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Effective activation of the proenzyme form of the urokinase-type plasminogen activator (pro-uPA) by the cysteine protease cathepsin L

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Increased levels of both the cysteine protease, cathepsin L, and the serine protease, uPA (urokinase-type plasminogen activator), are present in solid tumors and are correlated with malignancy. uPA is released by tumor cells as an inactive single-chain proenzyme (pro-uPA) which has to be activated by proteolytic cleavage. We analyzed in detail the action of the cysteine protease, cathepsin L, on recombinant human pro-uPA. Enzymatic assays, SDS-PAGE and Western blot analysis revealed that cathepsin L is a potent activator of pro-uPA. As determined by N-terminal amino acid sequence analysis, activation of pro-uPA by cathepsin L is achieved by cleavage of the Lys^{158}-Ile^{159} peptide bond, a common activation site of serine proteases such as plasmin and kallikrein. Similar to cathepsin B (Kobayashi et al., J. Biol. Chem. (1991) 266, 5147–5152) cleavage of pro-uPA by cathepsin L was most effective at acidic pH (molar ratio of cathepsin L to pro-uPA of 1:2,000). Nevertheless, even at pH 7.0, pro-uPA was activated by cathepsin L, although a 10-fold higher concentration of cathepsin L was required. As tumor cells may produce both pro-uPA and cathepsin L, implications for the activation of tumor cell-derived pro-uPA by cathepsin L may be considered. Different pathways of activation of pro-uPA in tumor tissues may coexist: (i) autocatalytic intrinsic activation of pro-uPA; (ii) activation by serine proteases (plasmin, kallikrein, Factor XIIa); and (iii) activation by cysteine proteases (cathepsin B and L).

Pro-uPA; Urokinase; Cysteine protease; Cathepsin B; Cathepsin L; N-Terminal amino acid sequence analysis

1. INTRODUCTION

Elevated levels of proteases are present in solid tumors of the breast, ovary, colon, stomach, prostate and lung [1–8]. Collagenases, cathepsins, plasmin and plasminogen activators are found at various locations; in the cytoplasma, on the cell surface or released into the extracellular space [8–10]. These proteases not only degrade tissue components and constituents of the extracellular matrix but also activate proenzymes and thus help to support tumor cell invasion and metastases [11]. An enzymatically inactive proenzyme form of the urokinase-type plasminogen activator (pro-uPA) is secreted by stroma cells and by tumor cells [8,12]. Pro-uPA binds to high affinity receptors located on the tumor cell surface and is then converted into the enzymatically active two-chain form of uPA (HMW-uPA) by the proteases plasmin, kallikrein or cathepsin B [13–16]. Tumor cell invasion and metastasis is a complex multistep process which ultimately depends on the net balance of various biochemical properties of the tumor cell (e.g. protease activity, growth factors, steroid hormones, immunogenicity, cell adhesion, attachment factors). Elevated levels of the cysteine proteases, cathepsin B, L and uPA, have been correlated with increased malignancy [3,4,5,8,17–22]. This may be due to the fact that cysteine proteases efficiently convert soluble or tumor cell receptor-bound pro-uPA to catalytically active two-chain HMW-uPA [16]. Increased content of uPA in tumor tissues of the breast even allows to discriminate between those patients having a low or a high risk to eventually develop metastases [4,5,21,22]. We now report that in addition to cathepsin B, another potent lysosomal cysteine protease, cathepsin L, also cleaves pro-uPA with high efficiency to render catalytically active HMW-uPA.

Abbreviations: BSA, bovine serum albumin; GFD, growth factor-like domain; E-64, trans-epoxysuccinyl-L-leucylamino-(guanidino)-butane; HPLC, high-performance liquid chromatography; HMW-uPA, high molecular weight form of uPA; mAb, monoclonal antibody; pro-uPA, proenzyme form of uPA (enzymatically inactive); uPA, urokinase-type plasminogen activator.

