H., Flick, M. J., Daugherty, C. C., and Degen, J. L. (1995) *Genes Dev. 9*, 794−807], we have now demonstrated and identified a component capable of initiating the cascade by activating pro-uPA. The urine from Plg −/− mice contained active two-chain uPA as well as a proteinase capable of activating exogenously added pro-uPA. The active component was purified and identified by mass spectrometry-based peptide mapping as mouse glandular kallikrein mGK-6 (true tissue kallikrein). The pro-uPA converting activity of the mGK-6 enzyme, as well as its ability to cleave a synthetic substrate for glandular kallikrein, was inhibited by the serine proteinase inhibitor leupeptin but not by other serine proteinase inhibitors such as aprotinin, antithrombin III, or α_1-antitrypsin. We suggest that mouse glandular kallikrein mGK-6 is an activator of pro-uPA in the mouse urinary tract in vivo. Since this kallikrein is expressed in a number of tissues and also occurs in plasma, it can also be considered a candidate for a physiological pro-uPA activator in other locations.

Proteolytic enzymes are involved in degradation of the extracellular matrix during the migration of cells through tissue barriers. Tissue degradation and cell migration occur in cancer invasion as well as in normal physiological processes such as trophoblast invasion, morphogenesis, inflammation, and wound healing. The plasminogen activation (PA)[1] system plays an important role in many of these degradative events (for reviews, see refs *1* and *2*). Furthermore, it may undertake specific cleavage reactions in the activation of certain growth factors (*3*) and other proteolytic systems (*4*). The central target for the activation processes is the pro-enzyme plasminogen, which is present in high amounts in plasma and other extracellular fluids and is activated by either of two plasminogen activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). The activation product, plasmin, is an efficient proteinase with a relatively broad, trypsin-like activity. Therefore, the stores of plasminogen represent a large, resting proteolytic potential which becomes activated locally under specific conditions. It follows that the mechanisms of this activation process play a trigger role in the whole proteolytic system. uPA is capable of cleaving plasminogen at the specific peptide bond critical for activation and plays a central role in the pericellular degradation of extracellular matrix proteins, mentioned above, in addition to a role in fibrinolysis (*1, 5, 6*). uPA is secreted as a single polypeptide chain protein which undergoes specific cleavages during the progress of the cascade. This single-chain form, pro-uPA, is a real pro-enzyme with virtually no intrinsic activity (*7*), and the active two-chain form is obtained upon cleavage after Lys^{158}. This activation can be efficiently mediated by plasmin, resulting in a strong feedback amplification of the system in the presence of plasminogen as soon as the first active enzyme molecules are formed. This

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[1] Abbreviations: PA, plasminogen activation; uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; ATF, amino-terminal fragment of uPA; LMW-uPA, low molecular weight two-chain uPA; mGK-6, the protein product from the mouse glandular kallikrein-6 gene; NGF, nerve growth factor; EGF-BP, epidermal growth factor binding protein; hK1, human true tissue kallikrein; hK2, human prostatic kallikrein; PSA, prostate-specific antigen; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; PAI-1, plasminogen activator inhibitor type 1.

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property of the pro-uPA/plasminogen system directs the focus of attention to the initiation of the cascade, but the physiological initiation mechanism is unknown and represents a major obstacle in our understanding of the PA system.

In the two-chain uPA molecule, the NH_2-terminal A-chain is connected to the B-chain by a single disulfide bond. The A-chain contains an epidermal growth factor (EGF)-like domain that mediates the binding to the cell surface receptor, uPAR (*8*), whereas the B-chain contains the catalytic site. An additional plasmin cleavage site is located after Lys^{135}. Cleavage at this site produces an amino-terminal fragment (ATF) and a low molecular weight catalytic fragment (LMW-uPA) which is devoid of uPAR binding activity.

A surprising finding with gene-inactivated mice deficient in plasminogen (Plg −/−) has been that active two-chain uPA is present in the urine (*9*). The existence of one or more pro-uPA activators, different from plasmin and functioning as initiators of the cascade, has long been speculated. Several proteinases including cathepsins B, C, G, and L (*10*−*13*), human mast cell tryptase (*14*), human T cell-associated serine proteinase-1 (*15*), and plasma kallikrein (*16*) have been shown to activate pro-uPA in vitro. However, it is uncertain whether these activators play a physiological role because no data from in vivo systems have been available, mainly due to technical difficulties associated with the confounding abundance of plasminogen in these systems. The Plg −/− mice provide a very useful tool in the search for in vivo activators since the proteolytic profiles of tissues from these mice can be investigated without the interference of plasmin.

We have purified the enzyme responsible for pro-uPA activation in urine from Plg −/− mice, and identified it as the mouse glandular kallikrein mGK-6 (also known as mouse true tissue kallikrein, mouse renal kallikrein, or mouse KLK1) which belongs to a subfamily of serine proteinases; the tissue kallikreins also denominated glandular kallikreins. The tissue kallikreins exhibit a narrow range of substrate specificities and have been described in various species to be involved in proteolysis of specific polypeptide precursors to yield the biologically active forms as exemplified by their ability to release vasoactive kinin (kallidin/bradykinin) from kininogen (reviewed in ref *17*). We now suggest that mGK-6 may act as a pro-uPA activator in vivo.

