Proteolytic Cleavage of Urokinase-Type Plasminogen Activator by Stromelysin-1 (MMP-3)†

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Received November 24, 1997; Revised Manuscript Received February 10, 1998

ABSTRACT: Matrix metalloproteinase-3 (MMP-3, or stromelysin-1) specifically hydrolyzes the Glu[143]−Leu[144] peptide bond in 45-kDa single-chain urokinase-type plasminogen activator (scu-PA) and in its two-chain (tcu-PA) derivative, yielding a 17-kDa NH_2-terminal domain comprising the u-PA receptor (u-PAR) binding site and a 32-kDa COOH-terminal moiety containing the serine proteinase domain of u-PA. The conversion is completely abolished in the presence of the MMP inhibitors EDTA or 1,10-phenanthroline. Biospecific interaction analysis indicates that binding of MMP-3 occurs through the 32-kDa fragment. The 32-kDa fragment derived from scu-PA (scu-PA-32k) has a specific activity of ≤500 IU/mg, but it can be activated with plasmin to a two-chain derivative (tcu-PA-32k) with a specific activity of 79 000 IU/mg. tcu-PA and tcu-PA-32k moieties derived from scu-PA-32k by plasmin or from tcu-PA by MMP-3 have comparable amidolytic activities toward the chromogenic substrate S-2444 (k_{cat}/K_m of 110 and 160 mM^{-1} s^{-1}, respectively) and similar plasminogen activating activities in a coupled chromogenic substrate assay. Specific binding of the 17-kDa NH_2-terminal domain to THP-1 monocytoid cells is completely abolished by competition with scu-PA but is not affected by scu-PA-32k (residual binding of 88 ± 9% (mean ± SEM; $n = 3$) with 25-fold molar excess). Thus, MMP-3 removes a functional NH_2-terminal u-PAR-binding domain from u-PA without affecting its enzymatic properties.

It has been suggested that the plasminogen/plasmin (fibrinolytic) system and the matrix metalloproteinase (MMP)[1] system are functionally interactive and cooperate in extracellular matrix degradation (*1*−*5*). The proenzyme plasminogen is converted into the active serine proteinase plasmin by tissue-type (t-PA) or urokinase-type (u-PA) plasminogen activator. Whereas t-PA is mainly involved in the dissolution of fibrin in the circulation, u-PA binds to a specific cellular receptor (u-PAR) and plays a role mainly in pericellular proteolysis (*6*). MMPs are also secreted as zymogens that are extracellularly activated by several proteinases and by organomercurial compounds. Active MMPs degrade most proteins and proteoglycans that constitute the extracellular matrix, and thereby play a role in normal tissue remodeling but also in pathological conditions such as arthritis, tumor invasion, and metastasis (*1*−*3*). Recently, it was shown that u-PA-mediated plasmin generation plays a role in media destruction and aneurysm formation during atherosclerosis, via activation of proMMP-3 (stromelysin-1), proMMP-9 (gelatinase B), proMMP-12 (metalloelastase), and proMMP-13 (collagenase-3) (*7*).

MMP-3 has been detected in cultures of medial or intimal smooth muscle cells and in fibroblasts, and mRNA expression was found in human atherosclerotic plaques, mainly associated with macrophages (*8*, *9*). The plasminogen activator u-PA is secreted as a single-chain molecule (scu-PA) that is converted into a more active two-chain derivative (tcu-PA) by cleavage of the Lys[158]−Ile[159] peptide bond with plasmin (*10*, *11*). u-PA contains several structural/functional domains, including an NH_2-terminal growth factor domain (comprising the u-PAR-binding sequence between amino acids 12 and 32 (*12*)), a kringle domain with unknown function, and a serine proteinase domain that contains the catalytic triad His[204], Asp[255], and Ser[356] (*6*). A 32-kDa single-chain derivative lacking the 143 NH_2-terminal amino acids has been reported with intact enzymatic activity and thrombolytic potential (*13*, *14*).

In the present study, we report that MMP-3 specifically cleaves the Glu[143]−Leu[144] peptide bond of u-PA, thereby removing the u-PAR-binding and kringle domains.

