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Inactive proenzyme to tissue-type plasminogen activator from human melanoma cells, identified after affinity purification with a monoclonal antibody

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The human 66 000 mol. wt. plasminogen activator (HPA66; tissue-type plasminogen activator) has been purified from melanoma cells by a one-step affinity method with a monoclonal antibody. HPA66 purified in this way consists mainly of a one-polypeptide chain form with small amounts (15%) of a form containing two polypeptide chains held together by one or more disulphide bridges. The one-chain form was converted to the two-chain form by catalytic amounts of plasmin. During the conversion, the enzyme activity of HPA66, as measured by an [125I]plasminogen conversion assay and with a chromogenic substrate, increased linearly with the percentage of the two-chain form. A linear regression analysis showed that all enzyme activity could be accounted for by the two-chain form, while the one-chain form had no measurable enzyme activity (detection limit ~5% of the activity of the two-chain form). Together with previous findings of inactive proenzymes to murine and human ~50 000 mol. wt. (urokinase-type) plasminogen activators, these findings indicate that plasminogen activators are generally formed from inactive one-chain proenzymes which are converted to active two-chain enzymes by limited proteolysis, thus demonstrating a third step in a cascade reaction leading to extracellular proteolysis.
Key words: plasminogen activator/proenzyme/serine protease/melanoma cells/thrombolysis

Introduction

Plasminogen activators are capable of initiating extracellular proteolysis with a broad trypsin-like substrate specificity by the enzymatic conversion of the extracellular zymogen plasminogen to the active protease plasmin (for reviews, see Astrup, 1975; Christman *et al.*, 1977; Reich, 1978; Collen, 1980). Two types of plasminogen-activating enzymes can be distinguished, as based on mol. wt., immunological reactivity, and data on the amino acid sequences of the proteins and the nucleotide sequences of the corresponding cDNA (Aoki and Kaulla, 1971; Unkeless *et al.*, 1974; Christman *et al.*, 1975; Danø and Reich, 1978; Granelli-Piperno and Reich, 1978; Åstedt, 1979; Vetterlein *et al.*, 1979; Danø *et al.*, 1980b; Rijken *et al.*, 1980; Roblin and Young, 1980; Wilson *et al.*, 1980; Rijken and Collen, 1981; Günzler *et al.*, 1982; Kaltoft *et al.*, 1982; Schaller *et al.*, 1982; Steffens *et al.*, 1982; Edlund *et al.* 1983; Nielsen *et al.*, 1983; Pennica *et al.*, 1983). One type, with a mol. wt. of ~50 K (the urokinase-type), seems to be involved in tissue degradation, among other functions (Beers *et al.*, 1975; Strickland *et al.*, 1976; Reich, 1978; Ossowski *et al.*, 1979). The other type, with a mol. wt. of ~70 K (the tissue-type), is supposed to be involved in thrombolysis (Matsuo *et al.*, 1981; Mattson *et al.*, 1981).

The murine and human plasminogen activators of the urokinase-type exist as inactive one-polypeptide chain proenzymes, convertible to their two-polypeptide chain counterparts by limited proteolysis (Skriver *et al.*, 1982; Nielsen *et al.*, 1982; Wun *et al.*, 1982b). The tissue-type plasminogen activators from both human and porcine sources are also known to exist in both a one-polypeptide chain and a two-polypeptide chain form (Rijken and Collen, 1981; Wallén *et al.*, 1981; Nielsen *et al.*, 1983). However, previously published investigations led to the conclusion that the one-polypeptide chain form of the tissue-type activator is not an inactive proenzyme, since it appeared to have considerable enzyme activity (Wallén *et al.*, 1981, 1982; Rijken *et al.*, 1982; Rånby, 1982; Rånby *et al.*, 1982). In the case of the human activator, the one-chain form was reported to be as active as the two-chain form (Rijken *et al.*, 1982). Recently, we purified the human tissue-type plasminogen activator (HPA66) by affinity chromatography with a monoclonal antibody (Nielsen *et al.*, 1983). We now present results showing that one-polypeptide chain HPA66 in preparations purified in this way is an inactive proenzyme, convertible to active two-polypeptide chain enzyme by catalytic amounts of plasmin.

