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Monoclonal antibody to human 66 000 molecular weight plasminogen activator from melanoma cells. Specific enzyme inhibition and one-step affinity purification

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Hybridomas producing a monoclonal IgG_1 antibody to a human plasminogen-activating enzyme with an apparent mol. wt. of 66 000 (66 K, HPA66) from human melanoma cells were obtained by fusion of NSI-Ag 4/1 mouse myeloma cells with spleen cells from a mouse immunized with a partially purified preparation of the enzyme. Screening for clones of hybridomas producing antibodies to HPA66 was performed with the impure enzyme preparation. A preliminary screening included enzyme-linked immunosorbent assay and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting; the final identification was based on inhibition of the enzymatic activity of HPA66 which was complete at high antibody concentrations. No inhibition of three other human and murine plasminogen activators or of plasmin was observed. Employing a one-step affinity procedure with the antibody coupled to Sepharose, HPA66 was purified ~200-fold from conditioned medium from the melanoma cells with a yield of 79%. The purified HPA66 was homogeneous as evaluated by SDS-PAGE. Electrophoresis under reducing conditions indicated that it consisted of one polypeptide chain. The binding constant between the antibody and ^{125}I-labelled HPA66 was $\sim 2.5 \times 10^9$ l/mol. The antibody did not bind to a variety of other plasminogen activators, including 52-K and 36-K human enzymes and 48-K and 75-K murine enzymes. Previously, a monoclonal antibody against another enzyme was derived by the sole use of enzyme inhibition for screening. The present study represents a modification of this procedure that can be used when antibody-unrelated inhibitors of the enzyme are present in hybridoma culture fluid.

Key words: plasminogen activator/monoclonal antibody/melanoma cells/protease/enzyme inhibition

Introduction

Conversion of the nearly ubiquitous extracellular zymogen plasminogen into the active protease plasmin is catalyzed by plasminogen-activating enzymes (for reviews, see Astrup, 1975; Christman *et al.*, 1977; Reich, 1978). At present, mammalian plasminogen-activating enzymes seem to fall into two distinct types differing in mol. wt., immunological reactivity and possibly in biological function (Aoki and Kaulla, 1971; Unkeless *et al.*, 1974b; Rifkin *et al.*, 1974; Christman *et al.*, 1975; Granelli-Piperno and Reich, 1978; Danø and Reich, 1978; Åstedt, 1979; Vetterlein *et al.*, 1979; Danø *et al.*, 1980b; Roblin and Young, 1980; Rijken *et al.*, 1980; Wilson *et al.*, 1980; Matsuo *et al.*, 1981; Rijken and Collen, 1981; Kaltoft *et al.*, 1982). One type with a mol. wt. of ~50 K (the urokinase type) seems primarily involved in tissue degradation and remodelling in normal physiological processes (Beers *et al.*, 1975; Strickland *et al.*, 1976; Reich, 1978; Ossowski *et al.*, 1979), as well as in connection with the invasion of cancer cells into other tissues (see Reich, 1978). The other type of plasminogen-activating enzymes with a mol. wt. of ~70 K (originally designated as tissue activators) is supposed to be involved in thrombolysis (Matsuo *et al.*, 1981; Mattsson *et al.*, 1981). A final clarification of the functions of the two types of plasminogen activators and their mutual relationship, however, will require further comparative biochemical and histochemical studies.

We have previously reported the derivation by the hybridoma method (Köhler and Milstein, 1975) of a monoclonal antibody to a human plasminogen activator of the ~50 K mol. wt. type (Kaltoft *et al.*, 1982). This antibody proved valuable for specific enzyme inhibition (Kaltoft *et al.*, 1982), for purification of the enzyme and the corresponding proenzyme (Kaltoft *et al.*, 1982; Nielsen *et al.*, 1982), and for immunocytochemical studies (Danø *et al.*, 1982). With the aim of performing parallel studies of the ~70 K mol. wt. plasminogen activators, we have now also developed a monoclonal antibody against an enzyme of this type (HPA66) derived from cultured human melanoma cells.

