Sensitive assay for plasminogen activator of transformed cells

(fluorogenic substrate/serine proteases/plasmin/active-site titrant/fluorescein ester)

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ABSTRACT A sensitive *in situ* assay for the plasminogen activator of transformed cells is described; it uses the fluorogenic molecule 3',6'-bis(4-guanidinobenzoyloxy)-5-(N'-4-carboxylphenyl)thioureidospiro[isobenzofuran-1(3H),9'-(9H)xanthen]-3-one. This fluorescein derivative is an excellent active-site titrant of the esterase activity of plasmin. When transformed cells are incubated with purified plasminogen and the resulting plasmin is titrated with the fluorogenic substrate, the amount of plasmin formed is linearly proportional to time and cell number. The assay is sensitive enough to detect quantitatively the plasminogen activator activity of as few as 250 transformed cells. This substrate should be useful in studying quantitatively the correlation between increased levels of plasminogen activator activity and cellular transformation and as a general active site titrant of serine proteases.

Enhanced proteolytic activity of tumors was observed in 1925 by Fischer (1). He found that chicken sarcomas growing *in vitro* could lyse fibrin clots, whereas normal cell explants could not. Recently (2-5) this fibrinolytic activity of tumors was shown to be the consequence of a serum factor (plasminogen) and a cell factor (plasminogen activator). 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* (2, 6-9) but also of cells involved in tissue remodeling and migration (10). Furthermore, plasminogen activator synthesis has been shown to be inducible by tumor promoters (11, 12) and carcinogens (13) and to be modulated by the presence of physiological concentrations of hormones (14-18).

A major difficulty 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 proteolysis by high concentrations of plasmin results in the degradation of plasmin, plasminogen, and the cell surface. A quantitative assay is required so that the amount of plasminogen activator in different cell lines can be meaningfully compared. The most widely used assays, the hydrolysis of casein (6) and fibrin (19) and the solubilization of ¹²⁵I-labeled fibrin (2), are rate assays. Both casein and fibrin overlays are used in plaque assays, in which 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 ¹²⁵I-labeled fibrin by plasmin. However, this assay is not ideal. The ¹²⁵I-labeled fibrin substrate is insoluble. Neither the nature and number of cleavages required of plasmin to release an ¹²⁵I-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, 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 (20). The kinetics are nonlinear and complex, thereby precluding a quantitative analysis of the data.

To measure the amount of plasmin produced by the plasminogen activator of transformed cells, an active site titrant is required that will react quickly and stoichiometrically with plasmin, exhibiting a physical change that can be sensitively detected. *p*-Nitrophenyl *p*-guanidinobenzoate (NPGb) is kinetically one of the best active site titrants of serine proteases (21, 22). In the presence of NPGb, plasmin is rapidly acylated ($k_2 = 0.46 \text{ sec}^{-1}$) and then slowly deacylated ($k_3 = 5.25 \times 10^{-6} \text{ sec}^{-1}$). Furthermore, NPGb is relatively stable in aqueous buffers. These and other characteristics of NPGb fulfill most of the criteria of an ideal active site titrant, except one. The major problem lies in the detectability of the product of the reaction, *p*-nitrophenol. The lowest concentration of *p*-nitrophenol that can be measured with an accuracy of better than 2% is 1 μM .

Here we describe the use of a different active site titrant of the esterase activity of plasmin. It is an analogue of NPGb in which a derivative of fluorescein is substituted for *p*-nitrophenol. It is similar to NPGb in that it exhibits the same excellent kinetic characteristics in an active site titration. It is different from NPGb in that by substitution of chromophores the detectability of the product in an active site titration is increased six orders of magnitude, from 1 μM for *p*-nitrophenol to 1 pM for the fluorescein derivative.

MATERIALS AND METHODS

Materials. The synthesis, partial purification, and initial characterization of 3',6'-bis(4-guanidinobenzoyloxy)-5-(N'-4-carboxylphenyl)thioureidospiro[isobenzofuran-1(3H),9'-(9H)xanthen]-3-one, hereafter abbreviated FDE for FluoresceinDiEster, have been described (23). Stock solutions of 0.5

Abbreviations: FDE, 3',6'-bis(4-guanidinobenzoyloxy)-5-(N'-4-carboxylphenyl)thioureidospiro[isobenzofuran-1(3H),9'-(9H)xanthen]-3-one; FME, 5-(N'-4-carboxylphenyl)thioureido-3'-(4-guanidinobenzoyloxy)-6'-hydroxyspiro[isobenzofuran-1(3H),9'-(9H)xanthen]-3-one; P_i/NaCl, phosphate buffered saline; NPGb, *p*-nitrophenyl *p*-guanidinobenzoate.