MATERIALS AND METHODS

The Plg gene-targeted mice employed in this study (*9*) were back-crossed to C57BL/6J or outbred NIHS mice, respectively, for six generations. Urine samples were taken directly from the bladder of anaesthetized male mice or were collected as void urine. The urine was placed on dry ice immediately after collection and stored at − 80 °C.

Purified Proteins, Inhibitors, and Proteinase Substrates. Purified human single-chain uPA was purchased from Technoclone (Vienna, Austria). Purified recombinant non-glycosylated human pro-uPA, expressed in bacteria and used only for MALDI-MS, was a kind gift from Dr. P. Sarmientos, Farmitalia Carlo Erba (Milan, Italy). Mouse single-chain uPA was purified from the medium of 3T3/MSV-LO cultures by affinity chromatography. Human PAI-1, thrombin, and the serine proteinase inhibitors α_1-antitrypsin, antithrombin III, aprotinin (Trasylol), and leupeptin were from Calbiochem (La Jolla, CA). Pro-uPA was labeled with ^{125}I using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (IODO-GEN)(*18*). The chromogenic substrates for uPA, L-pyroglutamylglycyl-L-arginine-*p*-nitroanilide (<Glu-Gly-Arg-pNA; S-2444), and for glandular kallikreins, H-D-valyl-L-leucyl-L-arginine-*p*-nitroanilide (H-D-Val-Leu-Arg-pNA; S-2666), respectively, were purchased from Chromogenics AB (Stockholm, Sweden).

Mono Q Anion-Exchange Chromatography. Anion-exchange chromatography was performed using a "SMART" HPLC system fitted with a Mono Q HR 5/5 column (Pharmacia Biotech, Uppsala, Sweden). The column was equilibrated with 20 mM Tris-HCl, pH 7.4 (buffer A), before application of 2 mL of urine pooled from three male C57BL/6J Plg −/− mice. Prior to application, the urine was dialyzed against buffer A and centrifuged at 20000*g* for 10 min. Bound proteins were eluted from the column using a linear salt gradient (0−0.5 M NaCl in buffer A) at a flow rate of 0.5 mL/min. Fractions were analyzed by SDS−PAGE followed by silver staining. The purified mGK-6 was quantitated by UV absorption spectroscopy using the theoretical molar extinction coefficient of A_{280}(1 M, 1 cm) = 44 300.

Western Blot Analysis. Protein samples were separated by SDS−PAGE on a 10% slab minigel under reducing or nonreducing conditions, and electroblotted onto an Immobilon P membrane (Millipore). Membranes were blocked with 1% skimmed milk powder and incubated with rabbit polyclonal antibodies against mouse uPA (*19*). The secondary, detection antibody was alkaline phosphatase-conjugated porcine anti-rabbit Ig (Dako A/S, Glostrup, Denmark).

Cleavage of Radiolabeled Pro-uPA and Detection of Active Two-Chain uPA. Human ^{125}I-pro-uPA (1 nM) was incubated with urine or purified protein samples in 20 mM Tris-HCl, pH 7.4, 0.1% Tween-80, overnight at 37 °C. In some experiments, human PAI-1 (50 nM) was then added, and the mixture was incubated for another 1 h as above to allow complex formation between active uPA and PAI-1. The samples were analyzed by SDS−PAGE on a 10% gel under reducing conditions and autoradiography. Reaction products were quantitated using a PhoshoImager and ImageQuant version 3.3 software (Molecular Dynamics, Sunnyvale, CA).

Chromogenic Proteinase Assays. The assays were performed in 96-well MaxiSorp surface plates (Nunc, Roskilde, Denmark) in a total reaction volume of 200 μL using 20 mM Tris, pH 7.4, 0.1% Triton X-100 for dilution of all samples. All samples were tested in duplicate. In the pro-uPA activation assay, human pro-uPA (40 nM) or mouse pro-uPA (100 nM) was incubated for 2 h at 37 °C with 2 μL samples of fractions from the ion-exchange chromatography described above. In some experiments, various proteinase inhibitors were added simultaneously as specified. After incubation, the samples were 40-fold diluted, essentially stopping any continued pro-uPA conversion during the following amidolytic detection step. The amidolytic activity of the formed active uPA was then followed after addition of the substrate S-2444 (0.4 mM) by readings of the absorbance at 405 nm every 5 min for 1 h at 37 °C. The change in absorbance was a linear function of time, and the rate was calculated. In the assay for glandular kallikrein, 2 μL fraction samples were mixed with proteinase inhibitors

as specified and the substrate S-2666 (0.02 mM), after which the activity was measured as described above.

Protein Identification by Mass Spectrometric Peptide Mapping and Database Searching. Stained protein bands were cut out of electrophoretic gels and prepared for mass spectrometric analysis. Briefly, the protein-containing gel pieces were rinsed, reduced, and alkylated prior to proteolytic digestion with trypsin, all performed in-gel (*20*). The unseparated peptide mixtures generated in this way were analyzed by delayed extraction matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry on a Bruker REFLEX instrument (Bruker Daltonics, Bremen, Germany) (*21*). Protein identification was performed by submitting the measured lists of tryptic peptide masses for sequence database searches using PeptideSearch software and a nonredundant sequence database (EMBL/EBI).