Results

Derivation and characterization of anti-HPA66

The Bowes melanoma cell line produces plasminogen activator with a mol. wt. of 60–70 K (Rifkin *et al.*, 1974; Danø and Reich, 1978; Rijken and Collen, 1981). Analysis of conditioned culture fluid from the subline of these cells used in the present study indicated that it only contained one molecular form of plasminogen activator, having a mol. wt. of 66 K (see Figure 2). This enzyme was partially purified by affinity chromatography with concanavalin A-Sepharose and zinc-chelate-Sepharose, as described by Rijken and Collen (1981). In the resulting preparation, a protein with an electrophoretic mobility indistinguishable from HPA66 constituted ~25%, as evaluated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This preparation was used for immunization of a BALB/c mouse.

The previously reported derivation of a monoclonal antibody against a 52 K mol. wt. human plasminogen activator was based on a screening procedure that only included inhibition of the enzyme (Kaltoft *et al.*, 1982). Mouse serum and conditioned medium from hybridoma cells contain, however, inhibitors of the assay of HPA66 that are not related to antibody production and are present in amounts that complicate the use of a similar procedure for this enzyme. Therefore, a preliminary screening was performed with enzyme-linked immunosorbent assay (ELISA) and SDS-PAGE followed by immunoblotting.

Serum from the immunized mouse showed, as measured by ELISA, a higher titer of antibodies binding to the proteins of the immunization preparation than preimmunization serum. SDS-PAGE followed by immunoblotting showed the

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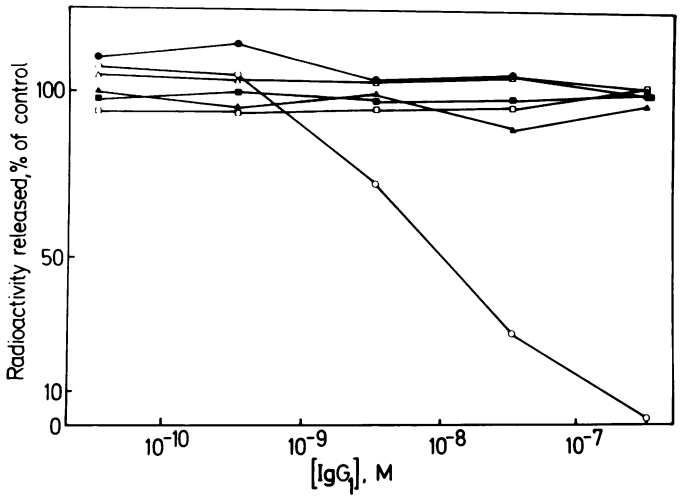

Fig. 1. Inhibition of HPA66 by anti-HPA66 IgG_1. Release of radioactivity from [^{125}I]fibrin was measured after 1 h incubation at 37°C. The assay wells contained in 0.55 ml of buffer, pH 8.1: 1 μg of human plasminogen, monoclonal anti-HPA66 IgG_1, as indicated, and one of the following plasminogen activator preparations: conditioned medium from melanoma cells containing 0.0065 Ploug units HPA66 (○); 0.009 Ploug units of purified 52 K human plasminogen activator (●); 0.008 Ploug units of purified 36 K human plasminogen activator (△); 0.006 Ploug units of purified 48 K murine plasminogen activator (▲); monoclonal IgG_1 anti-trinitrophenyl was substituted for anti-HPA66 in assays with 0.0065 Ploug units HPA66 (□); finally, plasminogen was replaced by 80 ng of human plasmin and no plasminogen activator was added (■). Each point represents the mean of two determinations. In the control assays, 4000 – 12 000 c.p.m. per well were released, which corresponds to 5 – 15% of the total radioactivity in each well.

presence of antibodies binding to several proteins in the immunization preparation, among these a protein with an electrophoretic mobility indistinguishable from that of HPA66.