MATERIALS AND METHODS

Proteins, Reagents, and Assays. Recombinant human scu-PA (saruplase) was a kind gift of Grünenthal GmbH (Aachen,

† This study was supported by grants from the Flemish Fund for Scientific Research (FWO, contract G.0126.96) and from the IUAP (contract P4/34).

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[1] Abbreviations: MMP-3, matrix metalloproteinase-3 (stromelysin-1, EC 3.4.24.17); u-PA, urokinase-type plasminogen activator (urokinase, EC 3.4.21.73); scu-PA, single-chain u-PA; tcu-PA, two-chain u-PA derived from scu-PA by cleavage of the Lys[158]−Ile[159] peptide bond with plasmin; scu-PA-32k, 32-kDa fragment of scu-PA lacking the 143 NH_2-terminal amino acids; tcu-PA-32k, 32-kDa fragment of tcu-PA lacking the 143 NH_2-terminal amino acids; ATF-17k, 17-kDa NH_2-terminal fragment of u-PA obtained by treatment with MMP-3; MAb, monoclonal antibody; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; S-2444, Glp-Gly-Arg-*p*-nitroanilide; S-2403, D-Glp-Phe-Lys-*p*-nitroanilide.

S0006-2960(97)02870-5 CCC: $15.00
Published on Web 04/28/1998

Germany). Stock solutions of tcu-PA were prepared by maximal activation at 37 °C of scu-PA (final concentration, 100 μM) with plasmin (enzyme/substrate ratio of 1/200) for 10 min in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.038 M NaCl and 0.01% Tween 80. Plasmin was neutralized by addition of ≥10-fold molar excess of aprotinin, and tcu-PA was isolated by chromatography on benzamidine−Sepharose, as described previously (*15*). Glycosylated recombinant scu-PA and the plasmin- resistant mutant scu-PA-Glu^{158} were obtained and characterized as described elsewhere (*16*). Recombinant scu-PA-32k, expressed in Chinese hamster ovary cells, was purified by chromatography on zinc chelate−Sepharose and immunoadsorption on the insolubilized MAb 4D1E8 (*14*). tcu-PA-32k was prepared by treatment with plasmin, as described above. Before use, all single-chain u-PA moieties were depleted of trace amounts of tcu-PA activity by chromatography on benzamidine−Sepharose.

Human Glu-plasminogen and plasmin were obtained and characterized as described elsewhere (*16, 17*). Recombinant human proMMP-3 (57-kDa native form) and MMP-3 (23.5-kDa truncated form, lacking the COOH-terminal domain) were a kind gift of H. Nagase (Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS) (*18, 19*). Protein concentrations were determined with the BCA protein assay (Pierce, Rockford, IL).

The chromogenic substrates S-2444 for u-PA and S-2403 for plasmin were purchased from Chromogenix (Antwerp, Belgium), and aprotinin (Trasylol) was from Bayer (Leverkusen, Germany). The International Reference Preparation for u-PA (66/46) was obtained from the National Institute for Biological Standards and Control (Hertfordshire, U.K.). The matrix metalloproteinase inhibitor 1,10-phenanthroline was purchased from Sigma Chemie (Bornem, Belgium). The human THP-1 cell line was obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium containing 2 mM L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

SDS−PAGE without reduction or after reduction with 1% dithioerythritol was performed on 10−15% gradient gels using the Phast System (Pharmacia) and staining with Coomassie brilliant blue. Apparent molecular masses were determined from reduced gels, by comparison with a protein calibration mixture consisting of phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Densitometric scanning of SDS−PAGE gels was performed with the Gel-scan accessory of the Beckman DU60 spectrophotometer.

NH_2-terminal amino acid sequence analysis was performed on an Applied Biosystems model 477A protein sequencer (Foster City, CA), interfaced with an Applied Biosystems model 120A on-line analyzer. Samples were electrophoresed on 10−20% Tris-HCl Ready Gels (Mini-Protean II Cell; BioRad, Hercules, CA) and blotted on ProBlott membranes (Applied Biosystems).