Results

Conversion of one-chain to two-chain HPA66

HPA66 was purified from conditioned medium from a human melanoma cell line by affinity chromatography with a monoclonal anti-HPA66 IgG antibody immobilized on Sepharose (Nielsen *et al.*, 1983). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the preparations under non-reducing conditions showed only one Coomassie blue stainable band, its migration corresponding to a mol. wt. of 66 000 (66K; see Figure 1). Under reducing conditions, the main part of the Coomassie blue-stainable material in the preparations also migrated at 66 K mol. wt., but a small amount migrated as a broad, lightly-stained band at ~35 K mol. wt. (Figure 1). When the preparations were incubated with catalytic amounts of plasmin, the HPA66 was converted to a form co-migrating with the original band in SDS-PAGE under non-reducing conditions, but migrating as a broad band with mol. wt. ~35 K under reducing conditions (Figure 1). This conversion was prevented by the omission of plasmin from the incubations, or by the addition of the plasmin inhibitor trasylol with the plasmin (data not shown). Spectrophotometric scanning indicated that the ~35 K mol. wt. band in the plasmin-treated preparations contained approximately the same amount of Coomassie blue-stainable

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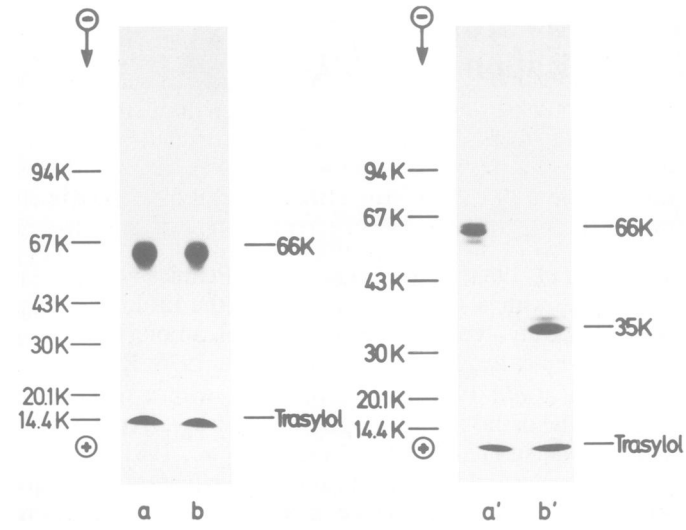

Fig. 1. Conversion of one-chain HPA66 to two-chain HPA66 by plasmin. Affinity-purified HPA66 (20 μg/ml) was incubated with plasmin (0.1 μg/ml) for 240 min at 37°C in 0.1 M Tris HCl, pH 8.1, 0.1% Triton X-100, 0.2 M NaCl. Both incubated and non-incubated samples were then made 50 μg/ml with respect to trasylol. Samples containing 10 μg HPA66 (one- + two-chain forms) before (**a,a'**) and after (**b,b'**) the incubation were submitted to SDS-PAGE in slab gels under non-reducing (**a,b**) and reducing (**a',b'**) conditions. The gels were stained with Coomassie blue. The positions of mol. wt. markers are indicated.