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mg/ml were prepared in redistilled dimethylformamide. Further dilutions were made into phosphate-buffered saline ($P_i/NaCl$) before each experiment. Plasminogen was purified from dog plasma by the method of Deutsch and Mertz (24). Prior to each experiment, stock solutions were prepared in $P_i/NaCl$. Dog plasmin was obtained by the activation of plasminogen by urokinase that had been coupled to Sepharose beads (25). Urokinase was purchased from Leo Pharmaceuticals (Helsingborg, Sweden). $P_i/NaCl$ contained 0.137 M NaCl, 2.68 mM KCl, 8.08 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. NPGB was purchased from ICN and dissolved in dimethylformamide to a concentration of 1.0 mg/ml. Further dilutions were made in $P_i/NaCl$ before each experiment.

Cell Culture. The RT4-71-D2 cell line was a kind gift from N. Sueoka of the University of Colorado, Boulder (26, 27). 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), penicillin at 100 units/ml, and streptomycin at 100 mg/ml. The cells were passaged twice weekly at semiconfluency, using trypsin/EDTA in Hanks' balanced salt solution (GIBCO). The number of cells in a suspension was determined by using a Coulter Counter.

Assay Procedure. Sixteen hours prior to 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 three times with $P_i/NaCl$. To each microwell, 0.05 ml of $P_i/NaCl$ 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 $P_i/NaCl$ containing 0.75 μ g of FDE. After 2 min at 25°C, 20 μ g of NPGB was added to quench the reaction. The magnitude of the fluorescence was then determined. Fluorescence was measured with a Perkin-Elmer MPF-44A fluorescence spectrophotometer with a Universal digital readout. The excitation and emission wavelengths were 491 nm 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 minus the magnitude of the fluorescence in a blank containing only FDE and NPGB. ΔF is proportional to the plasmin concentration.

RESULTS

Design of FDE. FDE was designed as an active site titrant of serine proteases (Fig. 1). It is an analogue of NPGB in which a fluorescein derivative is substituted for *p*-nitrophenol. FDE is nonfluorescent because the fluorescein moiety is in the lactone state. In the presence of a serine protease, however, one of the guanidinobenzoyl esters is hydrolyzed, yielding 5-(*N'*-4-carboxyphenyl)thioureido-3'-(4-guanidinobenzoyloxy)-6'-hydroxyspiro[isobenzofuran-1(3*H*), 9'-[9*H*]xanthen]-3-one, hereafter abbreviated FME for FluoresceinMonoEster. FME should be highly fluorescent, because the fluorescein moiety is in the quinone state.

FDE Is a Substrate for Plasmin. Plasmin is a serine protease. Bender *et al.* (28) have proposed the following three-step mechanism to explain the kinetics of ester bond hydrolysis by serine proteases:

FIG. 1. 3',6'-Bis(4-guanidinobenzoyloxy)-5-(*N'*-4-carboxyphenyl)thiureidospiro[isobenzofuran-1(3*H*),9'-[9*H*]xanthen]-3-one, the structure of FDE in $P_i/NaCl$ at pH 7.2.

in which E is plasmin; S is FDE; ES is the adsorptive enzyme-substrate complex; ES' is the acyl-enzyme complex, *p*-guanidinobenzoyl-plasmin; P_1 is FME; and P_2 is *p*-guanidinobenzoic acid.

