Binding of Plasminogen to Extracellular Matrix*

(Received for publication, October 23, 1985)

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We have previously demonstrated that plasminogen immobilized on various surfaces forms a substrate for efficient conversion to plasmin by tissue plasminogen activator (t-PA) (Silverstein, R. L., Nachman, R. L., Leung, L. L. K., and Harpel, R. C. (1985) J. Biol. Chem. 260, 10346–10352). We now report the binding of human plasminogen to the extracellular matrix synthesized in vitro by cultured endothelial cell monolayers. The binding was specific, saturable at plasma plasminogen concentrations, reversible, and lysine-binding site-dependent. Functional studies demonstrated that matrix immobilized plasminogen was a much better substrate for t-PA than was fluid phase plasminogen as shown by a 100-fold decrease in $K_m$. Activation of plasminogen by t-PA and urokinase on the matrix was equally efficient. The plasmin generated on the matrix, in marked contrast to fluid phase, was protected from its fast-acting inhibitor, $\alpha_2$-plasmin inhibitor. Matrix-associated plasmin converted bound Glu into Lys-plasminogen, which in turn is more rapidly activated to plasmin by t-PA. The extracellular matrix not only binds and localizes plasminogen but also improves plasminogen activation kinetics and prolongs plasmin activity in the subendothelial microenvironment.

The serine protease plasmin plays an important regulatory function in a variety of physiological and pathophysiological processes (1). In addition to its intravascular fibrinolytic properties (2), plasmin is also involved in modifying the extracellular environment during ovulation (3), embryogenesis (4), inflammation (5), wound healing (6), and neoplasia (7, 8).

In the fluid phase, plasminogen activation by tissue plasminogen activator (t-PA*) (9) is kinetically unfavorable with a $K_m$ greater than the plasma plasminogen concentration (10).

* This work was supported by National Heart, Lung, and Blood Institute Grant HL38829 (Specialized Center of Research in Thrombosis) and Grant HL30849 from the National Institute of Health. Additional support was provided by the Krakower Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: t-PA, tissue plasminogen activator; LBS, lysine-binding sites; TSP, thrombospondin; $\alpha_2$PI, $\alpha_2$-plasmin inhibitor; EACA, $\epsilon$-amino-$\delta$-caproic acid; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polycrylamide gel electrophoresis; RFU, relative fluorescence units; ELISA, enzyme-linked immunosorbent assay; HEFES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Immobilization of plasminogen on fibrin (11, 12) via lysine-binding sites (LBS) (13, 14) and also of t-PA dramatically improves kinetic parameters and makes plasminogen activation possible at circulating plasminogen concentrations.

The extracellular matrix produced by endothelial cells is a complex array of glycoproteins and glycosaminoglycans that contribute to the formation of the basement membrane (15), a barrier to penetration of cells and macromolecules through the vessel wall (16). Local destruction of the basement membrane by hydrolytic enzymes including plasmin allows cellular migration through the heavily cross-linked matrix network (17). Plasmin specifically cleaves thrombospondin (18), fibronectin (19), laminin (19), and von Willebrand Factor (20) and also exposes matrix components for degradation by other enzymes (21).

Endothelial cells grown under in vitro conditions secrete matrix constituents including fibronectin (22), collagen (23), laminin (24), von Willebrand Factor (25), and thrombospondin (TSP) (26). These adhesive glycoproteins together with proteoglycans are incorporated into the matrix network, adherent to the culture plate. Such matrix-coated surfaces have been used as model systems to study the effects of degradative enzymes secreted by malignant and nonmalignant cells on matrix components (27).

We have previously demonstrated that plasminogen binds with high affinity to TSP (28) and histidine rich glycoprotein (29) immobilized on polystyrene microtiter wells or Sepharose beads. In this fibrin free system, t-PA specifically bound to the TSP-plasminogen complex (30) and the efficiency of plasminogen activation by t-PA was greatly enhanced compared to the fluid phase (31). Furthermore, the generated plasmin was protected from its fast-acting inhibitor, $\alpha_2$-plasmin inhibitor ($\alpha_2$PI). We have now extended these studies to a more biologically relevant surface, endothelial cell extracellular matrix. Our studies demonstrate that plasminogen binds specifically and saturably to the extracellular matrix and is activated to plasmin in a kinetically favorable manner by t-PA. Plasmin generated on the matrix is protected from inhibition by $\alpha_2$PI.

