A Quantitative Assay for the Activation of Plasminogen by Transformed Cells in Situ and by Urokinase†

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ABSTRACT: An in situ quantitative assay for the plasminogen activator of transformed cells is described in which as few as 3500 transformed cells are incubated with purified plasminogen for various time intervals and the amount of plasmin formed is then quickly titrated with the new fluorogenic, active-site titrant 3′,6′-bis(4-guanidinobenzoyloxy)-5-[*N*′-(4-carboxyphenyl)thioureido]spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one. The kinetics of plasminogen activation are linear and obey the Michaelis–Menten rate equation. The $K_{m(app)}$ and V_{max} for the plasminogen activator of a rat neurotumor cell line with the two forms of dog plasminogen fractionated by lysine–Sepharose column chromatography are determined. The $K_{m(app)}$ for fraction I plasminogen is 6.6 μM and for fraction II plasminogen 3.4 μM. No secreted plasminogen activator is observed during the assay, indicating that under these conditions the plasminogen activator is cell associated. The assay is also used to show that lysine is a competitive inhibitor of transformed cell plasminogen activator. As a result of the ability to measure the plasminogen activator activity of cells quantitatively, the role of this enzyme in a variety of physiological processes can be more easily elucidated. The kinetics of activation of two forms of dog plasminogen by human urokinase, a soluble as opposed to cell-associated plasminogen activator, were also analyzed in a two-step assay. The rate of plasmin formation is linear and shown to obey the Michaelis–Menten rate equation. For the activation of fraction I plasminogen, the $K_{m(app)}$ is 31.7 μM and the k_{cat} is 1.98 s^{-1}; for the activation of fraction II plasminogen, the $K_{m(app)}$ is 19.2 μM and the K_{cat} is 1.86 s^{-1}. The assay conditions are flexible so that factors which influence the rate and extent of activation, e.g., fibrin and lysine, can now be characterized quantitatively. The apparent Michaelis constants for the transformed cell-associated plasminogen activator are 5-fold less than those for urokinase and are close to the in vivo concentration of plasminogen.

The enhanced proteolytic activity of tumors was originally observed by Fischer (1925) who found that chicken sarcomas growing in vitro could lyse fibrin clots whereas normal cell explants could not. Recently, this fibrinolytic activity of tumors was shown to be the consequence of a serum factor, plasminogen, and a cell factor, plasminogen activator (Ossowski et al., 1973; Unkeless et al., 1973, 1974; Quigley et al., 1974). Thus, in Fischer's experiments, the plasminogen activator of the sarcoma cells cleaved the plasminogen in the serum to plasmin, which then lysed the fibrin clots. Normal chicken cells exhibited little or no plasminogen activator activity. Since these observations, increased levels of plasminogen activator activity have been found to be characteristic not only of malignant cells growing in vitro (Unkeless et al., 1973; Goldberg, 1974; Christman & Acs, 1974; Rifkin et al., 1974; Pollack et al., 1975) but also of cells involved in tissue remodeling and migration (Ossowski et al., 1975; Vassalli et al., 1977), ovulation and trophoblast implantation (Beers et al., 1975; Strickland & Beers, 1976; Strickland et al., 1976), and differentiation (Sherman et al., 1976; Topp et al., 1976). Furthermore, plasminogen activator synthesis has been shown to be inducible by tumor promoters (Vassalli et al., 1977; Wigler & Weinstein, 1976), DNA damage (Miskin & Reich, 1980), and carcinogens (Hamilton et al., 1976) and to be modulated by the presence of physiological concentrations of hormones (Beers et al., 1975; Strickland & Beers, 1976; Granellipiperno et al., 1978; Vassalli et al., 1976; Lecroix et al., 1977).

A major problem in studying the cell-associated plasminogen activator has been the lack of an assay for plasmin that is both sensitive and quantitative. A sensitive assay is required because if high concentrations of plasmin are allowed to accrue before assaying, the plasmin will begin to degrade itself, plasminogen, and the cell surface. A sensitive assay also allows low levels of plasminogen activator activity to be detected. A quantitative assay is required so that the amount of plasminogen activator in different cell lines or in the same cell line under different physiological conditions can be meaningfully compared. The most widely used assays, the hydrolysis of casein (Goldberg, 1974) or fibrin (Jones et al., 1975a,b) and the solubilization of ^{125}I-labeled fibrin (Unkeless et al., 1973), are double rate assays. Both casein and fibrin overlays are used in plaque assays where clear lysis zones are formed around cells that have plasminogen activator activity. Although this type of assay is sensitive, it is not quantitative, because the relationship between the size of the lysis zone and the amount of plasminogen activator activity is not known. Many important experiments demonstrating increased levels of plasminogen activator activity in transformed cells have been performed by using the assay that measures the solubilization of ^{125}I-labeled fibrin by plasmin. This assay, however, is not ideal. The ^{125}I-labeled fibrin substrate is insoluble. Neither the nature and number of cleavages required of plasmin to release an ^{125}I-labeled fragment nor the exact dependence of the rate of the reaction upon the state of the fibrin is known. Large differences are observed in the amount of fibrin degradation products released when the same cell line is repeatedly assayed under apparently identical conditions because of isotope decay,

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changes in the clottability of fibrinogen upon storage, differences in sera, etc. Even the effective plasminogen concentration in such experiments is unknown because plasminogen binds to fibrin (Wiman & Wallen, 1977). The kinetics are nonlinear and complex, thereby precluding a quantitative analysis of the data.