Identification of the Pro-uPA Cleavage Site. Mass spectrometry analyses of fragments obtained after enzymatic cleavage of pro-uPA were done by MALDI-MS (Voyager, PerSeptive Biosystems). Pro-uPA (2 μM) was cleaved with mGK-6 (100 nM), plasmin (1 nM), or thrombin (20 nM) in 0.1 M NH_4HCO_3 overnight at 37 °C. Cleavage products (approximately 10 pmol) were dried by vacuum evaporation and redissolved in 5 μL of 6 M guanidinium chloride, 0.5 M Tris-HCl, pH 8.0, 2 mM EDTA, 20 mM dithiothretiol. The samples were then boiled for reduction of disulfides and subsequently alkylated with 50 mM iodoacetamide. All spectra were recorded after sample deposition (10 pmol of uPA) in α-cyano-4-hydroxycinnamic acid according to ref *22*. The spectra were calibrated internally using either the matrix ions or added internal protein calibrants (apomyoglobulin and α-cobra toxin).

Kinetic Assays. Conversion of pro-uPA to two-chain uPA was analyzed by electrophoresis followed by scanning of Coomassie-stained protein bands to quantitate the reaction products. Pro-uPA was incubated with mGK-6 in 20 mM Tris, pH 7.4, 0.1% Tween-80, at 37 °C, and after the time period indicated, the reaction was stopped by addition of Laemmli sample buffer. The samples were subjected to SDS−PAGE (10% gel under reducing conditions), and gels were stained with 0.1% Coomassie Blue R250. The gels were scanned in an HP ScanJet 4C scanner, and the reaction products were quantitated using the ImageQuant software specified above.

RESULTS

Two-Chain uPA Is Present in the Urine from Plg-Deficient Mice. Mouse urine has been shown to contain large amounts of uPA. In freshly voided urine from normal mice, the uPA is mainly present in the active two-chain form (*23*). To investigate the molecular form of uPA in the urine from Plg −/− mice, bladder and void urine was analyzed by Western-blotting (Figure 1 and data not shown). The approximate proportions of single-chain and two-chain uPA can be evaluated after SDS−PAGE under reducing conditions whereas parallel electrophoresis under nonreducing conditions reveals an additional cleavage reaction leading to the formation of a low molecular fragment with an M_r of approximately 32 000 (LMW-uPA). The levels of two-chain uPA in wild-type and Plg −/− mice were similar and accounted for the major part of the uPA present (Figure 1A).

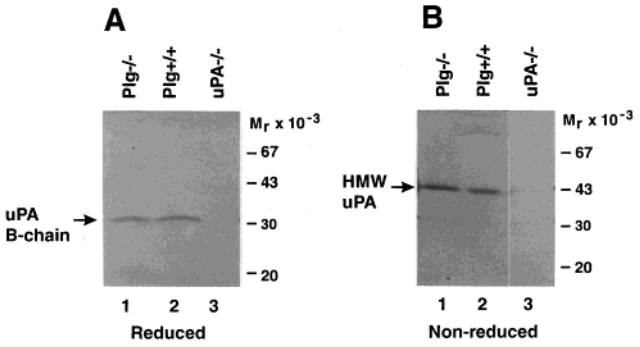

FIGURE 1: Two-chain uPA is present in Plg −/− mouse urine. Western blot detection of uPA under reducing (panel A) or nonreducing (panel B) conditions. Bladder urine (10 μL) from a Plg −/− mouse (lane 1), a wild-type (Plg +/+) mouse (lane 2), or a uPA −/− mouse (lane 3) was analyzed by SDS−PAGE on a 10% Mini gel and blotted onto PVDF membranes. The uPA was detected using an anti-uPA rabbit polyclonal antibody followed by alkaline phosphatase conjugated porcine anti-rabbit IgG. The M_r's of molecular weight marker proteins are indicated.

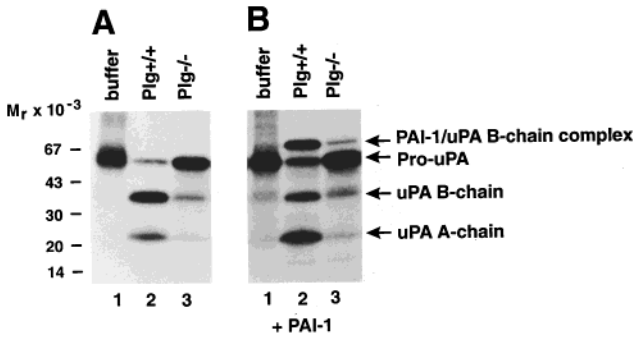

FIGURE 2: Urine from Plg −/− mice contains a proteolytic activity that activates pro-uPA. Human ^{125}I-pro-uPA (1 nM) was incubated in buffer alone (lane 1) or in bladder urine from a Plg +/+ mouse (lane 2) or from a Plg −/− mouse (lane 3) overnight at 37 °C. One set of samples was analyzed directly (panel A) while another set of samples was incubated for a further 1 h with 50 nM human PAI-1 to allow complex formation with active uPA (panel B). The samples were analyzed by SDS−PAGE on a 10% Mini gel under reducing conditions followed by autoradiography. The M_r's of molecular weight marker proteins are indicated.