Association rate constants (k_{ass} in M^{-1} s^{-1}) and dissociation rate constants (k_{diss} in s^{-1}) for the interaction between (pro)-MMP-3 and different u-PA moieties were determined by real-time biospecific interaction analysis using the BIAcore instrument (Pharmacia, Biosensor AB). ProMMP-3 or MMP-3 were immobilized on the surface of sensor chip CM5 using the Amine Coupling kit (Pharmacia), and binding of u-PA moieties was evaluated as described in detail elsewhere (*20*).

The 17-kDa NH_2-terminal fragment (ATF-17k) was labeled with ^{125}I to a specific radioactivity of 0.21 μCi/μg using the Iodogen method (*21*).

Proteolytic Cleavage of u-PA by MMP-3. scu-PA or tcu-PA (final concentration, 5 μM) were incubated with MMP-3 (final concentration, 500 nM) at 37 °C in 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, 10 mM $CaCl_2$, and 0.01% Tween 80. At different time intervals (0−3 h), samples were removed from the incubation mixtures, EDTA (final concentration, 25 mM) was added to stop the reaction, and SDS−PAGE was performed under nonreducing conditions. The amounts of the different molecular forms of u-PA in the samples were determined by densitometric scanning of the gels and expressed as a percentage of the maximal value obtained.

Activation of Single-Chain u-PA Moieties by Plasmin. scu-PA-32k or scu-PA (final concentration, 1 μM) were incubated at 37 °C with plasmin (final concentration, 10 nM) in 0.05 M phosphate buffer, pH 7.4, containing 0.038 M NaCl, 0.01% Tween 80, and 5% glycerol. At timed intervals (0−30 min), aliquots were removed and generated urokinase activity was measured with S-2444 (final concentration, 0.3 mM) after 50-fold dilution. Initial rates (0−5 min) for the conversion of single-chain to two-chain u-PA moieties were determined in the same way, but using plasmin at a final concentration of 2 nM.

Functional Activity of Two-Chain u-PA Moieties. Specific amidolytic activities of u-PA moieties were determined directly with S-2444 (final concentration, 0.3 mM). Specific plasminogen-activating activities were determined indirectly with S-2403 (final concentration, 0.3 mM) in mixtures of plasminogen (final concentration, 0.25 μM, which is well below the K_m of about 80 μM (*14*)) and tcu-PA moieties (final concentration, 0−0.18 ng/mL) after incubation at 37 °C for 60 min in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.038 M NaCl and 0.01% Tween 80. Specific activities were expressed in IU/mg of protein by comparison with tcu-PA, calibrated against the International Reference Preparation for u-PA.

The kinetic parameters of the two-chain u-PA moieties (final concentration, 10 nM) for hydrolysis of S-2444 (final concentration, 0.02−1.0 mM) in 0.05 M phosphate buffer, pH 7.4, containing 0.038 M NaCl and 0.01% Tween 80 at 37 °C, were determined by Lineweaver−Burk analysis.

Binding of u-PA Moieties to uPAR. Human THP-1 cells were plated in 96-well microtiter plates at a density of 200 000 cells per well and incubated overnight in regular culture medium containing phorbol 12-myristate 13-acetate (final concentration, 10^{-7} M). The cultures were washed with RPMI medium containing 0.1% BSA to remove nonadherent cells. The cells were treated with 50 mM glycine-HCl buffer, pH 3.0, containing 100 mM NaCl for 3 min at room temperature to dissociate surface-bound endogenous u-PA, and neutralized with 0.2 vol of 500 mM HEPES buffer, pH 7.5, containing 100 mM NaCl. The cells were washed three times, and 200-μL volumes of RPMI with 0.1% BSA were added containing 8 nM ^{125}I-labeled ATF-17k without or with increasing concentrations of competitor (4−200 nM scu-PA or scu-PA-32k). After incubation for 2 h