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2. MATERIALS AND METHODS

2.1. *Materials*

Purified human cathepsin L (EC 3.4.22.15; M_r = 28,000; 208 μg/ml) and cathepsin B (EC 3.4.22.1; M_r = 28,000; 400 μg/ml) were obtained from Medor (Herrsching/München, Germany). Human cathepsin L was purified by the manufacturer according to the method described in [42,43]. E-64, an inhibitor of cathepsin B and L, and human plasmin were from Sigma (München, Germany). Recombinant human pro-

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uPA (Saruplase) expressed in *Escherichia coli* was donated by Grünenthal (Stolberg, Germany). Naturally occurring glycosylated HMW-uPA (Ukidan) purified from human urine was from Serono (Freiburg, Germany). The chromogenic substrate pyro-Glu-Gly-Arg-pNA was purchased from Bachem Biochemica (Heidelberg, Germany), and aprotinin (Antagosan) was from Behring-Werke (Marburg, Germany).

2.2. *Methods*

2.2.1. Digestion of pro-uPA by cathepsin B and L or plasmin

Pro-uPA was incubated with increasing concentrations of cathepsin B, L or plasmin, respectively (75 min, 37°C). For digestion, pro-uPA was dissolved at pH 4.5 and 7.0, respectively, in 15 mM sodium phosphate buffer containing 1.5 mM EDTA and 2.5 mM cysteine. Digestion of pro-uPA with plasmin was performed in 0.1 M Tris-HCl containing 50 mM NaCl, pH 7.0. Reactions with cathepsin B and L, respectively, were terminated by the addition of E-64 (final concentration of 10 μM). Digestion of pro-uPA with plasmin was stopped by the addition of 50 k.I.U. aprotinin/ml. Samples were assayed for uPA enzymatic activity (at 405 nm) towards the synthetic substrate pyro-Glu-Gly-Arg-pNA in 50 mM Tris-HCl containing 40 mM NaCl, pH 8.8. uPA activity was calculated in Ploug units. Active-site titration of cathepsin L has not been performed. Samples (5–10 μg of protein per lane) were also separated by SDS-PAGE (15% acrylamide) according to Laemmli [23] in a Mini-Protean II apparatus (Bio-Rad). Selected samples were electrophoretically transferred to a fluorotrans membrane (0.2 μm; Pall, Dreieich, Germany). The membrane was blocked with 2% BSA in 20 mM Tris-HCl containing 0.125 M NaCl, pH 7.5 (TBS). The membrane was incubated with mAb #3471 (American Diagnostica, Greenwich, NJ), specific for a peptide sequence within the growth factor-like domain of uPA (uPA_{17-32}), as described [16]. Molecular weight markers (in kDa) used (Bio-Rad) were phosphorylase *b* (97.4), BSA (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), soybean trypsin inhibitor (21.5) and lysozyme (14.4).

2.2.2. Reverse-phase HPLC and N-terminal amino acid sequence analysis

1 mg pro-uPA was incubated with 1 μg cathepsin L (pH 4.5, 37°C, 1 and 2 h, respectively) and also at pH 7.0 (4 h, 37°C). For another experiment, 1 mg pro-uPA was digested with 0.1 μg cathepsin L at pH 4.5 (4 h, 37°C) in the buffer described above. Reduction of disulfide bonds was achieved by boiling (2 min) of the digested pro-uPA in 6 M guanidine hydrochloride (Merck, Darmstadt, Germany), 3% (v/v) 2-mercaptoethanol, 10 mM Tris-HCl, pH 8.5 and pyridylethylated with 4.5% (v/v) 4-vinylpyridine (23°C, 1.5 h; Fluka AG, Buchs, Switzerland) and then the reaction was stopped by adjusting the pH of the buffer to 2.0–3.0 with HCl. The peptides were separated on a Pharmacia-LKB HPLC system equipped with a Pep-S/C-18 column (Pharmacia-LKB) operating at 23°C with a flow rate of 1.0 ml/min. The column was equilibrated with 0.1% trifluoroacetic acid in H_2O. Elution of peptides was achieved by a linear gradient of 10–50% acetonitrile/0.1% trifluoroacetic acid within 60 min. N-Terminal amino acid sequence analysis was performed with a gas phase sequencer (470 A Protein Sequencer, Applied Biosystems) following the manufacturer's instructions. Each peptide sample was submitted to 8 Edman degradation cycles.