This two-chain uPA was predominantly present in the form of high molecular weight uPA in both wild-type and Plg-deficient mice (Figure 1B), although the urine from some mice had a higher proportion of LMW-uPA [approximately 20% of the total uPA as based on visual inspection (not shown)]. As a control of antibody specificity, a urine sample from a uPA −/− mouse (*6*) was included. No staining was seen in this case (Figure 1A,B). These results indicate the presence of a plasmin-independent proteolytic activity capable of cleaving pro-uPA, which is in accordance with the findings originally described (*9*).

The Urinary Tract of Plg-Deficient Mice Contains a Proteolytic Activity Capable of Activating Pro-uPA. Since the urine from Plg −/− mice contained two-chain uPA, an important question to address was whether the responsible pro-uPA activator was also excreted in the urine, since this would facilitate its purification. The presence of putative pro-uPA activator(s) was investigated by measuring the ability of urine samples from Plg −/− mice to cleave ^{125}I-labeled pro-uPA. Radiolabeled pro-uPA was incubated with urine samples from a Plg −/− mouse and a wild-type mouse, respectively. The urine from the wild-type mouse had a

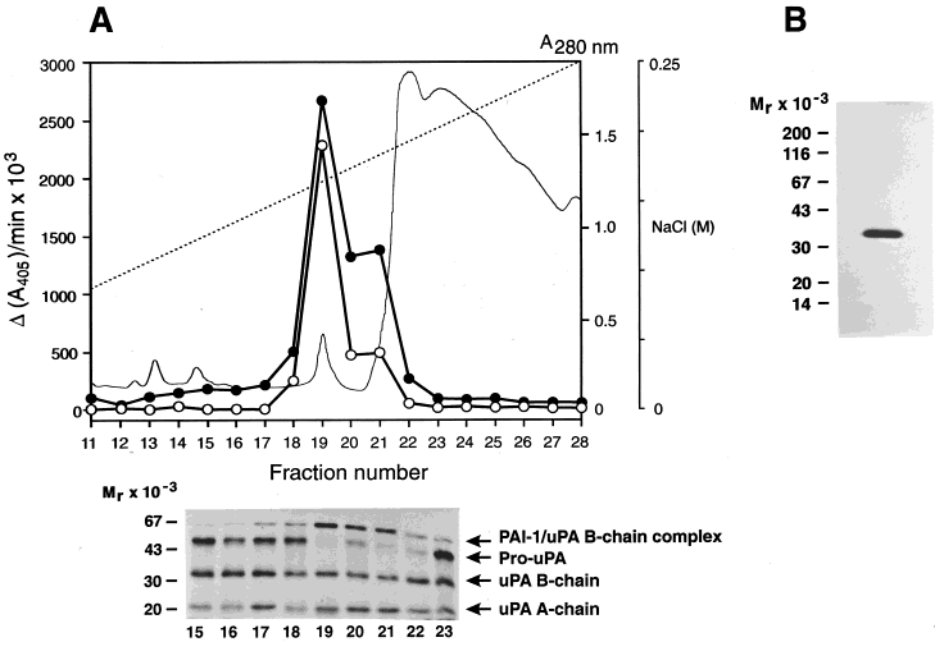

FIGURE 3: Purification of urinary glandular kallikrein by anion-exchange chromatography. Panel A: Plg −/− mouse urine (2 mL) was dialyzed against 20 mM Tris-HCl, pH 7.4, and applied to a Mono Q anion-exchange column. Proteins were eluted using a linear gradient of 0−0.5 M NaCl (- - -) in the same buffer at a flow rate of 0.5 mL/min, recording the absorbance at 280 nm (—). Fractions were collected and assayed for pro-uPA activating activity using exogenously added pro-uPA and the chromogenic substrate S-2444 (●). This activity is expressed as the ΔA_{405}/min obtained in each fraction after 2 h of pro-uPA activation. Parallel samples, prepared in the same manner but without pro-uPA, all had substrate conversion rates below 200×10^3 AU/min (results not shown). Glandular kallikrein activity was measured using the chromogenic substrate S-2666 and expressed as the conversion rate of this substrate (○). The activation cleavage of radiolabeled pro-uPA (1 nM) obtained after incubation overnight at 37 °C with 2 μL each of fractions 15−23 followed by incubation with PAI-1 (50 nM) for 1 h is shown below the graph. For each sample, activation was analyzed by SDS−PAGE on a 10% gel under reducing conditions and autoradiography. Panel B: Fraction 19 from the anion-exchange chromatography was analyzed by SDS−PAGE on a 10% gel under reducing conditions. The gel was silver stained. The single protein band with an apparent M_W of 35 000 was excised from a corresponding gel stained with Coomassie blue and identified by mass spectrometry-based peptide mapping as the true tissue kallikrein, mouse glandular kallikrein, mGK-6 (Table 1). The M_r's of molecular weight marker proteins are indicated.