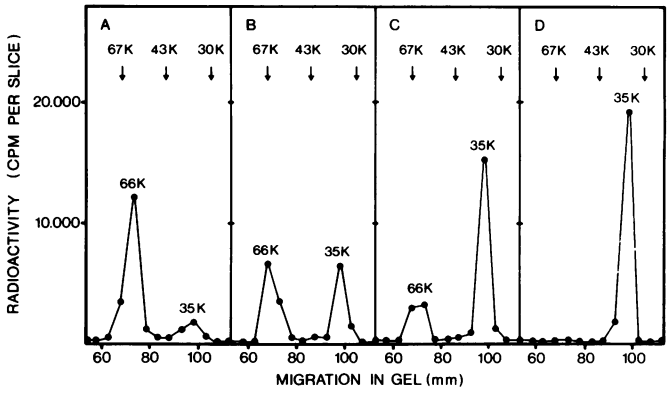

Fig. 2. Quantitation of the relative amounts of one-chain and two-chain HPA66 by [^{3}H]DFP-labelling and SDS-PAGE under reducing conditions. Affinity-purified HPA66 was incubated with plasmin as described in the legend to Figure 1 for 0 (**A**), 20 (**B**), 45 (**C**) or 240 min (**D**). At these time points, the solutions were made 50 μg/ml with respect to trasylol. Aliquots were labelled with 90 μM [^{3}H]DFP for 24 h at 20°C, and submitted to SDS-PAGE under reducing conditions. The gel lanes were cut into 0.5 cm slices, and the tritium content of each slice determined by liquid scintillation spectrometry. The positions of the mol. wt. marker proteins are indicated by arrows. The total radioactivity found in the two peaks varied <10% in the four cases. In addition, the tritium in the two peaks always accounted for >95% of the total amount of protein bound tritium in the gels. From the radioactivity in each peak, the percentages of two-chain HPA66 in the preparations were calculated as 16 (**A**), 43 (**B**), 71 (**C**) and 97 (**D**), respectively (see text).

material as the 66 K + 35 K mol. wt. bands in the original preparations.

These findings lead to the conclusion that the affinity-purified HPA66 preparation contained mainly a one-polypeptide chain form which was converted by limited proteolysis to a form consisting of two polypeptide chains, each with mol. wt. of ~35 K. They also suggest that the small amount of ~35 K mol. wt. material seen in the affinity-purified preparation after reduction is identical with the two-polypeptide chain form of HPA66 produced by limited proteolysis.

Table I. Effect of DFP on the enzyme activity of different HPA66 preparations, as measured by the ^{125}I-labelled fibrin plate assay with small amounts of plasmin added

Relative content of two-chain HPA66 (% of total HPA66)	Incubation	Radioactivity released in assay (c.p.m.)
16%	None	1530 ± 248
	Without DFP	1718 ± 68
	With DFP	90 ± 6
97%	None	2226 ± 255
	Without DFP	2187 ± 243
	With DFP	47 ± 10

Preparations containing mainly one-chain HPA66 or mainly two-chain HPA66 were obtained as described in the legend to Figure 2. The preparations were the same as those used for the experiments in Figure 2A and D, respectively. Samples were taken for ^{125}I-labelled fibrin plate assay before or after incubations of aliquots containing 1 μg/ml of total HPA66 (one- + two-chain form) with 90 μM DFP (or, as controls, without DFP). Before the assay, the samples were diluted 5000-fold. The assays were performed with 14 ng/ml of plasmin added to each assay well. Radioactivity released in parallel control assays without plasminogen activator (~600 c.p.m.) has been subtracted. The total radioactivity in the [^{125}I]fibrin plates was ~60 000 c.p.m. Means and standard deviations for two parallel assays of each sample are indicated. Separate control experiments demonstrated that DFP, in the concentrations in which it was transferred to the assay buffer with the HPA66 samples, did not inhibit the assays.

Quantitation of one-chain and two-chain HPA66 by [^{3}H]diisopropylfluorophosphate incorporation

Affinity-purified HPA66 preparations, which had or had not been incubated with plasmin, were labelled with [^{3}H]diisopropylfluorophosphate (DFP) and submitted to SDS-PAGE under reducing conditions, followed by scintillation counting of gel slices. The labelling profiles are shown in Figure 2. Without incubation with plasmin, two peaks were observed, with electrophoretic mobilities corresponding to 66 K and 35 K, respectively. Plasmin treatment gradually decreased the 66 K mol. wt. peak and increased the 35 K peak, until after 240 min nearly all radioactivity was found in the latter. Incubation for longer periods (up to 24 h) did not change the labelling profile any further. Under non-reducing conditions, all the incorporated [^{3}H]DFP migrated in the 66 K position, independently of whether the preparation was plasmin-treated or not. These findings show that both the one- and two-chain HPA66 forms incorporated [^{3}H]DFP, and that the 35 K mol. wt. Coomassie blue-stainable material in the affinity-purified preparation not treated with plasmin (Figure 1a') really represents the two-chain HPA66 form.

