Purification of Zymogen to Plasminogen Activator from Human Glioblastoma Cells by Affinity Chromatography with Monoclonal Antibody†

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ABSTRACT: Incorporation of the serine protease active site reagent diisopropyl fluorophosphate (DFP) into a plasminogen activator with an M_r of approximately 52 000 released from cultured human glioblastoma cells was strongly enhanced by incubation with plasmin. This observation led to the isolation of an inactive form of the enzyme from serum-free conditioned culture fluid by affinity chromatography on a column of a Sepharose-bound monoclonal antibody raised against urokinase. An 831-fold purification was obtained with a yield of 41%. The purified molecule was homogeneous as evaluated by polyacrylamide gel electrophoresis with sodium dodecyl sulfate ($NaDodSO_4$), having one stainable band under nonreducing as well as reducing conditions with an M_r of approximately 52 000. It was unable to activate plasminogen, but catalytic amounts of plasmin converted it into active enzyme. After $NaDodSO_4$–polyacrylamide gel electrophoresis, the active enzyme showed one band under nonreducing conditions, but after reduction, two bands with M_r values of approximately 20 000 and 32 000 were observed. The active enzyme incorporated [^{3}H]DFP into the approximately M_r 32 000 band, while no incorporation was observed into the inactive form. These findings show that the M_r 52 000 human plasminogen activator exists in a proenzyme form consisting of a single polypeptide chain that by proteolysis between half-cystine residues is converted into the active enzyme consisting of two chains with molecular weights of approximately 20 000 and 32 000, the active site being on the latter chain. The results are consistent with the active form of the enzyme being identical with the higher molecular weight form of urokinase, and together with recent observations that a murine plasminogen activator is released from sarcoma virus transformed cells as an inactive proenzyme, they suggest that zymogens to plasminogen activators are of more general occurrence.

Plasmin is a trypsin-like serine protease with a broad substrate specificity. Its zymogen, plasminogen, is present in most extracellular body fluids and can be activated by another class of serine proteases, plasminogen activators [for a review, see Christman et al. (1977)]. Cellular release of plasminogen activators thus constitutes a means of obtaining strongly amplified extracellular proteolysis, and this mechanism seems to play a central role in a variety of physiological and pathological processes involving tissue degradation, e.g., postlactational mammary gland involution (Ossowski et al., 1979), invasive growth of trophoblasts (Strickland et al., 1976), ovulation (Beers et al., 1975), inflammation (Unkeless et al., 1974b; Reich, 1978), and malignant neoplasia (Unkeless et al., 1973, 1974a; Goldberg, 1974; Christman & Acs, 1974; Rifkin et al., 1974; Danø & Reich, 1978; Jaken & Black, 1979; Roblin & Young, 1980; Goldfarb & Quigley, 1980), as well as in the solubilization of thrombi within the vascular system (Astrup, 1975; Matsuo et al., 1981; Mattsson et al., 1981).

In each animal species, there exist at least two types of plasminogen activators which can be distinguished, e.g., by electrophoretic mobility and immunological reactivity (Unkeless et al., 1974b; Rifkin et al., 1974; Christman et al., 1975; Granelli-Piperno & Reich, 1978; Danø & Reich, 1978; Astedt, 1979; Danø et al., 1980b; Wilson et al., 1980; Kaltoft et al., 1982), and which probably have different biological functions, an approximately M_r 70 000 type being implicated in thrombolysis (Matsuo et al., 1981; Mattsson et al., 1981) and an approximately M_r 50 000 type in other processes, e.g., tissue degradation (Ossowski et al., 1979).

In accordance with the amplificaton involved in the activation reaction, plasminogen activators are only present in biological fluids in very low concentrations. This has complicated their purification and characterization. We have previously reported the selection of a murine cell line transformed by sarcoma viruses which releases relatively high amounts of an approximately M_r 48 000 plasminogen activator and the complete purification of the enzyme from this source (Danø et al., 1980a). Recently, we reported that this murine plasminogen activator is released from serum-starved cells as an inactive proenzyme, which is converted to the active enzyme by limited proteolysis catalyzed by plasmin (Skriver et al., 1982). We now report the purification of a zymogen to an approximately M_r 52 000 plasminogen activator (HPA52) released from cultured human glioblastoma cells by the use of affinity chromatography with a monoclonal antibody developed (Kaltoft et al., 1982) against urokinase.

