THE DEVELOPMENT AND APPLICATIONS
OF A FLUORESCENCE ASSAY
FOR TRANSFORMED CELLS

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Abstract

An in vitro fluorescence assay is described that can quantitatively measure the level of plasminogen activator activity present in a cell line. Fluorescein DiEster, a fluorescent substrate for the esterase activity of plasmin, is used to quantitate the amount of plasmin generated by the cellular plasminogen activator during the period of time cells are incubated with plasminogen. The assay has many advantages over other transformation assays currently used because it has greater sensitivity, yields quantitative and reproducible results, and utilizes procedures which can be performed quickly and easily. The assay was used in experiments designed to measure the second-order rate constant for the attack of NPGB on the plasminogen activator, identify the rate-limiting step in the activation of plasminogen, measure the maximal velocity and Michaelis Constant of the plasminogen activator of two tumorigenic cell lines, and compare the levels of plasminogen activator activity expressed in tumorigenic and non-tumorigenic cell lines.
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I. Introduction

A. Transformation Assays (Not Measuring Plasminogen Activator Activity)

A cell in culture is defined as malignantly transformed when injection of cells from the culture into a suitable host leads to the formation of tumors. Scientists investigating the mechanisms and factors involved in the transformation of cells realize that their ability to obtain information is dependent upon their ability to identify a transformed cell.

In order for an assay for transformation to be useful to an investigator, it must detect a property that has been shown to be closely correlated with transformation. It must also have the requisite sensitivity, and must be a reproducible and quantitative test. In addition, the techniques and materials employed in the assay must not be prohibitively expensive and should not require a great deal of time to perform. Preferably the procedures should be relatively simple to perform and not cause any damage to the cells being assayed, so that additional experiments can be performed upon the same cells.

There are many assays currently used to detect transformed cells. Each method measures a property that is associated with transformed cells. One property used to identify transformed cells is their altered morphology and growth pattern. Dr. Charles Heidelberger, et al., have extended the initial investigations of Berwald and Sachs, by showing that cell cultures of C3H mouse cells treated with carcinogenic hydrocarbons contain regions where the cells grow to high cell density and are piled up into multilayered arrays called foci. The cells isolated from the foci were tumorigenic while cells isolated from the monolayer regions of the same culture did not produce tumors upon injection into syngeneic hosts. This observation is the basis of the focus formation assay for transformed cells. Nontransformed cells which were incubated with a carcinogen for a given time period are cultured for six weeks. The cells are then fixed with methanol and stained with Giemsa and transformation is quantitated on
the basis of the number of observable foci. This method has neither the requisite sensitivity nor simplicity that a transformation assay must have to be useful. Its usefulness is limited due to the time and expense involved in culturing cells for six weeks prior to quantitation. The criteria for identifying transformation are rather subjective, and the assay does not have the required reproducibility since the measured transformation efficiencies are very dependent upon the culture conditions, such as the number of cells seeded and serum concentration in the medium.

The ability of transformed cells to grow in soft agar is another property that has been used to identify transformed cells. A subclone of BHK 21 C13 cells was isolated that is able to undergo several rounds of cell division in soft agar (microcolony formation). The assay procedure involves incubating the cloned cells after exposure to a chemical carcinogen, oncogenic virus, or x-ray irradiation in soft basal agar containing complete culture medium for four weeks. Microscopically visible colonies are assumed to have arisen from transformed cells. The usefulness of this assay is also limited by the delay and expense due to the need to culture the cells for four weeks in the agar. In addition, the subclone of BHK 21 C13 cells used in the assay exhibit many of the properties of transformed cells. Injection of 10,000 untreated cells into syngeneic hosts does induce the formation of tumors.

It should also be stated that the results of tests for mutagenic agents and for the occurrence of mutagenesis are not sufficient to identify a substance as a carcinogen or a cell as transformed. Several carcinogens have been identified that are not mutagenic to mammalian cells. In addition, the results of tests applied to bacterial systems may not be valid for mammalian cells. Many of the carcinogenic polycyclic aromatic hydrocarbons require metabolic activation by microsomal enzymes to form electrophilic species before they will react with any cellular macromolecules. A substance that is
capable of transforming a mammalian cell after activation would not be detected using bacterial systems.

B. Plasminogen Activator

1. Role in Transformation and Tumorigenicity

Fischer's initial observation that primary explants of viral sarcomas in chickens rapidly lyse plasma clots, whereas explants of normal connective tissue do not, makes apparent another property of transformed cells that is measured in assays for transformed cells. Recently, evidence has accumulated that transformed cells express higher levels of an enzyme that can activate plasminogen and that this enzyme plays an important role in the initiation and/or maintenance of the transformed state.