Here we describe the development of a sensitive and quantitative, indirect assay for the plasminogen activator of transformed cells and for urokinase. We measure the product of activation, plasmin, by using the new fluorogenic, active-site titrant 3′,6′-bis(4-guanidinobenzoyloxy)-5-[*N*′-(4-carboxyphenyl)thioureido]spiro[isobenzofuran-1(3*H*),9′-[9*H*]-xanthen]-3-one (FDE, fluorescein diester),[1] as described in the preceding paper in this issue (Livingston et al., 1981). Conditions have been found in a two-step assay procedure in which the amount of plasmin formed is proportional to time and in which the rate of plasminogen activation obeys the Michaelis–Menten rate equation. The $K_{m(app)}$ and V_{max} of the cell-associated, as opposed to secreted, plasminogen activator of a rat neurotumor clonal subline with two forms of purified dog plasminogen are determined. Also, the inhibition of the transformed cell-associated plasminogen activator by lysine is quantitatively characterized. The $K_{m(app)}$ and k_{cat} of urokinase with the two forms of purified dog plasminogen are also determined.

Materials and Methods

Materials. The synthesis, purification, and characterization of FDE are described in the preceding paper in this issue (Livingston et al., 1981). Stock solutions of 1 mM were prepared in redistilled dimethylformamide. Further dilutions were made into phosphate-buffered saline before each experiment. Aprotinin or Trasylol was purchased from Mobay Chemical Corp. Urokinase was purchased from Leo Pharmaceuticals. Phosphate-buffered saline contained 0.137 M NaCl, 2.68 mM KCl, 8.00 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.91 mM $CaCl_2$, and 0.49 mM $MgCl_2$ at pH 7.2. *p*-Nitrophenyl *p*-guanidinbenzoate (NPGB) was purchased from ICN and dissolved in redistilled dimethylformamide at a concentration of 3 mM. Further dilutions were made in phosphate-buffered saline before each experiment. Lysine–Sepharose 4B and cyanogen bromide activated Sepharose 4B were purchased from Pharmacia. Diisopropyl fluorophosphate and benzamidine hydrochloride hydrate were purchased from Aldrich. ϵ-Aminocaproic acid was purchased from Calbiochem. All electrophoresis reagents were purchased from Bio-Rad. The discontinuous pH, $NaDodSO_4$–polyacrylamide gel system described by Laemmli was used (Laemmli, 1970). Densitometer tracings were obtained by using an Ortec densitometer.

Cell Culture. The RT4-71-D2 cell line was a kind gift from Dr. N. Sueoka of the University of Colorado, Boulder, CO (Imada & Sueoka, 1978; Imada et al., 1978). The cells were cultured in 100 × 15 mm petri dishes (Lux) in a Forma Scientific Incubator set at 37 °C and 5% CO_2 atmosphere. The culture medium used was Dulbecco's modified Eagle's medium (Gibco) with 10% fetal calf serum (Gibco), 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were passaged twice weekly at semiconfluency by using trypsin–EDTA in Hank's balanced salt solution (Gibco). The number of cells in a suspension was determined by using a Coulter counter.

Assay Procedure for the Cell-Associated Plasminogen Activator. Sixteen hours prior to performing an assay, 0.10-mL aliquots of a cell suspension in complete culture medium were placed into microwells (Costar 6.4 mm diameter tissue culture cluster dishes). After this seeding period, the medium was removed, and the cells were washed 3 times with phosphate-buffered saline. To each microwell, 0.05 mL of phosphate-buffered saline containing plasminogen was added. After incubation at 37 °C for the indicated time intervals, the 0.05-mL aliquots were withdrawn and added to 1.0-mL solutions of phosphate-buffered saline (PBS) containing 1 μM FDE. After 2 min at 25 °C, the magnitude of the fluorescent burst was measured by using a Perkin-Elmer MPF-44A fluorescence spectrophotometer with a Universal digital readout. The excitation and emission wavelengths were 491 and 514 nm, respectively, both set with a bandwidth of 4 nm. The change in fluorescence (ΔF) is equal to the magnitude of the fluorescence in the sample cuvette minus the magnitude of the fluorescence in a reference cuvette containing the same concentrations of FDE and plasminogen but in which the plasminogen had been incubated in microwells without cells. ΔF is proportional to the plasmin concentration.

Purification and Characterization of Dog Plasminogen. Dog plasminogen was purified from plasma by a modification of the method of Castellino & Sodetz (1976). Fresh blood was collected in the presence of A-C-D (74.8 mM sodium citrate, 38.1 mM citric acid, and 0.136 M glucose) such that there was 15 mL of A-C-D per 100 mL of blood. The blood was centrifuged at 4 °C for 30 min at 27000*g* to remove particulate matter. The supernatant, 1000 mL, was applied to a 1.7 by 70 cm column of lysine–Sepharose at a flow rate of 24 mL/h. The column was washed with PBS_A (PBS minus calcium and magnesium) until the A_{280} of the effluent was less than 0.05. Plasma proteins were eluted by washing the column with a solution containing 0.3 M phosphate and 0.003 M EDTA, pH 7.4, until the A_{280} of the effluent was less than 0.05. The plasminogen was eluted by a 480-mL linear gradient of ϵ-aminocaproic acid from 0 to 0.03 M with 0.1 M phosphate and 0.003 M EDTA, pH 7.4, at a flow rate of 24 mL/h. To inhibit irreversibly any contaminating plasmin or plasminogen activator, the two fractions of plasminogen isolated from the column were incubated at 4 °C for 30 min with 0.004 M diisopropyl fluorophosphate (DFP). The plasminogen was precipitated to remove DFP and ϵ-aminocaproic acid by adding 0.31 g of $(NH_4)_2SO_4$ per mL of solution, stirring at 4 °C for 30 min, and then centrifuging at 4 °C for 30 min at 30000*g*. The precipitate was dissolved in less than 4 mL of PBS, and that solution was desalted by chromatography on a 3 × 30 cm column of Sephadex G-25. The peak fractions were pooled and concentrated by filtration under nitrogen pressure with an Amicon ultrafiltration membrane. The plasminogen, at concentrations greater than 8 mg/mL, was stored in aliquots at −70 °C. The yield from 1000 mL of plasma was about 50 mg of fraction I plasminogen and 100 mg of fraction II plasminogen.