With the binding conditions used (90 μM DFP for 24 h at 20°C), the enzyme activity of both untreated and plasmin-treated preparations was inhibited >95% as assayed by the [^{125}I]fibrin method with small amounts of plasmin added (Table I). The addition of plasmin makes the activity measured in this assay nearly independent of the percentages of the one-chain and two-chain forms of HPA66 in the preparation (see below). This indicates that both the active site in the two-chain form and the corresponding site in the one-chain form became almost completely saturated with DFP under these conditions. In addition, the incorporation

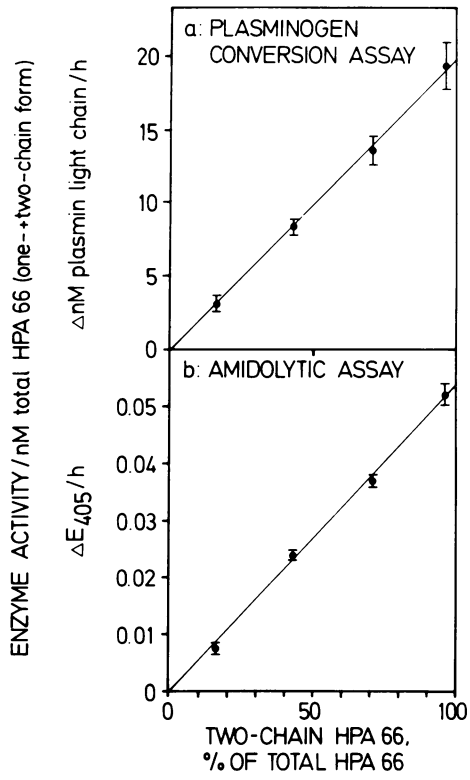

Fig. 3. The enzyme activity of preparations containing different percentages of one- and two-chain HPA66. The HPA66 preparations were non-[^{3}H]-DFP-treated aliquots of those used for the experiment described in the legend to Figure 2. Enzyme activity of each preparation measured by conversion of [^{125}I]plasminogen to [^{125}I]plasmin (**a**) and amidolysis of L-pyro-glutamyl-glycyl-L-arginine-*p*-nitroanilide (**b**) was determined as described in Materials and methods. Means ± SE for the enzyme activities are indicated in the figure. The standard errors of the determinations of the relative amount of the two-chain form appeared to be negligible in the present context. The parameters of the lines drawn were determined by weighted linear regression analysis. F-tests on the square sum of the residuals showed that the experimental values did not differ significantly from the values expected from the constructed lines ($P > 0.05$ in both assays). The parameters of the lines (± SE) were as follows: (a) interception with the ordinate − 0.15 ± 0.58, slope 0.20 ± 0.01; (b) interception with the ordinate − 0.00029 ± 0.00107, slope 0.00054 ± 0.00002.

of [^{3}H]DFP into both one-chain and two-chain HPA66 was inhibited >98% by 100 mM *p*-amino-benzamidine (data not shown), indicating that only the above-mentioned sites incorporated DFP (Danø and Reich, 1978). Futhermore, when the HPA66 preparation not treated with plasmin was preincubated for 24 h at 20°C before the addition of [^{3}H]DFP, this did not result in any change in the distribution of radioactivity between the two peaks, demonstrating the absence of conversion of one-chain HPA66 to two-chain HPA66 under the conditions for the DFP labelling. These findings demonstrate that [^{3}H]DFP labelling followed by SDS-PAGE under reducing conditions and scintillation counting is a valid method for quantitation of the percentages of the two forms in preparations containing both.