a. Clones derived from HT1080, a human fibrosarcoma cell line, were shown to have fibrinolytic activity. Cells derived from tumors in hamsters, induced by the injection of clones of the HT1080 cells with low fibrinolytic activity, showed a marked increase in their fibrinolytic activity. 12

b. Plasminogen activator is expressed in a number of cell lines transformed by carcinogens or oncogenic viruses and is not expressed in the corresponding normal cell line. 13,14,15 Chicken embryo fibroblasts (CEF), transformed by Rous sarcoma virus expressed high levels of plasminogen activator while their normal counterparts did not. CEF cultures infected with a temperature-sensitive strain of the Rous sarcoma virus did not exhibit fibrinolytic activity when cultured at the nonpermissive temperature. Fibrinolytic activity did appear 8-10 hr after the infected cultures were shifted to the permissive temperature. 16

c. Clone B5/59 of mouse melanoma B16 cells are highly tumorigenic in C57 Bl./6J mice. When those cells are cultured in the presence of bromodeoxyuridine (BrdU), a thymidine analogue that is incorporated into cellular DNA, their tumorigenicity and fibrinolytic activity (measured using the
I$^{125}$-fibrin assay) exhibit a parallel decrease. Tumorigenicity and plasminogen activator expression are restored when the cells are cultured for three to five days in the absence of BrdU. 17

The fibrinolytic activity of transformed cells may enhance the transformed cells' ability to proliferate and their capacity to spread and to invade normal tissues. The proteolytic activity of the activated plasmin could alter the cell membranes or other components in a manner that would induce or enhance the transformed properties. Proteolysis enhances the agglutinability of cells by plant lectins 18 which is another property characteristic of transformed cells. In addition, a variety of cell surface proteins and receptors are altered by proteolysis. 19 Trypsinization of nuclear components was shown to increase the activity of DNA polymerase 20 and extent of DNA methylation. 21 A tumor must induce neovascularization in order for it to continue to proliferate and invade normal tissue. 22 Plasmin plays an important role in inflammation which stimulates the formation of granulation tissue and the vascularization of tissues (see Section C.1.).

2. Assays for Plasminogen Activator

The evidence that plasminogen activator is expressed in transformed cells and that it appears to play an important role in maintaining the transformation properties has led to the development of a number of transformation assays that measure plasminogen activator activity. The fibrin overlay method measures the level of plasminogen activator as a function of the number of lysis zones appearing in a fibrin-agarose overlay containing acid treated serum placed upon a layer of cells in a petri dish. 23 This assay requires incubation periods of 18-48 hr and its reliability is questionable. High backgrounds were observed in petri dishes that did not contain any cells and in those that contained normal cells, especially when the incubation was for extended time periods. The casein overlay technique is a similar type of assay.
The level of plasminogen activator in a cell line is measured by examining the number of plaques appearing in a casein-agarose overlay. This assay does not have the requisite sensitivity. Experiments are routinely performed using 500,000 to 1 million cells and incubation periods of 12-48 hr. Another problem with both of the overlay techniques is that they are difficult to quantitate. It is not advisable to quantitate the assay on the basis of the number of observed lysis zones alone as is done for the fibrin overlay. The dimensions of each lysis zone are also dependent upon the amount of plasminogen activator. The relative scale of 0 to 5 applied to the results of the casein overlay experiment precludes its being used as a quantitative assay.

Currently the assay for plasminogen activator that is most extensively used is the \( ^{125}\)I-fibrin technique. This assay measures the amount of radioactivity released into solution as a result of the proteolytic activity of plasmin upon \( ^{125}\)I-fibrin coating the surface of a petri dish. Many important experiments demonstrating that the plasminogen activator is present in transformed cells have been performed using this method, yet there is clearly a need for another technique. The results of experiments that have utilized this technique are neither reproducible nor quantitative. The \( ^{125}\)I-fibrin substrate is insoluble, the nature and number of cleavages required to release a fragment are not known, and the dependence of the rate of the reaction upon the state of the fibrin is uncertain. The kinetics are thus very complex and prevent a quantitative interpretation of the results of the assay. "Repeated assays of the same cell line may vary in absolute activity...." This assay also does not have the necessary sensitivity. The experiments using this method are routinely performed upon 200,000 to 3 million cells. In addition, the procedures for preparing the \( ^{125}\)I-fibrin petri dishes are rather complex and batches of plates prepared at different times will not be the same.
The most recently developed technique is the direct fluorescence assay for the plasminogen activator. The amide bond between a tripeptide, Cbz-Gly-Gly-Arg, and the fluorescent, 7-amino-4-methyl coumarin, is cleaved by urokinase and the plasminogen activator activity secreted by transformed cells into culture medium. Although it has been shown that the fluorescence is dependent upon the volume of the cell culture concentrate being assayed, it has not been shown that the amount of activator detected in the cell culture concentrate is dependent upon the number of cells in the culture or time of incubation. The assay does not have the requisite sensitivity. The activator is present in very minute amounts and there is no amplification of its activity by plasmin. The culture fluid for the assay is obtained from cultures of 10 million cells incubated for 12-hr periods. In addition, the procedure requires acidification and ammonium sulfate precipitation of the harvest fluid. This may cause spurious results. If an inhibitor, bound to the activator and preventing its expression, is released by the acidification, then plasminogen activator activity will be detected even though it may not be expressed in the intact cell.