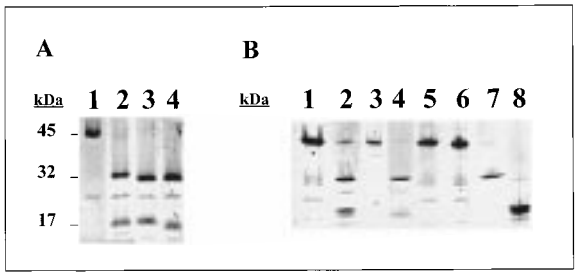


FIGURE 1: SDS−PAGE on 10−15% gradient gels under reducing (A) or nonreducing (B) conditions of the u-PA moieties generated upon incubation of scu-PA or tcu-PA at 37 °C for 3 h with MMP-3. Panels A and B, lane 1, scu-PA; lane 2, scu-PA treated with MMP-3; lane 3, tcu-PA; lane 4, tcu-PA treated with MMP-3. Lanes 5 and 6 in panel B represent scu-PA treated with MMP-3 in the presence of EDTA or 1,10- phenanthroline, lane 7 shows the purified 32-kDa fragment, and lane 8 shows the purified 17-kDa fragment.

at 37 °C, the cells were washed three times with RPMI containing 0.1% BSA and lysed with 100 μL of 1 N NaOH. The lysate of each well was counted for bound radiolabel. Measurements were corrected for background using values obtained with similarly treated wells without cells (usually around 180 cpm).

RESULTS

Proteolytic Cleavage of u-PA by MMP-3. Incubation of scu-PA or tcu-PA (nonglycosylated forms) with MMP-3 (enzyme/substrate ratio of 1/10) resulted in quantitative conversion to two non-disulfide-bonded lower M_r fragments (32 and 17 kDa), as shown by SDS−PAGE under nonreducing or reducing conditions (Figure 1). Addition of EDTA (final concentration, 25 mM) or 1,10-phenanthroline (final concentration, 1 mM) to the incubation mixture abolished the proteolytic cleavage, confirming that it is MMP-dependent. Similar results were obtained with recombinant glycosylated scu-PA (not shown). Densitometric scanning of SDS−PAGE gels of samples taken at different time points revealed a comparable time course for the hydrolysis of scu-PA and tcu-PA, with complete conversion occurring within 3 h (Figure 2). Hydrolysis of scu-PA or tcu-PA by MMP-3 was evident from a parallel decrease of the concentration of intact u-PA (45 kDa) and an increase of the 32- and 17-kDa fragments.

NH_2-terminal amino acid sequence analysis revealed specific cleavage of the Glu^{143}−Leu^{144} peptide bond, both in scu-PA and in tcu-PA (Table 1). Indeed, the 32-kDa fragment obtained from scu-PA (scu-PA-32k) starts at Leu^{144}, and the 17-kDa fragment (ATF-17k) represents the NH_2-terminal domain starting with Ser^1. As expected, the 32-kDa fragment obtained from tcu-PA (tcu-PA-32k) contains two NH_2-terminal sequences in comparable amounts, one starting at Leu^{144} and one at Ile^{159}. The lower M_r band, migrating as a doublet of approximately 17 kDa on SDS−PAGE, was heterogeneous: the upper band (ATF-17k (1)) corresponds to the NH_2-terminal Ser^1, and the lower band (ATF-17k (2)) contains two sequences, generated by cleavage of the Asn^2−Glu^3 and Glu^3−Leu^4 peptide bonds, respectively. These amino acid sequence data are compatible with the pattern seen on SDS−PAGE under nonreducing or reducing conditions (Figure 1). Under nonreducing conditions only the 32- and 17-kDa fragments are observed for both scu-PA and tcu-PA, whereas under reducing conditions the M_r of the 32-kDa fragment of tcu-PA, but not that of scu-PA, decreases by approximately 1 kDa, corresponding to removal of the sequence Leu^{144}−Lys^{158}, which is disulfide-bonded to the proteinase domain.