Materials and Methods

Chemicals and Reagents. Urokinase (reagent grade and reference standard) and bovine pancreatic trypsin inhibitor (BPTI; Kunitz inhibitor, Trasylol) were kind gifts from Leo Pharmaceutical Co., Ballerup, Denmark, and Bayer AG, Wupperthal, West Germany, respectively. Fetal bovine serum and Dulbecco-modified Eagle's medium were obtained from Grand Island Biological Co., Grand Island, NY. Protein A–Sepharose and cyanogen bromide activated Sepharose 4B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Rabbit IgG anti-mouse IgG was from DAKO Immunoglobulins, Copenhagen, Denmark. All other chemicals and reagents were as previously described or of the commercially best available grade.

Cells and Cell Culture. A cell line derived from a human glioblastoma [UCT/gl 1; see Wilson et al. (1980)] was maintained as a monolayer culture essentially as described

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previously in 150-mm plastic petri dishes (Falcon Plastics, Oxnard, CA) using Dulbecco-modified Eagle's medium supplemented with 10% fetal bovine serum (Danø et al., 1980a). For preparation of culture fluid for purification of plasminogen activator, the cells were grown to confluence [(5–8) × 10^4 cells/cm^2] in serum-supplemented medium. The cultures were then washed 3 times with a buffer containing 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ and incubated in 25 mL of serum-free medium for 7 days with intervals of 2 or 3 days between medium changes. Hybridoma cells producing mouse IgG_1 anti-human M_r 52000 plasminogen activator (HPA52-2-21-27 cells) were maintained as a suspension culture in Dulbecco-modified Eagle's medium as previously described (Kaltoft et al., 1982).

Assay for Plasminogen Activator. Plasminogen activator was assaed enzymatically by the ^{125}I-labeled fibrin plate method by using Multiwell Disposo trays (Linbro Scientific Co., New Haven, CT) coated with 20 μg (50000–65000 cpm) of fibrin/well (Danø et al., 1980a). Each assay well contained 0.5 mL of 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.1, 0.25% gelatin, 0.1% Triton X-100 (assay buffer), and 1 μg of human plasminogen. A reference standard preparation of urokinase was used to calibrate each assay. The assays were linear with enzyme concentration over the range 0.002–0.005 Ploug unit. All samples and the standard were diluted to contain an amount within these limits in 25–50 μL, and this volume was added to the assay mixture. All dilutions were performed with assay buffer. Bovine ^{125}I-labeled fibrin was prepared as described by Unkeless et al. (1973). Human plasminogen was prepared from fresh plasma by two cycles of affinity chromatography (Deutsch & Mertz, 1970; Danø & Reich, 1979). The plasminogen preparations were pure as evaluated by polyacrylamide gel electrophoresis with sodium dodecyl sulfate ($NaDodSO_4$), containing two Coomassie Blue stainable bands with an M_r of approximately 92000 (Danø & Reich, 1975). The detection limit for possible contaminating proteins was <2% of the plasminogen content. In some experiments, the plasminogen used in the assay was supplemented with human plasmin (13.2 ng/mL final concentration in the assay). The plasmin was generated from plasminogen by incubation with urokinase in 50% gycerol as described previously (Danø & Reich, 1979).

Purification of Monoclonal Antibodies. Mouse IgG_1 anti-HPA52 was precipitated from 150 mL of hybridoma cell culture fluid with $(NH_4)_2SO_4$ (27% w/v final concentration), and the mixture was left standing for 16 h at 4 °C. The precipitate was then resuspended in 50 mL of 0.1 M sodium phosphate, pH 8.0 (application buffer), dialyzed extensively against the same buffer, and applied to a column (5 mL, 12 × 43 mm) of protein A–Sepharose. The column was washed with 80 mL of application buffer and eluted with 15 mL of 0.1 M sodium acetate, pH 4.0, and 0.15 M NaCl. The column was developed at a flow rate of 40 mL/h at 4 °C. The eluate was neutralized by addition of 0.1 volume of 1 M Tris-HCl, pH 9.0. It contained pure IgG as evaluated by $NaDodSO_4$–polyacrylamide gel electrophoresis under nonreducing conditions (a single Coomassie Blue stainable band with an M_r of approximately 150000). The IgG concentration was determined by spectrophotometry at 280 nm by using an extinction coefficient, $E^{1\%,1cm}_{280nm}$ of 14. The yield of IgG anti-HPA52 was 3.0 mg. This represented 51% of the IgG originally present in the culture fluid as evaluated by immunodiffusion against rabbit IgG anti-mouse IgG.