C. Plasminogen and Plasmin

1. Properties and Mechanism of Activation

Plasminogen is the zymogen for plasmin that is present in high concentrations, up to 0.1 mg/ml, in the plasma. Plasmin is a trypsin-like endopeptidase that cleaves lysyl-lysine bonds, has a wide range of substrates, and performs a number of important physiological functions. It is capable of: 1) activating the complement system through its ability to convert $C_1$ to its active form and split anaphylatoxin ($C_{3a}$) from $C_3$; 2) enhance the formation of kinins which are potent vasodilators through its catalysis of the conversion of factor XIIa to factor XIIIf which is a prekallikrein activator; and 3) induce fibrinolysis of blood clots.
Plasmin is responsible for maintaining the circulation of blood in blood vessels and aids in inflammation due to its ability to enhance the formation of kinins.\textsuperscript{27}

Plasminogen is composed of a single peptide chain with an asparagine residue at the carboxy terminus.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{plasminogen.png}
\caption{Plasminogen (from Christman, Silverstein, and Acu, p. 95).}
\end{figure}

Two major forms of the enzyme have been isolated from human plasma. Glu-plasminogen (M.W. 89,000-92,000) which has a glutamine residue at the amino terminus and Lys-plasminogen (M.W. 81,000-95,000) which has a lysine residue at the amino terminus. Proteolytic cleavage at bond 1 in Fig. I.1 converts Gly-Pmg to Lys-Pmg with the release of an activation peptide (M.W. 6,000-9,000). Activation of plasminogen occurs when there is cleavage of an
internal Arg-Val bond (#2 in Fig. I.1). Plasmin is composed of two peptide chains: a heavy chain (HC) derived from the amino terminus and a light chain (LC) derived from the carboxy terminus, held together by a single disulfide bond.

<table>
<thead>
<tr>
<th>Component</th>
<th>Molecular Weight</th>
<th>Amino Terminal Residue (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-Pmg</td>
<td>89,000 - 92,000</td>
<td>Glu (0.94)</td>
</tr>
<tr>
<td>Lys-Pmg</td>
<td>81,000 - 85,000</td>
<td>Lys (0.88)</td>
</tr>
<tr>
<td>HC (Glu)</td>
<td>64,000 - 69,000</td>
<td>Glu (0.91)</td>
</tr>
<tr>
<td>HC (Lys)</td>
<td>58,000 - 63,000</td>
<td>Lys (0.85)</td>
</tr>
<tr>
<td>L</td>
<td>23,000 - 26,000</td>
<td>Val (0.90)</td>
</tr>
<tr>
<td>p</td>
<td>6,000 - 9,000</td>
<td>Glu (0.83)</td>
</tr>
</tbody>
</table>

(From Violand and Castellino, p. 3908.)

Enzymes capable of activating plasminogen have been isolated from a variety of different tissues,\textsuperscript{28} from plasma, and from human urine. Urokinase, a serine protease (M.W. 54,000) isolated from human urine,\textsuperscript{29} is the plasminogen activator that has been best characterized. There has been a controversy over whether urokinase can catalyze the conversion of Glu-Pmg to Lys-Pmg (i.e., cleavage of bond 1 in Fig. I.1) or if plasminogen is capable of autoactivation through the cleavage of bond 2 in Fig. I.1.\textsuperscript{30} The results of experiments performed on the activation of human plasminogen in the presence of trasyloI\textsuperscript{31} and rabbit plasminogen in the presence of bovine trypsin inhibitor\textsuperscript{32} by insolubilized urokinase demonstrate that the following scheme for plasminogen activation is most probable:

![Diagram of plasminogen activation](image-url)
Although Lys-Pmg is more rapidly activated than Glu-Pmg and is found when Glu-Pmg is activated by urokinase, it is not an obligatory intermediate in the activation mechanism. Plasminogen can also be activated by other proteases. Streptokinase forms a complex with plasminogen that catalyzes the activation of plasminogen. Most recently, a plasminogen activator has been isolated from pig heart (M.W. 54,000). The plasminogen activator (M.W. 50,000) purified from SV40 transformed hamster cells had a high cystein content indicating it has many disulfide bridges.

2. Kinetics of Serine Proteases

Plasminogen and the plasminogen activator are serine proteases. An intermediate acyl enzyme complex is formed as a result of the interaction of the catalytic serine residue on the enzyme and the acyl portion of the substrate. This intermediate must be included in the rate equations for the reactions catalyzed by these enzymes:

\[
\begin{align*}
E + S & \xrightleftharpoons[k_1]{k_2} ES \xrightarrow{k_3} ES' + P_1 \\
& \quad \text{acyl enzyme}
\end{align*}
\]

\[
E = \text{enzyme} \\
ES = \text{noncovalent enzyme-substrate complex} \\
S = \text{substrate} \\
ES' = \text{acylated intermediate} \\
P_1, P_2 = \text{first and second reaction products}
\]

\[
K_s = \frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_1}
\]

In the steady state, the equation for the velocity of the reaction will have the same form as the Michaelis-Menten equation:

\[
\begin{align*}
(A) \quad V &= \frac{d[P_1]}{dt} = \frac{d[P_2]}{dt} = \frac{(k_2 k_3)}{(k_2 + k_3)} [E_0][S] \\
& = \frac{k_{\text{cat}}[E_0][S]}{K_{\text{mapp}} + [S]}
\end{align*}
\]
(B) \[ k_{\text{cat}} = \frac{k_2 k_3}{k_2 + k_3} \]

(C) \[ K_{\text{mapp}} = K_s \left( \frac{k_3}{k_2 + k_3} \right) \]

(D) \[ V_{\text{mapp}} = k_{\text{cat}}[E_0] \]

The macroscopic constants, \( V_{\text{mapp}} \), \( k_{\text{cat}} \), and \( K_{\text{mapp}} \) can be obtained by the same methods that are used for enzymes for which Michaelis-Menten assumptions apply.