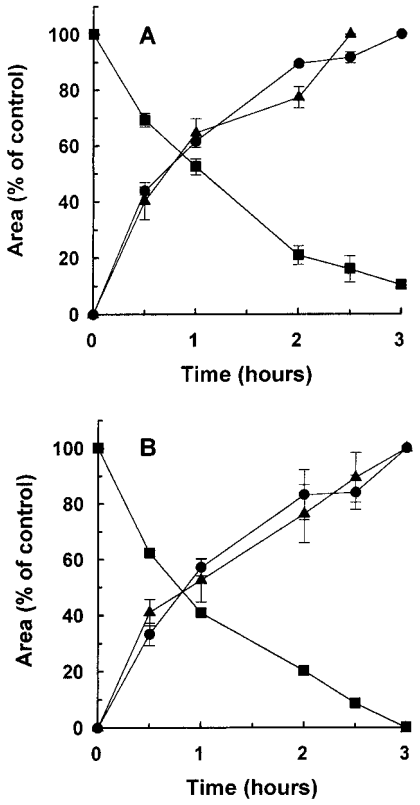


FIGURE 2: Time course of the hydrolysis of scu-PA (panel A) or tcu-PA (panel B) by MMP-3 (enzyme/substrate ratio of 1/10). The amount of 45- (■), 32- (●) and 17- kDa u-PA moiety (▲) at different time points was determined by densitometric scanning of nonreduced SDS−PAGE and is expressed as a percentage of the control (maximal) value. The data represent mean ± SEM of three separate experiments.

The 32- and 17-kDa fragments were purified to homogeneity by chromatography on benzamidine−Sepharose or on MAb-4D1E8−Sepharose for the fragments derived from tcu-PA or scu-PA, respectively (*14*, *15*) (Figure 1B, lanes 7 and 8). The specific activities of the scu-PA-32k and scu-PA moieties against S-2444 were <500 IU/mg.

Functional Characterization of the u-PA Cleavage Products. Binding of different u-PA moieties to proMMP-3 and to MMP-3 was evaluated by biospecific interaction analysis. The affinity of scu-PA-32k for binding to both proMMP-3 and MMP-3 was comparable to that of intact scu-PA or active site-blocked tcu-PA, whereas the purified 17-kDa NH_2-terminal fragment (ATF-17k) did not bind (Table 2). Binding to scu-PA-Glu^{158} also occurred, confirming that the interaction with MMP-3 does not require prior conversion to tcu-PA. These data thus indicate that the binding site for MMP-3 is contained within the 32-kDa u-PA fragment.

The time course for the activation with plasmin (enzyme/substrate ratio of 1/100) of scu-PA-32k obtained by digestion of scu-PA with MMP-3 was very similar to that for activation of the scu-PA-32k moiety prepared by recombinant DNA technology (Figure 3). Maximal activation resulted in specific activities of approximately 79 000 and 100 000

Table 1: NH_2-Terminal Amino Acid Sequence Analysis of scu-PA and tcu-PA Treated with MMP-3

	amino acid (recovery in pmol) in cycle							
protein	1	2	3	4	5	6	7	8
scu-PA								
scu-PA-32k	Leu (75)	Lys (66)	Phe (80)	Gln (68)	(Cys) (−)	Gly (58)	Gln (50)	Lys (33)
ATF-17k	Ser (12)	Asn (39)	Glu (47)	Leu (48)	His (27)	Gln (36)	Val (38)	Pro (30)
tcu-PA								
tcu-PA-32k	Leu (32)	Lys (23)	Phe (33)	Gln (13)	(Cys) (−)	Gly (14)	Gln (7)	Lys (8)
	Ile (38)	Ile (39)	Gly (26)	Gly (25)	Glu (18)	Phe (27)	Thr (6)	Thr (9)
ATF-17k (1)	Ser (2)	Asn (8)	Glu (6)	Leu (5)	His (4)	Gln (4)	Val (4)	Pro (4)
ATF-17k (2)	X (−)	Leu (6)	His (6)	Gln (4)	Val (8)	Pro (8)		
	Leu (−)	His (2)	Gln (4)	Val (7)	Pro (6)	X (−)		