The kinetics of hydrolysis of FDE by plasmin (Fig. 2A) are

FIG. 2. Kinetics of hydrolysis of FDE by plasmin (A) and the titration of increasing concentrations of plasmin by FDE (B). (A) To a 1-ml solution of $P_i/NaCl$ containing FDE at a concentration of 0.846 μ M, 1.25 μ g of dog plasmin was added and the increase in fluorescence was measured at 25°C in a Perkin-Elmer MPF-44A fluorescence spectrophotometer connected to a Hewlett-Packard x-y recorder with time base. (B) To 1.7 mM solutions of FDE in $P_i/NaCl$, an equal volume (0.05 ml) of $P_i/NaCl$ containing plasmin at the indicated concentrations was added and mixed. After 10 min at 25°C, 0.9 ml of $P_i/NaCl$ was added and the change in fluorescence (ΔF) was measured.

similar to the kinetics of hydrolysis of NPCGB by plasmin (21, 22). The "burst," due to the rapid formation of the *p*-guanidinobenzoyl-plasmin complexes and the concomitant release of the highly fluorescent FME, is complete within 45 sec. Thereafter, a steady state is reached during which only a slight linear increase in fluorescence is observed. This indicates that the acyl-enzyme intermediate is stable (half-life = 37 hr; unpublished observation) and that $k_2 \gg k_3$. Thus, the magnitude of the fluorescence change (ΔF) that occurs within the first 2 minutes is directly proportional to the amount of plasmin present. A standard curve in which increasing amounts of plasmin are titrated with FDE is shown in Fig. 2B.

Use of FDE to Measure the Plasminogen Activator Activity of Transformed Cells. Experiments were then performed to determine whether FDE could be used to measure indirectly the plasminogen activator activity of transformed cells by an active site titration of the plasmin formed after incubation of cells with plasminogen. The model cell line used is a clonal subline, RT4-71-D2, of a neurotumor, RT4, induced by the subcutaneous injection of ethylnitrosourea into newborn BDIX rats (26, 27). The cells are tumorigenic when injected into syngeneic BDIX rats. To measure the plasminogen activator activity of these cells quantitatively, assay conditions had to be found in which the amount of plasmin formed is proportional to time and cell number. A two-step procedure was eventually adopted (see *Materials and Methods*) in which transformed cells growing in microwells are first incubated with aliquots of purified plasminogen in $P_i/NaCl$. The aliquots are then removed and the resultant plasmin is quickly titrated with FDE. ΔF is directly proportional to the plasmin concentration.

The kinetics of plasminogen activation by the RT4-71-D2 cell line at a constant plasminogen concentration are linear and proportional to cell number (Fig. 3A). After 90 min, however, the velocity begins to decrease in the presence of 15,000 cells. The rate of plasminogen activation usually begins to decrease once a critical concentration of plasmin has formed. This decrease appears to result from proteolytic action by high concentrations of plasmin on itself, plasminogen, and the cell surface. When the plasminogen concentration is varied at a constant cell number, the kinetics of plasminogen activation are also linear (Fig. 3B).

Control experiments were performed to determine the amount of contaminating plasmin in the plasminogen preparations and to determine the extent of plasminogen-independent hydrolysis of FDE. Plasminogen solutions were incubated in microwells that did not contain cells (Fig. 3A), and $P_i/NaCl$ solutions that did not contain plasminogen were incubated in microwells containing cells (Fig. 3B). The solutions were then titrated with FDE. The results indicate a small amount of contaminating plasmin in the plasminogen preparations and a small amount of plasmin-independent hydrolysis of FDE. The minimal ΔF observed in these control experiments indicate, however, that the hydrolysis of FDE requires both a cell factor (plasminogen activator) and plasminogen.

Additional proof that the activity of a plasminogen activator is being measured under the assay conditions is shown in Fig. 4. $P_i/NaCl$ solutions containing a constant amount of plasminogen and increasing concentrations of NPCGB were incubated with 10,000 cells for 60 or 90 min and then assayed for plasmin activity with FDE. The ΔF decreases as the NPCGB concentration increases or as the time of incubation increases, indicating that the hydrolysis of FDE is dependent upon the activity of a serine protease.

To determine the sensitivity of the FDE assay for the plasminogen activator activity of transformed cells, various numbers of cells, ranging from 250 to 20,000, were incubated with

FIG. 3. Effects of cell number (A) and the plasminogen concentration (B) on the kinetics of plasminogen activator activity. (A) Each microwell contained plasminogen at 0.696 mg/ml; $\blacksquare$, 15,000 cells; $\circ$, 7000 cells; $\triangle$, 0 cells. (B) Each microwell contained 9000 cells. The plasminogen concentrations were: $\triangle$, 0.2 mg/ml; $\circ$, 0.1 mg/ml; $\blacktriangle$, 0 mg/ml. After the indicated time intervals, the amount of plasmin produced was titrated with FDE.

plasminogen for a fixed time and the amount of plasmin produced was then titrated with FDE (Fig. 5). One set of experiments was performed using plasminogen at 0.745 mg/ml and

FIG. 4. Inhibition of plasminogen activator activity by NPCGB. Each microwell contained 10,000 cells, plasminogen at 0.388 mg/ml, and the indicated concentrations of NPCGB. Incubation was for 90 min ($\bullet$) or 60 min ($\circ$). After the indicated time intervals, the amount of active plasmin was titrated with FDE.