pronounced proteolytic effect on pro-uPA with conversion of approximately 90% of the pro-uPA under the conditions used, leading to the accumulation of free B-chain and A-chain under reducing conditions (Figure 2 A). The major part of this activity most likely represented plasmin since it was efficiently inhibited by aprotinin as well as by α_2-antiplasmin (results not shown). Interestingly, the Plg −/− urine also contained a pro-uPA converting activity, though this activity was lower than that observed in wild-type mice, leading to approximately 15% conversion under the conditions of this experiment. To confirm that the resulting cleavage product was indeed proteolytically active uPA, its ability to bind the plasminogen activator inhibitor, PAI-1, was tested. This inhibitor binds to the active two-chain uPA, forming an SDS-stable complex, while it does not bind pro-uPA (*24*) or the uPA variant that is obtained after thrombin cleavage [cleavage occurring between Arg^{156} and Phe^{157} (*16*)] (result not shown). When PAI-1 was added to the sample, an SDS-stable complex consisting of PAI-1 and uPA B-chain was seen in both cases (Figure 2B). Even though this experiment was performed as a qualitative determination of complex formation, the amounts of complex formed indeed reflected the difference in pro-uPA conversion levels between wild-type and Plg −/− urine described above as judged by scanning (results not shown). There was no difference in the results obtained in this assay, or in the immunoblot assay described above, using void or bladder urine. In addition, analysis of urine samples from mice of different backgrounds, C57BL/6J or outbred NIHS mice, did not show any differences between the two types of mice with respect to amounts of two-chain uPA and pro-uPA converting activity. These results indicate that mouse urine does contain a plasmin-independent activator of pro-uPA.

The Active Pro-uPA Converting Enzyme in Plg-Deficient Mouse Urine Is Glandular Kallikrein mGK-6. To identify the pro-uPA activator, the proteins in the Plg −/− mouse urine were separated by anion-exchange chromatography. The eluted fractions were tested for pro-uPA activating proteolytic activity by a chromogenic assay using conversion of exogenous purified pro-uPA and measurement with a uPA-specific substrate. One major peak of pro-uPA activating activity was seen, eluting at 0.2 M NaCl (Figure 3 A). To ensure that this result reflected activation of the added pro-uPA and not merely the isolation of an activity cleaving the *p*-nitroanilide substrate, a parallel assay using samples without added pro-uPA was performed. In this control experiment, all active fractions had a conversion activity lower than 15% of that seen when pro-uPA was added (results not shown). The identification of the pro-uPA activating fraction was verified using the ^{125}I-pro-uPA activation assay (Figure 3A, bottom). Whereas these experiments were most conveniently done with human pro-uPA which is available with virtually undetectable amounts of active uPA, all results were confirmed using murine pro-uPA, having a somewhat higher background activity (results not shown).

The fraction containing the highest pro-uPA converting activity was analyzed by SDS−PAGE followed by silver

Table 1: Identification of the Pro-uPA Activator by Mass Spectrometry[a]

measured mass (Da)	theoretical mass (Da)[b]	residue no.	sequence
1162.600	1162.637	246−254	(R)VLNFNTWIR(E)
1347.650	1347.680	34−44	(K)NSQPWQVAVYR(F)
1670.780	1670.773	165−177	(K)YEYPDELQCVNLK(L)
1733.880	1733.889	148−164	(K)LGSTCLASGWGSITPVK(Y)
2446.270	2446.380	126−147	(R)LKKPADITDVVKPIDLPTEEPK(L)
2655.190	2655.255	48−70	(K)YQCGGILLNANWVLTAAHCHNDK(Y)

[a]The protein band of the active fraction 19 from anion exchange chromatography was excised from a Coomassie-stained SDS gel and subjected to "in gel" trypsin digestion. Fragments obtained after cleavage were analyzed by MALDI-MS. Peptide masses refer to the protonated monoisotopic molecules (MH+). Cysteine is *S*-carboxamidomethyl-Cys, and methionine is unmodified. [b] Theoretical masses were derived from the trEMBL peptide molecular mass database identifying mouse glandular kallikrein, mGK-6 (trEMBL Database = Q61855, Swiss Protein Database = P15947).

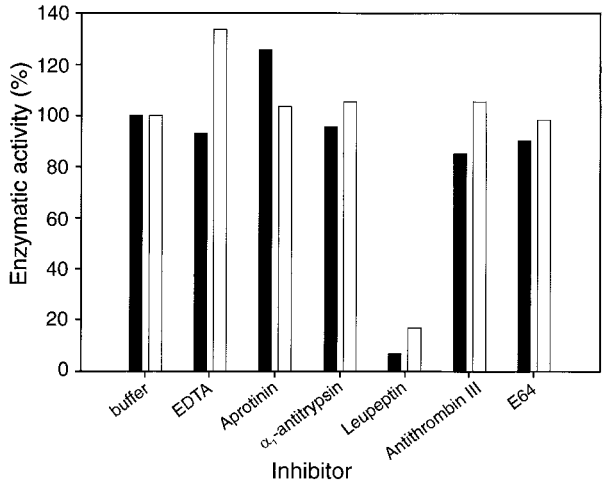

FIGURE 4: Inhibition profile of urinary glandular kallikrein. Fraction 19 from the anion-exchange chromatography was analyzed for pro-uPA activating activity (■) and glandular kallikrein activity (□) in the presence of various proteinase inhibitors using the substrates and conditions described in the legend to Figure 3A. The results are expressed as enzymatic activity [$\Delta(A_{405})$/min × 10^3] in percent of the maximal activity obtained after incubation with buffer alone. The inhibitors were added in the following concentrations: aprotinin, 10 μM; EDTA, 10 mM; leupeptin, 200 μM; antithrombin III and α_1-antitrypsin, 400 nM; E64, 10 μM.