The macroscopic constants for serine proteases are functions of several microscopic constants. Determination of the microscopic constants requires following the kinetics of the reaction before it reaches the steady state.

The kinetics of reactions catalyzed by serine proteases under presteady state conditions differ from those observed for Michaelis-Menten enzymes. There is a rapid formation of the acyl-enzyme intermediate with release of \( P_1 \), referred to as the "burst," followed by a slower rate of deacylation and release of \( P_2 \). The following biphasic curve is observed when the release of \( P_1 \) is followed with time:

---

Fig. I.2 Burst kinetics of a serine protease (from Zeffren and Hall, p. 75).
This curve is described by the following equation:

\[ [P_1] = At + \pi (1 - e^{-bt}) \]

where

\[(F) \quad A = \frac{k_{cat} [E_0][S]}{[S_0] + K_{mapp}} \]

\[(G) \quad \pi = \frac{[E_0]\left(\frac{k_2}{k_2 + k_3}\right)}{(1 + K_{mapp}/[S_0])^2} \]

\[ [E_0] = \text{initial enzyme concentration} \]

\[ [S_0] = \text{initial substrate concentration} \]

\[(H) \quad b = \frac{(k_2 + k_3)[S_0] + k_3K_s}{K_s + [S_0]} \]

\[(I) \quad \Delta_t = \pi e^{-bt} \]

If \( k_3 \ll k_2 \) then \( K_{mapp} \ll [S_0] \) and the burst size \( \pi \) will be equal to the active enzyme concentration. Examination of the substrate dependence of the \( \Delta_t \) term will enable determination of the microscopic constants.

3. Serine Proteases: Inhibition by NPGB

Nitrophenyl-\( \rho \)-Guanidino Benzoic Acid (NPGB) is a potent inhibitor of serine proteases such as trypsin, plasmin, and thrombin. The \( \rho \)-guanidino-benzoyl-enzyme (PGB-enzyme) complex is very stable and its rate of deacylation is extremely slow. The deacylation rate for the PGB-plasmin complex formed from NPGB is \( 5.25 \times 10^{-6} \text{ sec}^{-1} \) \( (\pm 0.05) \).\(^{36}\) The second-order rate constant for the attack of NPGB on a serine protease can be determined from data measuring the percent of active enzyme as a function of the time and concentration of NPGB.\(^{37}\) Due to the slow rate of deacylation of the PGB-enzyme complex, it is assumed that the inhibition by NPGB is irreversible. The following equations describe the formation of the PGB-enzyme complex.
(A) \[ E + \text{NPGB} \rightarrow E \cdot \text{PGB} \]

\[ E \equiv \text{serine protease} \]
\[ E \cdot \text{NPGB} \equiv \text{PGB - enzyme complex} \]

(B) \[ \frac{d[\text{PGB}]}{dt} = k_2[E][\text{NPGB}] = k_{\text{obs}}[E] \]

(C) \[ k_{\text{obs}} = k_2[\text{NPGB}] \]

The conservation equation is:

(D) \[ [E_0] = [E] + [EI] \]

Rearranging (B) using (D) yields:

(E) \[ \frac{d[E \cdot I]}{[E]} = k_{\text{obs}} dt = \frac{d[E \cdot I]}{[E_0] - [E \cdot I]} \]

Which can be integrated:

(F) \[ -\ln([E_0] - [EI]) \left| \frac{[E]}{[E_0]} \right| = k_{\text{obs}} t \bigg|_0 \]

(G) \[ -\ln([EI]) \left| \frac{[E]}{[E_0]} \right| = k_{\text{obs}} t \bigg|_0 \]

\[ \% \text{ activity} = \frac{[E]}{[E_0]} \times 100 \]

The percent activity can be expressed as a function of time:

(H) \[ -\ln(\%/100) = k_{\text{obs}} t \]
The slope of a graph of $-\ln(x)$ vs time is the $k_{obs}$. To verify the results, the $k_{obs}$ can be measured using different concentrations of NPGB. The slope of a graph of $k_{obs}$ as a function of NPGB concentration is the second-order rate constant for the attack of NPGB on the enzyme.

D. Description of Cell Lines Used in Experiments

The RT4-71-D2, RT-D3(AC24-2) and RT-E4(AC24-3) cell lines used in the experiments were developed by Dr. N. Sueoka of the University of Colorado, Boulder. They are clonal sublines of a neurotumor, RT4, induced by the subcutaneous injection of ethyl nitrosourea. After two months, cultures of the RT4 neurotumor cells consist of cells displaying four distinct morphologies: AC, B, D, and E. The B, D, and E cell lines are the products of the in vitro differentiation of an AC stem cell. This phenomenon is referred to as "cell type conversion." The RT4-71-D2 subline was tumor derived and the RT-D3(AC24-2) and RT-E4(AC24-3) sublines were derived from a single stem cell.

