Plasminogen activators catalyse conversion of inhibitor from fibrosarcoma cells to an inactive form with a lower apparent molecular mass

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Purified ~54 kDa plasminogen activator inhibitor from human fibrosarcoma cells was converted to an inactive form with slightly higher electrophoretic mobility by incubation with catalytic amounts of urokinase-type or tissue-type plasminogen activator. Serine proteinase inhibitors and a monoclonal antibody against urokinase-type plasminogen activator inhibited the conversion, indicating that it was caused by plasminogen activator-catalyzed proteolysis. These findings represent the first demonstration of a well-defined protein apart from plasminogen, constituting a substrate for plasminogen activators.

Plasminogen activator *Urokinase* *Enzyme inhibitor* *(HT-1080 fibrosarcoma cell)*

1. INTRODUCTION

Plasminogen activators catalyse the cleavage of a single peptide bond in plasminogen, thereby converting it to the active proteinase plasmin. Two types of plasminogen activators exist. They are both serine proteinases, and products of different genes, and presumably have different biological functions. u-PA is among other functions supposed to be involved in tissue degradation and t-PA is a key enzyme in thrombolysis (for reviews, see [1–5]).

Inhibitors of plasminogen activators have been identified in a variety of tissues and cell types ([6–24], see [5,25] for reviews). We have recently reported that the human fibrosarcoma cell line HT-1080 releases an ~54 kDa protein that inhibits u-PA and t-PA, but not plasmin, and has the same mobility in SDS-PAGE as plasminogen activator inhibitors present in endothelial cells and blood platelets [26,27]. We now report that catalytic amounts of u-PA and t-PA convert this inhibitor to an inactive form with a slightly higher electrophoretic mobility.

Abbreviations: u-PA, urokinase-type plasminogen activator; pro-u-PA, proenzyme to urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

Conditioned serum-free culture fluid from the human fibrosarcoma cell line HT-1080 was prepared as reported for other cell lines [28], except that the cells were cultured in the presence of 10^{-6} M dexamethasone to increase inhibitor production [26]. Human pro-u-PA was prepared from the conditioned culture fluid of HT-1080 cells by affinity chromatography with a monoclonal antibody [29]. Active u-PA, plasminogen and plasmin were prepared as described [30,31]. Rabbits were immunized with plasminogen activator inhibitor purified from conditioned culture fluid from HT-1080 cells by affinity chromatography on concanavalin A-Sepharose [17,26] followed by preparative SDS-PAGE [26]. Antibodies were purified from rabbit serum using affinity

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chromatography on protein A-Sepharose as described [32].

The plasminogen activator enzyme activity was determined by the ^{125}I-fibrin plate assay as described [28], using 1 h incubation at 37°C, 500 μl assay buffer consisting of 0.1 M Tris-HCl, pH 8.1, 0.1% Triton X-100, 0.25% gelatin and urokinase (LEO, Ballerup, Denmark) as a standard. This assay was modified for quantitation of inhibitor as described [27]. Before assay, inhibitor preparations (~80 μg/ml) were treated with 0.1% SDS (final concentration) for 1 h at 25°C to enhance inhibitory activity [23]. Triton X-100 was then added to a final concentration of 1%.

SDS-PAGE was carried out in slab gels with a linear 6–16% polyacrylamide concentration gradient as described [33]. When necessary samples were concentrated by trichloroacetic acid precipitation before electrophoresis. The gels were either stained with Coomassie blue or processed for reverse zymography for the detection of u-PA inhibitors by a modification [14,26] of the zymographic technique described by Granelli-Piperno and Reich [34]. Inhibitors diffused into an agarose gel containing u-PA, plasminogen and fibrin, and their presence was revealed by lysis-resistant zones. Protein concentration in purified concentrated inhibitor preparations was estimated with the folin-Ciocalteu phenol reagent. All reagents were as described [26,28,30,32,35,36] or of the best grade commercially available.

3. RESULTS

~54 kDa human plasminogen activator inhibitor from serum-free culture fluid from the HT-1080 cell line was purified by chromatography on a Sepharose column coupled with rabbit antibodies against the inhibitor. The purified preparation contained one Coomassie blue-stainable band after SDS-PAGE under non-reducing (fig.1) as well as reducing (not shown) conditions, indicating that the inhibitor consists of one polypeptide chain. The apparent molecular mass as based on the electrophoretic mobility was ~54 kDa under both non-reducing and reducing conditions. Under non-reducing conditions the electrophoretic mobility of the stained band was indistinguishable from that of the inhibitory activity as determined by reverse zymography (fig.1).