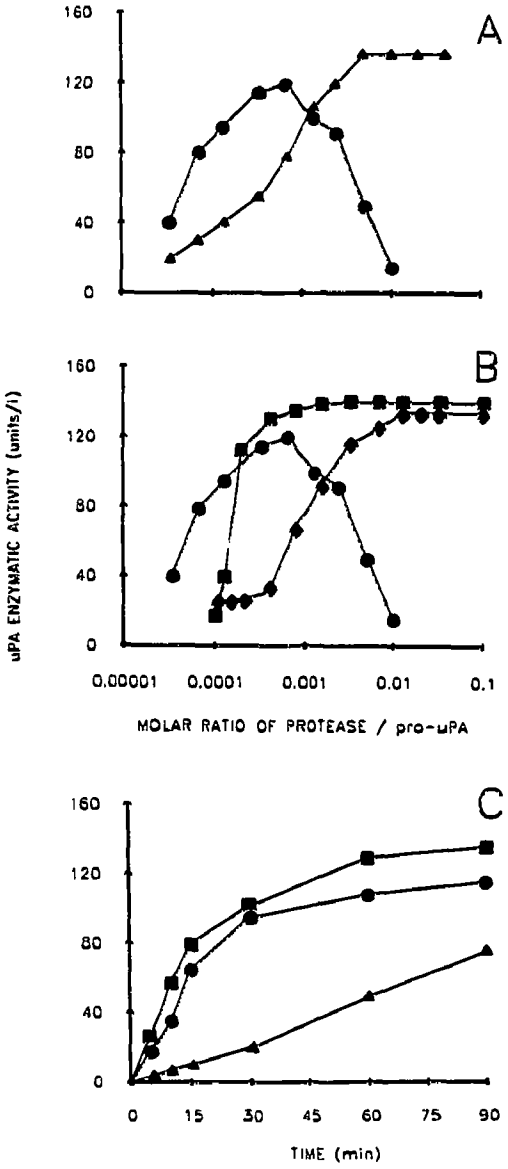

Fig. 1. Proteolytic activation of pro-uPA by cathepsin B, L or plasmin. (A) Dose-dependent activation of pro-uPA (1 mg/ml) by cathepsin L at pH 4.5 (●) or pH 7.0 (▲) (75 min, 37°C) was assayed by measuring the amidolytic activity of uPA towards pyro-Glu-Gly-Arg-pNA. (B) Dose-dependent activation of pro-uPA (1 mg/ml) by cathepsin B (◆) and cathepsin L (●) at pH 4.5, and by plasmin (■) at pH 7.0 (75 min, 37°C). (C) Time-dependent activation of pro-uPA by cathepsin L at pH 4.5 (●) and pH 7.0 (▲), and by plasmin at pH 7.0 (■) at a molar ratio of protease to pro-uPA of 1:1,600. At indicated time points aliquots were removed for enzymatic assays. The reaction was terminated by E-64 (cathepsin L) and aprotinin (plasmin), respectively. Conditions were as described in Fig. 1A.