Two major forms of dog plasminogen separate on a lysine–Sepharose column in a gradient of ϵ-aminocaproic acid (Figure 1). Since the physical differences between these two forms have not been determined, the first form eluted from the column is called fraction I and the second form fraction II. $NaDodSO_4$–polyacrylamide gel analysis (Figure 1) indicates that the apparent molecular weight of fraction I is

[1] Abbreviations used: FDE, 3′,6′-bis(4-guanidinobenzoyloxy)-5-[*N*′-(4-carboxyphenyl)thioureido]spiro[isobenzofuran-1(3*H*),9′-[9*H*]-xanthen]-3-one; FME, 5-[*N*′-(4-carboxyphenyl)thioureido]-3′-(4-guanidinobenzoyloxy)-6′-hydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]-xanthen]-3-one; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl fluorophosphate; PBS, phosphate-buffered saline.

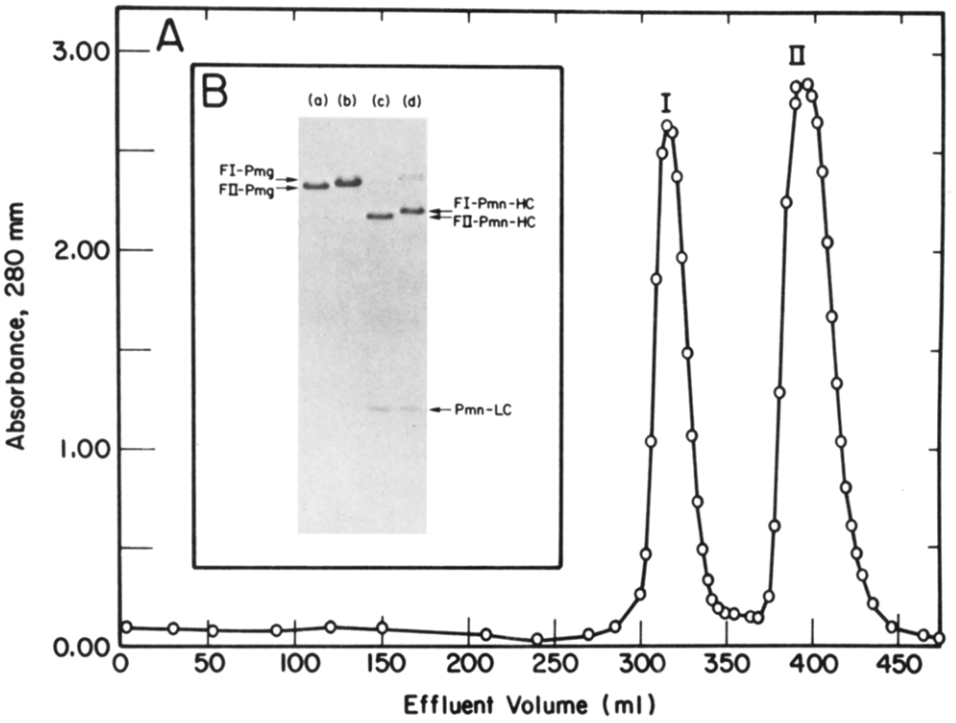

FIGURE 1: Elution of fraction I and fraction II dog plasminogen from a lysine–Sepharose column (A) and $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of their activation by human urokinase (B). (A) Fraction I and fraction II dog plasminogen are eluted from a lysine–Sepharose column by a gradient of ϵ-aminocaproic acid as described under Materials and Methods. (B) Solutions (0.1 mL) of phosphate-buffered saline containing 0.07 mg of fraction I and fraction II plasminogen were incubated for 10 min at 37 °C with 5 Plough units of urokinase and 60 kallikrein inhibitor units of aprotinin. Aliquots of these solutions and also of fraction I and of fraction II plasminogen each containing 0.014 mg of protein were analyzed by electrophoresis on a 10% $NaDodSO_4$–polyacrylamide slab gel. Slot a contains fraction II plasminogen, slot b contains fraction I plasminogen, slot c contains activated fraction II plasminogen, and slot d contains activated fraction I plasminogen.

95 000 and of fraction II is 90 000. In the presence of aprotinin, a plasmin inhibitor, urokinase can activate both forms of plasminogen to plasmin (Figure 1). The apparent molecular weight of the plasmin heavy chain from fraction I plasminogen is 85 000 and from fraction II plasminogen is 80 000. With both forms of plasminogen, the light chain has an apparent molecular weight of 24 000.

Determination of the Activatable Plasminogen Concentration. The concentration of activatable plasminogen was determined by activation with urokinase. The activation reaction in 0.1 mL of PBS at room temperature contained 22.4 Plough units of urokinase and 0.02 mg of plasminogen. At 5-min intervals, 0.01-mL aliquots were removed, and the plasmin produced was titrated in 1 mL of 2.33×10^{-6} M FDE in PBS. Under these activation conditions, the amount of plasmin formed did not increase after a 20-min incubation with fraction II plasminogen and after a 30-min incubation with fraction I plasminogen. The fraction of activatable plasminogen was calculated by dividing the concentration of plasmin formed after activation by the concentration of plasminogen before activation (determined by absorbance using an extinction coefficient $E^{1\%}_{280}$ of 15.6). For most preparations of fraction I and fraction II dog plasminogen, the fraction of activatable plasminogen is between 0.6 and 0.8. Plasminogen concentrations reported here are concentrations of activatable plasminogen.

Determination of the Initial Velocities and Steady-State Kinetic Constants for the Activation of Plasminogen by Urokinase. A two-step procedure was used to monitor the kinetics of activation of plasminogen by urokinase. Activation reactions in 0.1 mL of phosphate-buffered saline, pH 7.2, contained plasminogen concentrations ranging from 0.5 to 6 mg/mL and 5.6 Plough units of urokinase. After various time intervals, 0.02-mL aliquots were withdrawn, and the amount of plasmin formed was measured by titration in 1 mL of 2.33×10^{-6} M FDE in PBS. Conversion of relative fluorescence units to molar concentrations of active sites was described previously (Livingston et al., 1981). The initial velocities were calculated by using a computer-performed least-squares program to find the slope of the lines defined by the time points. The $K_{m(app)}$ and V_{max} were obtained by using a computer-performed direct linear plot program from the method described by Eisenthal & Cornish-Bowden (1974).