Various in vivo and in vitro tests were used to characterize the clonal sublines. The AC and D sublines were shown to be tumorigenic when injected into syngeneic BDIX rats, while the B and E subclones were not. There was a low level of plasminogen activator activity and a relatively substantial amount of LETS protein in the nontumorigenic subclones. In the tumorigenic subclones, the amount of plasminogen activator activity was high, and there was very little LETS protein.
II. Fluorescein DiEster Assay for Plasminogen Activator

A. Superiority of the FDE Assay

The FDE fluorescein assay for plasminogen activator was developed in response to the need for a transformation assay that is sensitive enough to detect a small number of transformed cells, reproducible, quantitative, simple to perform, does not require a long time period, and does not harm the cells being assayed. FDE is a substrate for the esterase activity of plasmin. This molecule has several properties that make it an excellent substrate to be used in a sensitive assay for plasmin. The high quantum yield of fluorescein and efficiency of light absorption of fluorescein enable very low levels of plasmin to be detected. The half-life of the PGB-plasmin complex is $1.90 \times 10^5$ sec formed from NPGB indicating that the PGBA ester of fluorescein will also be a good substrate for plasmin. The monoester is fluorescent and thus only one cleavage is necessary for the generation of the fluorescent species. To prevent the molecule from entering the cell, the $p$-amino benzoic acid is coupled to the isothiocyanate to add charge to the molecule.

![Chemical Structure](image)

**Fig. II.A** Fluorescein DiEster  
M.W. 848 g/mole
The FDE fluorescence assay for plasminogen activator uses relatively simple procedures that do not require a great deal of time to perform. The cells to be tested are incubated with a solution of plasminogen for a certain time period; the plasminogen solution is then removed and added to a solution of FDE. The fluorescence change is dependent upon the number of cells being assayed, plasminogen concentration, and time of incubation. The quantitative nature of the assay makes it possible to measure the kinetic parameters of the plasminogen activator activity of a cell line. The sensitivity of the assay is apparent from the fact that a signal can be detected when the assay is performed on a microwell in which 100 cells are seeded.

B. Materials and Methods

1. Cell Culture

The RT4-71-1 [AC], RT4-D3(AC24-2) [D3], and RT4-E4(AC24-3) [E4] cell lines used in this paper were supplied by Dr. N. Sueoka of the University of Colorado, Boulder. They are cultured in 100 x 15 mm petri dishes (LUX) in a Forma Scientific Incubator set at 37°C, 5% CO₂. The culture medium is Dulbecco's Modified Eagle's Medium (DME) (from GIBCO) with 10% fetal calf serum (GIBCO) and antibiotics (penicillin 100 units/ml; streptomycin 100 mcg/ml). The medium for culture of the E4 cell line contains 12.5% horse serum (ISI Biologicals) and 2.5% fetal calf serum. The cells are passaged two times a week using trypsin-EDTA in Hank's balanced salt solution (GIBCO). After washing with 5.0 ml of the trypsin solution to remove cell debris, the cells are then incubated for 5 min at 37°C in 1.5 ml of the trypsin-EDTA solution. The trypsinization is terminated by the addition of 2.0 ml of the culture medium, and the suspension is added to a large volume of the culture medium. The number of cells in a cell suspension is determined using a Coulter Counter.
2. Plasminogen

The plasminogen used in the assays for plasminogen activator is β-plasminogen isolated from dog blood using a modification of the procedure developed by Deutsch and Mertz.\textsuperscript{42,43} The plasminogen is eluted from a lysine sepharose 4B column using a linear gradient of 0 to 0.03 M ε-amino caproic acid in 0.10 M KPO\textsubscript{4} buffer (pH 7.4). There are two peaks in the lysine-sepharose 4B column elution profile and the second peak consists of pure β-plasminogen (Fig. II.1). A sephadex column (G-25 medium grade) equilibrated with 0.10 M ammonium bicarbonate is used to separate the ε-amino caproic acid from the plasminogen. The plasminogen is then lyophilized and the powder is stored at -20°C.

The plasminogen solutions used in the plasminogen activator assays are prepared by dissolving the lyophilized plasminogen in PBS. The concentration is determined by measuring the absorbance of the solution at 280 nm using a Beckman ACTA CIII spectrophotometer. The extinction coefficient is 1.56 ml/mg-cm for a 1 cm pathlength at 280 nm.

3. Assay Methods

Dulbecco's phosphate buffered saline with calcium and magnesium at pH 7.3 (PBS)\textsuperscript{44} is the buffer in which the cells are incubated during an assay. The nitrophenyl β-guanidine benzoate HCl (ICN) [NPGB] is dissolved in dimethyl formamide (DMF) to a concentration of 1.00 mg/ml. The FDE stock solutions are prepared by dissolving the FDE in DMF to a concentration of 0.50 mg/ml.

