

The Plasminogen System and Cell Surfaces: Evidence for Plasminogen and Urokinase Receptors on the Same Cell Type

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Abstract. The capacity of cells to interact with the plasminogen activator, urokinase, and the zymogen, plasminogen, was assessed using the promyeloid leukemic U937 cell line and the diploid fetal lung GM1380 fibroblast cell line. Urokinase bound to both cell lines in a time-dependent, specific, and saturable manner ($K_d = 0.8\text{--}2.0$ nM). An active catalytic site was not required for urokinase binding to the cells, and 55,000-mol-wt urokinase was selectively recognized. Plasminogen also bound to the two cell lines in a specific and saturable manner. This interaction occurred with a K_d of $0.8\text{--}0.9$ μM and was of very high capacity ($1.6\text{--}3.1 \times 10^7$ molecules bound/cell). The interaction of plasminogen with both cell types was partially sensitive to trypsinization of the cells and required an unoccupied high affinity lysine-binding site in the ligand. When plasminogen was added to the GM1380 cells, a line with high intrinsic plasminogen

activator activity, the bound ligand was comprised of both plasminogen and plasmin. Urokinase, in catalytically active or inactive form, enhanced plasminogen binding to the two cell lines by 1.4–3.3-fold. Plasmin was the predominant form of the bound ligand when active urokinase was added, and preformed plasmin can also bind directly to the cells. Plasmin on the cell surface was also protected from its primary inhibitor, α_2 -antiplasmin. These results indicate that the two cell lines possess specific binding sites for plasminogen and urokinase, and a family of widely distributed cellular receptors for these components may be considered. Endogenous or exogenous plasminogen activators can generate plasmin on cell surfaces, and such activation may provide a mechanism for arming cell surfaces with the broad proteolytic activity of this enzyme.

EXPRESSION of specific proteinase activities at cell surfaces is a phenotypic feature of a variety of cells and is a frequently elicited response of cells to stimulation and transformation (22, 32, 33, 36). Plasminogen activators (PA)¹ are among the most broadly distributed of such cell surface proteinases (27, 37, 39, 40). Urokinase (UK) is representative of one of the two major types of PA enzymes. This type of PA is expressed by a variety of cells including many tumor cells (3, 27, 37) and activated mononuclear cells (27, 33, 36). UK is highly specific for plasminogen and can activate the zymogen to plasmin by cleavage of a single peptide bond (26). Plasmin, in addition to its primary role in the proteolysis of fibrin, is a broad spectrum proteinase which may participate in such general functions as cell migration and differentiation, tissue remodeling, and inflammation (9, 17, 25). Control of UK synthesis and secretion and production of specific inhibitors of PA and plasmin are identified

mechanisms for regulation of the proteolytic activity of the plasminogen system. Recently, evidence has been provided for the presence of specific binding sites for UK on cell surfaces, suggesting a previously unconsidered mechanism for the regulation of the plasminogen system. Vassalli et al. (34) showed the presence of specific binding sites for UK on the U937 cell line which has certain monocyte-like properties, and on human peripheral blood monocytes; Del Rosso et al. (6) demonstrated the interaction of this ligand with 3T3 cells and a Rous sarcoma transformed line of 3T3 cells; and Stoppelli et al. (29) demonstrated that the UK binding capacity of U937 cells could be regulated with phorbol myristate acetate. Taken together, these studies provide direct evidence for the existence of cell surface binding sites for UK. The results of these studies differ with regard to the requirement for catalytically active UK in the interactions. U937 cells bind both active and diisopropyl fluorophosphate-treated UK (34), and this interaction is apparently mediated by the amino-terminal region of the ligand (29). In contrast, binding to the 3T3 cells requires an active catalytic site in the enzyme (6).