Table 2: Apparent Affinity Constants of proMMP-3 and MMP-3 for Binding to Different Molecular Forms of u-PA[a]

	proMMP-3			MMP-3		
ligand	k_{ass} ($\times 10^3$) (M^{-1} s^{-1})	k_{diss} ($\times 10^{-3}$) (s^{-1})	K_A ($\times 10^6$) (M^{-1})	k_{ass} ($\times 10^3$) (M^{-1} s^{-1})	k_{diss} ($\times 10^{-3}$) (s^{-1})	K_A ($\times 10^6$) (M^{-1})
scu-PA	33 ± 1.5	8.8 ± 0.4	3.8 ± 0.06	67 ± 15	6.5 ± 0.6	9.9 ± 1.0
scu-PA-Glu[158]	21 ± 1.2	9.3 ± 0.5	2.2 ± 0.12	67 ± 10	9.6 ± 0.9	6.9 ± 0.53
scu-PA-32k	17 ± 3.7	8.2 ± 0.9	2.1 ± 0.67	53 ± 14	8.4 ± 1.7	6.3 ± 0.75
tcu-PA-EGR[b]	30 ± 3.3	5.8 ± 1.0	5.1 ± 0.90	60 ± 3.2	13 ± 1.8	4.8 ± 0.52
ATF-17k	ND[c]			ND		

[a] Data are mean ± SEM of 3 determinations. [b] tcu-PA with its active site blocked by Glu-Gly-Arg-CH_2Cl. [c] ND: no binding detectable.

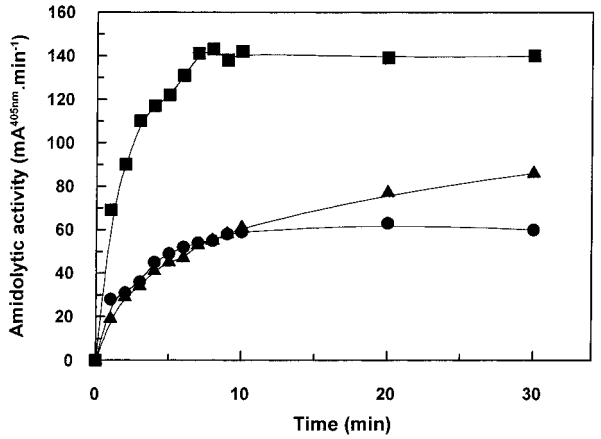


FIGURE 3: Time course of the activation by plasmin (enzyme/substrate ratio of 1/100) of scu-PA-32k obtained from scu-PA by treatment with MMP-3 (●), of scu-PA-32k prepared by recombinant DNA technology (▲), or of scu-PA (■). The amidolytic activity determined with S-2444 after 50-fold dilution of samples is plotted versus time.

IU/mg, respectively, as compared to 117 000 IU/mg for scu-PA treated with plasmin under the same experimental conditions. SDS−PAGE under reducing conditions of samples removed from the incubation mixtures after 30 min showed nearly quantitative conversion of scu-PA to tcu-PA and of scu-PA-32k to a slightly lower M_r species, compatible with cleavage of the Lys[158]−Ile[159] peptide bond (not shown).

From the initial rates (0−5 min) of the activation by plasmin, similar catalytic efficiencies (k_{cat}/K_m) were calculated for scu-PA-32k derived from scu-PA by MMP-3 (0.09 ± 0.01 μM^{-1} s^{-1}) and for scu-PA-32k obtained by recombinant DNA technology (0.13 ± 0.006 μM^{-1} s^{-1}), as compared to 0.60 ± 0.01 μM^{-1} s^{-1} for scu-PA (mean ± SD; $n = 3$). In this calculation, we have neglected the actual substrate concentration used (1 μM scu-PA moiety) as compared to the K_m of plasmin for hydrolysis of scu-PA or scu-PA-32k (about 4 μM (*14*)).