Sixteen hr prior to performing an experiment, 0.100 ml aliquots of a cell suspension in complete culture medium are placed into microwells (Costar tissue culture cluster dishes of 6.4 mm diameter). After the seeding period, the medium is removed and the cells are washed three times with PBS. To each microwell 0.050 ml of PBS containing plasminogen is added. After incubation at 37°C for the indicated time intervals, 0.045 ml aliquots are withdrawn
and added to 1.0 ml solutions of PBS containing 0.75 µg of FDE. After 2 min at 25°C, 20 µg of NPGB was added to quench the reaction. The fluorescence was then measured using a Perkin-Elmer MPF-44A fluorescence spectrophotometer. The excitation wavelength was 491 nm with a slit width of 4 nm and the emission wavelength was 514 nm with a slit width of 4 nm. The sample sensitivity was 30. The change in fluorescence (ΔF) is determined by subtracting the background fluorescence of the 1.0 ml solution of PBS containing FDE from the fluorescence observed after the addition of the 0.045 ml aliquot that was incubated in the microwells.

4. Estimation of Kinetic Parameters

Due to the variability in the estimation of the $K_m$ and $V_{max}$ when the various graphical methods are used, the $K_m$ and $V_{max}$ were estimated using the method of Cleland. The initial velocity was determined from the slope of the line drawn through the data points from least squares analysis.

C. Results

1. Experiments Demonstrating the Sensitive and Quantitative Nature of the Assay

The time course of the fluorescence increase, occurring when a plasminogen solution which had been incubated in microwells containing RT5-71-02 cells assayed in an FDE solution, was monitored (Fig. 11.2). The kinetic profile exhibits a burst that is characteristic of serine proteases. The burst, due to the rapid formation of the p-guanidine-benzoate-plasmin complex with release of the fluorescent monoester, is completed within the first 45 sec of the reaction. After the burst, there is no further increase in the fluorescence within the 2-min period monitored. This indicates that the acyl-intermediate formed when plasmin reacts with FDE is very stable and has a long half-life. This was expected since the half-life of the PGB-plasmin complex formed from NPGB also has a long half-life. The fluorescence change that occurs within a 2-min period is a measure of the burst size and can be used to measure the amount of active

-19-
plasmin generated from plasminogen during the incubation. The observed fluorescence change is not increased when the FDE concentration is increased, indicating the plasmin is saturated with the FDE concentrations used in the assay.

Experiments were performed to test if the FDE assay could quantitatively measure the amount of plasminogen activator. The results of assays performed on plasminogen solutions incubated in microwells containing RT4-71-D2 cells shows the AF is linearly dependent upon the time the plasminogen solutions are incubated in the microwells. When the plasminogen concentration is held constant, the increase in AF with time is proportional to the number of cells (Fig. II.3A). For a constant number of cells, the increase in AF with time is proportional to the plasminogen concentration (Fig. II.3B). Incubation of the cells with solutions containing different plasminogen concentrations shows that the activity of the plasminogen activator is dependent upon the concentration of its substrate, plasminogen, and that it can be saturated with substrate (Fig. II.4). When the time of incubation and the plasminogen concentration are constant, the AF is linearly dependent upon the number of cells seeded. The sensitivity of the assay is apparent from the fact that a quantitative signal is detected from plasminogen solutions incubated in microwells into which as few as 250-2,500 cells were seeded (Fig. II.6).

Control experiments were performed to test the background due to active plasmin in the plasminogen solutions or FDE hydrolysis that is not plasminogen dependent. Plasminogen solutions, incubated in microwells that did not contain any cells and PBS solutions that did not contain any plasminogen which was incubated in microwells containing cells, were assayed. The minimal fluorescence change observed in these control experiments indicate that FDE hydrolysis requires a cell factor, the plasminogen activator, and plasminogen in the incubation medium. PBS solutions containing plasminogen and NPGB were incubated in microwells containing cells and then assayed. There is a decrease
In the AF as the NPGB concentration increases, which indicates that the FDE hydrolysis is dependent upon the activity of a serine protease (Fig. II.5).

2. Determination of the Second-Order Rate Constant for the Attack of NPGB upon the Plasminogen Activator

RT4-7i-D2 cells were preincubated with solutions containing various concentrations of NPGB and then washed three times with PBS to remove any unbound NPGB prior to being assayed for plasminogen activator. There is a decrease in the observed AF due to the inhibition of the plasminogen activator by NPGB. The extent of the inhibition was dependent upon the NPGB concentration and the amount of time the cells were preincubated with the NPGB solution (Fig. II.7). The inhibition by NPGB indicates that plasminogen activator is a serine protease. I assume that the PGB-plasminogen activator complex has a very long half-life similar to the intermediate acyl enzyme complexes formed between NPGB and other serine proteases. The slow rate of deacylation of the PGB-activator complex makes the inhibition of the plasminogen activator by NPGB effectively irreversible for the time period of the assay. The first-order rate constant \( k_{obs} \) was determined for three different NPGB concentrations (Fig. II.7) using the analysis presented in I.C.3. The second-order rate constant was then calculated to be \( 9 \times 10^3 \text{ M}^{-1} \text{sec}^{-1} \) (Fig. II.8).