After hybridization of spleen cells from the mouse with myeloma cells (NSI-Ag 4/1), the mixture was distributed into 48 wells. Growth of hybridoma cells was observed in all wells, and culture fluid from 16 of the wells gave a positive reaction in ELISA with the protein preparation used for immunization. Cells from three strongly ELISA-positive wells were transferred, cloned and recloned, resulting in three stable ELISA-positive clones. One of these clones produced antibody which reacted only with a 66-K protein in the immunization preparation, as demonstrated by the immunoblotting technique. By immunodiffusion, the antibody was found to be of the IgG_1 subclass. It was purified to homogeneity, as evaluated by SDS-PAGE, and was then found to inhibit completely the enzymatic activity of HPA66 present in conditioned medium from the melanoma cells (see Figure 1), thus confirming that the antibody reacted with this enzyme. This clone was selected for the subsequent studies and designated anti-HPA66-1.

Inhibition of enzymatic activity of HPA66 by anti-HPA66

The inhibition of the enzymatic activity of HPA66 was assayed in a radiolabelled fibrin plate assay, which involves the activation of plasminogen and the subsequent degradation of [^{125}I]fibrin by the plasmin formed. 50% inhibition was obtained at $\sim 10^{-8}$ M of anti-HPA66 IgG_1, and complete inhibition at $\sim 3 \times 10^{-7}$ M (Figure 1).

To test the specificity of the inhibition of HPA66, similar assays were performed with human 52 K mol. wt. plasminogen activator and its 36 K degradation product,

Table I. Purification of HPA66 from human melanoma cells by monoclonal antibody coupled to Sepharose

Fraction	Volume ml	Protein mg	Total activity (Ploug units[a])	Specific activity (Ploug units/mg[a])	Yield %
Conditioned culture fluid applied to column	1340	170	81 000	465	–
Run-through	1340	ND[b]	3400	ND[b]	4
Wash	40	ND[b]	45	ND[b]	0
Eluate	10	0.65	64 000	98 000	79

Conditioned medium from human melanoma cells was applied at a flow rate of 60 ml/h to a Sepharose column (1.5 x 2.3 cm) coupled with 4.2 mg monoclonal anti-HPA66 IgG_1. The column was washed with 40 ml of 0.1 M Tris HCl, pH 8.1, 0.1% Triton X-100 and 1 M NaCl. Elution was done at a flow rate of 12 ml/h with 0.1 M glycine HCl, pH 2.5, 0.1% Triton X-100, 0.5 M NaCl. Fractions of 1 ml were collected in tubes containing 100 μl of 1 M Tris HCl, pH 9.0. The temperature was 4°C throughout the procedure.

[a]Due to the lack of a suitable standard preparation of HPA66, the indicated specific activities are in arbitrary units, based on the use of a urokinase preparation as a standard in the ^{125}I-labelled fibrin plate assay. Since the activity of HPA66 is greatly stimulated by fibrin, while that of urokinase is not (Wallén, 1978), the indicated specific activities are not directly comparable to those found in assay systems without fibrin.

[b]Not determined.

and with murine 48 K plasminogen activator. None of these enzymes were inhibited by anti-HPA66 IgG_1 in concentrations up to 3×10^{-7} (Figure 1), indicating that these enzymes were at least 100-fold less sensitive than HPA66 to the inhibitory effect of the antibody. The latter findings also indicate that HPA66 itself, and not plasmin, was the target for the effect of the antibody in the HPA66 assay. This conclusion was confirmed by the direct finding that anti-HPA66 IgG_1 did not inhibit preformed plasmin (Figure 1).