Results

Design of the Assay. For studies on the plasminogen activator activity of transformed cells, a two-step assay procedure was designed. Transformed cells growing in microwells are incubated with aliquots of plasminogen in PBS for time intervals ranging from 30 min to 2 h. For titration of the amount of plasmin formed during the incubation, the aliquots are removed from the microwells and added to solutions of FDE. Two minutes later, the magnitude of the fluorescent burst (ΔF) is measured.

The model cell line chosen to study the plasminogen activator activity of transformed cells was the RT4-71-D2 cell line (Imada & Sueoka, 1978; Imada et al., 1978). This clonal subline was derived from a neurotumor, RT4, induced by the subcutaneous injection of ethylnitrosourea into newborn BDIX rats. The cells are tumorigenic when injected into syngeneic BDIX rats, have a relatively small amount of a 260 000 molecular weight cell surface protein, which is probably the large external transformation-sensitive or LETS protein, and have been shown to exhibit high levels of plasminogen activator activity.

Validation of the Assay. The kinetics of hydrolysis of FDE by aliquots of plasminogen that had been incubated for 45 min at 37 °C in microwells seeded with 3500 RT4-71-D2 cells are

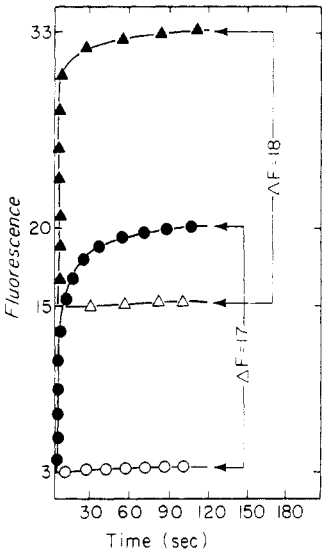

FIGURE 2: Titration of the plasmin produced upon incubating transformed cells with plasminogen. Microwells seeded with 3500 RT4-71-D2 cells were incubated with either 0.05 mL of phosphate-buffered saline or 0.05 mL of phosphate-buffered saline containing 1.25 mg/mL fraction II plasminogen. After 45 min at 37 °C, the 0.05-mL aliquots were removed and added to 1-mL solutions of phosphate-buffered saline containing either 0.2 or 0.6 μM FDE. Fluorescence was measured with an X–Y recorder containing a time base. Titration in 0.2 μM FDE of the aliquots incubated with cells that contained phosphate-buffered saline (○) or phosphate-buffered saline plus plasminogen (●). Titration in 0.6 μM FDE of the aliquots incubated with cells that contained phosphate-buffered saline (△) or phosphate-buffered saline plus plasminogen (▲).

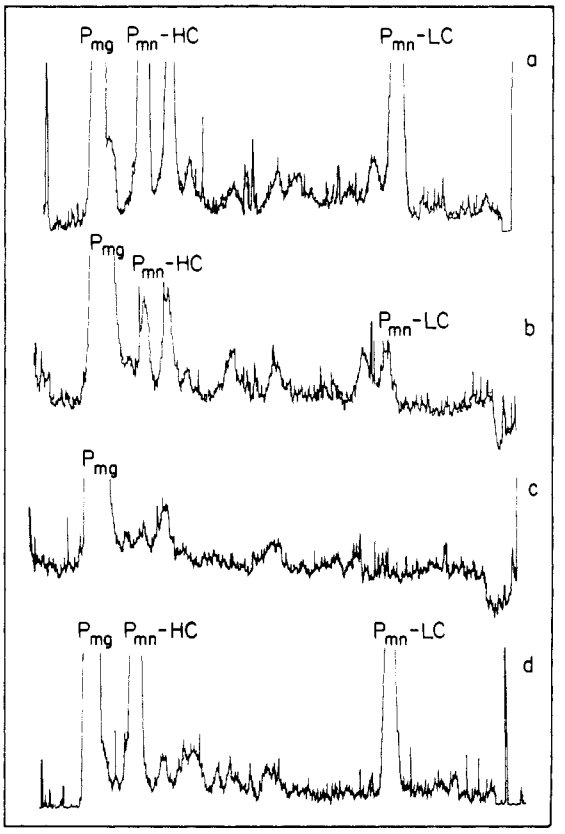

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of the activation of plasminogen by the plasminogen activator of transformed cells and by urokinase. Solutions (0.1 mL) of phosphate-buffered saline containing 0.70 mg/mL fraction II plasminogen were incubated for 10 min at 37 °C with 5 Plough units of urokinase in the presence (d) or absence (a) of 60 kallikrein inhibitor units of aprotinin. Solutions (0.05 mL) of phosphate-buffered saline containing 0.70 mg/mL fraction II plasminogen were incubated for 45 min at 37 °C in microwells in the presence (b) or absence (c) of 3500 RT4-71-D2 cells. Aliquots of each of these solutions containing 14 μg of protein were analyzed by electrophoresis on a 10% $NaDodSO_4$–polyacrylamide slab gel. Each slot was scanned in the densitometer at the same sensitivity. P_{mg} is plasminogen, P_{mn}-HC is the plasmin heavy chain, and P_{mn}-LC is the plasmin light chain.

shown in Figure 2. This kinetic profile is typical of an active-site titration of plasmin by FDE (Livingston et al., 1981). No hydrolysis of FDE is observed if aliquots of PBS without plasminogen are incubated with transformed cells and then titrated with FDE. Incubation of the same amount of plasminogen in empty microwells followed by titration with FDE yields no fluorescent "burst" (data not shown). The hydrolysis of FDE under these conditions is dependent upon the presence of a serine protease because if high concentrations of *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) are present during the incubation of plasminogen with cells, no fluorescent "burst" is observed upon titration with FDE (Liu et al., 1980).