In this study, we show for the first time that nucleated cells

1. *Abbreviations used in this paper:* 6-AHA, 6-aminohexanoic acid; DIP-plasmin and DIP-UK, diisopropylphosphoryl-plasmin and -urokinase obtained by treating plasmin or UK with 5 mM diisopropyl fluorophosphate; PA, plasminogen activator; UK, urokinase.

bind plasminogen with very high capacity. We verify the presence of specific UK binding sites on two cell types: a catalytically active enzyme is not required for these interactions. These same cells not only bind plasminogen and UK, but also UK influences plasminogen binding to the cells, raising the possibility of a plasmin-generating system on cell surfaces. Evidence is also presented to indicate that plasmin may remain cell surface associated, suggesting a mechanism to arm the cell with the broad proteolytic activity of this enzyme. Finally, cell-bound plasmin is protected from its primary inhibitor, α_2 -antiplasmin.

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Materials and Methods

Proteins

Glu-plasminogen was purified from fresh frozen human plasma by affinity chromatography on lysine-Sepharose followed by gel filtration (7). The characteristics of the plasminogen used in this study in both nonlabeled and radiolabeled form have been previously described (19). UK, predominantly of 55,000 mol wt, was purified from Winkinase (125,000 CTA U/mg), provided by Winthrop Laboratories, New York, NY, by affinity chromatography on benzamidine-Sepharose (37). ^{125}I -UK was prepared by a modified chloramine T labeling procedure (16) with specific activities of 1–3 $\mu\text{Ci}/\mu\text{g}$. As reported by Pannell and Gurewich (23), radioiodination of UK resulted in some loss of enzymatic activity. As determined by the capacity of ^{125}I -UK to activate plasminogen as measured with the tripeptide substrate S2251 (30) (Helena Laboratories, Beaumont, TX), the radiolabeled preparations used in this study retained 23–34% of enzymatic activity. Although Coomassie Blue staining after SDS PAGE showed only minimal amounts of low molecular weight UK within the preparations used, autoradiography of ^{125}I -UK indicated the presence of this species. Therefore, for selected experiments, particularly for Scatchard analyses, low molecular weight UK within the ^{125}I -UK preparation was removed by gel filtration on Sephadex G75. DIP-UK was prepared by treating ^{125}I -UK or UK with 5 mM diisopropyl fluorophosphate for 60 min at 22°C. Using a synthetic substrate assay (30), the extent of inactivation by diisopropyl fluorophosphate was at least 95%. α_2 -Antiplasmin (lot No. 5/21/85) was purchased from American Diagnostica Inc., Greenwich, CT.

Cell Culture and Ligand Binding Assays

Two model cell lines were used in this study: the mycoplasma-free human myeloid leukemic cell line, U937, was provided by Dr. Hillel Koren of Duke University and was used to permit comparison of the UK binding data with that of Vassalli et al. (34); the diploid fetal lung fibroblast cell line, GM 1380, was obtained from the Genetic Mutant Repository, Camden, NJ. Both cell lines express cell associated PA activity as determined on ^{125}I -fibrin plate assays in the presence of plasminogen (33). The PA activity of the GM 1380 cells was 5–10-fold greater than that of the U937 cells; i.e., 10 times more U937 cells were required to release the same amount of radioactivity from the fibrin plates within the same time period (3 h).

U937 cells were grown in RPMI 1640 containing 10% FCS. For use in ligand binding assays, the cells were washed three times in RPMI 1640 and resuspended to a final concentration of $1 \times 10^7/\text{ml}$ (as determined with a hemocytometer) in RPMI 1640 containing 0.05 M HEPES, pH 7.4, and 0.1% BSA. ^{125}I -UK or ^{125}I -plasminogen was added, maintaining a total vol of 0.2–1.0 ml. At selected time points, triplicate 50- μl aliquots were removed from the mixtures, layered onto 300 μl of 20% sucrose, and centrifuged for 2 min in a Beckman microfuge. Tips of the centrifuge tubes were amputated and counted for radioactivity. On the basis of protein determinations of cell pellets, recoveries of the U937 cells exceeded 95%. For studies with the GM 1380 cells, the cells were seeded into six (3.4 cm i.d.) or 12 (2.2 cm) Costar wells (Costar, Cambridge, MA) at 4 or 1×10^5 cells/well, respectively. The cells were grown in DME containing 10% FCS for 3–4 d at which time confluence was reached. Before use, the medium was removed, and the cells were washed twice with DME. Ligand binding was measured in DME containing 0.1% BSA, maintaining a total vol of 1 ml in the 6-well and 0.4 ml in the 12-well plates. Radiolabeled ligands were added and, at selected time