On this basis it was calculated that the affinity-purified HPA66 preparation used for the experiment shown in Figure 2 contained 84% of the one-chain form and 16% of the two-chain form before plasmin treatment, and that the one-chain form was completely converted to the two-chain form by incubation for 240 min with plasmin.

Enzyme activity of one- and two-chain HPA66

As measured by the standard [^{125}I]-labelled fibrin plate assay for plasminogen activation, the activity of the HPA66 preparations fully converted to the two-chain form was ~4-fold higher than that of the affinity-purified preparation before plasmin treatment (data not shown). It is not likely, however, that this difference reflects the real difference in enzyme activity caused by the different content of the one- and two-chain forms in the preparations, since the plasmin formed during the assay of the untreated preparations (containing a mixture of the two forms) will convert some of the one-chain form to two-chain form. In support of this was the finding of nearly identical activities of the two preparations when the assay mixtures were supplemented with 14 ng/ml of plasmin before the start of the assay (see Table I).

To compare the enzyme activities of the two forms, therefore, two assays were used, during which conversion of one-chain HPA66 to two-chain HPA66 by plasmin could be excluded: an [^{125}I]plasminogen conversion assay (Danø and Reich, 1975, 1979) and an amidolytic assay with the substrate L-pyroglutamyl-glycyl-L-arginine-*p*-nitroanilide (S-2444; Wun *et al.*, 1982a). The principle in the plasminogen conversion assay is the measurement of the conversion of [^{125}I]plasminogen to [^{125}I]plasmin by the electrophoretic separation of [^{125}I]-plasminogen from the heavy and light chains of [^{125}I]plasmin (Danø and Reich, 1975, 1979). To inhibit conversion of one-chain to two-chain HPA66 during the assay, a high concentration of trasylol was added. Trasylol inhibits plasmin, but not plasminogen activators (Danø and Reich, 1975, 1979; unpublished results). In the amidolytic assay, the activity of HPA66 in hydrolyzing the terminal amide bond in the tripeptide S-2444 is determined by measuring the ensuing increase in absorbancy at 405 nm. Here, too, trasylol was present during the assay. In both types of assays, the amount of substrate converted was linearly related to time and the total HPA66 concentration for all preparations tested. As measured by these assays, the enzyme activity of the affinity-purified HPA66 preparations increased ~6-fold after 240 min plasmin treatment. Incubation with plasmin for longer periods (up to 24 h) did not result in any further changes in enzyme activity.

A check was made that there was no conversion of one-chain to two-chain HPA66 during these assays. For the amidolytic assay, the [^{3}H]DFP labelling technique showed that there were no differences in the percentages of the two forms before and after incubation (detection limit ~1% of the total amount of HPA66). For the [^{125}I]plasminogen conversion assay, it was found that there was no difference in the amidolytic activities before and after incubation (detection limit ~5%).

In Figure 3, the enzyme activities of four HPA66 preparations containing different percentages of the one- and two-chain HPA66 forms are plotted as a function of their content of two-chain HPA66 as measured by the [^{3}H]DFP labelling technique. With both assays, there was a linear increase in enzyme activity with the percentage of the two-chain form in the preparation, and in both cases the interception with the ordinate of the line did not differ significantly from zero. This means that with both types of assays, all enzyme activity could be accounted for by the amount of two-polypeptide chain HPA66, while the one-polypeptide chain HPA66 did not have detectable enzyme activity (detection limit ~5% of the activity of the two-chain form). One-chain HPA66 can

therefore be considered as an inactive proenzyme and was designated pro-HPA66.