3. Determination of the Rate-Limiting Step in the Activation of Plasminogen

Because of the sensitivity of the FDE fluorescence assay, experiments could be performed to identify the rate-limiting step in the activation of plasminogen by the plasminogen activator. Equation (A) for the plasminogen activator (PA) and its substrate, plasminogen (Pmg):

\[
\begin{align*}
PA + Pmg & \xrightarrow{k_1} PA:Pmg \xrightarrow{k_2} \text{noncovalent enzyme-substrate complex} \xrightarrow{k_3} \text{acylated-enzyme complex} \\
& \xrightarrow{k_{1}} \text{PA} + Pm \xrightarrow{k_{1}} \text{PA} + Pm
\end{align*}
\]

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If \( k_2 \ll k_3 \), then a saturating plasminogen concentration will lead to a buildup of the noncovalent intermediate. If \( k_3 \gg k_2 \), then a saturating plasminogen concentration will lead to a buildup of the covalent acyl-enzyme complex. To distinguish between the two possibilities, RTA-72-D2 cells were preincubated with PBS solutions containing 1.42 mg/ml of plasminogen for 15 min. The preincubated plasminogen solutions were removed from the cells and assayed 0, 15, 30 and 45 min after removal from cells (Fig. II.9). There is no increase in the AP with time after the plasminogen solutions are removed from the cells indicating the cells do not secrete plasminogen activator into the medium. The microwells containing cells which were preincubated with plasminogen were washed three times with PBS to remove any unbound plasminogen and then incubated with one of the following solutions:

a) Fresh 0.025 ml PBS solutions containing 1.42 mg/ml Pmg.

This was to show that the cells are still capable of activating plasminogen. This control experiment can be compared to the assays performed on microwells into the same number of cells with solutions containing the same concentration of plasminogen. The washing procedures cause the release of a small percentage of the cells from the microwell surface, but most of the cells remain attached.

b) fresh PBS solutions. No active plasmin was detected when these solutions were assayed.

The results are consistent with the formation of the acyl-enzyme intermediate being the rate-limiting step. If the decylation of the covalent complex was rate-limiting, active plasmin would be released when the cells were incubated in PBS after preincubation with plasminogen solutions. If formation of the acyl enzyme was rate-limiting, the noncovalent intermediate would accumulate when the cells were incubated with saturating plasminogen. When the plasminogen solution was replaced with the PBS solution, the equilibrium would shift toward the unbound plasminogen. No active plasmin should be detected when
PBS solutions are added to microwells containing cells preincubated with plasminogen.

4. Characterization of the Plasminogen Activator in the RT4-71-D2 and RT-D3(AC24-2) Cell Lines

Because the AF is a measure of the amount of active plasmin produced during the incubation, the rate of increase in AF is a measure of the rate that plasminogen is activated to plasmin by cellular plasminogen activator. Assays were performed upon solutions containing various concentrations of plasminogen that were incubated in microwells containing cells to determine the initial rate of plasminogen activation. To measure the $K_{m,app}$ and $V_m$, the RT4-71-D2 cells, plasminogen activator, and plasminogen solutions incubated in microwells containing 4,000 (and 8,000) cells were incubated with the PBS solutions containing 0.10-1.5 mg/ml of plasminogen for 20 min and 40 min (15 min and 30 min) (Fig. II.10). The RT-D3(AC24-3) cells were characterized by assaying plasminogen solutions incubated in microwells containing 2,000, 4,000 and 6,000 cells for 48 min, 30 min, and 20 min, respectively (Fig. II.1). The data for the two cell lines was normalized to cell number and analyzed by the method of Cleland (Table II.1).

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>$K_{m,app}$ (mg/ml)</th>
<th>$V_m$</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT4-71-D2</td>
<td>0.600 + 0.136</td>
<td>0.152 + 0.013</td>
<td>5.76 x 10^{-5}</td>
</tr>
<tr>
<td>RT-D3(AC24-3)</td>
<td>0.543 + 0.116</td>
<td>0.574 + 0.047</td>
<td>1.32 x 10^{-3}</td>
</tr>
</tbody>
</table>

Within the uncertainty of the measurement, the plasminogen activator in both cell lines has the same $K_{m,app}$, but the $V_{max}$ of the RT-D3(AC24-3) cells is 3.8 times greater than that of the RT4-71-D2. This result clearly demonstrates the advantages of using an assay method which is quantitative. The
\(^{125}\)-fibrin assay was unable to detect any differences in the amount of plasminogen activator found in tumor and stem cell derived clonal sublines.\(^ {39} \)

When measuring the plasminogen activator activity of a cell line, the \(V_{\text{max}}^1\) is the critical parameter to measure. There are two factors which determine the \(V_{\text{max}}\): the rate the individual enzyme molecule can convert substrate to product \((k_{\text{cat}})\) and the amount of enzyme present \([E_0]\). I believe that differences in the amounts of plasminogen activator activity between cell lines is due to differences in the accessibility to substrate and amount of enzyme in the cell membrane. If the plasminogen activator is shielded or inhibited by a cell-associated molecule, plasminogen will not be able to bind to it and little plasminogen activator will be detected. Uncovering the factors regarding the activity of the plasminogen activator will yield valuable information about the malignant transformation of a cell.