EXPERIMENTAL PROCEDURES

Materials—Lysine agarose was purchased from Pharmacia, $[^{38}S]$ methionine and $[^{3}H]$proline from New England Nuclear, $\epsilon$-$\alpha$-amino-$\delta$-caproic acid (EACA) and d-phenyl-d-alanyl-d-prolyl-l-arginine chloromethylketone from Calbiochem-Behring and bovine serum albumin (BSA) from Pentex. Tissue culture media were purchased from Mallinckrodt Bioproducts, heparin and gelatin from Sigma and tissue culture plates from Falcon. Calf skin gelatin was obtained from Gelatin Products. Other chemicals were purchased from Sigma or obtained from suppliers cited in the text.

Purified Proteins—Plasminogen was prepared from acid citrate dextrose anticoagulated plasma by affinity chromatography on lysine-

10765
agaroase as described by Deutsch and Mertz (32). All purification steps were performed in the presence of 0.01% soybean trypsin inhibitor. Glu- and Lys-plasminogen were further separated by rechromatography on a lysine-agaroase column using a 0-20 mM EACA gradient (33). By comparison on 7.5% SDS-PAGE, the first peak was shown to contain pure Glu-plasminogen form I whereas the second peak corresponded to Lys-plasminogen (34). Recombinant t-PA was generously provided by Genentech, Inc., human high molecular weight urokinase by Sterling Winthrop Research Institute.

Radioiodo Labeling—Plasminogen was labeled with I125 by the modified chloramine-T method (36) as previously described (31).

Preparation of Extracellular Matrices—Human umbilical vein endothelial cells (HUVECs) were used to prepare extracellular matrices. HUVECs were harvested from umbilical cords by standard techniques (37). Immunofluorescence purified anti-plasminogen Fab, segments (35) did not react by enzyme-linked immunosorbent assay (ELISA) with purified human fibrogenin, albumin, fibronectin, thrombospondin or Factor VII1R:Ag (28). Anti-human t-PA serum was a generous gift from Dr. Nils Bang, Eli Lilly, Indianapolis, IN. Dr. F. Castellino (Notra Dame University, South Bend, IN).

Cell Culture—Human umbilical vein endothelial cells were obtained from umbilical veins by mild collagenase treatment as previously described (38). Cells were cultured in 75-cm2 tissue culture flasks coated with calf skin gelatin (2 mg/ml) and passed into 96-well plates (Falcon) at passage 2-4. Cells were maintained at 37 °C in a humidified 93% air, 7% CO2 atmosphere in complete culture medium, consisting of Medium 199 supplemented with 25 mM HEPES, pH 7.4, 2 mM l-glutamine, 100 units/ml penicillin, 100 

RESULTS

Plasminogen Binding to the Endothelial Cell Matrix—An ELISA system was developed to measure plasminogen binding to endothelial cell matrices deposited on the bottom of 96-well plates. Matrix composition determined both by incorporation of [3H]proline and by anti-von Willebrand Factor immunoreactivity was found to be nearly identical among cultures from different individuals, passage number, and pooled human serum lots. After a 4-day culture in complete medium, 2900 ± 300 cpm (mean ± S.E.) labeled matrix proteins were eluted per well with 2 M NaOH. Binding of Glu- and Lys-plasminogen to matrix-coated wells was specific and was saturated with an approximate 1.0 and 0.2 μM, respectively (Fig. 1). Under the same conditions, neither albumin nor fibrogenin showed significant binding to the matrix. 10 mM tranexamic acid (data not shown) or 10 mM EACA strongly inhibited the binding of Glu-plasminogen to the fibrin free surface of endothelial cell matrices (Fig. 2) suggesting the involvement of LBS in plasminogen matrix interactions. Specific EACA inhibitable binding saturated at a concentration of 1 μM Glu- or Lys-plasminogen (Figs. 1 and 2). 10–20% of Glu-plasminogen binding was unaffected by EACA and was considered nonspecific. Similarly, 50–90% of Lys-plasminogen binding was inhibited by 10 μM EACA (data not shown). At 37 °C, the amount of Glu-plasminogen bound increased rapidly and reached a maximum after 3 h (Fig. 3A). The binding of Glu-plasminogen to the extracellular matrix was reversible by EACA; 60% of total counts were released from the matrix after a 12-h incubation (Fig. 3B). A 500-fold excess of cold plasminogen reduced the binding of 125I-plasminogen to the matrix by 80%. Ca2+ did not effect plasminogen binding to the matrix (data not shown).