Purification of Plasminogen Activator. For purification of plasminogen activator from conditioned culture fluid, monoclonal mouse IgG_1 anti-HPA52 was coupled to Sepharose by incubation of 1.2 mg of the purified antibody preparation with 2 mL (swelled volume) of cyanogen bromide activated Sepharose in 4 mL of 0.25 M sodium bicarbonate, pH 8.5, and 0.5 M NaCl (coupling buffer) for 16 h at 4 °C. By this procedure, 86% of the protein was bound to the Sepharose. The column was treated with 1 M ethanolamine and washed extensively, alternately with coupling buffer and with 0.1 M sodium acetate, pH 4.0, and 0.5 M NaCl. A 2-mL column (16 × 10 mm) was equilibrated with 0.1 M Tris-HCl, pH 7.6, and 0.1% Triton X-100, and culture fluid from glioblastoma cells was applied at 30 mL/h at 4 °C. This temperature was maintained throughout the purification. The column was washed with 10 mL of 0.1 M Tris-HCl, pH 7.6, and 0.1% Triton followed by 40 mL of the same buffer supplemented with 1 M sodium chloride and 1 mM $ZnCl_2$ and then eluted with 24 mL of 0.1 M glycine hydrochloride, pH 2.5, 0.5 M NaCl, 1 mM $ZnCl_2$, and 0.1% Triton. The rate for washing and elution was 12 mL/h. Elution of the column was performed by reverse flow, the flow direction being changed after the application of the first 5 mL of washing buffer. The eluate was collected in 1-mL fractions which were assayed enzymatically for plasminogen activator. Enzyme-containing fractions were pooled and adjusted to pH 6.8 by addition of a double volume of 0.1 M Tris-HCl, pH 7.8, 0.1% Triton X-100, and 1 mM $ZnCl_2$, and a second purification procedure identical with the first was performed, except that the rate during elution was 2 mL/h. The M_r 52000 plasminogen activator from urokinase was purified by chromatography on anti-HPA52 IgG_1–Sepharose to homogeneity (evaluated by $NaDodSO_4$–polyacrylamide gel electrophoresis) as described previously (Kaltoft et al., 1982).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis with $NaDodSO_4$ was performed in a stacking system of slab gels as described previously (Danø & Reich, 1978), using a gradient of 6–16% polyacrylamide and a potential difference of 60 V for 16–18 h. Gels were stained with 0.1% Coomassie Blue. In each gel, the following mixture of marker proteins was electrophoresed: human plasminogen (M_r 92000), bovine serum albumin (M_r 67000), ovalbumin (M_r 45000), trypsinogen (M_r 24000), β-lactoglobulin (M_r 18400), and lysozyme (M_r 14300). Enzymatic activity of plasminogen activators in $NaDodSO_4$–polyacrylamide gels was detected by layering the gels over an agarose gel containing plasminogen and fibrin (Granelli-Piperno & Reich, 1978; Danø et al., 1980a). Plasminogen activators diffused into the agarose gel and activated plasminogen to plasmin which caused visible fibrinolysis zones. Labeling of proteins with [^3H]DFP and detection of the labeled proteins in gels by fluorography were performed as described previously (Danø & Reich, 1978).

Miscellaneous Procedures. Protein concentrations were estimated by spectrophotometry at 280 nm (immunoglobulins) or by the method of Lowry et al. (1951). When Triton X-100 was present, $NaDodSO_4$ was added during the latter procedure to prevent precipitation (Danø et al., 1980a). Plasminogen activator was extracted as described previously (Unkeless et al., 1974a) from the glioblastoma cells with 0.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.1 (100 μL of buffer per 10^6 cells).