3. RESULTS AND DISCUSSION

3.1. *Cleavage of pro-uPA by cathepsin L yields enzymatically active HMW-uPA*

Both pro-uPA and cathepsin L are generated by human tumor cells and released into the extracelluiar space [8,10,24,25]. In intracellular lysosomes cathepsin L exerts its maximum catalytic capacity at acidic pH, similar to cathepsin B [26]. To investigate the proteolytic action of cathepsin L on pro-uPA, recombinant pro-uPA was therefore incubated with various concentrations of purified cathepsin L at pH 4.5 (Fig. 1A). Evidently, cathepsin L activates pro-uPA with high efficiency. Maximum activation was achieved at a molar ratio of cathepsin L to pro-uPA of 1:2,000 to 1:7,000.

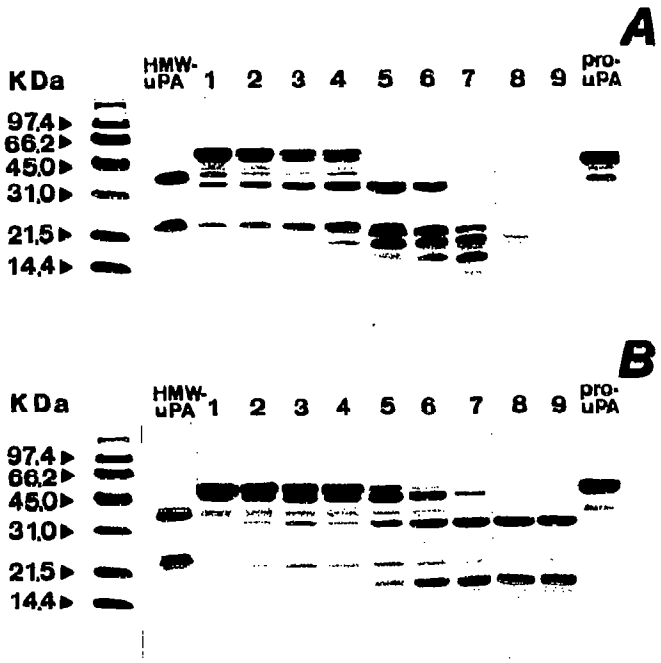

Fig. 2. SDS-PAGE analysis of cleavage of pro-uPA by cathepsin L. The SDS-PAGE (reducing conditions) displays the dose-dependent cleavage of pro-uPA (1 mg/ml) by cathepsin L at (A) pH 4.5 and (B) pH 7.0, at various molar ratios of cathepsin L to pro-uPA (75 min, 37°C): (lane 1) 48,000; (lane 2) 24,000; (lane 3) 16,000; (lane 4) 8,000; (lane 5) 1,600; (lane 6) 800; (lane 7) 400; (lane 8) 200; (lane 9) 100. Marker proteins are indicated in kDa. Please, note that the molecular weight of the B-chain of urinary HMW-uPA (Ukidan) which was used as a reference protein is slightly higher than that of recombinant pro-uPA due to glycosylation.