FIG. 5. Plasminogen activator activity as a function of the number of cells. Microwells were seeded with the indicated number of cells and incubated with 0.725 mg/ml of plasminogen for 120 min (●) or 225 min (○). After the indicated time intervals, the amount of plasmin produced was titrated with FDE. ΔF divided by the incubation time t is plotted as a function of the number of cells seeded in the microwells, on two scales.

a 120-min incubation, another set with the same amount of plasminogen and a 225-min incubation. The ΔF was then normalized with respect to the time of incubation and plotted versus cell number. The result is a straight line from 20,000 cells to 250 cells through 0. This experiment also indicates the assay is linear with time and proportional to cell number and that it is sensitive enough to detect a quantitative fluorescence signal from the plasmin produced by as few as 250 transformed cells.

DISCUSSION

The fluorogenic active-site titrant for plasmin, FDE, can be used to measure the *in situ* activity of plasminogen activator of transformed cells. The assay is quick, sensitive, and quantitative. The kinetics of plasminogen activation by transformed cells are linear and proportional to cell number. The activity of a cell line can easily be characterized within an hour, using only 4000 cells per assay. For those cell lines exhibiting low levels of plasminogen activator activity, the assay conditions can easily be altered to increase sensitivity by either using more cells per assay or lengthening the incubation time with plasminogen. The second step of the assay, the active-site titration, is absolutely quantitative, takes 2 min to perform, and requires only one measurement to determine the plasmin concentration.

Although the structures of FDE and FME have not yet been confirmed by chemical methods, the usefulness of this fluorescein derivative as an active-site titrant of serine proteases has clearly been demonstrated. Heretofore, the most sensitive fluorogenic substrates for serine proteases have been the coumarin derivatives—e.g., 4-methylumbelliferyl *p*-guanidinobenzoate (29) and Cbz-Gly-Gly-Arg-7-amino-4-methylcoumarin (30). Fluorescein has an extinction coefficient 5-fold greater than that of umbelliferone, has a quantum yield at least twice as great, and absorbs maximally at 491 nm where the xenon lamp output is twice that at 345 nm, the absorption maximum for umbelliferone. Thus, the detectability of FME is at least 20-fold greater than that of umbelliferone. Also, flu-

orescein is completely colorless and nonfluorescent in the lactone state, whereas the coumarin esters exhibit some intrinsic fluorescence.

No attempt was made in the experiments with transformed cells to analyze the data quantitatively. ΔF is not comparable in different experiments. The dog plasminogen used was purified by the method of Deutsch and Mertz (24). Different preparations contain different ratios of the two forms of the zymogen. Because these two forms are activated at different rates by the plasminogen activator of transformed cells (5), the effective plasminogen concentration in each enzyme preparation is different. In a subsequent communication we shall show that by using purified fraction I or fraction II plasminogen, the $K_{m(\text{app})}$ and V_{max} for the activation of plasminogen by different cell lines can be obtained.

The major advantages of our assay for the plasminogen activator activity of transformed cells are: (i) The assay amplifies plasminogen activator activity. This is achieved by using plasminogen as the substrate for the activator and assaying for plasmin production. (ii) The assay is performed with purified components—i.e., plasminogen in P_i/NaCl —thereby avoiding the variables involved in using either casein or fibrin with serum. (iii) The assay can detect low concentrations of plasmin. At high concentrations, proteolysis by plasmin destroys itself, plasminogen, and the cell surface. (iv) 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. (v) 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 that modulate plasminogen activator activity. Such inhibitors may not prevent antibodies from binding and may be removed after partial purification. (vi) The assay conditions are such that the cells being assayed are not destroyed, so that further experiments can be performed with them.