Results

Enhanced [^3H]DFP Labeling of Plasminogen Activator by Preincubation with Plasmin. We have previously reported how labeling with [^3H]DFP followed by $NaDodSO_4$–polyacrylamide gel electrophoresis and fluorography can be used to identify serine proteases in biological fluids (Danø & Reich,

Table I: Purification of Pro-HPA52 by Affinity Chromatography with Anti-HPA52 IgG_1-Sepharose [a]

fraction	volume (mL)	protein concn (mg)	activity (Ploug units) [b]	sp act. (Ploug units/mg)	yield (%)
culture fluid applied to anti-HPA52-Sepharose, column 1	6170	376	47509	126	100
run through, column 1	6170	ND [c]	5553	ND	12
0.1 M Tris-HCl, pH 7.6, wash, column 1	40	ND	50	ND	0.1
0.1 M Tris-HCl, pH 7.6, and 0.5 M NaCl wash, column 1	80	ND	1368	ND	3
0.1 M Gly·HCl, pH 2.5, and 0.5 M NaCl eluate from column 1, diluted and applied to column 2	104	0.887	22360	25209	47
run through, column 2	312	ND	56	ND	0.1
0.1 M Tris-HCl, pH 7.6, wash, column 2	10	ND	31	ND	0
0.1 M Tris-HCl, pH 7.6, and 0.5 M NaCl wash, column 2	20	ND	764	ND	2
0.1 M Gly·HCl, pH 2.5, and 0.5 M NaCl eluate, column 2	20.8	0.187	19584	104727	41

[a] See Materials and Methods for details of purification procedure. Four independent purification runs were performed with column 1, and the eluates from these runs were pooled and applied to column 2. Numbers referring to column 1 are added from the four runs. [b] Assayed with plasmin-supplemented plasminogen. [c] ND, not determined.

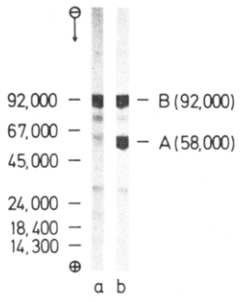

FIGURE 1: Fluorogram after $NaDodSO_4$–polyacrylamide gel electrophoresis of culture fluid from glioblastoma cells treated with [^{3}H]DFP (a) directly or (b) after preincubation with plasmin. Culture fluid (20 mL) containing 28 Ploug units of plasminogen activator was used for each experiment. The culture fluid was concentrated 10-fold, dialyzed against 0.1 M Tris–sulfate, pH 7.4, and incubated for 1 h at 20 °C with or without plasmin (0.1 μg/mg), followed by incubation with [^{3}H]DFP (10 μM, 6.5 Ci/mmol) for 16 h at 20 °C. The samples were electrophoresed in slab gels with 6–16% polyacrylamide, and fluorography was performed with an exposure time of 5 days. In control experiments in which the incubation with [^{3}H]DFP was preceded by incubation for 1 h with 100 μM *p*-nitrophenyl *p*-guanidinobenzoate, bands A and B were not visible (results not shown). The location of marker proteins and the estimated M_r values of the ^{3}H-labeled bands based on their electrophoretic mobilities are indicated.

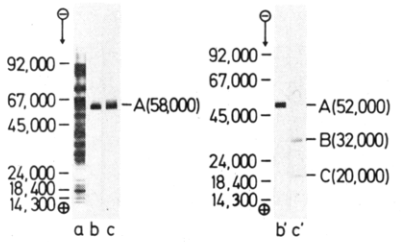

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis under (a–c) nonreducing and (b′ and c′) reducing conditions of (a) protein from 6 mL of crude conditioned culture fluid from glioblastoma cells, (b and b′) 6 μg of pro-HPA52, and (c and c′) 6 μg of HPA52. Pro-HPA52 (3.4 μg/mL) purified from serum-free conditioned culture fluid from glioblastoma cells was converted to HPA52 by incubation with plasmin (0.2 μg/mL) as described in Table I. Samples were electrophoresed, and the gel was stained with Coomassie Blue. Reduction was performed immediately before electrophoresis by addition of β-mercaptoethanol (5% final concentration) followed by heating to 100 °C for 2 min.