The kinetic parameters for the hydrolysis of S-2444 by tcu-PA-32k derived from tcu-PA by MMP-3 treatment or

Table 3: Kinetic Constants for the Hydrolysis of S-2444 by Different tcu-PA Moieties

moiety		K_m	k_{cat}	k_{cat}/K_m	
M_r (kDa)	origin[b]	(mM)	(s^{-1})	(mM^{-1} s^{-1})	r[a]
32	tcu-PA/MMP-3	0.063	10	160	0.995
32	scu-PA/MMP-3/plasmin	0.057	6.4	110	0.991
32	scu-PA-32k/plasmin	0.063	13	200	0.996
45	scu-PA/plasmin	0.071	12	170	0.996

[a] r, correlation coefficient determined by linear regression analysis of Lineweaver−Burk plots. [b] The 32-kDa tcu-PA moieties were obtained by treatment of tcu-PA with MMP-3, by treatment of scu-PA with MMP-3 followed by hydrolysis of the resulting scu-PA-32k with plasmin, or by treatment of recombinant scu-PA-32k with plasmin. The intact 45-kDa tcu-PA moiety was obtained by treatment of scu-PA with plasmin.

obtained by treating scu-PA with MMP-3 followed by hydrolysis of the resulting scu-PA-32k moiety with plasmin were comparable to those for tcu-PA and for tcu-PA-32k obtained by treatment of recombinant scu-PA-32k with plasmin (Table 3) (catalytic efficiencies ranging between 110 and 200 mM^{-1} s^{-1}).

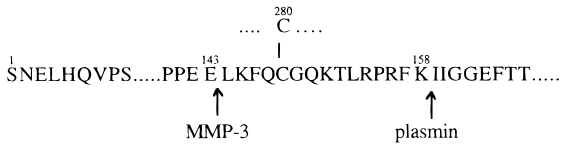
The specific activities of the tcu-PA moieties, as determined in a plasminogen-coupled chromogenic substrate assay by comparison with the International Reference Preparation for u-PA, were 72 000 ± 4 500 or 108 000 ± 7 600 IU/mg for tcu-PA-32k derived from tcu-PA by MMP-3 or obtained by treating scu-PA with MMP-3 followed by hydrolysis of the scu-PA-32k moiety with plasmin, as compared to 120 000 ± 12 000 or 76 000 ± 4 800 IU/mg for tcu-PA or tcu-PA-32k obtained by treatment of recombinant scu-PA-32k with plasmin (mean ± SEM; $n = 4-7$). The rates of plasmin generation in this assay were also comparable: in mixtures of 0.1 ng/mL tcu-PA moiety and 0.25 μM plasminogen, plasmin generation within 60 min amounted to 9.3, 14, 15, and 9.6 nM, respectively.

Binding of u-PA Cleavage Products to uPAR. Specific binding of ^{125}I-labeled ATF-17k to THP-1 cells was ob-

served, which was reduced in a concentration-dependent manner by addition of scu-PA but not of scu-PA-32k. At equimolar concentration of competitor to ligand, residual binding was (mean ± SEM; $n = 3$) 35 ± 8% with scu-PA and 86 ± 5% with scu-PA-32k; at 25-fold molar excess of scu-PA-32k, residual binding was 88 ± 9%, whereas no residual binding was detected with a 25-fold excess of scu-PA.

DISCUSSION

MMP-3 belongs to the stromelysin class of MMPs; its known substrates include proteoglycans, different types of collagen, fibronectin, gelatin, and laminin (*4*). In addition, MMP-3 degrades cross-linked fibrin by hydrolysis of the $Gly^{404}-Ala^{405}$ peptide bond in the γ-chain (*22*). In the present study, we report that MMP-3 specifically hydrolyzes the $Glu^{143}-Leu^{144}$ peptide bond in u-PA, yielding an NH_2-terminal 17-kDa fragment containing the u-PAR-binding sequence and a COOH-terminal 32-kDa fragment containing the serine proteinase domain. The NH_2-terminal amino acid sequences of the generated fragments (Table 1) indeed are compatible with single cleavage at Glu^{143} in scu-PA and in tcu-PA. This is shown schematically in the following sequence:



A similar 32-kDa scu-PA moiety (scu-PA-32k) has previously been observed in the conditioned medium of the human lung adenocarcinoma cell line CALU-3 (*13*) and in monkey kidney cells (*23*). scu-PA-32k, with NH_2-terminal Leu^{144}, has subsequently also been produced by recombinant DNA technology (*14*). In cultured human kidney cells, cleavage of the $Glu^{143}-Leu^{144}$ peptide bond in scu-PA was demonstrated by the putative metalloproteinase PUMP-1 or matrilysin (*24*).