Affinity purification of HPA66 with anti-HPA66

The monoclonal anti-HPA66 IgG_1 was coupled to Sepharose and used for affinity purification of HPA 66 from conditioned culture fluid from the melanoma cells by a column procedure (Table I). 96% of the enzymatic activity was bound to the column at neutral pH. After extensive washing, 79% of the applied activity was eluted at pH 2.5. The eluate contained pure HPA66, as evaluated by SDS-PAGE. Under non-reducing as well as reducing conditions, one Coomassie blue stainable band was seen (Figure 2), indicating that the molecule consists of one polypeptide chain. Under non-reducing conditions, the electrophoretic mobility of the stained bands was indistinguishable from that of the enzymatic plasminogen activator activity, as determined by zymography (Figure 3). The apparent mol. wt., as based on the electrophoretic mobility relative to the marker proteins, was 66 K. The purification factor in this one-step purification procedure was ~200.

Binding between HPA66 and anti-HPA66

Purified HPA66 was labelled with ^{125}I. Fixed concentrations of the labelled enzyme and anti-HPA66 IgG_1 were incubated with varying concentrations ($1.4 \times 10^{-10} - 1.4 \times 10^{-7}$ M) of unlabelled HPA66, followed by precipitation with a second antibody. Based on a Scatchard plot, the apparent binding constant was found to be 2.5×10^9 l/mol (five concentrations; correlation coefficient – 0.93).

The specificity of the binding of anti-HPA66 IgG_1 to

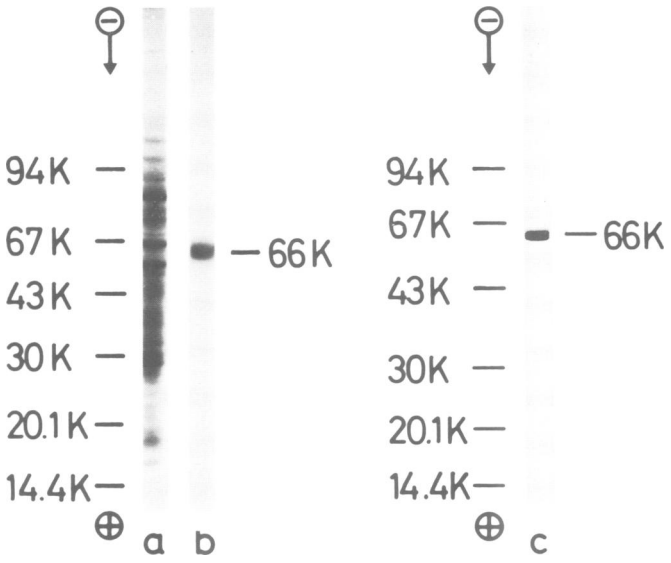

Fig. 2. SDS-PAGE of preparations of HPA66 before and after purification. **Lane (a)**, proteins from 4 ml of crude culture fluid from melanoma cells. **Lanes (b)** and **(c)**, each 13 μg of purified HPA66 electrophoresed under **(b)** non-reducing and **(c)** reducing conditions. Reduction was performed immediately before electrophoresis by addition of 2-mercaptoethanol (5% final concentration) followed by heating to 100°C for 2 min. The mobilities of marker proteins and the apparent mol. wt. of HPA66 as evaluated by its relative electrophoretic mobility are indicated. The marker proteins were 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). It should be noted that SDS-PAGE only allows an estimate of the mol. wt. of proteins (Weber and Osborn, 1975). The mol. wt. of 66 K estimated in this experiment is not significantly different from that of 72 K estimated by Rijken and Collen (1981) for purified Bowes melanoma cell plasminogen activator. In that study, the electrophoretic mobility of the enzyme under reducing conditions was slightly higher than that of albumin, in agreement with the results in **lane c**.

HPA66 in relation to other plasminogen activators was investigated by zymography of plasminogen activator samples before and after passage through a column of anti-HPA66 IgG_1-Sepharose. Under these conditions, anti-HPA66 IgG_1 bound HPA66, but not the human 52 K and 36 K plasminogen activators. Nor did the antibody bind two murine plasminogen activators with mol. wts. of 48 K and 75 K (Figure 3).