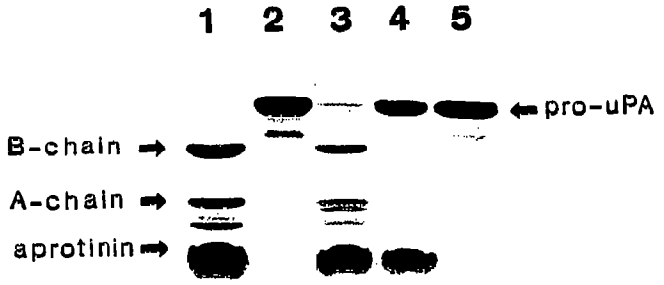

Fig. 3. Cleavage of pro-uPA by cathepsin L is inhibited by the cysteine protease inhibitor E-64 but not by the serine protease inhibitor aprotinin. As demonstrated by SDS-PAGE, cleavage of pro-uPA is caused by the cysteine protease cathepsin L and is not due to contaminating serine proteases. This is evident by the fact that the cysteine protease inhibitor E-64 completely prevented pro-uPA activation whereas the serine protease inhibitor aprotinin was without effect. Activation strictly depended on the presence of a reducing agent (e.g. cysteine). A molar ratio of protease to pro-uPA of 1:1,600 was applied (60 min, 37°C). The pH value for cathepsin L digestion was 4.5; for plasmin digestion 7.0. (Lane 1) pro-uPA in the presence of plasmin, then addition of aprotinin; (lane 2) pro-uPA in the presence of cathepsin L, no cysteine added; (lane 3) pro-uPA in the presence of cathepsin L, cysteine and aprotinin; (lane 4) pro-uPA in the presence of cathepsin L, cysteine, aprotinin and E-64; (lane 5) pro-uPA in the presence of cathepsin L, cysteine and E-64.

The activation of pro-uPA at pH 4.5, however, is only transient and depends on the ratio of cathepsin L to pro-uPA. At higher concentrations of cathepsin L irreversible degradation of both the A- and B-chain of uPA occurs (Fig. 2A). Activation of pro-uPA by cathepsin L takes also place at pH 7.0 (Figs. 1A and 2B). In contrast to pH 4.5, activation requires a higher dose of cathepsin L at neutral pH (molar ratio of cathepsin L to pro-uPA of 1:200). Under this condition optimum activation of pro-uPA but no degradation of the A- and B-chain of uPA was observed (Fig. 2B). No activation of pro-uPA by cathepsin L was observed at alkaline pH (>pH 8.0). Activation of pro-uPA by cathepsin L (pH 4.5) is as efficient as activation by plasmin (pH 7.0) (Fig. 1B). Optimum activation of pro-uPA by cathepsin B (pH 4.5) requires a relatively high concentration of cathepsin B; the course of activation parallels that of cathepsin L at pH 7.0 (Fig. 1A). Time-dependent activation of pro-uPA by cathepsin L (pH 4.5 and 7.0) and by plasmin (pH 7.0) are depicted at a molar ratio of protease to pro-uPA of 1:1,600 (Fig. 1C); maximum activation of pro-uPA was achieved within 1 h by plasmin and by cathepsin L at pH 4.5.

To demonstrate the specificity of cathepsin L action on pro-uPA several control experiments (SDS-PAGE) were carried out (Fig. 3). Pro-uPA was incubated with cathepsin L in the presence or absence of the thiol reagent cysteine, the cysteine protease inhibitor E-64 and the serine protease inhibitor aprotinin, respectively. Cleavage of pro-uPA by cathepsin L at pH 4.5 requires a reducing agent such as cysteine; cleavage is prevented by E-64. Cathepsin L action on pro-uPA is not affected by aprotinin indicating that trace contaminations of cathepsin L with serine proteases are not responsible for the cleavage of pro-uPA. Similar results were obtained at pH 7.0. No cleavage of pro-uPA by cathepsin L was observed at alkaline pH (>8.0). As reported by the authors [16], treatment of pro-uPA with cathepsin D does not lead to significant activation of pro-uPA, therefore possible trace contaminations of the cathepsin L preparation with the aspartic protease cathepsin D are negligible. Prior to SDS-PAGE, aliquots of the samples were subjected to determination of amidolytic activity in order to confirm the SDS-PAGE results (data not shown). The loss of amidolytic activity of uPA at pH 4.5 at high concentration of cathepsin L is due to the high proteolytic potential of cathepsin L. Published data characterize cathepsin L as a very potent lysosomal endopeptidase [27–29]. Cathepsin B is a less active endopeptidase although the amino acid sequences of both cathepsins B and L clearly show that they have evolved from a common ancestor [30,31]. There are two other lysosomal cysteine proteases known, cathepsin H and S, which are closely related to the cathepsins B and L [44]. Cathepsin S also degrades a variety of protein

Table I

Identification of cathepsin L cleavage sites in pro-uPA by N-terminal amino acid sequence analysis