1978). During an attempt to use this approach on culture fluid from glioblastoma cells, it was observed that the [^{3}H]DFP labeling of a protein with an electrophoretic mobility corresponding to an approximate M_r of 58 000 (band A) was strongly enhanced by incubation of the culture fluid with small amounts of plasmin before the [^{3}H]DFP labeling (Figure 1). The [^{3}H]DFP labeling was inhibited by incubation with *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) prior to [^{3}H]DFP incubation. This identified the protein as a trypsin-like serine protease. An analysis for plasminogen activator activity by layering of a polyacrylamide gel over an agarose gel containing plasminogen and fibrin showed plasminogen activator activity with the same electrophoretic mobility as the [^{3}H]DFP-labeled band A (result not shown). Band B with an approximate M_r of 92 000 also represented a serine enzyme, as shown by competition with NPGB. This enzyme was not a plasminogen activator, and its labeling by [^{3}H]DFP was not enhanced by plasmin. It had an electrophoretic mobility similar to that of plasmin, but in contrast to findings with plasmin (L. S. Nielsen, unpublished experiments), bovine pancreatic trypsin inhibitor (BPTI) did not inhibit the incorporation of [^{3}H]DFP into the band B enzyme, and it is as yet unidentified.

In combination with a recent finding that oncogenic virus-transformed mouse cells release a plasminogen activator as an inactive proenzyme which can be activated by plasmin (Skriver et al., 1982), these observations indicated that also the human glioblastoma cells release plasminogen activator as a zymogen which tentatively was designated M_r 52 000 human plasminogen proactivator (pro-HPA52), because under reducing conditions it has an electrophoretic mobility corresponding to an M_r of approximately 52 000 (see Figure 2b′).

Purification of Pro-HPA52. We have previously found that the enzymatic activity of plasminogen activator in culture fluid from these glioblastoma cells is inhibited by a monoclonal IgG_1 antibody developed against human urokinase (anti-HPA52 IgG_1), that plasminogen activator in these cells can be immunohistochemically stained by anti-HPA52 IgG_1, and that plasminogen activator from these cells is absorbed to anti-HPA52 IgG_1–Sepharose (Kaltoft et al., 1982; Danø et al., 1982). Attempts were therefore made to purify the presumed proenzyme by affinity chromatography on this type of column. Previously, it was found that mouse plasminogen proactivator can be assayed in a modification of the ^{125}I-labeled fibrin plate assay in which plasminogen is supplemented with plasmin (Skriver et al., 1982). This assay method was therefore used to monitor the purification of pro-HPA52, and as shown in Table I, an 831-fold purification and a yield of 41% were obtained by two cycles of chromatography on anti-HPA52 IgG_1–Sepharose. Triton X-100 and Zn^{2+} were added to the buffers used for the purification because of previous experience with hydrophobic adsorption and proteolytic degradation, respectively, during purification of murine plasminogen activator (Danø et al., 1980a). Figure 2a,b shows stained $NaDodSO_4$–polyacrylamide gels after electrophoresis of crude conditioned culture fluid and the purified pro-HPA52 prep-

Table II: Effect of Preincubation with Plasmin on Plasminogen Activator Activity of Purified Pro-HPA52 [a]

preincubation			plasminogen activator assay	
pro-HPA52 (μg/mL)	plasmin (μg/mL)	BPTI (μg/mL)	plasminogen (μg/mL)	radioactivity released (cpm) [c]
1.4	0	0	2	644
1.4	0.06	0	2	7047
1.4	0.06 [b]	0	2	589
1.4	0.06	0.6	2	608
1.4	0.06	0.6 [b]	2	5635
1.4	0.06	0	0	68
0	0.06	0	2	21

[a] Pro-HPA52 purified as described in Table I was preincubated with plasmin as indicated for 4 h at 37 °C in 0.1 M Tris-HCl, pH 8.1, 0.1% Triton X-100, 40 mM Arg, and 0.8 mM Zn^{2+}. The samples were then diluted 40 000-fold and assayed in the radiolabeled fibrin plate assay by using plasminogen at the indicated concentrations. [b] Added at the end of the preincubation period. In all other cases, plasmin and BPTI were added at the start of the preincubation period. [c] Radioactivity released in parallel control assays without sample (<550 cpm) has been deducted.

aration, the latter having only one stained band.