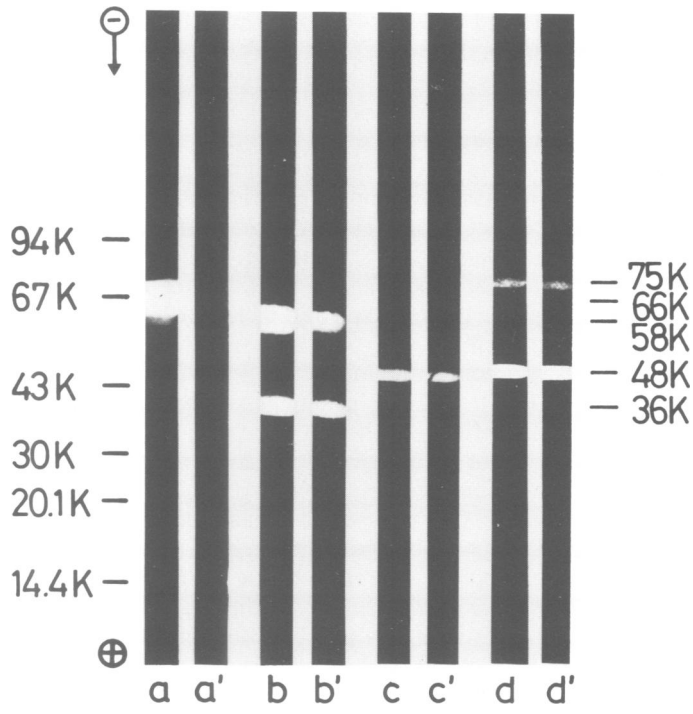

Fig. 3. Binding of various types of plasminogen activators to Sepharose columns coupled with anti-HPA66 IgG_1. Before **(lanes a, b, c and d)** and after **(lanes a', b', c' and d')** passage of columns, samples were electrophoresed in a slab SDS-polyacrylamide gel and the gel was layered over an agarose gel containing fibrin and plasminogen. Plasminogen activators diffused into the agarose gel, here activating plasminogen to plasmin and causing visible lysis zones. The following plasminogen activator preparations were passed through the columns: **a'**, 2 ml of culture medium from human melanoma cells containing 120 Ploug units of plasminogen activator; **b'**, 150 Ploug units of urokinase in 2 ml of 0.1 M Tris HCl, pH 8.1; **c'**, 2 ml of culture fluid from murine sarcoma virus-transformed murine 3T3-cells containing 14 Ploug units of plasminogen activator: **d'**, 1.5 ml of murine lung homogenate containing 2.0 Ploug units of plasminogen activator. In **lanes a,a'**: 2 μl; in **b,b'**: 1 μl; in **c,c'**: 7 μl; in **d,d'**: 50 μl of the respective samples were electrophoresed. Control experiments in which plasminogen was omitted from the agarose gel showed no lysis zones. The mol. wt. of marker proteins and the approximate mol. wt. of the plasminogen activators as evaluated by their electrophoretic mobility are indicated. Under the non-reducing conditions used in this experiment, the 52 K human plasminogen activator has an electrophoretic mobility corresponding to 58 K. 0.5 ml Sepharose columns (9 x 8 mm) coupled with 120 μg anti-HPA66 IgG_1 were used throughout. The columns were developed by gravity at 4°C.

Discussion

This study illustrates the derivation of a monoclonal antibody against an enzyme which was only available in an impure preparation, by the use of an initial screening with ELISA and SDS-PAGE/immunoblotting, and a final selection based on enzyme inhibition. Previously, a monoclonal antibody against another enzyme was derived by the sole use of enzyme inhibition for screening (Kaltoft *et al.*, 1982). The present study represents a modification of this principle that can be used when antibody-unrelated inhibitors of the enzyme are present in serum and/or hybridoma culture fluid.

The antibody did not bind and/or inhibit either other human and murine plasminogen activators or human plasmin. In addition, the finding that HPA66 could be purified to homogeneity from culture fluid from melanoma cells by affinity chromatography with the antibody coupled to Sepharose, indicates that the antibody did not react with any other protein present in detectable amounts in this culture fluid.