staining which demonstrated the presence of a single band with an apparent M_r of 35 000 under reducing conditions (Figure 3B). The protein band was excised from a corresponding gel stained with Coomassie blue and subjected to mass spectrometry-based peptide mapping. This analysis unambiguously identified the protein as the glandular kallikrein mGK-6 (Table 1). Based on this finding, an amidolytic chromogenic assay using a substrate for glandular kallikrein was performed on the fractions from the ion exchange chromatography. The pro-uPA activating fraction completely coincided with the fraction of glandular kallikrein activity (Figure 3A). In addition, both the uPA converting activity and the amidolytic glandular kallikrein activity were inhibited by the serine proteinase inhibitor leupeptin whereas other serine proteinase inhibitors such as aprotinin, antithrombin III, or α_1-antitrypsin, the metalloproteinase inhibitor EDTA, or the cysteine proteinase inhibitor E64 had no inhibitory effect (Figure 4). Further evidence that mGK-6 was indeed the enzyme responsible for pro-uPA activation in the raw material was provided by the finding that the pro-uPA activation activity in crude Plg −/− urine was likewise efficiently inhibited by leupeptin (result not shown).

mGK-6 Activates Pro-uPA by Cleavage between Lys[158] and Ile[159]. To determine the mGK-6 cleavage site in pro-uPA, a mass spectrometry analysis was performed on the uPA A-chain after enzymatic cleavage. Samples of pro-uPA were incubated with the isolated mGK-6, plasmin or thrombin. Plasmin activates pro-uPA by cleavage between Lys[158] and Ile[159] (*25*). In contrast, thrombin cleaves between Arg[156] and Phe[157], generating a catalytically inactive variant of two-chain uPA, as mentioned above (*16*). Both recombinant pro-uPA expressed in bacteria and pro-uPA expressed in mammalian cells were included in this study, due to the complicating factor of O-glycosylation (a fucose moiety on Thr[18] in the A-chain) in mammalian pro-uPA (*26*). Prior to mass analysis, the uPA cleavage products were analyzed by SDS−PAGE under reducing conditions. Two bands, migrating with apparent M_r's of approximately 29 000 and 18 000 corresponding to the A-chain and B-chain of uPA, respectively, were observed irrespective of whether plasmin, thrombin, or purified mGK-6 was used in the cleavage reaction (results not shown). The actual molecular masses of the uPA A-chains, determined by MALDI-MS, are listed in Table 2. It is evident that mGK-6 generates an A-chain identical to that obtained after plasmin cleavage but clearly different from the thrombin-cleaved fragment. None of the molecular masses determined differed more than 0.1% from the theoretical masses whether bacterially expressed pro-uPA or mammalian expressed pro-uPA was analyzed. We therefore conclude that mGK-6, like plasmin, activates pro-uPA by cleavage between Lys[158] and Ile[159].

Kinetic Analysis of Pro-uPA Activation by mGK-6. The catalytic efficiency of mGK-6-mediated activation of pro-uPA was determined. When pro-uPA was incubated with mGK-6, conversion of the pro-enzyme occurred in a time-dependent manner (Figure 5). The pro-uPA activation was a linear function of the mGK-6 concentration when the molar excess of pro-uPA was greater than 5 (data not shown). The kinetics of pro-uPA activation were determined by incubating increasing concentrations of pro-uPA with a constant concentration of mGK-6. The activation reaction was concentration-dependent and saturable (Figure 6 B) and seemed to follow simple Michaelis−Menten kinetics in the substrate concentration range studied (Figure 6C). Based on the Lineweaver−Burk plot, the kinetic constants were determined. K_m and k_{cat} for the activation of pro-uPA by mGK-6 were found to be 6 μM and 0.02 s^{-1}, respectively, giving a catalytic efficiency (k_{cat}/K_m) of 3.3 × 10^3 M^{-1} s^{-1}.

DISCUSSION

A central question in the understanding of proteolytic cascade systems is the identification of the physiological initiators of the cascade. In the case of the uPA−plasmin

Table 2: Molecular Mass Analysis of Fragments Obtained after Enzymatic Cleavage[a]

	human uPA A-chain				
	expressed in bacteria		expressed in mammalian cells		
enzyme	measured mass (Da)	theoretical mass (Da)	measured mass (Da)	theoretical mass (Da)	N-terminus uPA A-chain
mGK-6	18702.3	18704.1	18842.6	18850.2	1−158
plasmin	18703.8	18704.1	18863.4	18850.2	1−158
thrombin	18422.6	18428.8	18589.0	18574.9	1−156

[a] The molecular masses of the uPA A-chains measured after cleavage of 10 pmol of pro-uPA with mGK-6, plasmin, or thrombin were determined by MALDI-MS and are listed along with the theoretical average masses for comparison. Molecular masses refer to the protonated (MH+) fragments.