Incubation of Pro-HPA52 with Plasmin. It was previously found that when murine plasminogen proactivator is assayed in the ^{125}I-labeled fibrin plate assay by using plasminogen that was not supplemented with plasmin, no plasminogen activator activity was found, while active murine plasminogen activator showed activity when assayed under these conditions (Skriver et al., 1982). In accordance with these findings, no radioactivity was released when pro-HPA52, purified as described in Table I, was assayed with plasminogen alone. As shown in Table II, however, when pro-HPA52 was incubated with catalytic amounts of plasmin before the assay, the plasminogen was activated, and radioactive fibrin was solubilized. Control experiments showed (Table II) that if the plasmin was added at the end of the preincubation period, it had no effect on the radioactivity which was subsequently released in the assay. The effect of plasmin was therefore due to a direct action of plasmin on the plasminogen activator preparation during the incubation period. Furthermore, the action of plasmin could be inhibited by addition of BPTI during the preincubation period, while BPTI added after the preincubation period showed no effect (Table II). These findings are consistent with pro-HPA52 being an inactive proenzyme which can be activated by plasmin.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis of Pro-HPA52 and HPA52. After electrophoresis of the pro-HPA52 preparation under reducing conditions, the gel showed one stained band. The electrophoretic mobility of this band corresponded to an M_r of approximately 52 000 (Figure 2b′) in contrast to the apparent M_r of approximately 58 000 under nonreducing conditions (Figure 2b). Even in strongly overloaded gels, there were no indications of a minor polypeptide chain being split from pro-HPA52 by the reduction, and the difference in the estimated M_r of the protein obtained under the two sets of conditions probably reflects a lesser effect of reduction on unfolding of pro-HPA52 than of the marker proteins (primarily ovalbumin and bovine serum albumin) used to estimate its M_r. Estimation of M_r by $NaDodSO_4$–polyacrylamide gel electrophoresis presupposes a complete unfolding of the peptide chains of the test protein as well as of the marker proteins (Weber & Osborn, 1975). Therefore, the estimate of the M_r of pro-HPA52 as being 52 000 as obtained under reducing conditions appears to be the most reliable.

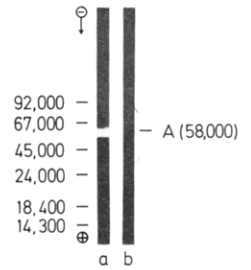

FIGURE 3: Detection of plasminogen activator activity in pro-HPA52 after $NaDodSO_4$–polyacrylamide gel electrophoresis. A sample containing 2 ng of purified pro-HPA52 was electrophoresed in a 6–16% polyacrylamide gel, and the gel was sandwiched between two agarose gels, one (a) containing fibrin and plasminogen (plasmin supplemented) and the other (b) containing fibrin alone. After incubation for 4 h, lysis in the agarose gels was visualized by staining with amido black.

After conversion of pro-HPA52 to HPA52 by incubation with plasmin, electrophoresis under nonreducing conditions still showed one stainable band with an electrophoretic mobility indistinguishable from that of pro-HPA52 (Figure 2c). However, when HPA52 was electrophoresed under reducing conditions, two stainable bands (B and C) with approximate M_r values of 32 000 and 20 000, respectively, were obtaind (Figure 2c′). The electrophoretic mobility of bands A–C was indistinguishable from that of the bands obtained when the higher molecular weight form of plasminogen activator in urokinase after affinity purification with monoclonal antibodies (Kaltoft et al., 1982) was electrophoresed in parallel.

For evaluation of the relationship of the stained protein band A in Figure 2b and the enzymatic activity of the purified pro-HPA52 preparation as assayed with plasmin-supplemented plasminogen, a small amount of pro-HPA52 was electrophoresed under conditions identical with those used for the experiment described in Figure 2. The polyacrylamide gel was washed with Triton X-100 and layered between two agarose gels, one containing plasminogen (plasmin supplemented) and fibrin and the other containing fibrin alone. As shown in Figure 3, a lysis zone was observed in the plasminogen-containing agarose gel, while no lysis was observed in the plasminogen-free agarose gel. This shows the presence of a plasminogen-activating enzyme. Its electrophoretic mobility was exactly like that of the stained band A in Figure 2b, indicating identity of the stained protein and the plasminogen activator. Taken together, the findings in Table II and Figures 2 and 3 show that pro-HPA52 consists of one peptide chain with an approximate M_r of 52 000 and that this molecule can be converted by plasmin to HPA52, which consists of two polypeptide chains with M_r values of approximately 32 000 and 20 000, held together by disulfide bridges.