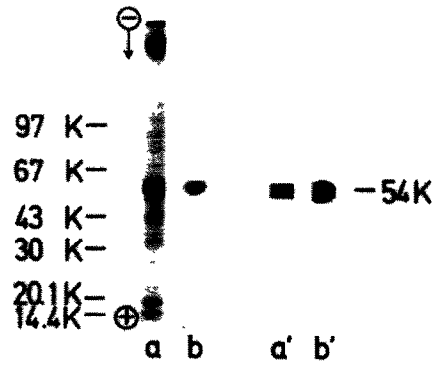

Fig.1. SDS-PAGE followed by Coomassie blue staining (a,b) or reverse zymography (a',b') of plasminogen activator inhibitor from culture fluid of HT-1080 cells before (a,a') and after (b,b') purification by affinity chromatography on a column of Sepharose-conjugated rabbit antibodies against the inhibitor. 250 ml culture fluid from dexamethasone-treated HT-1080 cells were applied to a 25 ml Sepharose column containing 250 mg rabbit anti-inhibitor IgG and equilibrated with 0.1 M Tris-HCl, pH 8.1, 0.01% Tween 20. The column was washed with 50 ml equilibration buffer, followed by 80 ml of the same buffer supplemented with 1 M NaCl. Elution was performed with 0.1 M CH_3COOH, pH 2.9, 1 M NaCl, 0.01% Tween 20 in 5 ml fractions into tubes containing 0.75 ml of 1 M Tris-HCl, pH 9.0. The eluate contained 101 μg protein and 52% of the inhibitory activity applied to the column. 1.5 ml (a) or 40 μl (a') culture fluid and 10 μg (b) or 0.2 μg (b') purified protein were applied to the gel. The positions of M_r-marker proteins are indicated.

Incubation of the purified SDS-treated inhibitor with catalytic amounts of u-PA led to its dose-dependent conversion to a form with a slightly increased mobility in SDS-PAGE under non-reducing (fig.2) as well as reducing (not shown) conditions. No conversion was observed when SDS-treatment of the inhibitor was omitted, when *p*-aminobenzamidine, *p*-nitrophenyl-*p'*-guanidinobenzoate (both inhibitors of u-PA; see [33]), or a monoclonal antibody to u-PA (previously found to inhibit the proteolytic activity of u-PA [29]) was added, or when u-PA was substituted by its proenzyme. Bovine pancreatic trypsin inhibitor did not inhibit the conversion caused by incubation with u-PA (fig.2). t-PA caused a similar conversion of the inhibitor, while plasmin caused a conversion to

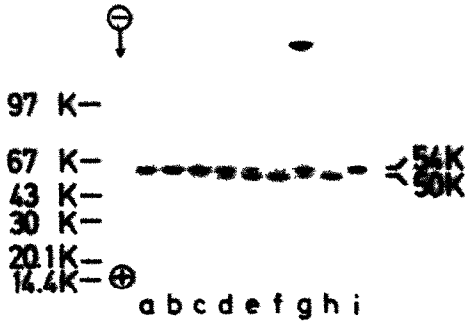

Fig.2. SDS-PAGE of inhibitor incubated without plasminogen activator (b), with u-PA (a,c–h) or pro-u-PA (i). Inhibitor purified as described in the legend to fig.1 was preincubated without (a) and with SDS (b–i), followed by incubation (500 μl total volume, 4 μg inhibitor/ml) for 2 h at 37°C in 0.1 M Tris-HCl, pH 8.1; 0.1% Triton X-100 with u-PA: 0.0008 (c), 0.004 (d), 0.02 (e), 0.1 μg/ml (a,f–h), 0.1 μg/ml pro-u-PA (i) and 10 μg/ml monoclonal anti-u-PA IgG clone 2 (g) or 25 μg/ml bovine pancreatic trypsin inhibitor (h). After electrophoresis the gel was stained with Coomassie blue. Incubation of sample f with *p*-aminobenzamidine (5 mM) or *p*-nitrophenyl-*p'*-guanidinobenzoate (100 μM) gave results identical to those shown in lane g. A monoclonal antibody of irrelevant specificity (anti-trinitrophenyl) had no effect on the conversion (not shown). When u-PA in sample f was substituted with t-PA (0.1 μg/ml) identical results were obtained (not shown). Incubation of sample b with plasmin (0.2 μg) resulted in a partial conversion to several polypeptides with M_r values below 45000. This effect was completely abolished by bovine pancreatic trypsin inhibitor (25 μg/ml). Incubation of sample b with less than 0.02 μg/ml plasmin did not lead to detectable conversion. Inhibitor preparations which were preincubated with SDS and not incubated with u-PA were indistinguishable from that shown in lane a.