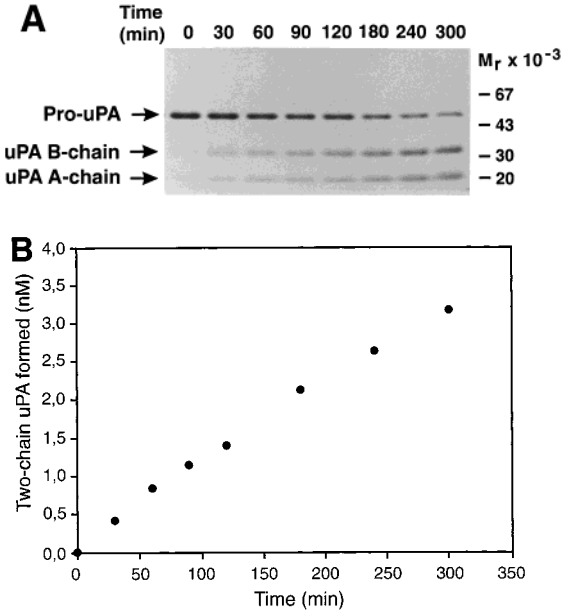

FIGURE 5: Time course of mGK-6-mediated pro-uPA activation. Pro-uPA (5.3 μM) was incubated for the indicated periods of time with purified mGK-6 (150 nM) in 20 mM Tris-HCl, 0.1% Tween-80, pH 7.4. Conversion of single-chain pro-uPA to two-chain uPA was analyzed by SDS−PAGE under reducing conditions (panel A), and the reaction products were quantitated by densitometry (panel B). The conversion is expressed as the amount of two-chain uPA formed (nanomolar) as a function of time (hours). The trace amount of two-chain uPA present in the pro-uPA when no mGK-6 was added (see panel A) was subtracted from all data points.

system, both of the two key enzymes are secreted as inactive zymogens, and it has long been speculated that other proteinases are involved in an initial step by activating pro-uPA. Since plasmin itself is a potent pro-uPA activator and plasminogen is present in large amounts in most extracellular environments, it has been difficult to search for alternative physiological activators. The possibility of including samples from plasminogen-deficient mice in our studies has given us a unique opportunity to undertake this task. The existence and in vivo activity of one or more pro-uPA converting enzymes different from plasmin could thus be inferred directly from the finding that disruption of the plasminogen gene had no impact on the levels of active uPA in urine. Based on our identification of the enzyme responsible for pro-uPA activation in Plg −/− mouse urine as mGK-6, a member of the tissue kallikrein family, we now consider this enzyme a candidate initiator of the uPA-mediated pathway of plasminogen activation in the mouse urinary tract in vivo.

mGK-6 mRNA is expressed in various mouse tissues including kidney, pancreas, pituitary gland, and submandibular glands (*27*). Furthermore, mGK-6 can be detected in the lactating mammary gland (*28*), plasma, and urine (*29*). Mouse uPA is also widely distributed, e.g., in pancreas, kidney, the gastrointestinal tract, involuting mammary glands, placenta, lungs (*30*), plasma, and urine (*23*, *31*). In the kidney, both uPA (*30*) and glandular kallikrein (*32*) are expressed in the distal tubules. Therefore, it can be suggested that the two enzymes coexist in the tubular renal system, making molecular interaction and pro-uPA activation likely in this location. In the view of the occurrence of mGK-6 in several tissues as well as in plasma, this enzyme could be considered a candidate for a physiological pro-uPA activator also in other locations. Indeed, we have identified a pro-uPA activating activity in kidney extracts from Plg −/− mice and found that this activity was inhibited by leupeptin (K. List, unpublished result), thus making it likely that it does represent glandular kallikrein. It cannot be excluded though, that also other proteinases contribute to the activation process in this and other organs. A clarification of this question must await the development of methods for monitoring uPA activity and pro-uPA activation in complex systems such as intact organs and tissues. An approach which in the future could also be valuable in the investigations of the physiological relevance of mGK-6 is the generation of mice deficient in both plasminogen and mGK-6.

In the mouse, the tissue kallikreins are encoded by a large highly homologous multigene family located on chromosome 7 (*33*) including genes encoding mGK-6, γ- and α-subunits of nerve growth factor (NGF) (*27*), and epidermal growth factor binding protein (EGF-BP) (*34*).

Many studies concerning the physiological functions of tissue kallikreins have focused on their kininogenase activity (reviewed in refs *17* and *35*), but their involvement in processing of other substrates has received increasing attention in recent years. Thus, substrates cleaved in vitro by these enzymes include low-density lipoprotein (*36*) and prolactin (*35*, *37*) and pro-collagenases which can be activated by tissue kallikrein (*38*). A role of tissue kallikreins in uPA-dependent plasminogen activation has been suggested based on reports that other members of this enzyme family can activate pro-uPA in vitro, i.e., EGF-BP (*39*) and γ-NGF (*40*) in the mouse. In the human tissue kallikrein gene family, three members have so far been identified encoding true tissue kallikrein (hK1), prostatic kallikrein (hK2), and prostate-specific antigen (PSA) (*41*). Indeed, human prostatic kallikrein, hK2, is capable of activation of pro-uPA in vitro (*42*). The ability to activate pro-uPA is not a general property of the tissue kallikrein family members though, since neither PSA (*42*) nor hK1 (K. List, unpublished data) seems to be active in this process.