The amount of plasminogen activator in tumorigenic and nontumorigenic clonal sublines was compared by assaying PBS solutions containing 1.25 mg/ml of plasminogen incubated in microwells containing 10,000 RT4-E4(AC24-3), RT-D3(AC24-2), and RT4-71-D2 cells (Fig. II.12). The tumorigenic sublines had much higher levels of plasminogen activator activity than the nontumorigenic sublines which expressed minimal activity just above the background signal. This data supports the contention that tumorigenic cells express plasminogen activator while nontumorigenic cells do not.
FOOTNOTES


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Figure II.1  Profile for elution of dog plasminogen from a DEAE column in 0.1 M KPO₄ buffer (pH 7.4) with a gradient of L-amino caproic acid. The limiting buffers are 0.1 M KPO₄ (pH 7.4) and 0.1 M KPO₄ (pH 7.0) with 0.03 M L-amino caproic acid. Each fraction has a volume of 3.0 ml. The two peaks in the profile are due to the elution of Glu-plasminogen (1) and Lys-plasminogen (11).

Figure II.2  Reaction of plasmin with FDE exhibits burst kinetics. Microwells containing 3,500 RT4-71-D2 cells were incubated with PBS solutions and PBS solutions containing plasminogen at a concentration of 1.25 mg/ml for 45 min. The increase in fluorescence was monitored as a function of time while the solutions were assayed. Assays were in 1.0 ml PBS solutions containing 0.75 μg FDE(A) and 2.25 μg FDE(B).

Figure II.3  The kinetics of plasminogen activator activity as a function of
a) the number of cells and b) the plasminogen concentration:
   a) Each microwell contained a plasminogen concentration of
      0.696 mg/ml; □ 29,000 cells; ■ 15,000 cells; ○ 7,000 cells;
      ▲ 0 cells.
   b) Each microwell contains 9,000 cells; the plasminogen concentra-
      tion was □ 0.5 mg/ml; ▲ 0.20 mg/ml; ○ 0.10 mg/ml;
      ▲ 0 mg/ml.

Figure II.4  Saturation of plasminogen activator activity with plasminogen as
a function of the number of cells. Plasminogen at the indicated concentra-
trations was incubated for 90 min with □ 10,000 cells; ○ 5,000
   cells; ▲ 0 cells.

Figure II.5  Inhibition of the AF by NPGB. Each microwell contained 10,000
cells, 0.388 mg/ml of plasminogen, and the indicated concentrations of
NPGB. Incubation was for □ 90 min or ○ 60 min.
Figure 11.6  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 (○) 0.337 mg/ml plasminogen for 225 min. The data was then normalized to time.

Figure 11.7  Inhibition of plasminogen activator by NPGB. Microwells seeded with 8,000 RT4-71-D2 cells were incubated with 0.200 ml of PBS containing the indicated concentrations of NPGB for 3, 6, 9 and 12 min. After removal of the PBS containing NPGB, the cells were washed three times with PBS and then assayed for plasminogen activator activity by incubation with 0.05 ml PBS solutions containing 0.67 mg/ml of plasminogen for 30 min. One hundred percent activity was defined as the fluorescence change observed when cells which were incubated with 0.200 ml of PBS for 3, 6, 9 and 12 min were assayed for plasminogen activator: (▲) 4.5 x 10^{-5} M NPGB, (○) 3.0 x 10^{-5} M NPGB, (★) 1.5 x 10^{-5} M NPGB.

Figure 11.8  Determination of the second-order rate constant for the attack of NPGB upon the plasminogen activator: the slope of the least squares line drawn through the data points is the reported value for the second-order rate constant.

Figure 11.9  Determination of the rate-limiting step in the activation of plasminogen. Microwells seeded with 5,000 RT4-71-D2 cells were washed three times prior to preincubation with 0.025 ml PBS solutions containing 1.42 mg/ml of Pmg for 15 min. The preincubated plasminogen solution was then assayed 0, 15, 30 and 45 min after it was removed from the cells (▲). The cells which were preincubated with the plasminogen solutions were washed three times with PBS and fresh 0.025 ml volumes of PBS (▲) and PBS containing 1.42 mg/ml Pmg (■) were placed in the microwells. These were assayed after the indicated time of incubation. Microwells
containing cells that were not preincubated with plasminogen were also assayed after incubation with 0.025 ml PBS solutions containing 1.42 mg/ml of plasminogen (□).

Figure II.10  Hanes' plot for RT4-71-1 cells. Microwells which were seeded with 4,000 (and 8,000) RT4-71-1 cells in their 13th (11th) passage were assayed for plasminogen activator. The cells were incubated with PBS solutions containing plasminogen concentrations ranging from 0.10-1.50 mg/ml for 20 min (15 min) and 40 min (30 min).

Figure II.11  Hanes' plot for RT-D3(AC24-2) cells. Microwells were seeded with 2,000, 4,000 and 6,000 RT-D3(AC24-2) cells and fixed time. Plasminogen activator assays were performed after 48, 30 and 20 min as described in the Materials and Methods section to determine the initial velocity.

Figure II.12  Comparison of the amount of PA in the RT4-71-D2, RT-E4(AC24-3) and RT-D3(AC24-2) subclones. 0.05 ml PBS solutions containing 1.25 mg/ml Pmg were incubated in microwells seeded with 10,000 cells for the indicated time periods: (▲) RT-E4(AC24-3), (■) RT4-71-D2, (□) RT-D3(AC24-2).
Fig. II.9