This high degree of specificity is analogous to that of a monoclonal antibody to the 52 K mol. wt. human plasminogen activator (Kaltoft *et al.*, 1982; Nielsen *et al.*, 1982; Danø *et al.*, 1982), and implies that the anti-HPA66 IgG_1 can be used to distinguish between HPA66 and other types of plasminogen activator in enzymatic assays of biological fluids. The specificity is also crucial for the possible use of the antibody for immunoassays of HPA66 and immunohistochemical studies of the enzyme.

The existence of a monoclonal antibody that inhibits the enzymatic activity of HPA66 is in good agreement with the described inhibition of the 52 K mol. wt. human plasminogen activator by a monoclonal antibody (Kaltoft *et al.*, 1982). Furthermore, two additional inhibitory monoclonal antibodies to the 52 K activator have now been derived (unpublished observation). It should, however, be noted that all these monoclonal antibodies were selected for inhibition. The use of alternative screening methods (e.g., ELISA with pure enzyme) might lead to identification of monoclonal an-

tibodies that bind to, but do not inhibit, the enzyme. Monoclonal antibodies with this property have been reported in at least one enzyme system (Frackelton and Rotman, 1980).

The one-step purification of HPA66 described in the present study compares very favorably with previously published procedures for partial or complete purification of this type of enzyme with respect to both simplicity and yield (Aoki, 1974; Cole and Bachmann, 1977; Radcliffe and Heinze, 1978; Wallén *et al.*, 1978; Binder *et al.*, 1979; Rijken *et al.*, 1979; Allen and Pepper, 1981; Rijken and Collen, 1981; Wallén *et al.*, 1981). Furthermore, it should be noted that the one-step purification procedure minimizes the possibility of enzyme degradation. For the 52 K plasminogen activator, a similar gentle affinity-purification with a monoclonal antibody led to the identification of an inactive one-chain proenzyme that is converted by limited proteolysis with plasmin to an active form consisting of two polypeptide chains held together by one or more disulphide bridges (Nielsen *et al.*, 1982). Similar findings were reported for the murine 48 K plasminogen activator (Skriver *et al.*, 1982). HPA66 has also been described as existing both in a one- and a two-chain form (Rijken and Collen, 1981; Wallén *et al.*, 1981), and, in agreement with a previous report on a porcine plasminogen activator with a similar mol. wt. (Wallén *et al.*, 1981), the one-chain form of HPA66 as purified in the present study can be converted to a two-chain form by catalytic amounts of plasmin (unpublished results). In contrast to the findings for the one-chain form of the 52 K plasminogen activator, the one-chain form of HPA66 purified in the present study is active as assayed in the radiolabelled fibrin plate assay. It remains, however, to be investigated whether this is because the molecule is active in itself, or because it is activated by factors present during the assay.

Materials and methods

Materials

The following materials were obtained from the indicated sources: protein A-Sepharose, concanavalin A-Sepharose and cyanogen-bromide-activated Sepharose (Pharmacia, Uppsala, Sweden); Freund's incomplete adjuvant (Statens Seruminstitut, Copenhagen, Denmark); rabbit IgG anti-mouse immunoglobulins, either horseradish peroxidase-conjugated or not (Dako Immunoglobulins, Copenhagen, Denmark); hybridoma screening reagent (β-galactosidase conjugated to $F(ab')_2$-fragments of sheep IgG anti-mouse immunoglobulins) (Bethesda Research Laboratories, MD); Millipore nitrocellulose paper GSWP 000 10 (Millipore, Molsheim, France); urokinase (Leo Pharmaceutical, Ballerup, Denmark); Costar trays (Costar, MD); Immuno Plates (Nunc, Roskilde, Denmark); Ouchterlony trays (Meloy, VA). All other materials were those described previously (Danø *et al.*, 1980a, 1980b; Kaltoft *et al.*, 1982; Nielsen *et al.*, 1982), or of the best commercially-available grade.