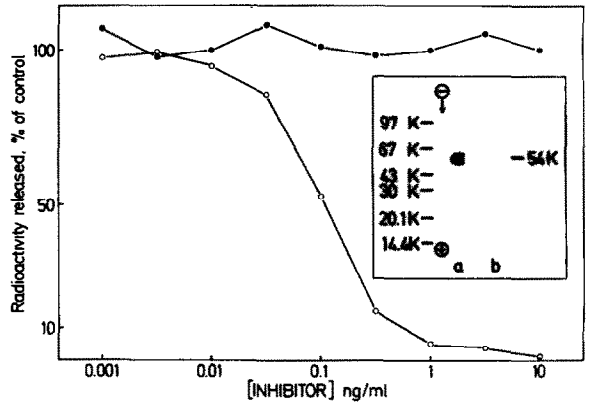

Fig.3. Effect of non-converted (○) and converted (●) inhibitor on ^{125}I-fibrin plate assay for u-PA. SDS-treated inhibitor (9 μg/ml) was incubated for 2 h at 37°C in assay buffer without (○) or with (●) 0.1 μg/ml u-PA and passed twice through a 1-ml column of 1 mg monoclonal anti-u-PA IgG clone 2 coupled to Sepharose. Analysis by SDS-PAGE of the preparations gave the results shown in fig.2 lanes b and f, respectively. The protein content was determined by spectrophotometric scanning of Coomassie blue stained gels, using purified non-converted inhibitor as a standard [27]. Inhibitor in various concentrations was preincubated with urokinase (200 mPU/ml) for 10 min at 4°C in assay buffer, diluted 10-fold with this buffer and assayed in the ^{125}I-fibrin plate assay. The final concentration of inhibitor during the assay is indicated. Control assays were performed identically except that inhibitor was omitted. In the control assay ~7000 cpm per well were released corresponding to ~12% of the total radioactivity in each well. Background values without u-PA added (~500 cpm) have been subtracted. Inset: Reverse fibrin-agarose zymography of the non-converted (a) and converted (b) inhibitor preparations. 50 ng protein was electrophoresed in each lane.

several polypeptides that migrated faster than the one formed by the plasminogen activators. Results similar to those shown in fig.2, lanes a and f, were obtained with u-PA and an inhibitor preparation purified by affinity chromatography with a monoclonal antibody that neutralized its inhibitory activity (L.S. Nielsen, unpublished).

The converted form of the inhibitor did not inhibit u-PA activity as measured by the ^{125}I-fibrin plate assay or by zymography (fig.3). Neither did it inhibit t-PA in the ^{125}I-fibrin plate assay under similar conditions (not shown).

4. DISCUSSION

This study demonstrates that plasminogen activators catalyse conversion of HT-1080 plasminogen activator inhibitor to an inactive form with an electrophoretic mobility in SDS-PAGE corresponding to an apparent M_r slightly lower than that of the non-converted form. The conversion caused by u-PA was inhibited by inhibitors of

this enzyme (including the active site titrant *p*-nitrophenyl-*p'*-guanidinobenzoate) indicating that it was due to proteolysis directly involving the active site of u-PA. On the basis of the present data, it cannot be determined whether the observed change in electrophoretic mobility during conversion is only due to a decrease in M_r as conformational changes may also contribute. For a further clarification of this question, studies on the amino acid sequence of the two forms of the inhibitor are needed. Such studies are in progress, but have been hampered by the fact that the active form is N-terminally blocked (A. Henschen and L.S. Nielsen, unpublished).

These findings provide the first unequivocal demonstration of a well-defined protein apart from plasminogen acting as a substrate for plasminogen activators. The existence of such substrates has been suggested previously [37,38], although the nature of the putative protein substrates was not determined. Our results raise the possibility that plasminogen activator inhibitors have been involved in the effects observed in these studies.

The present findings seem to indicate that the mechanism of action of this inhibitor deviates from the standard mechanism for inhibition of proteinases by protein inhibitors, where proteolysis of the inhibitors does not proceed to completion and where the modified forms of the inhibitors are active (reviews [39,40]). In these respects, this plasminogen activator inhibitor seems closer to some members of the α_1-proteinase inhibitor class [40].

The HT-1080 inhibitor forms an SDS-resistant complex with the active form of u-PA, but not with pro-u-PA [26]. The u-PA/inhibitor complex has an apparent plasminogen activator activity as revealed by zymography after SDS-PAGE, where plasminogen activators diffuse into and cause lysis zones in agarose gels containing plasminogen and fibrin [26]. Similar findings have been reported for complexes of plasminogen activators and inhibitors from other sources ([13,15–20,23]; T.D. Gelehrter, personal communication). The present results offer a likely explanation for the apparent activity of the complexes. During the zymography, the plasminogen activator degrades the inhibitor to its inactive form, the complex is dissociated and the plasminogen activator regains its activity.

The physiological role of the ~54 kDa protein described here remains to be clarified. Apart from its possible function as a physiological inhibitor of plasminogen activators, it may be speculated that either itself, its ~50 kDa conversion product or the putative released peptide, might have other functions.

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