Discussion

The finding that the one-chain form of HPA66 had no detectable activity, either with respect to its natural substrate plasminogen or to an artificial chromogenic substrate, is in contrast to previous reports of enzyme activity of one-chain forms of this type of plasminogen activator. Thus, it was previously reported (Rijken *et al.*, 1982) that one-chain HPA66 and two-chain HPA66 had identical activities. The HPA66 used in the previous study was purified from the same melanoma cell line as the one used in the present study, and assayed by the same type of [^{125}I]plasminogen conversion assay. It was, however, purified by a relatively complicated procedure, in contrast to the simple one-step affinity purification with a monoclonal antibody used in the present study. The most likely explanation for the conflicting results is, therefore, that in the study of Rijken *et al.* (1982) pro-HPA66 was activated (but not converted to the two-chain form) during the more complicated purification procedure, or that a factor able to activate pro-HPA66 in the presence of trasylol was co-purified with the HPA66. Similar considerations hold for studies reported on the enzyme activity of one- and two-chain forms of the ~70 K mol. wt. porcine plasminogen activator, where the one-chain form appeared to have considerable activity (~1/3 – 1/9 of that of the two-chain form) in an amidolytic assay (Wallén *et al.*, 1981,1982; Rånby *et al.*, 1982) and in a coupled plasminogen activator/plasmin assay (Rånby, 1982). In the latter case, conversion of one-chain to two-chain form by plasmin during the assay may also have contributed to the apparent activity of the one-chain form. It therefore seems likely that in addition to its simplicity the affinity purification with a monoclonal antibody is also more effective and/or gentle than the hitherto-used procedures, and therefore will prove valuable for purifying the proenzyme, whether it is produced by human cell cultures or by the expression of HPA66-cDNA in bacteria (Edlund *et al.*, 1983; Pennica *et al.*, 1983).

The pro-HPA66 preparation used in the present study contained ~15% of the active enzyme. We were not able to reduce this fraction significantly, either by prolonged serum starvation of the cells or by the addition of trasylol to the medium during the cultivation and to the buffers used for the purification (unpublished results). At present, it is not known whether the contaminating active enzyme is released by the melanoma cells or whether it is formed from pro-HPA66 in the culture fluid or during the purification by a process that is not inhibited by trasylol.

The existence of an inactive proenzyme to the tissue-type plasminogen activator is analogous to the recently reported existence of inactive proenzymes to murine and human urokinase-type plasminogen activators (Skriver *et al.*, 1982; Nielsen *et al.*, 1982; Wun *et al.*, 1982b). It now appears likely that plasminogen activators in general are released from cells as inactive one-polypeptide chain proenzymes that are converted to active two-polypeptide chain enzymes by limited proteolysis, and that plasmin-mediated extracellular proteolysis has the general character of a cascade reaction with at least three steps. It should be noted that initiation of this process will require initiating factors which are as yet unknown.

A striking difference between the proenzyme forms of the urokinase-type activators and the tissue-type activators is the lack of incorporation of DFP in the former type (Nielsen *et al.*, 1982; Skriver *et al.*, 1982; Wun *et al.*, 1982b), in contrast to the findings with the tissue-type activator in the present study. In this respect, pro-HPA66 resembles the proenzymes to the blood coagulation factors VII (Kerr *et al.*, 1978) and X (Nemerson, 1976).

It is noteworthy that the demonstration of inactive proenzymes to plasminogen activators points to the existence of serious pitfalls in the measurements of plasminogen activators in biological samples by enzymatic assays. Such measurements may be strongly influenced by the presence of trace amounts of plasmin and/or plasmin inhibitors. This means that an internal standard of purified proenzyme should be used to control the validity of such assays. Alternatively, the development of immunoassays for plasminogen activators might bypass the influence of activating and inhibiting substances.

Several findings, e.g., on the relative fibrinolytic activity of the two types of plasminogen activators (Matsuo *et al.*, 1981; Mattson *et al.*, 1981) and on their immunocytochemical distribution (unpublished results) point to the tissue-type activator being involved in thrombolysis. Tissue-type plasminogen activator immunoreactivity is thus present in endothelial cells of several types of blood vessels. Most likely, the form released from the endothelial cells is the proenzyme, and this form might be more advantageous than the active enzyme for clinical trials of tissue-type plasminogen activator for thrombolytic therapy.