Cell culture

The myeloma line NSI-Ag 4/1 (Köhler and Milstein, 1976) was a gift from Georges Köhler, Basel Institute for Immunology. These cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. A human melanoma cell line (Bowes) was a gift from D.B.Rifkin, New York University, NY. This line and a line of sarcoma virus transformed mouse 3T3-cells (Danø *et al.*, 1980a) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. Conditioned serum-free culture fluid was prepared from confluent monolayer cultures (Danø *et al.*, 1980a).

Partial purification of HPA66 for immunization and screening

HPA66 was partially purified from conditioned serum-free culture fluid, essentially as described by Rijken and Collen (1981), by the consecutive use of affinity chromatography with concanavalin A-Sepharose and zinc-chelate-Sepharose. The latter was prepared as described by Porath *et al.* (1975). All buffers contained 0.005% Triton X-100. The purity of the plasminogen activator in the eluate from the zinc-chelate-Sepharose column was ~25%, as judged from spectrophotometric scanning at 600 nm after SDS-PAGE followed by Coomassie blue staining. For immunization purposes, the preparation was dialysed against distilled water containing 0.005% Triton X-100, and lyophilized.

Immunization and hybridization

Immunization of a BALB/c mouse with HPA66 and hybridization of spleen cells with NSI-Ag 4/1 myeloma cells were performed essentially as described (Kaltoft *et al.*, 1982). Hybridomas were cloned and recloned by limiting dilution.

ELISA

ELISA was performed according to Engvall and Perlmann (1972). The partially purified HPA66 preparation [100 ng HPA66 in 50 μl 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl (PBS)] was incubated for 16 h in the wells of Immuno Plates. The wells were washed with PBS, incubated for 2 h with 50 μl mouse serum or hybridoma culture fluid in appropriate dilutions with PBS, and washed with PBS containing 1.5 mM magnesium chloride, 2 mM 2-mercaptoethanol, and 0.05% sodium azide. The wells were then incubated for 2 h with 50 μl β-galactosidase-conjugated $F(ab')_2$ fragments of sheep anti-mouse IgG in PBS, and reacted for 1 h with 50 μg *p*-nitrophenyl-β-D-galactoside dissolved in 50 μl 50 mM sodium phosphate, pH 7.2, 1.5 mM magnesium chloride, 100 mM 2-mercaptoethanol. The reaction was stopped by addition of 50 μl 0.5 M sodium carbonate.

Immunoblotting

After SDS-PAGE (see below) of the immunization preparation, the proteins were transferred electrophoretically (10 V, 250 mA for 16 h at room temperature) from the polyacrylamide gel to nitrocellulose paper (Burnette, 1981). The transfer buffer used was 0.125 M Tris HCl, 0.19 M glycine, 20% (v/v) methanol, 0.1% (w/v) SDS, pH 8.6.

The nitrocellulose paper was washed in 0.05 M Tris HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100 (TBS-Triton) for 15 min at room temperature and incubated for 30 min with TBS-Triton containing human serum albumin (10 mg/ml). The paper was then washed 2 x 15 min in TBS-Triton. Vertical lanes were cut out and incubated overnight at 4°C with culture supernatants from the hybridomas. The lanes were washed in TBS-Triton 3 x 15 min, incubated for 1 h at room temperature with peroxidase-conjugated rabbit IgG anti-mouse immunoglobulins (diluted 1:50 in TBS-Triton), and washed 3 x 10 min in 0.05 M Tris HCl, pH 7.6. The peroxidase reaction was then performed with 0.5 mg/ml of di-aminobenzidine in 0.01% H_2O_2 for 5 min at room temperature.

Plasminogen activator assay

Plasminogen activator activity was assayed using the ^{125}I-labelled fibrin plate method, as described (Danø *et al.*, 1980a). A standard preparation of urokinase was used to calibrate each assay.