HPLC - fraction	N-terminal amino acid sequence	Component (of)
A 1	39 **G↓G Q H C E I D K**	A-chain
	44 E↓I D K S K T C Y	A-chain
A 2	159 **K↓I I G G E F T T**	B-chain
B 1	1 **S N E L H Q V P**	A-chain
	6 **H↓Q V P S N C D C**	A-chain
B 2	159 **K↓I I G G E F T T**	B-chain
	162 G↓G E F T T I E N	B-chain
C 1	6 **H↓Q V P S N C D C**	A-chain
	60 **R↓G K A S T D T M**	A-chain
C 2	394 **S↓H F L P W I R S**	B-chain
	391 T↓R V S H F L P W	B-chain
C 3	6 **H↓Q V P S N C D C**	A-chain
	1 S N E L H Q V P	A-chain
C 4	162 **G↓G E F T T I E N**	B-chain
	166 T↓T I E N Q P W F	B-chain
	159 K↓I I G G E F T T	B-chain

↓ denotes the cathepsin L cleavage site; major sequences are shown in bold face; the position of the first residue is indicated.

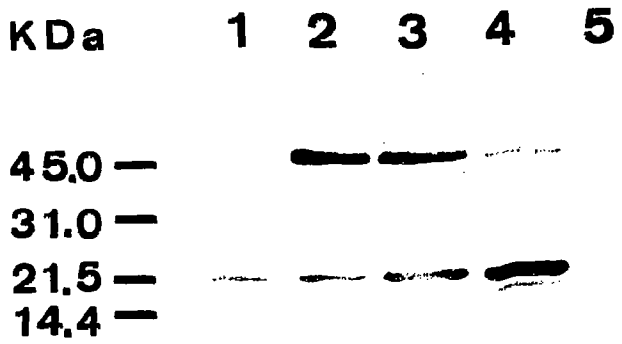

Fig. 4. Western blot analysis of pro-uPA cleavage by cathepsin L or plasmin. Pro-uPA was treated with cathepsin L at pH 4.5 (60 min, 37°C; lanes 4 and 5) or pH 7.0 (lanes 2 and 3) or with plasmin at pH 7.0 (lane 1). The samples were subjected to SDS-PAGE and electrotransfer as described in Materials and Methods. The transfluoromembrane was blocked with 2% BSA and then incubated with mAb #3471, which is directed to the receptor binding domain within the growth factor-like domain of uPA [16]. Note that only intact pro-uPA and the A-chain(s), but not the B-chain, of uPA were stained. The molar ratios of protease to pro-uPA were as follows: (lane 1) 1:2,500; (lane 2) 1:8,000; (lane 3) 1:1,600; (lane 4) 1:8,000; (lane 5) 1:400.

substrates, including collagen with a high specific activity, and is considered to play an important role in intracellular protein breakdown [45–47]. The two cysteine proteases, cathepsin B and L, seem likely to account for most of the contribution of this group of enzymes to physiological and pathophysiological proteolysis [32,33].

Pro-uPA which has been activated by cathepsin L (HMW-uPA) still binds to uPA receptors on tumor cells indicating an intact binding domain within GFD of HMW-uPA. Binding of active HMW-uPA to tumor cell receptors was confirmed by flow cytofluorometry [16,41]. HMW-uPA effectively competed for binding of fluorescent FITC-pro-uPA to receptors on U-937 cells (data not shown). Unimpaired GFD can also be demonstrated (Western blot analysis) by use of mAB #3471 which is directed to the peptide sequence 17–32 within GFD representing the receptor binding domain (Fig. 4). MAb #3471 only recognizes intact uPA_{17-32} within pro-uPA and the A-chain of HMW-uPA [16], and does not react with the B-chain of HMW-uPA [16].