Effect of DFP on Pro-HPA52 and HPA52. For a further evaluaton of pro-HPA52 as an inactive proenzyme, the two preparations were incubated with 10 μM DFP and, after a 1000-fold dilution, assayed for plasminogen activator in the ^{125}I-labeled fibrin plate assay using plasmin-supplemented plasminogen. Table III shows that under these conditions, DFP nearly completely inhibited the HPA52 preparation, while no inhibition of the pro-HPA52 preparation was observed. These findings indicate that the potentially active site in pro-HPA52 is inaccessible to DFP, in contrast to the active site in HPA52. This interpretation was further substantiated by the finding that [^{3}H]DFP was incorporated into HPA52 but not into pro-HPA52 as detected by $NaDodSO_4$–polyacrylamide gel electrophoresis followed by fluorography. After electrophoresis of [^{3}H]DFP-labeled pro-HPA52 under reducing conditions, radioactivity was detected in the M_r 32 000

Table III: Effect of Incubation with DFP on Enzymatic Activity of Pro-HPA52 and HPA52 As Measured in the ^{125}I-Labeled Fibrin Plate Assay with Plasmin-Supplemented Plasminogen [a]

sample	[DFP] during preincubation (μM)	radioactivity released in assay (cpm) [b]
pro-HPA52	10	6731
pro-HPA52	0	6662
HPA52	10	369
HPA52	0	12562

[a] Pro-HPA52 and HPA52 were prepared as described in the legend to Figure 2, and samples containing 1.7 Ploug units (pro-HPA52) or 3.2 Ploug units (HPA52) in 150 μL of assay buffer were incubated for 16 h at 20 °C with 10 μM DFP (or, as a control, without DFP). After a 1000-fold dilution, the samples were assayed for plasminogen activator activity by the ^{125}I-labeled fibrin plate method, using plasmin-supplemented (13.2 ng/mL final concentration in assay) plasminogen. [b] Radioactivity released in parallel control assays without sample (1682 cpm) has been deducted.

polypeptide, but not in the M_r 20000 polypeptide, indicating that the active site of the enzyme is located on the former chain (results not shown).

Intracellular Pro-HPA52. When Triton X-100 extracts of the glioblastoma cells were assayed with plasmin-supplemented plasminogen, they contained 5.2 Ploug units of plasminogen activator per mL, while an assay with plasminogen, but without plasmin, showed activity corresponding to only 0.1 Ploug unit/mL, indicating that the intracellular form of the enzyme is pro-HPA52. These results were obtained with cells grown in serum-supplemented medium. Similar results were obtained with serum-starved cells.

Discussion

The present study shows that the human glioblastoma cells release a plasminogen activator with an approximate M_r of 52000 in an inactive zymogen form. This finding is in good agreement with recent studies from this laboratory showing that virus-transformed murine cells release a plasminogen activator with an approximate M_r of 48000 in a zymogen form (Skriver et al., 1982). The existence of a zymogen to a human plasminogen activator of the urokinase type has previously been proposed by Bernik et al. (1974, 1981) and Nolan et al. (1977), who found that the plasminogen-activating activity in culture fluid from human cells was enhanced by incubation with trypsin. In those studies, purified enzyme was not available in sufficient amounts to prove the existence of a proactivator. The present studies do, however, support the hypothesis that the effect of trypsin in those studies was due to activation of a proenzyme to plasminogen activator.

As evaluated by $NaDodSO_4$–polyacrylamide gel electrophoresis, pro-HPA52 was purified to homogeneity by affinity chromatography on Sepharose columns with monoclonal antibodies developed against an approximately M_r 52000 enzyme (Kaltoft et al., 1982), which is the highest molecular weight form of the plasminogen activators present in commercial urokinase. This enzyme is by itself an active plasminogen activator, as evidenced, for example, by its ability to incorporate [^{3}H]DFP (Danø & Reich, 1978). The two-chain structure of HPA52 closely resembles that of the M_r 52000 enzyme in urokinase (Soberano et al., 1976). Together with the immunological cross-reaction between the urokinase enzyme and HPA52 and the identical electrophoretic mobility of the enzymes and their two polypeptide chains, this finding is consistent with HPA52 and the higher molecular weight form of urokinase being identical. A final clarification of this question, however, awaits studies on amino acid sequences.