Additional proof that plasminogen is activated to plasmin by the RT4-71-D2 cells under the assay conditions is obtained by analyzing the activation reaction by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 3). A densitometer tracing of the gel slot which contains purified fraction II plasminogen that had been incubated at 37 °C for 40 min in empty microwells is shown in Figure 3c. When the same amount of plasminogen was incubated at 37 °C for 40 min in microwells seeded with 12000 RT4-71-D2 cells and similarly analyzed, the heavy and light chains of plasmin are clearly discernible (Figure 3b). The large band to the right of the heavy chain and the large band to the left of the light chain are probably degradation products of plasminogen which resulted from proteolysis by plasmin. This is inferred by analyzing the products of the activation of plasminogen by urokinase in the presence of the plasmin inhibitor aprotinin (Figure 3d). Here, only the heavy and light chains of plasmin are present. When activation by urokinase occurs in the absence of the plasmin inhibitor, the two additional bands appear in Figure 3a. The two additional bands are probably degradation products of plasminogen rather than plasmin, because the amount of plasmin formed in the presence and absence of a plasmin inhibitor is the same and the ratio of heavy chain mass to light chain mass in both cases is the same as the ratio of their molecular weights.

Kinetics of Plasminogen Activator Activity. The effect of the plasminogen concentration on the kinetics of plasminogen activation by 8000 RT4-71-D2 cells is shown in Figure 4. The rate of activation is constant for 30 min and then begins to decrease. This decrease in the initial velocity is always greater and begins to occur more quickly at the higher plasminogen concentrations. If the incubation with plasminogen is continued beyond 60 min, some of the cells will detach from the microwell surface. Under the same conditions but in the absence of plasminogen, the cells do not detach. The cells also do not detach in the presence of high concentrations of plasmin if the plasmin inhibitors, aprotinin or NPGB, are present (data not shown). Thus, this decrease in the initial velocity appears to be the result of plasmin digesting plasminogen and the cell surface.

For determination of the Michaelis–Menten parameters for the activation of plasminogen by transformed cells, aliquots of fraction I or fraction II dog plasminogen were incubated in microwells seeded with 20000 RT4-71-D2 cells. The plasminogen concentrations ranged from 0.2 to 1.3 mg/mL. After 30 min, the aliquots were withdrawn, and the amount of plasmin formed was titrated with FDE. The data are shown in Figure 5 in the form of a direct linear plot (Eisenthal &

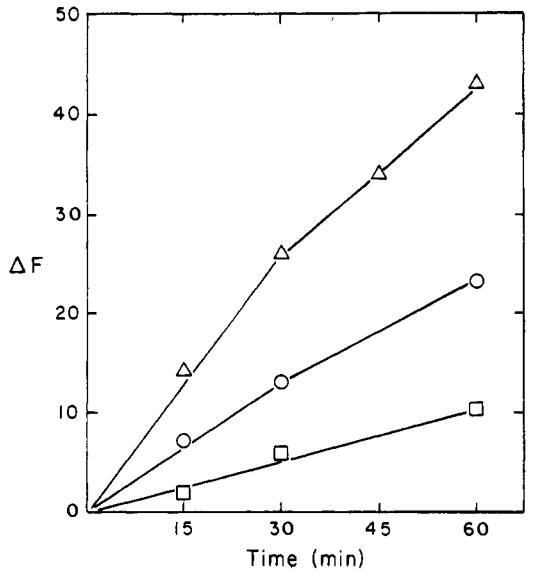

FIGURE 4: Effect of the plasminogen concentration on the kinetics of plasminogen activation by transformed cells. Microwells were seeded with 8000 RT4-71-D2 cells and incubated for the indicated time intervals with 0.05-mL solutions of phosphate-buffered saline containing fraction II plasminogen at concentrations of (□) 0.10, (○) 0.50, and (△) 1.25 mg/mL. The amount of plasmin formed was then titrated with FDE.

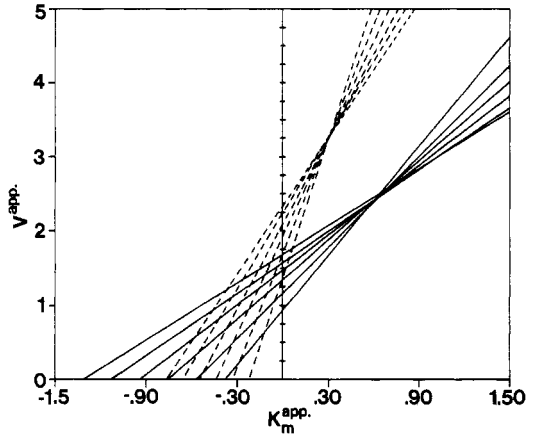

FIGURE 5: Direct linear plot of the initial rate of plasminogen activation by the RT4-71-D2 cells as a function of the plasminogen concentration. Microwells seeded with 20000 RT4-71-D2 cells were incubated with 0.05-mL aliquots of fraction I plasminogen (solid lines) or fraction II plasminogen (dashed lines). After 30 min at 37 °C, the aliquots were withdrawn, and the amount of plasmin produced during the incubation was titrated with FDE. The dimensions of the intial velocities are ΔF units/[(min) (10000 cells)].

Cornish-Bowden, 1974). The $K_{m(app)}$ for fraction I plasminogen is 0.63 mg/mL, and the $K_{m(app)}$ for fraction II plasminogen is 0.31 mg/mL. For comparison of relative values of k_{cat} with different forms of plasminogen and the same cell line and for comparison of the values of V_{max} with the same form of plasminogen and different cell lines, V_{max} can be normalized to the activity exhibited by 10000 cells. The V_{max}^N with fraction I plasminogen is 2.4 ΔF units/[(10000 cells) (min)] and that for fraction II plasminogen is 3.3 ΔF units/[(10000 cells) (min)].