3.2. *Cleavage of pro-uPA by cathepsin L occurs between Lys^{158} and Ile^{159}*

N-Terminal amino acid sequence determination was applied in order to identify the exact position(s) of the cleavage site(s) in cathepsin L-treated pro-uPA (Table I). pro-uPA was treated with cathepsin L at pH 7.0 (molar ratio of cathepsin L to pro-uPA of 1:1,600) and pH 4.5 (molar ratio of cathepsin L to pro-uPA of 1:16,000), respectively, and then, after reduction and alkylation, the resulting uPA polypeptides were separated by reverse-phase HPLC (Fig. 5A,B). At a higher concentration of cathepsin L (molar ratio of cathepsin L to pro-uPA of 1:1,600; pH 4.5) degradation of both the A- and the B-chain of uPA occurred (Fig. 5C). This is in agreement with the SDS-PAGE pattern depicted in Fig. 2. The major peaks were collected and then subjected to N-terminal amino acid sequence analysis. The A-chain polypeptide eluted first (31–33% acetonitrile), followed by the B-chain (40–42% acetonitrile) (Fig. 5A,B).

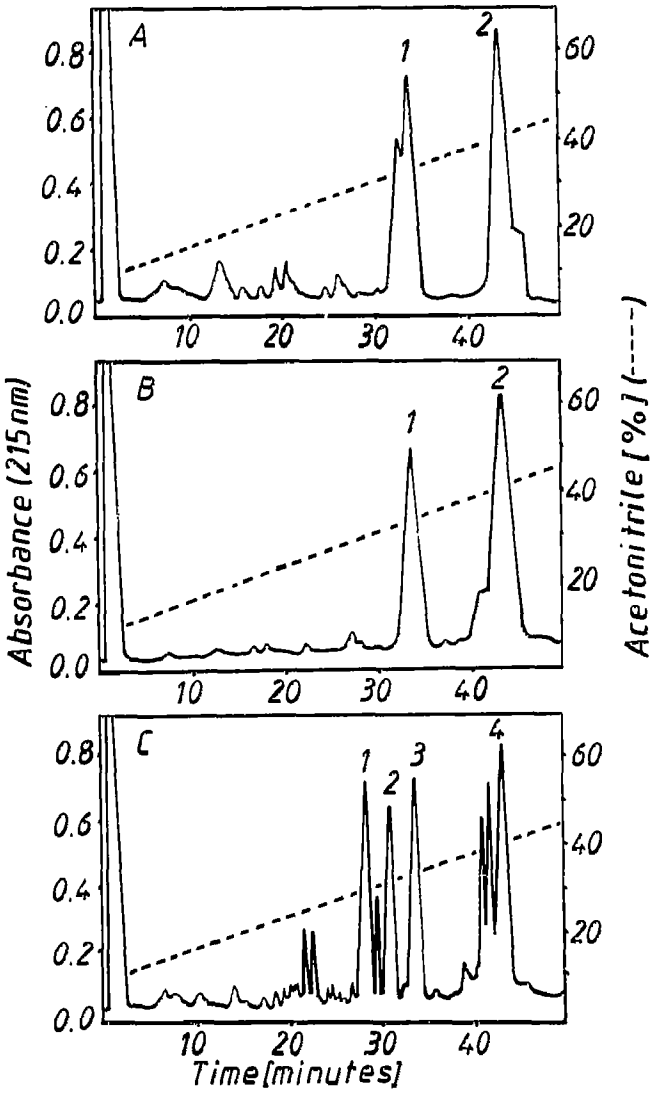

Fig. 5. Reverse-phase HPLC of pro-uPA cleaved by cathepsin L. uPA fragments derived from pro-uPA by digestion with cathepsin L were analyzed by reverse-phase HPLC. 500 μg of digested uPA peptides were separated. The numbered peaks were collected and submitted to N-terminal amino acid sequence determination. The N-terminal sequences (8 steps) of polypeptides contained in these peaks are given in Table I. (A) 4 h, 37°C, pH 7.0. Molar ratio of cathepsin L to pro-uPA of 1:1,600. (B) 4 h, 37°C, pH 4.5. Molar ratio of cathepsin L to pro-uPA of 1:16,000. (C) 2 h, 37°C, pH 4.5. Molar ratio of cathepsin L to pro-uPA of 1:1,600.