Inhibition of Glu-Plasminogen Binding by EACA—Since plasminogen interacted with the gelatin coating used for cell culture, it was necessary to show that the observed plasminogen binding to the extracellular matrix did not occur to exposed gelatin not covered by matrix proteins. Unlike matrix, Glu-plasminogen binding to gelatin (Fig. 4) was only 60% inhibitable, even at highest (50 mM) EACA concentrations tested. 50% inhibition occurred at 750 μM EACA sug-
Plasminogen Binding to Matrix

10767

FIG. 1. Binding of Glu- and Lys-plasminogen, albumin, and fibrinogen to endothelial cell matrices. Human umbilical vein endothelial cells were cultured in 96-well plates and removed as described under "Experimental Procedures." The adherent matrices were incubated with 0.1% BSA for 1 h at 37 °C. After washing Lys-plasminogen (A), Glu-plasminogen (C), albumin (E), or fibrinogen (B) were added in triplicate wells and incubated for 3 h at 37 °C. Binding was detected with the appropriate antibodies using the ELISA assay system as described under "Experimental Procedures." Color generation was followed after the addition of the substrate p-nitrophenyl phosphate in a Titertek multiscan photometer. The reaction was expressed as the enzymatic activity of the bound alkaline phosphatase (\(\text{A}_{405}\) nm min\(^{-1}\)). The values represent the mean of triplicate studies.

FIG. 2. Glu-plasminogen binding to the endothelial cell matrix in the presence of EACA. Matrices were incubated at 37 °C for 1 h with 0.1% BSA. After washing, Glu-plasminogen was added at the indicated concentrations and incubated at 37 °C for 3 h in the absence (○) and presence (●) of 10 mM EACA. The amount of bound plasminogen was quantified by ELISA using monospecific alkaline phosphatase-coupled antibodies. Specific binding (●) was calculated by subtracting EACA noninhibitable binding (○) from total binding (○). The values represent the mean of triplicate studies.

FIG. 3. Time dependence and reversibility of Glu-plasminogen binding to the matrix. Endothelial cell matrices were prepared as described and incubated with 0.1% BSA for 1 h at 37 °C. A, time dependence of Glu-plasminogen binding to the endothelial cell matrix. Plasminogen (50 pg/ml) was added in the absence and presence of 10 mM EACA. At indicated time points, wells were washed and antiplasminogen antibodies coupled to alkaline phosphatase were added. Specific binding was determined as in Fig. 2 and expressed as a percentage of maximal binding after 3 h incubation. The values represent the mean of triplicate studies. B, reversibility of Glu-plasminogen binding. Matrices were incubated with 0.2 ml of \(125^\text{I}\)-Glu-plasminogen (100 pg/ml; 10\(^6\) cpm/pg) for 3 h at 37 °C. After washing, 0.2 ml of 10 mM EACA in TBS/Tween was added, and 20-µl aliquots were removed from triplicate wells at each timepoint and counted. Total counts released into the supernatant were calculated and expressed as percentage of maximal cpm released after overnight incubation with 2 M NaOH.

FIG. 4. Inhibition of Glu-plasminogen binding by EACA. Glu-plasminogen (50 µg/ml) was added to wells covered with matrix (●) or gelatin (○) together with increasing amounts of EACA and the binding measured as described in Fig. 1 with an alkaline phosphatase-conjugated antiplasminogen antibody. All measurements were performed in triplicate wells. The stippled line indicates 50% inhibition of Glu-plasminogen binding.

gestating the contribution of low affinity LBS in the interaction between Glu-plasminogen and gelatin. In contrast, only 40–60 µM EACA were necessary to prevent Glu-plasminogen binding to the matrix. Qualitative differences between Glu-plasminogen gelatin and Glu-plasminogen matrix interactions most likely account for the 15-fold increase in the EACA concentration necessary to reduce 50% of Glu-plasminogen binding to gelatin.