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1. Fischer, A. (1925) *Arch. Entwicklungsmech. Org.* **104**, 210-219.
2. Unkeless, J. C., Tobia, A., Ossowski, J. P., Rifkin, D. B. & Reich, E. (1973) *J. Exp. Med.* **137**, 85-111.
3. Ossowski, L., Unkeless, J. C., Tobia, A., Quigley, J. P., Rifkin, D. B. & Reich, E. (1973) *J. Exp. Med.* **137**, 112-126.
4. Unkeless, J., Danø, K., Kellerman, G. M. & Reich, E. (1974) *J. Biol. Chem.* **249**, 4295-4305.
5. Quigley, J. P., Ossowski, L. & Reich, E. (1974) *J. Biol. Chem.* **249**, 4306-4311.
6. Goldberg, A. R. (1974) *Cell* **2**, 95-102.
7. Christman, J. & Acs, G. (1974) *Biochim. Biophys. Acta* **340**, 339-351.
8. Rifkin, D. B., Loeb, J. N., Moore, G. & Reich, E. (1974) *J. Exp. Med.* **139**, 1317-1326.
9. Pollack, R., Risser, R., Conlon, S., Freedman, V., Shin, S.-I. & Rifkin, D. B. (1975) in *Proteases and Biological Control*, Cold Spring Harbor Conferences on Cell Proliferation, eds. Reich, E., Rifkin, D. B. & Shaw, E. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 2, pp. 885-899.
10. Ossowski, L., Quigley, J. & Reich, E. (1975) in *Proteases and Biological Control*, Cold Spring Harbor Conferences on Cell Proliferation, eds. Reich, E., Rifkin, D. B. & Shaw, E. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 2, pp. 901-913.
11. Wigler, M. & Weinstein, I. B. (1976) *Nature (London)* **259**, 232-233.
12. Vassalli, J. D., Hamilton, J. & Reich, E. (1977) *Cell* **11**, 695-705.
13. Hamilton, J., Vassalli, J. D. & Reich, E. (1976) *J. Exp. Med.* **144**, 1689-1694.
14. Graneliperno, A., Vassalli, J. D. & Reich, E. (1978) *J. Exp. Med.* **147**, 223-234.
15. Vassalli, J. D., Hamilton, J. & Reich, E. (1976) *Cell* **8**, 271-281.
16. Lecroix, M., Smith, F. E. & Fritz, I. B. (1977) *Mol. Cell. Endocrinol.* **9**, 227-236.
17. Strickland, S. & Beers, W. H. (1976) *J. Biol. Chem.* **251**, 5694-5762.
18. Beers, W. H., Strickland, S. & Reich, E. (1975) *Cell* **6**, 387-394.
19. Jones, P., Benedict, W., Strickland, S. & Reich, E. (1975) *Cell* **5**, 323-329.
20. Wiman, B. & Wallen, P. (1977) *Thromb. Res.* **1**, 213-222.
21. Chase, T. & Shaw, E. (1967) *Biochem. Biophys. Res. Commun.* **29**, 508-514.
22. Chase, T. & Shaw, E. (1969) *Biochemistry* **8**, 2212-2224.
23. Mangel, W. F., Livingston, D. C., Brocklehurst, J. R., Liu, H.-Y., Peltz, G. A., Cannon, J. F., Leytus, S. P., Wehrly, J. A., Salter, B. L. & Mosher, J. L. (1980) *Cold Spring Harbor Symp. Quant. Biol.* **44**, 669-680.
24. Deutsch, D. G. & Mertz, E. T. (1970) *Science* **170**, 1095-1096.
25. Castellino, F. J. & Sodetz, J. M. (1976) *Methods Enzymol.* **45**, 273-286.
26. Imada, M. & Sueoka, N. (1978) *Dev. Biol.* **66**, 97-108.
27. Imada, M., Sueoka, N. & Rifkin, D. B. (1978) *Dev. Biol.* **66**, 109-116.
28. Bender, M. L., Begue-Canton, M. L., Blakeley, R. L., Brubacher, L. J., Feder, J., Gunter, C. R., Kézdy, F. J., Kilheffer, J. V., Jr., Marshall, T. H., Miller, C. G., Roeske, R. W. & Stoops, J. K. (1966) *J. Am. Chem. Soc.* **88**, 5890-5913.
29. Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A. & Elmore, D. T. (1973) *Biochem. J.* **131**, 107-117.
30. Zimmerman, M., Quigley, J. P., Ashe, B., Dorn, C., Goldfarb, R. & Troll, W. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 750-753.