Electrophoresis

SDS-PAGE was carried out under reducing or non-reducing conditions in slab gels with a 6–16% linear concentration gradient of polyacrylamide (Danø *et al.*, 1980a). Enzymatic activity of plasminogen activators in the gels was detected by diffusion into agarose gels containing fibrin and plasminogen (Granelli-Piperno and Reich, 1978; Danø *et al.*, 1980a). Isoelectric focussing was performed as described (Danø *et al.*, 1980a).

Characterization and purification of antibodies

Class and subclass of the antibody were determined by immunodiffusion using pre-prepared Ouchterlony trays. For purification of the monoclonal antibody, 200 ml of conditioned culture fluid from hybridomas was applied to a 5 ml protein A-Sepharose column (12 x 43 mm). The column was washed with 30 ml 0.1 M Tris HCl, pH 8.1. Elution was performed with 0.1 M sodium acetate, pH 4.0, 0.15 M NaCl. Fractions of 2 ml were collected in tubes containing 200 μl 1 M Tris HCl, pH 9.0. The yield of anti-HPA66 IgG_1 was >90%. The column was developed throughout at a flow rate of 12 ml/h at 4°C. The eluted IgG was pure, as evaluated by SDS-PAGE and isoelectric focussing. The IgG concentration in the purified preparation was determined by spectrophotometry at 280 nm ($A^{1\%}_{280}$ = 14). Concentrations of IgG in impure solutions were determined by single radial immunodiffusion using purified IgG as a standard.

Binding between [^{125}I]HPA66 and anti-HPA66

Completely purified HPA66 (Table I, Figure 2b,c) was labelled with ^{125}I by the iodogen method (Fraker and Speck, 1978). [^{125}I]HPA66 was separated from free ^{125}I by affinity chromatography with anti-HPA66 IgG_1-Sepharose. The specific activity of the labelled HPA66 was 4.4 Ci/nmol.

The ^{125}I-labelled HPA66 (2.5 pM) was incubated with purified anti-HPA66 IgG_1 (1.3 nM) and various concentrations of unlabelled HPA66, in 0.25 ml 0.1 M Tris HCl, pH 8.1, 0.1% Triton X-100 (assay buffer). An IgG_1 antibody of irrelevant specificity (anti-human 52 K mol. wt. plasminogen activator) was added to make the total IgG_1 concentration 67 nM during the incuba-

tions. After 4 h, rabbit IgG anti-mouse immunoglobulin was added (final dilution 1:40). The mixture was incubated overnight, the precipitate spun down, washed twice with assay buffer and the radioactivity determined in a gamma spectrometer.

Miscellaneous procedures

Monoclonal anti-HPA66 IgG_1 was coupled to cyanogen bromide-activated Sepharose 4B, as reported (Kaltoft *et al.*, 1982).

The 52 K human plasminogen activator was purified from urokinase by affinity chromatography with a monoclonal antibody to this enzyme, as described (Kaltoft *et al.*, 1982). The 36 K human plasminogen activator (a degradation product of the 52 K activator), was purified from urokinase using affinity chromatography with the consecutive use of two Sepharose columns coupled with different monoclonal antibodies: anti-HPA52-1 IgG_1, which binds the 52 K but not the 36 K activator, and anti-HPA52-2 IgG_1, which binds both the 52 K and the 36 K activators (unpublished observation). Run-through from the anti-HPA52-1 IgG_1 column was applied to the anti-HPA52-2 IgG_1 column. The 36 K activator was eluted from this column at pH 2.5.

Purified 48 K murine plasminogen activator, human plasminogen, human plasmin, monoclonal IgG_1 anti-trinitrophenyl, monoclonal IgG_1 anti-human 52 K plasminogen activator and Triton X-100 extract of mouse lung tissue were prepared as described (Unkeless *et al.*, 1974a; Danø and Reich, 1975; Danø *et al.*, 1980a, 1980b; Kaltoft *et al.*, 1982; Skriver *et al.*, 1982).

Protein concentrations were estimated by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. When Triton X-100 was present, this method was modified as described by Bonsall and Hunt (1971).

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