Materials and methods

Materials

The following materials were obtained from the indicated sources: [^{3}H]diisopropylfluorophosphate (6.0 Ci/mmol) and Na [^{125}I] (15.9 mCi/μg iodine) (The Radiochemical Centre, Amersham); L-pyroglutamyl-glycyl-L-arginine-p-nitroanilide (S-2444; KABI, Stockholm); Lumasolve and Lipoluma (Luma, Basel); *p*-amino-benzamidine (Merck, Darmstadt). All other materials were those described previously (Danø *et al.*, 1980a, 1980b; Kaltoft *et al.*, 1982; Nielsen *et al.*, 1982; Skriver *et al.*, 1982; Nielsen *et al.*, 1983), or of the best grade commercially available.

Cell culture

A human melanoma cell line (Bowes) producing HPA66 was a gift from D.B. Rifkin, New York University, NY. This line was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Conditioned serum-free culture fluid was prepared from confluent monolayer cultures (Danø *et al.*, 1980a; Nielsen *et al.*, 1983).

Purification of HPA66

HPA66 was purified from conditioned medium of the Bowes melanoma cell line by affinity chromatography with a monoclonal anti-HPA66 IgG_1 antibody immobilized on Sepharose (Nielsen *et al.*, 1983). The HPA66 content of the purified preparation was determined by the ^{125}I-labelled fibrin plate assay with 14 ng/ml of plasmin added to each assay well (see below), using an HPA66 standard preparation, the protein concentration of which was determined as described by Bonsall and Hunt (1971).

Electrophoresis

To concentrate dilute protein samples before electrophoresis, the proteins were precipitated with 7% trichloroacetic acid (TCA); the pellets were washed once with 7% TCA and twice with acetone, and dried. SDS-PAGE was carried out under reducing or non-reducing conditions in slab gels with a 6 – 16% linear concentration gradient of polyacrylamide (Danø and Reich, 1978). The following mol. wt. markers were used: rabbit phosphorylase b (94 K), bovine serum albumin (67 K), ovalbumin (43 K), carbonic anhydrase (30 K), soybean trypsin inhibitor (20.1 K) and α-lactalbumin (14.4 K). The gels were stained with Coomassie blue. Spectrophotometric scanning at 600 nm of individual gel lanes was performed with a SP8-150 Pye-Unicam Spectrophotometer.

DFP-treatment of HPA66

0.2 – 1 μg of HPA66 was incubated with 20 nmol of DFP (^{3}H-labelled or not)

in a total volume of 225 μl 0.1 M Tris HCl, pH 8.1, 0.1% Triton X-100, 0.2 M NaCl and 12.5 μg/ml of trasylol, at 20°C for 24 h. When labelling was performed in the presence of *p*-amino-benzamidine, the latter was pre-incubated with HPA66 for 1 h before the addition of [^{3}H]DFP (Danø and Reich, 1978). The HPA66 was precipitated with 7% TCA together with 5 μg ovalbumin as described above, and submitted to SDS-PAGE. After electrophoresis and staining, each gel lane was cut into 0.5 cm slices. The tritium was extracted from the slices and determined by liquid scintillation counting, using a mixture of water, Lumasolve and Lipoluma (0.2:1:10).

Radiolabelled fibrin plate assay

The radiolabelled fibrin plate assay for plasminogen activators was performed as described (Nielsen *et al.* 1982). Each assay well contained 0.5 ml of 0.1 M Tris HCl, pH 8.1, 0.1% Triton X-100, 0.25% gelatin, 1 μg human glu-plasminogen and 10 – 100 pg of HPA66. In some assays, 14 ng/ml of human plasmin was added before the start of the experiment. Incubation was for 1 h at 37°C. Human glu-plasminogen was prepared from fresh human male plasma by a modification (Danø and Reich, 1979) of the method of Deutsch and Mertz (1970). Plasmin was generated from plasminogen by incubation with urokinase in 50% glycerol (Danø and Reich, 1979).