Cell-Associated, As Opposed to Soluble, Plasminogen Activator Activates Plasminogen during the Assay. Plasminogen activator has been shown to be secreted by transformed cells into the growth medium (Jones et al., 1975a,b). For determination of whether this soluble form is activating plasminogen during the assay, as opposed to a cell-associated form, the following experiment was performed. Cells growing in microwells were preincubated with aliquots of plasminogen

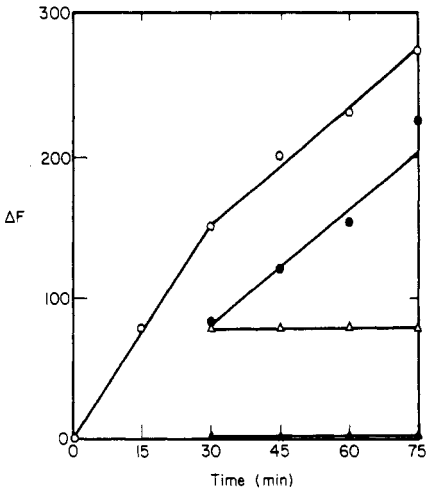

FIGURE 6: Determination of whether cell-associated or secreted plasminogen activator activates plasminogen during the assay. Microwells seeded with 16000 RT4-71-D2 cells were incubated with 0.05-mL aliquots of phosphate-buffered saline containing 1 mg/mL fraction II plasminogen. After a 15-min preincubation at 37 °C, the aliquots were removed and incubated in microwells containing no cells for the times indicated, before assaying for plasmin activity with FDE (△). Cells preincubated for 15 min with plasminogen were washed 3 times with phosphate-buffered saline and then incubated with either fresh aliquots of plasminogen (●) or phosphate-buffered saline (▲) for the indicated periods of time, before assaying for plasmin activity with FDE. The rate of activation of plasminogen was determined by incubating 0.05-mL aliquots of phosphate-buffered saline containing 1 mg/mL fraction II plasminogen in microwells seeded with 16000 RT4-71-D2 cells, removing the aliquots at the indicated times, and assaying the plasmin formed with FDE (○). Time zero is the beginning of the preincubation.

for 15 min. The aliquots were then removed and incubated for additional time intervals in empty microwells before being titrated for plasmin activity with FDE. If plasminogen activator had been secreted by the cells into the aliquots of plasminogen during the 15-min preincubation, further incubation of the aliquots in the absence of cells should result in additional activation. No increase in the amount of plasmin formed after the 15-min preincubation was observed (Figure 6). Thus, secreted plasminogen activator is not the major form of the enzyme-activating plasminogen during the period of the assay. Consistent with this conclusion is the observation that after removing the preincubation aliquots and washing the cells, the rate of plasminogen activation upon adding fresh aliquots of plasminogen is the same as during the preincubation (Figure 6). Thus, none of the activity can be removed from the cells by either preincubation or washing. A control experiment indicates that the hydrolysis of FDE by the activation aliquot is not due to the release of some factor from the cells that is induced by the presence of plasminogen or plasmin. If aliquots of PBS are incubated with cells which had been preincubated with plasminogen for 15 min and the cells washed, no fluorescence signal is detected upon titration of the PBS aliquots with FDE.

Inhibition of Transformed Cell-Associated Plasminogen Activator by Lysine. Lysine has been shown to be an inhibitor of the plasminogen activator activity secreted by transformed cells (Danø & Reich, 1975). For determination of whether lysine inhibits the cell-associated plasminogen activator and the type of inhibition by lysine, 40000 RT4-71-D2 cells were incubated with various concentrations of plasminogen and either 50 mM lysine, 25 mM lysine, or no lysine. The initial velocities for the activation of plasminogen were then measured. The data are presented as a Lineweaver–Burk plot (Figure 7A) and indicate that lysine is a competitive inhibitor

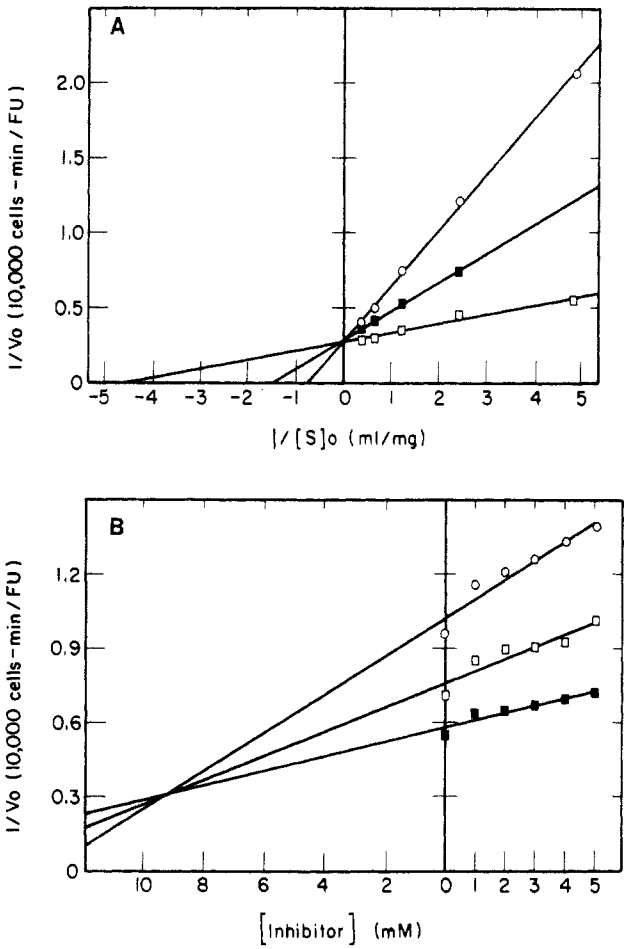

FIGURE 7: Inhibition of transformed cell plasminogen activator by lysine. (A) Microwells seeded with 40 000 RT4-71-D2 cells were incubated with 0.05-mL aliquots of phosphate-buffered saline containing the indicated concentrations of fraction II plasminogen and either (○) 50 mM lysine, (■) 25 mM lysine, or (□) no lysine. After 30 min at 37 °C, the amount of plasmin formed was titrated with FDE. The data are presented in the form of a Lineweaver–Burk plot. (B) Microwells seeded with 25 000 RT4-71-D2 cells were incubated with 0.05-mL aliquots of phosphate-buffered saline containing the indicated concentrations of lysine and fraction II plasminogen at concentrations of (○) 0.25, (□) 0.5, or (■) 1.0 mg/mL. After 30 min at 37 °C, the amount of plasmin formed was titrated with FDE. The data are presented in the form of a Dixon plot.