Kinetics of Glu-Plasminogen Activation on the Matrix—Using the fluorometric plasmin substrate, Glu-plasminogen

Experimental Procedures.
activation was studied and found to follow Michaelis-Menten kinetics. The overall reaction rate was second order due to a two-step mechanism consisting of the initial conversion of plasminogen to plasmin and subsequent substrate hydrolysis by the plasmin generated. The amount of plasmin formed was proportional to the amount of Glu-plasminogen bound, as shown in Fig. 5.

While in the fluid phase, Glu-plasminogen activation by urokinase occurred much more rapidly than activation by t-PA; on the matrix the rates of Glu-plasminogen activation by t-PA and urokinase were almost the same (Fig. 6). The matrix, similar to fibrin (40), was able to accelerate t-PA-mediated Glu-plasminogen activation to rates comparable to urokinase. Both the rate and the final amount of plasmin generated were approximately the same when equal units of t-PA and urokinase were used for matrix-immobilized Glu-plasminogen activation. The matrix therefore provided a highly favorable environment for t-PA-mediated plasminogen activation.

To determine the kinetic parameters of Glu-plasminogen activation by t-PA, a Lineweaver-Burke plot was constructed using matrix-immobilized Glu-plasminogen and fluid phase t-PA (Fig. 7). The absolute amount of Glu-plasminogen bound per well was derived from a standardized ELISA, and the initial rate of Glu-plasminogen activation was measured in parallel wells. A reaction volume of 38.5 µl was estimated by multiplying the area of the well by a unit height of 1 to determine the concentration of Glu-plasminogen on the matrix. Under these conditions, the apparent $K_m$ for the reaction was found to be 12 nM. t-PA binding to endothelial cell matrices was also demonstrated using an ELISA system similar to that developed to detect plasminogen binding. ² A Lineweaver-Burke plot was then constructed with fluid phase Glu-plasminogen and matrix-immobilized t-PA (Fig. 8). Activation of Glu-plasminogen by immobilized t-PA occurred with an apparent $K_m$ of 24 nM. The similarity between the two $K_m$ values suggested that the affinity between enzyme and substrate was independent of the sequence of addition of Glu-plasminogen and t-PA to the matrix. Compared to the fluid phase ($K_m$ of 2.5 µM, Fig. 7) the matrix surface decreased the $K_m$ by 100-fold and thereby allowed plasminogen activation to occur at plasma plasminogen concentrations.

The mechanism of activation on the matrix was further investigated by analysis of reaction intermediates. As shown in Fig. 9, matrix-immobilized Glu-plasminogen was rapidly converted into Lys-plasminogen (lanes 1-3). After 30 min a large proportion of Glu-plasminogen comigrated with Lys-plasminogen on SDS-PAGE (lane 2). At the same time, the appearance of plasmin light chain was observed after reduction (lane 4). After a 2-h activation little Glu-plasminogen

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² B. S. Knudsen, R. L. Silverstein, and R. L. Nachman, in preparation.
was added together with plot was obtained as described in Fig.

Matrix-coated wells were incubated with unreduced Glu- and Lys-plasminogen at a relative molecular system. Due to incomplete reduction some plasminogen comigrates easily identified although a faint band was detectable in the original gel at a relative molecular weight of 65.

was eluted from the matrix (lane 3); the predominant species was Lys-plasminogen. From amidolytic data approximately 20% of bound plasminogen was converted to plasmin at 2 h.

Protection from \( \alpha_2 \)PI—In the fluid phase plasmin was rapidly inhibited by its fast-acting inhibitor, \( \alpha_2 \)PI. 50% inhibition occurred at a molar ratio of approximately 1:1 (Table I). When Glu-plasminogen was first bound to the matrix and t-PA was added together with \( \alpha_2 \)PI, a 30-fold excess of \( \alpha_2 \)PI was necessary to achieve 50% inhibition. Even at a 100-fold excess of \( \alpha_2 \)PI, 20% of the total plasmin activity generated during the activation process was not affected during the 1 h assay period, whereas in the fluid phase inhibition occurred instantaneously.