The kinetic studies indicated that mGK-6 has a catalytic efficiency in activating pro-uPA of about 3×10^3 M^{-1} s^{-1}. This activation efficiency is comparable with that reported

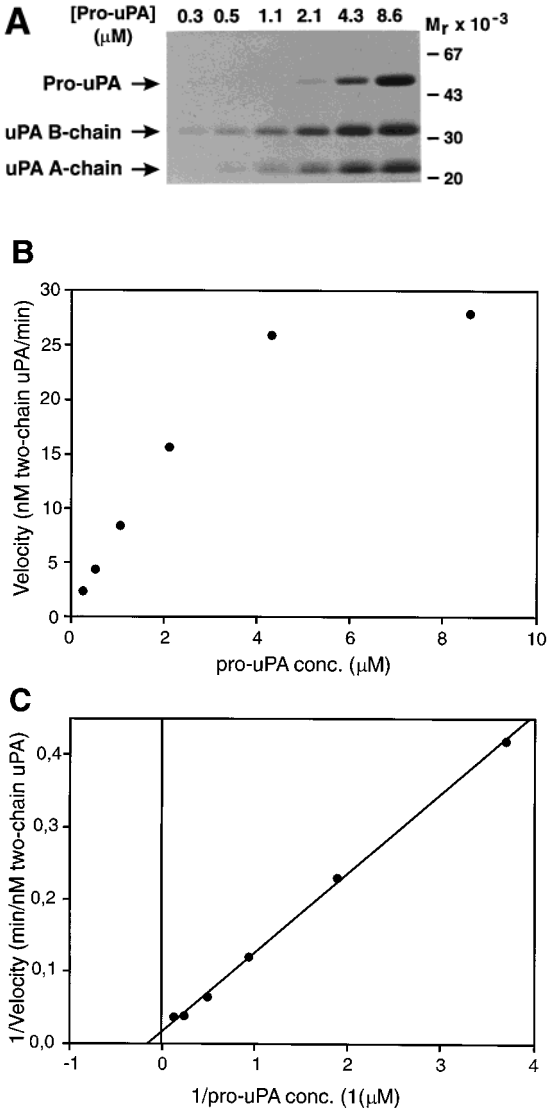

FIGURE 6: Kinetics of pro-uPA activation by mGK-6. Panel A: Varying concentrations of pro-uPA were incubated with mGK-6 (50 nM) in a total volume of 50 μL of 20 mM Tris-HCl, 0.1% Tween-80, pH 7.4, for 2 h at 37 °C. The samples were analyzed by SDS−PAGE on a 10% gel under reducing conditions, stained with Coomassie Blue R250, and quantitated by densitometric scanning. Panel B: The data obtained from panel A were plotted as the rate of two-chain uPA formation (nM two-chain uPA/min) as a function of pro-uPA concentration (μM). Panel C: The same data were replotted in the form of a Lineweaver−Burk plot. The trace amount of two-chain uPA present in the pro-uPA preparation when no mGK-6 was added was subtracted from all data points.

for mast cell tryptase, a potential pro-uPA activator according to in vitro studies (*14*). Since mGK-6 is clearly less efficient in this activation reaction than the other known in vivo activator plasmin (K_m 7.1 μM, k_{cat} 1.3 s^{-1}, catalytic efficiency 1.8×10^5 M^{-1} s^{-1}) (*25*), it can be speculated that the role of mGK-6 is to initiate the uPA-dependent proteinase cascade rather than to contribute to pro-uPA activation in the amplification phase.

There have been several reports that tissue kallikreins are highly expressed in human cancers. Prostate-specific antigen (PSA) is a tumor marker in prostate cancer and possibly in female breast cancer, whereas human prostatic kallikrein, hK2, may be useful as a marker for prostate cancer (reviewed in ref *43*). Due to the finding that hK2 activates pro-uPA in vitro (*42*) combined with the observation of increased uPA expression in prostatic cancer tissues (*44*), it has been speculated that tissue kallikrein-dependent activation of the PA system could be a means of action leading to the increased extracellular matrix degradation and migration related to cancer invasion (*42*).

The current study demonstrates that, at least in the mouse, a member of the tissue kallikrein family may indeed play a role in the PA system in vivo. Several studies on different components of the PA system have pointed out that this system is important in physiological and pathophysiological tissue remodeling events including wound healing (*45*−*47*), postlactational mammary gland involution (*48*, *49*), trophoblast implantation (*50*), and cancer invasion and metastasis. In cancer, histochemical studies show a specific expression of PA components in areas of tumor invasion (*2*). In addition, several studies, in vitro and in vivo, have demonstrated that various inhibitors of PA components inhibit the ability of cancer cells to invade and/or metastasize (*2*). The employment of Plg −/− mice in tumor model studies has demonstrated that plasminogen deficiency leads to a marked decrease in the formation of lung metastasis in mice transgenic for the Polyoma middle T antigen under the control of the mouse mammary tumor promoter (*51*, *52*). Also, growth of Lewis lung tumors is impaired in these mice (*51*). To efficiently inhibit the PA-mediated matrix-degradation processes, it may be beneficial to target an early step in the cascade system. The elucidation of novel critical components in the plasminogen activation cascade in vivo could be a significant step forward in the development of therapeutic strategies in this context.

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