The finding that plasminogen activators exist as proenzymes demonstrates an additional step in the cascadelike reaction involved in plasmin-mediated extracellular proteolysis. This step may have important regulatory functions, and it should be noted that initiation of plasminogen activation necessarily requires other molecules than plasminogen proactivators and plasminogen. As yet unknown initiating factors may therefore be crucial for the biological mobilization of plasminogen.

The present study also illustrates important methodological pitfalls in the detection of plasminogen activators in biological samples by the radiolabeled fibrin plate assay or other enzymatic methods. When the activators occur in the proenzyme form, their detection will be dependent on small amounts of plasmin (or other activating agents) being present during the assay, while, on the other hand, small amounts of contaminating inhibitors of plasmin (or of other activating agents) can prevent detection of the proenzymes.

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Glucose-6-phosphate Dehydrogenase from *Leuconostoc mesenteroides*. Isolation and Sequence of a Peptide Containing an Essential Lysine†

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ABSTRACT: Interaction of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* with pyridoxal 5′-phosphate and sodium borohydride leads to inactivation and modification of two lysine residues per enzyme dimer that are thought to bind glucose 6-phosphate [Milhausen, M., & Levy, H. R. (1975) *Eur. J. Biochem. 50*, 453–461]. The amino acid sequence surrounding this lysine residue is reported. Following tryptic hydrolysis of the modified enzyme, two peptides, each containing one pyridoxyllysine residue, were purified to homogeneity and subjected to automated Edman degradation. The sequences revealed that one of these, a heptapeptide, was derived from the other, containing 11 amino acids. Supporting evidence for the role of the modified lysine is provided in the following paper [Haghighi, B., & Levy, H. R. (1982) *Biochemistry* (second paper of three in this issue)]. End-group analysis of the native enzyme revealed that valine is the N-terminal and glycine the C-terminal amino acid and provides support for the identity of the enzyme's two subunits.

Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* was first isolated by DeMoss et al. (1953) and purified to homogeneity by Olive & Levy (1967). Its molecular weight is 103 700 (Olive & Levy, 1971), and the subunit molecular weight is 54 800 (Ishaque et al., 1974; Kawaguchi & Bloch, 1974). Kinetic studies suggested that a lysine residue in this enzyme may function in glucose 6-phosphate binding (Olive et al., 1971). Detailed studies on the interaction between the enzyme and pyridoxal 5′-phosphate (PLP)[1] showed that inhibition by PLP was competitive with respect to glucose 6-phosphate; in the presence of sodium borohydride this inhibition led to inactivation; N^6-pyridoxyllysine was identified after acid hydrolysis of the modified enzyme, and of the 74 lysine residues/mol of dimeric enzyme (Ishaque et al., 1974), only two were modified in the totally inactive enzyme (Milhausen & Levy, 1975). It was concluded from these studies that one unique lysine residue was pyridoxylated per subunit of *L. mesenteroides* glucose-6-phosphate dehydrogenase and that this lysine was probably involved in glucose 6-phosphate binding (Milhausen & Levy, 1975).

We now report the amino acid sequence surrounding this lysine residue. Two tryptic peptides, DIIA and DIIB, containing 7 and 11 amino acid residues, respectively, were isolated from pyridoxylated *L. mesenteroides* glucose-6-phosphate dehydrogenase and purified to homogeneity. Both DIIA and DIIB contain 1 mol of pyridoxyllysine/mol of peptide, and their amino acid sequence revealed that DIIA was derived from DIIB. This is the first report of the sequence of a peptide

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; PTH, phenylthiohydantoin; dansyl or Dns, 5-(dimethylamino)naphthalene-1-sulfonyl; Tris, tris(hydroxymethyl)aminomethane. For designation of flourescence excitation and emission wavelengths, see Haghighi & Levy (1982).

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