**DISCUSSION**

This study demonstrates that plasminogen specifically interacts with the endothelial cell matrix. The binding was saturable at plasma plasminogen concentrations, reversible, lysine-binding site-dependent, and greatly enhanced the rate of activation by t-PA. Matrix-immobilized plasminogen was a much better substrate for t-PA than fluid phase plasminogen as shown by a 100-fold decrease in \( K_m \). Activation of plasminogen by t-PA and double chain urokinase on the matrix was equally efficient. The plasmin generated on the matrix, in marked contrast to fluid phase, was protected from its fast-acting inhibitor \( \alpha_2 \)PI. Matrix-associated plasmin converted matrix-bound Glu- into Lys-plasminogen, which in turn is more rapidly activated to plasmin by t-PA (10). Thus, a powerful potentiation of plasmin production on the matrix was achieved.

The protection of plasmin from circulating inhibitors is an important factor for the prolongation of proteolytic activity since in the fluid phase plasmin is rapidly inactivated by various inhibitors (43, 44). The most potent inhibitor, \( \alpha_2 \)PI binds to plasminogen and plasmin LBS (35, 45). When these sites were already occupied by matrix proteins, even a 100-fold excess of inhibitor did not abolish all enzymatic activity, and matrix proteolysis still occurred.

The exact mechanism of plasminogen activation on a fibrin clot remains controversial (34, 42). Elution of plasminogen activation products from the fibrin surface suggests that Glu-plasminogen is directly converted to plasmin (34, 46). In addition it has recently been suggested that fluid phase activation of plasminogen in the plasma milieu results in the formation of Glu-plasmin-\( \alpha_2 \)PI complexes (47). It is of interest that gel analysis of matrix-bound activation products showed that the N-terminal cleavage of Glu- into Lys-plasminogen occurred quickly (Fig. 9). Therefore at least in this system, the hydrolysis of the peptide bond associated with the conversion of Lys-plasminogen into plasmin may be the rate-limiting step. Lys-plasminogen is an excellent substrate for t-PA and considerable activation takes place even in the fluid phase (43). It is therefore conceivable that the increase in the efficiency of plasmin generation on the matrix is due to a
much higher Lys-plasminogen/Glu-plasminogen ratio when compared to fluid phase as well as to conformational changes in Glu-plasminogen and t-PA.

The identity of the matrix plasminogen-binding site is not known. Exposed lysine or arginine residues are most likely involved in the interaction since binding was >80% inhibitable by EACA. Recently the interaction of plasminogen with immobilized but not with fluid phase fibronectin has been shown for an in vitro system (48). In addition endothelial cells are known to synthesize TSP and to incorporate it into their extracellular matrix (26). Immobilized TSP specifically binds plasminogen via high affinity LBS and enhances its activation in a fibrin-free environment (51). High affinity LBS are also involved in the binding of Glu-plasminogen to the matrix. TSP and/or fibronectin may therefore also function as plasminogen-binding sites in the matrix network, and their role in matrix-mediated plasminogen activation is under current investigation using matrices derived from different cell systems.

Mutual interaction between cells and matrix provides a dynamic reciprocity for the regulation of cellular movement, proliferation, and differentiation (49). Modification of specific matrix constituents by plasmin may change the effects of the matrix on cell function and may therefore play an important role in the control of cell matrix interactions. These interactions influence cellular protein synthesis (50), cell differentiation (51), and morphology (52) which in turn may lead to the production of a different matrix particularly during tissue remodeling, wound healing, and organ regeneration (53, 54). Changes in the matrix may not only increase the number of plasminogen-binding sites, but may also regulate plasminogen activation since the efficiency of plasminogen activation may vary with matrix composition. Plasminogen binding is not restricted to endothelial cell matrix as we have recently also demonstrated specific, saturable binding to smooth muscle cell matrix. In addition recent data from our laboratory suggest that plasminogen also binds to the endothelial cell surface.

In addition to its role in inflammation (5) and wound healing (6), plasmin may also mediate diapedesis of cells through the vessel wall (55). Activated macrophages (56) and endothelial cells (57) as well as certain tumor cell clones (58) secrete high levels of PAs so that plasminogen activation and plasmin formation occur efficiently in a localized area during cellular movement from the vessel lumen into the surrounding tissue. Furthermore, the generation of active plasmin on the matrix rather than on the cell surface (59) may be of advantage in the breakdown of vascular tissue during inflammation and metastasis. The extracellular matrix may therefore function as an important mediator of plasminogen binding and activation in various biological processes.

Acknowledgments—We would like to thank Barbara Ferris for excellent technical help and Dr. Eric Jaffe for help with the endothelial cell culture techniques.

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Plasminogen Binding to Matrix

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