On the basis of the known sequence of pro-uPA

[34,35], identification of major and minor cleavage sites was ensured (Table I). The major cleavage site is between Lys^{158} and Ile^{159}. This is the common activation site known for the serine proteases plasmin, kallikrein and trypsin [15] but also for the cysteine protease cathepsin B [16]. Another important cleavage site (Gly^{161}-Gly^{162}) was detected when pro-uPA was treated with a high concentration of cathepsin L at pH 4.5 for 2 h (peak 4 in Fig. 5C). The resulting HMW-uPA was enzymatically inactive and was found to contain a B-chain component (up to 80% of total) starting with the N-terminal sequence Gly-Glu-Phe-Thr-. In parallel, pro-uPA was incubated only for 1 h with cathepsin L under the same conditions as in Fig. 5C, the cleavage mixture was separated by reverse-phase HPLC without reduction and was then assessed for the cleavage site(s). N-Terminal amino acid sequence analysis revealed the major activation site between Lys^{158}-Ile^{159}, and a major cleavage site between Gly^{161}-Gly^{162} reflecting the transitory activation of pro-uPA by cathepsin L at acidic pH. The A-chain was cleaved between His^5-Gln^6.

Cathepsin L has a broad amino acid specificity [32,33]. The identified cleavage sites in cathepsin L-treated pro-uPA show that cathepsin L splits peptide bonds involving the carboxyl groups of glycine, lysine, threonine, serine, isoleucine, histidine, arginine or glutamic acid. The broad substrate specificity of cathepsin L has also been shown by other investigators. They demonstrated that cathepsin L is able to cleave the extracellular matrix protein collagen [27,36]. Cathepsin L has even been described as a potent elastase-like enzyme degrading elastin, another extracellular protein resistant to most proteases [37].

Cathepsin L may even play an important role in cell growth and cell transformation as the major excreted protein (MEP) of transformed mouse fibroblasts has been reported recently to be identical with the precursor molecule of cathepsin L (procathepsin L) [38–40]. MEP is also secreted by untransformed mouse cells in response to tumor promoters or growth factors. There is published evidence that treatment of NIH 3T3 cells with platelet-derived growth factor will cause selective secretion of pro-cathepsin L; other lysosomal enzymes were not detected in the media from PDGF-treated cells [20]. The procathepsin L molecule has a small amount of latent activity which is stimulated at low pH or by cleavage with another proteinase such as cathepsin D [48,49]. Procathepsins B and L were detected in media from premalignant and malignant colon tumor cell lines in equal amounts, but only medium from malignant cell lines contained mature cathepsins B and L, which were active against an isolated basement membrane matrix in vitro [18]. The finding of significant activity of mature cathepsin L at neutral pH, and the evidence for autoprocessing of procathepsin L to active cathepsin L at neutral pH in this work, argue for a possible role of cathepsin L in tumor invasiveness. Mature cathepsin L is not stable for long periods at neutral pH [50], yet we observed no autolysis at pH 7.0. It is possible that the presence of substrates can retard or prevent autolysis. It has recently been reported that the pH optimum of recombinant mature cathepsin L is higher than that published for human liver cathepsin L: thus cathepsin L preparations from different sources can possess activity at various pH profiles [50,51]. Our finding, namely the effective activation of pro-uPA by cathepsin L, may also be of importance for tumor spread and metastasis as uPA has been shown to be an important prognostic factor for increased relapse formation and shorter overall survival in breast cancer patients [4,5,21,22]. So far, detailed clinical studies on the interaction of cysteine proteases and pro-uPA/uPA levels in cancer patients and the outcome of the disease have not been reported.

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