points, fluid was removed by aspiration, and the cells were washed twice with DME. Cell-bound radioactivity was then extracted in 0.1% SDS. In selected experiments, the cell-bound radioactivity was extracted first with 1% deoxycholate and then with SDS to distinguish cell surface-associated from matrix-bound ligand (18). GM 1380 cell concentrations were determined on the basis of DNA content (8) of selected wells in each experiment, and DNA content was converted to cell number based on a determined value of 21 pg DNA per cell. For selected experiments, the U937 and GM 1380 cells were cultured for 3–4 d in FCS which had been acidified at pH 3.2 for 2 h to destroy certain plasmin inhibitors (33) and depleted of plasminogen by passage over lysine-Sepharose. By immunodiffusion analysis with an antiserum reactive with bovine plasminogen, the extent of plasminogen depletion exceeded 90%. In addition, for experiments in which the effect of the lysine analog, 6-aminohexanoic acid (6-AHA), on the interactions was tested, binding was measured in Hank's balanced salt solution (Gibco, Grand Island, NY) containing 0.03 M HEPES, pH 7.3, to exclude lysine from the medium. In binding experiments, cytotoxicity was determined by cell counting, cytotoxicity on the basis of trypan blue exclusion, and degradation of ligands by changes in the precipitability of radioactivity in 15% TCA. The latter analysis was performed by extracting the cell-bound ligand with 0.1% SDS at selected time points and subjecting the extract to precipitation with 15% TCA for 20–30 min at 4°C.

Molecules of ligand bound per cell were calculated based on the specific activities of each ligand. Specific binding was measured as the difference in the radioactivity bound to the cells in the presence and absence of a 50-fold molar excess of nonlabeled ligand. Dissociation constants (K_d s) were determined from specific binding isotherms using a nonlinear curve-fitting computer program, Ligand, of Munson and Rodbard (21) as previously applied in studies of plasminogen binding to platelets (19). Where indicated, results are reported as means \pm SD. Linear correlation coefficients were calculated with a TI-55-II calculator (Texas Instruments Inc., Dallas, TX).

SDS PAGE

Cell-bound ligands were extracted with 2% SDS which solubilized $\geq 95\%$ of the radioactivity and the extracts were boiled for 5 min before electrophoresis. SDS PAGE was performed on 8×10 cm vertical slab gels. UK was analyzed on 10% polyacrylamide gels under nonreducing conditions and plasminogen on 7% gels under reducing conditions in the buffer system of Laemmli (12). The plasmin/ α_2 -antiplasmin complex (see Fig. 7) was analyzed on 6% polyacrylamide gels in the buffer system of Weber and Osborn (38). Gels were stained with Coomassie Blue, dried, and autoradiograms were developed with XRP-1 film (Eastman Kodak, Rochester, NY). The autoradiograms were scanned using a Zeineh soft laser densitometer equipped