[^{125}I]Plasminogen conversion assay

The principles of this assay have already been described (Danø and Reich, 1975, 1979). Human glu-plasminogen was labelled with ^{125}I by the procedure of Helmkamp *et al.* (1960), using a 4-fold molar excess of iodine monochloride relative to plasminogen. A specific activity of ~0.6 μCi/μg plasminogen was obtained. For the assay, HPA66 was incubated for 60 min at 37°C in 20 μl of a buffer with 0.07 μM [^{125}I]plasminogen, 0.55 μM unlabelled plasminogen, 0.1 M Tris HCl, pH 8.1, 0.1% Triton X-100, 0.075 M NaCl and 500 μg/ml of trasylol. For each mixture of one-chain and two-chain HPA66 to be assayed, incubations were done in duplicate with five different HPA66 concentrations, the highest and the lowest differing by a factor of 5; in addition, a control without HPA66 was included. The reactions were stopped by cooling the incubation mixtures to 0°C and adding 20 μl 0.06 M Tris HCl, pH 8.1, 6% SDS. 10 μg plasminogen and 10 μg plasmin were added as markers for the subsequent gel electrophoresis. The percentage conversion of [^{125}I]plasminogen to [^{125}I]plasmin was determined by SDS-PAGE under reducing conditions (Danø and Reich, 1978), followed by staining of the gel, sectioning of each lane into 0.5 cm slices, and determination of the radioactivity in each slice by gamma spectrometry. For each incubation mixture, the percentages of the total radioactivity in each of the bands representing glu-plasminogen, glu-plasmin heavy chain and plasmin light chain were calculated after a correction for background radioactivity in the regions containing the two plasmin chains, as determined from the gel lanes with [^{125}I]plasminogen incubated in the absence of plasminogen activator. The relative radioactivity of the heavy and light chains of plasmin reflected the ratio of their mol. wts. (Christman *et al.*, 1977). The total HPA66 concentrations were between 0.06 and 1 μg/ml (0.9 – 15 nM) in all experiments, and were chosen so that the conversion obtained during a 60 min incubation at 37°C never exceeded 20% of the start concentration of plasminogen. Under these conditions, the conversion was linearly related to the time and the total HPA66 concentration for all HPA66 preparations. The activity of each HPA66 preparation was calculated by linear regression analysis as the slope of the line defined by the data pairs (total HPA66 concentration/plasmin light chain concentration), and expressed as Δ nM plasmin light chain x h^{-1} x (nM total HPA66)$^{-1}$.

Amidolytic assay

This assay was performed essentially as described by Wun *et al.* (1982a). 0.1 mM L-pyroglutamyl-glycyl-L-arginine-*p*-nitroanilide (S-2444) was incubated with HPA66 at total concentrations of one-chain and two-chain forms of 0.05 – 0.2 μg/ml for 2 – 4 h at 37°C in a buffer containing 0.1 M Tris HCl, pH 8.1, 0.1% Triton X-100 and 10 μg/ml of trasylol. The reaction was stopped by addition of SDS (0.5% final concentration) and the absorbancy at 405 nm was measured. For each HPA66 preparation, incubations were performed in duplicate with two different total HPA66 concentrations, differing by a factor of 2; in addition, a control without HPA66 was included. The HPA66 concentrations and incubation times were chosen so that the increase in absorbancy at 405 nm never exceeded 0.3. Under these conditions, the increase in absorbancy at 405 nm for each HPA66 preparation was linearly related to the time and the total HPA66 concentration. The activity of each HPA66 preparation was calculated by linear regression analysis as the slope of the line defined by the data pairs (total HPA66 concentration/absorbancy at 405 nm) and expressed as Δ absorbancy at 405 nm x h^{-1} x (nM total HPA66)$^{-1}$. The activity of plasmin with repect to amidolysis of S-2444 was found to be negligible in the present context (data not shown).

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