of plasminogen activator. For determination of the K_I for inhibition by lysine, 25 000 RT4-71-D2 cells were incubated with various concentrations of lysine and either 0.25, 0.5, or 1.0 mg/mL fraction II dog plasminogen. The initial velocities for the activation of plasminogen were then measured. The data are presented as a Dixon plot (Figure 7B). the K_I for the inhibition of the cell-associated plasminogen activator by lysine is 9.1 mM.

Kinetics of Plasminogen Activation by Urokinase. The kinetics of activation of fraction I and fraction II plasminogen by urokinase were monitored by a two-step assay (see Materials and Methods). Different concentrations of plasminogen were incubated with urokinase; aliquots were withdrawn after various time intervals, and the amount of plasmin in the aliquots was determined by an active-site titration with FDE. The kinetics of activation are linear and obey the Michaelis–Menten rate equation. The data are presented in Figure 8 in the form of a direct linear plot (Eisenthal & Cornish-Bowden, 1974). For the activation of fraction I plasminogen, the $K_{m(app)}$ is 3.17×10^{-5} M and the k_{cat} is 1.98 s^{-1}; for the activation of fraction II, the $K_{m(app)}$ is 1.92×10^{-5} M and the k_{cat} is 1.86 s^{-1}.

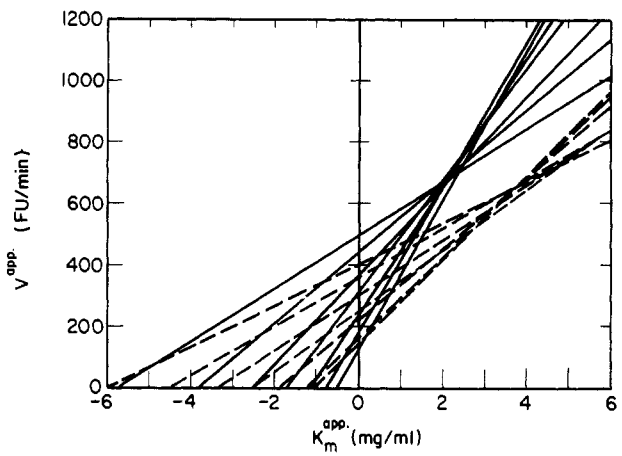

FIGURE 8: $K_{m(app)}$ and V_{max} for the activation of fraction I (dashed lines) and fraction II (solid lines) dog plasminogen by human urokinase. Activation reactions were performed at room temperature in 0.1 mL of phosphate-buffered saline containing 5.6 Plough units of urokinase. The fraction I plasminogen concentrations were 1.05, 1.22, 1.89, 2.52, 3.36, 4.48, and 5.98 mg/mL. The fraction II plasminogen concentrations were 0.50, 0.75, 1.12, 1.70, 2.55, 3.82, and 5.72 mg/mL. After 30- and 60-s time intervals, 0.02-mL aliquots were withdrawn, and the amount of plasmin formed was titrated in 1 mL of 2.33×10^{-6} M FDE in phosphate-buffered saline. The initial velocities were calculated by using a computer-performed least-squares program (see Materials and Methods). The data are presented in the form of a direct linear plot as described by Eisenthal & Cornish-Bowden (1974).

Discussion

The new fluorogenic, active-site titrant of plasmin, FDE, can be used in an in situ assay for the plasminogen activator activity of transformed cells. The assay is quick, sensitive, and quantiative. A cell line can easily be characterized within an hour by using as few as 3500 cells per assay. The kinetics of plasminogen activation by transformed cells are linear and conform to the Michaelis–Menten rate equation so that the $K_{m(app)}$ and V_{max} of the reaction can be determined. Since V_{max}^N is proportional to the amount of active enzyme per 10 000 cells, the amount of plasminogen activator of different cell lines and from cells grown under different conditions can now be meaningfully compared.

A two-step assay in which cells are first incubated with purified plasminogen for various time intervals and the resultant plasmin then quickly titrated with FDE allows for flexibility in the first part of the assay and for optimization of the titration conditions in the second part of the assay. For example, those cells with relatively small amounts of plasminogen activator activity can be incubated with plasminogen for longer periods of time. Alternatively, a larger number of cells can be assayed. Although the rate of spontaneous hydrolysis of FDE in aqueous buffers is slow ($t_{1/2}$ = 38 h at pH 7.2) and the rate of nonactive site induced hydrolysis minimal (Livingston et al., 1981), both of these variables can be negated by quickly titrating the amount of plasmin formed, in the absence of cells. A one-step assay in which both FDE and plasminogen are incubated with cells was rejected because cells may exhibit other esterase activities which would obscure the titration of plasmin and because plasminogen activator is a serine protease that may be inhibited by FDE.