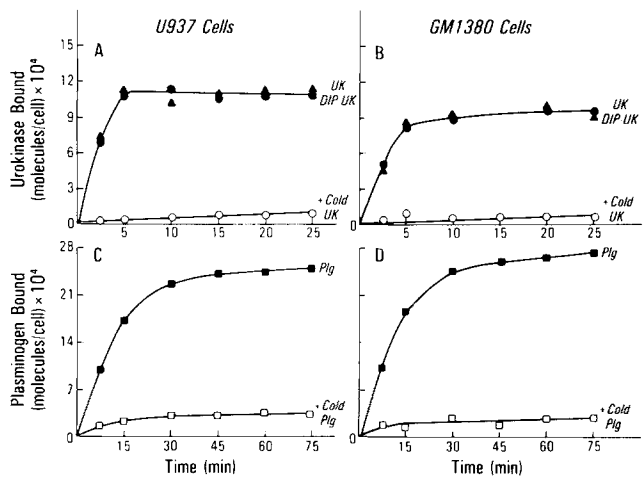


Figure 1. Time courses of ^{125}I -urokinase and ^{125}I -plasminogen binding to U937 and GM 1380 cells at 37°C. ^{125}I -UK in active or inactive (DIP-UK) form were added at 0.5 nM. Excess nonlabeled UK was used at 500 nM to assess the saturability of binding. ^{125}I -Plasminogen was added at 0.1 μM and nonlabeled plasminogen at 20 μM . For U937 cells, bound ligand from free ligand was separated by centrifugation through 20% sucrose. For the GM 1380 cells, the cell monolayer was washed by aspiration, and the bound ligand was extracted with SDS.

with an integrator (Biomed Instruments Inc., Fullerton, CA). Molecular weights were estimated relative to standard proteins obtained from Sigma Chemical Co. (St. Louis, MO).

Results

As shown in Fig. 1, the human myeloid leukemic cell line, U937, and the diploid fetal fibroblast cell line, GM 1380, bound ^{125}I -UK in a time-dependent manner at 37°C . With both cell lines, binding was rapid and reached apparent equilibrium within 5 min. DFP-treated ^{125}I -UK also bound to both cell lines, and the rate and extent of binding were similar to that observed with the untreated ligand. Binding of ^{125}I -UK to the cells was saturable as indicated by the capacity of excess nonlabeled UK to inhibit binding of the radiolabeled ligand. At the input concentration of ^{125}I -UK used, 0.5 nM, inhibition by a 50-fold molar excess of nonlabeled UK at a 30-min time point was 90% for the U937 cells and 92% for the GM 1380 cells. Binding of untreated or DFP-treated ^{125}I -UK was inhibited by either nonlabeled DIP-UK or active UK, exceeding 85% in all cases. As shown in Fig. 1, C and D, these same cell lines were also capable of binding ^{125}I -plasminogen. U937 and GM 1380 cells bound ^{125}I -plasminogen with a similar time course and apparent equilibrium was attained within 30–60 min. This interaction was also inhibited by excess nonlabeled plasminogen, indicating only low levels of nonsaturable binding.

The specificity of the interactions of ^{125}I -UK and ^{125}I -plasminogen with the cells was determined by assessing the capacity of a panel of unrelated proteins to inhibit binding of the radiolabeled ligands. As noted above and verified in Table I, a 50- to 100-fold molar excess of nonlabeled UK or plasminogen produced >83% inhibition of the binding of the respective radiolabeled ligands to either cell line. At a 100-fold or greater molar excess, myoglobin, immunoglobulin G, transferrin, fibrinogen, and ovalbumin produced <11% inhibition of the binding of ^{125}I -UK and <19% inhibition of ^{125}I -plasminogen binding to the two cell lines.

Table I. Specificity of Urokinase and Plasminogen Binding to U937 and GM1380 Cells

Nonlabeled proteins	Inhibition of binding (%)	
	U937	GM1380
	Urokinase	
Myoglobin	0 (+11)	0
IgG	7	10
Transferrin	0 (+8)	11
Fibrinogen	6	0 (+1)
Ovalbumin	0	0 (+6)
Urokinase	88	94
	Plasminogen	
Myoglobin	0	2
IgG	19	0
Transferrin	15	6
Fibrinogen	0 (+1)	3
Ovalbumin	0 (+6)	0 (+10)
Plasminogen	92	83

^{125}I -UK and ^{125}I -plasminogen were present at 2 nM and 100 nM, respectively. The nonlabeled proteins were at final concentrations of 2–10 μM . Binding at 37°C was measured after 30 min for ^{125}I -UK and 60 min for ^{125}I -plasminogen. The numbers in parentheses indicate the extent of increased UK- or plasminogen-binding to the cells in the presence of the added nonlabeled proteins relative to the absence of the proteins.