Studies on the substrate specificity of MMPs with the use of synthetic model peptides have indicated that stromelysin has an extended active site with preferences at positions P3, P2, P1, P1′, and P2′ of its substrate for the hydrophobic amino acids Pro, Leu, Ala, Nva (norvaline), and Trp, respectively (*25*, *26*). Structural studies by NMR (*27*) and X-ray diffraction analysis (*28*, *29*) of the catalytic domain of MMP-3 and determination of the crystal structure of the complex with TIMP-1 (*30*) have confirmed a large specificity pocket in MMP-3. On the basis of such studies, fluorogenic substrates for stromelysin and other MMPs were designed (*26*, *31*), of which some, e.g., (7-methoxycoumarin-4-yl) acetyl-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys[2,4-dinitrophenyl]-NH_2, are specific for MMP-3 (*32*). The P_1P_1' subsite in u-PA is different, however, from any previously reported MMP-3 cleavage site in proteins or synthetic substrates (*32*). This was also observed for the MMP-3 cleavage site in the γ-chain of cross-linked fibrin (His-His-Leu-Gly-Gly^{404}-Ala^{405}-Lys-Gln-) (*22*).

The 17-kDa u-PA moiety obtained by treatment of scu-PA with MMP-3 specifically binds to the u-PAR on THP-1 cells, as shown by competition with scu-PA but not with scu-PA-32k, indicating that the receptor-binding site remained intact. The 32-kDa moiety obtained by treatment of scu-PA with MMP-3 was shown to have functional properties similar to those of its counterpart produced by recombinant DNA technology. Thus, activation by plasmin occurs with a comparable efficiency, yielding the corresponding two-chain derivatives which have comparable specific amidolytic activity and plasminogen activating activity. Similarly, the 32-kDa moiety obtained by treatment of tcu-PA with MMP-3 has functional properties comparable to those of tcu-PA-32k obtained by treating scu-PA with MMP-3 followed by hydrolysis of the resulting scu-PA-32k moiety with plasmin or to those of tcu-PA-32k obtained by treatment of recombinant scu-PA-32k with plasmin. The enzymatic properties of these 32-kDa u-PA moieties are also very similar to those of the parent molecule scu-PA or tcu-PA.

Interestingly, hydrolysis of u-PA by MMP-3 removes the u-PAR-binding domain. Binding of u-PA to its cellular receptor u-PAR was shown to be involved in several functions of u-PA, including enhanced activation of scu-PA, internalization of u-PA−inhibitor complexes, and signal transduction promoting migration, adhesion, and growth of cells (*33*−*35*). However, homozygous u-PAR-deficient ($u\text{-}PAR^{-/-}$) mice appeared to be viable, healthy, and fertile. The u-PA-dependent plasminogen activating capacity of peritoneal macrophages was reduced in $u\text{-}PAR^{-/-}$ mice as compared to their wild-type littermates but was not abolished, suggesting some u-PAR-independent functional properties of u-PA (*36*).

Thus, on one hand, u-PA may play a role in the in vivo activation of proMMP-3 via generation of plasmin (*7*), and on the other hand, active MMP-3 may cleave the receptor-binding domain from u-PA. This does not necessarily result in an impaired potential of u-PA for activation of proMMP-3, as it was recently shown that activation of proMMP-12 and -13 by u-PA-mediated plasmin generation occurs in the absence of u-PAR (*37*). Our data thus indicate that MMP-3 specifically cleaves the $Glu^{143}-Leu^{144}$ peptide bond in u-PA, resulting in removal of the u-PAR-binding domain without significantly affecting the enzymatic properties. This interaction may play a role in regulation of cell-associated u-PA activity, and may represent a mechanism whereby u-PAR-dependent and u-PAR-independent functions of u-PA are controlled.

ACKNOWLEDGMENT

NH_2-terminal amino acid sequence analysis was performed through the courtesy of Dr. F. Van Leuven (Center for Human Genetics, University of Leuven, Belgium).

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BI9728708