Our assay for the plasminogen activator activity of transformed cells has several advantages: (1) The assay exploits the amplification of plasminogen activator activity by using plasminogen as the substrate and assaying the rate of plasmin formation. (2) The assay is performed with purified components, i.e., plasminogen in PBS, thereby avoiding the variables

Table I: Kinetic Constants for the Activation of Plasminogen

	cell-associated		urokinase	
plasminogen	$K_{m(app)}$ (μM)	V_{max} (ΔF units/[(10 000 cells) (min)]	$K_{m(app)}$ (μM)	k_{cat} (s^{-1})
fraction I	6.6	2.4	31.7	1.98
fraction II	3.4	3.3	19.2	1.86

involved in using casein or fibrin and serum. (3) The assay can detect low concentrations of plasmin. At high concentrations, proteolysis by plasmin destroys some of the components of the assay. (4) The assay for plasmin is an active-site titration, measuring the absolute amount of plasmin formed. Although a catalytic assay would result in even more amplification, a fixed time, stoichiometric assay is much easier to perform routinely and to interpret. (5) The assay measures the amount of plasminogen activator activity in situ rather than the number of plasminogen activator molecules titrated by antibodies or assayed after partial purification. This is especially important when studying the role of the cell-associated plasminogen activator because natural inhibitors may be present which modulate plasminogen activator activity. Such inhibitors may not prevent antibodies from binding and may be removed after partial purification. (6) The assay conditions are such that the cells being assayed are not destroyed, so that further experiments can be performed with them.

Purified fraction I or fraction II plasminogen, in which the amount of activatable protein is known, is required to obtain reproducible Michaelis–Menten parameters of transformed cell lines. Previously, we purified dog plasminogen from serum by the method of Deutsch & Mertz (1970). The macroscopic kinetic constants, however, varied with the enzyme preparation. Dog plasminogen purified by this procedure contains a mixture of two forms of the zymogen whose ratios varied from 1:3 to 3:1. Since the $K_{m(app)}$ with the RT4-71-D2 cell line for fraction I plasminogen is twice that for fraction II plasminogen, mixtures containing different ratios of the two forms of the zymogen will yield different macroscopic constants.

There are major differences and similarities between the activation of fraction I and fraction II dog plasminogen by the RT4-71-D2 cell line and by human urokinase (Table I). The $K_{m(app)}$ of the RT4-71-D2 cell line for fraction I plasminogen is almost twice that for fraction II plasminogen whereas the corresponding values for V_{max} are relatively close. Human urokinase, a soluble plasminogen activator, exhibits this same difference in $K_{m(app)}$ and similarity in V_{max} in activating fraction I and fraction II dog plasminogen. When the two plasminogen activators are compared to each other, the $K_{m(app)}$ for both fraction I and fraction II plasminogen with the cell-associated enzyme is 5-fold lower than with urokinase and is close to the in vivo concentration of plasminogen. Our yield of plasminogen from 1000 mL of dog plasma is about 150 mg. We are currently analyzing fraction I and fraction II dog plasminogen to determine the physical differences between them.

The important parameter in comparing the amount of plasminogen activator activity in various normal and transformed cell lines is V_{max}. It is dependent upon both the amount of active enzyme [E_0] and the rate at which each enzyme molecule converts plasminogen to plasmin (k_{cat}). Some of the factors which may influence V_{max} are the number of times the cell line has been passaged, the cell density before or after seeding into microwells, the time interval between seeding cells into microwells, the incubation with plasminogen, etc. The importance of these factors is now being investigated. The V_{max} reported here is normalized with respect to the number of cells seeded into microwells. The washing step prior to the addition of plasminogen is necessary to remove serum inhibitors, but it also removes some of the seeded cells. We are currently developing a procedure to count the cells after the incubation with plasminogen so that V_{max} can be normalized to the number of cells assayed.

The kinetics of activation of human plasminogen by human urokinase have previously been studied with coupled, double rate assays in which activation occurs in the presence of a plasmin substrate, e.g., N^{α}-benzoyl-L-arginine ethyl ester (Christensen & Mullertz, 1977; Christensen, 1977) or D-Val-Leu-Lys-*p*-nitroanilide (Wohl et al., 1980). The use of coupled, double rate assays to monitor the kinetics of activation has limitations. The kinetics are nonlinear, and the initial velocity of the reaction must be determined indirectly. Furthermore, the kinetic parameters for the interaction of plasmin with the synthetic substrate must be known since these values are used to determine the initial velocities for the activation of plasminogen. If the synthetic substrate reacts with the plasminogen activator, the kinetic parameters of that reaction must also be known. This may be difficult to do with plasminogen activators that are not available in sufficient quantity such as the plasminogen activator activity of transformed cells. Also, the affinity of the synthetic substrate for plasmin must be greater than its affinity for the plasminogen activator.

The two-step assay procedure described here to monitor the kinetics of plasminogen activation by urokinase yields data that are more easy to interpret and is much more flexible than a coupled, double rate assay. The kinetics are linear and thus directly yield the initial velocity. The first step of the assay, the activation reaction, can be performed under a variety of physiological conditions. The second step of the assay is designed solely to titrate the amount of plasmin formed in the first step. During the second step, the continued activation of plasminogen by urokinase is effectively quenched by dilution of the activation reaction aliquot into a solution of FDE. Since the $K_{m(app)}$ for plasminogen and urokinase is similar to the K_s for FDE and urokinase (Livingston et al., 1981), quenching occurs upon dilution because the FDE concentration becomes much greater than the plasminogen concentration, i.e., urokinase will react preferentially with FDE, resulting in its inhibition. A coupled assay in which FDE is present during the activation was not chosen because a low concentration of FDE relative to the plasminogen concentration would have to be used in order for FDE not to inhibit activation. This concentration of FDE would be well below the K_s for FDE and plasmin, and thus the plasmin produced would not be titrated immediately. The flexibility in the assay conditions should allow the factors that influence the rate and extent of plasminogen activation, e.g., fibrin and lysine, to be characterized quantitatively.

Plasminogen activators have been implicated in a wide variety of physiological processes, yet knowledge of their properties is still very limited. The assay we describe here should allow us to characterize quantitatively the behavior of these enzymes under a variety of conditions. For elucidation of their biological roles, inhibitors of plasminogen activator will be useful. Because plasminogen activator and plasmin are both serine proteases, screening for specific inhibitors of plasminogen activator is difficult. A two-step assay in which an active-site titrant of plasmin is used should facilitate the characterization of inhibitors, as exemplified by our quantitative characterization of the competitive inhibition of

transformed cell-associated plasminogen activator by lysine.

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