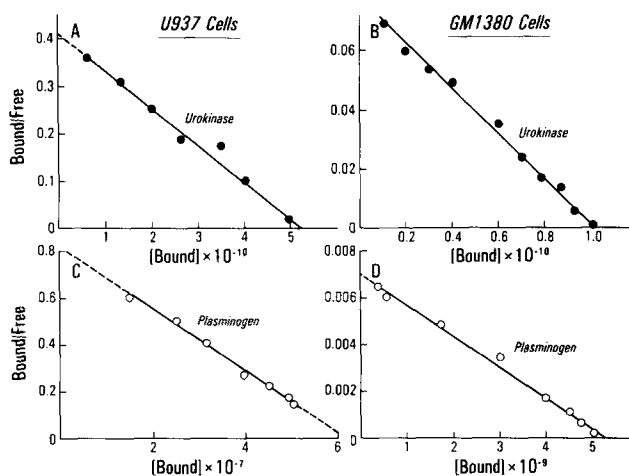


Figure 2. Scatchard plots for the binding of urokinase and plasminogen to the U937 and GM 1380 cells. The data points for these plots were derived from specific binding isotherms for the two ligands with each cell type. Binding isotherms for UK were constructed by adding varying amounts of ^{125}I -DIP-UK to the cells in the presence or absence of excess nonlabeled DIP-UK. Plasminogen binding isotherms were obtained by adding varying amounts of nonlabeled plasminogen (0.1–20 μM) and a constant amount of tracer ^{125}I -plasminogen (0.1 μM) to the cells. UK binding was measured at 30 min at 37°C . Plasminogen binding was measured at 60 min at 37°C .

Reversibility of the interactions of plasminogen and UK with the two cell lines was demonstrated. ^{125}I -Plasminogen was incubated with the cells for 30 min, and a 50-fold molar excess of nonlabeled plasminogen was then added. In the next 60 min, >75% of the ^{125}I -plasminogen bound to the U937 and GM 1380 cells was displaced. Under the same conditions, buffer alone caused a 25–35% dissociation of bound plasminogen. Thus, the reversibility of plasminogen binding to these cells is similar to that previously observed with platelets (19). In a similar experimental format, within 30 min, excess nonlabeled UK displaced >60% of the ^{125}I -UK prebound to the cells for 20 min. Equivalent (fourfold) dilution with buffer alone caused <5% dissociation, consistent with a high affinity interaction.

With evidence of specificity, saturability, and reversibility, binding parameters for the interactions of ^{125}I -UK (DFP-treated) and ^{125}I -plasminogen with the U937 and GM 1380 cells were derived. Two experimental formats were used to construct specific binding curves at 37°C for each ligand with each cell line. With ^{125}I -DIP-UK, varying concentrations of the radiolabeled ligand in the range of 0.1–20 nM were added to the cells in the presence or absence of 750 nM nonlabeled DIP-UK. Specific binding curves were then derived by subtracting binding observed in the presence of excess nonlabeled UK from that observed in its absence. To construct plasminogen binding isotherms, a constant amount of tracer ^{125}I -plasminogen at 0.1 μM was added in combination with increasing concentrations of nonlabeled plasminogen in the range of 0.1–20 μM plasminogen. This protocol was used to limit the addition of excessive radioactivity to the cells. Nonspecific binding was estimated experimentally from the residual binding in the presence of the highest concentration of nonlabeled plasminogen (20 μM) or as a fitted parameter in the Ligand computer program. Values estimated for the nonspecific binding of plasminogen were very

Table II. Parameters for the Binding of Plasminogen and Urokinase to the GM1380 and U937 Cells at 37°C

Parameter	GM1380		U937	
	UK	Plasminogen	UK	Plasminogen
K_d	0.8 ± 0.5 nM	0.9 ± 0.4 μ M	2.0 ± 1.6 nM	0.8 ± 0.5 μ M
Sites/Cell	$2.6 \pm 0.8 \times 10^5$	$3.1 \pm 0.4 \times 10^7$	$5.0 \pm 2.9 \times 10^4$	$1.6 \pm 1.4 \times 10^7$
Degradation*	1.6%/h	0.4%/h	2.0%/h	0.8%/h

These results are based on at least four determinations with each ligand and each cell line.

* Based on the decrease in precipitability in 15% TCA of the cell-associated ligands over a 3-h period at 37°C.

similar by these two approaches. The data obtained from these analyses were used to construct the Scatchard plots shown in Fig. 2. The binding of UK and plasminogen to both cell types was best described by straight lines as linear correlation coefficients exceeded $r = 0.99$. Thus, these results suggest the presence of a single class of binding sites with respect to affinity for each ligand with each cell line.

Values for the binding parameters obtained from the Scatchard plots have been summarized in Table II. UK bound to both the GM 1380 and U937 cells with a similar apparent K_d , but the GM 1380 cells had a fivefold higher capacity. The binding parameters obtained for the U937 cells ($K_d = 2$ nM; sites = 50,000) are similar to those reported by Vassalli et al. (34) ($K_d = 0.4$ nM; sites = 60,000). Plasminogen interacted with both cell lines with similar binding parameters. The K_d determinations for these interactions were the same, and the number of sites differed by less than twofold. The rates of degradation of bound ligand indicated in Table II are based upon the changes in the precipitability of the cell-bound radioactivity in 15% TCA during 3 h at 37°C. Under these conditions, neither plasminogen nor UK was significantly degraded by either cell line. FCS, which was used to maintain the two cell lines, contains plasminogen and plasmin inhibitors. To determine if their presence could influence the binding parameters measured, the cells were grown for 3–4 d in FCS depleted of plasminogen on lysine-Sepharose affinity columns and acidified to denature certain plasmin inhibitors (33). 125 I-Plasminogen and 125 I-UK binding to these cells was then measured, and Scatchard plots were constructed. The binding parameters determined were identical to those derived for cells grown in the untreated FCS.

The GM 1380 and U937 cells used in this study express PA activity. U937 cells have been shown to synthesize a PA inhibitor which forms a complex with UK that is at least partially stable in SDS (34, 35). Therefore, possible modifications of the 125 I-plasminogen and 125 I-UK ligands bound to the two cell lines were considered. 125 I-plasminogen and 125 I-UK were bound under equilibrium conditions to the two cell lines, and the bound ligands were extracted from the cells and subjected to SDS PAGE analyses (Fig. 3). Under reducing conditions, single chain plasminogen ($M_r = 92,000$) is readily distinguished from the plasmin heavy chain ($M_r = 60,000$). No plasmin was detected in the 125 I-plasminogen preparation added to the cells, and the ligand bound to the U937 cells remained predominantly plasminogen ($\sim 90\%$ based on densitometric scans). In contrast, partial activation of the ligand bound to the GM 1380 cells was observed. Based upon the estimated molecular weights of 94,000 for the upper band and 62,000 for the lower band, these derivatives were identified as plasminogen and the plasmin heavy

chain, and these were present in approximately equal quantities on the GM 1380 cells at 60 min. The extent of plasmin formation on the GM 1380 cells was time dependent. As established by densitometric scans of autoradiograms such as shown in Fig. 3, at 0.5 h, 71% of the cell-bound ligand was plasminogen, and this decreased to 33% by 4 h. This time-dependent change in plasmin formation was also observed in the unbound fraction but at a slower rate. Thus, the GM 1380 cells mediated a slow rate of plasmin formation in the free and cell-bound material. At no time point was formation of an SDS-stable complex between the plasmin light chain and a cellular inhibitor on the cells or in the soluble phase detected. To characterize the 125 I-UK bound to the cells, a ligand preparation containing both the high and low molecular weight forms of UK was used to determine if the cells could discriminate between these forms. As shown in Fig. 3, 55,000-mol-wt UK was selectively bound to the U937 and GM 1380 cells. Formation of an SDS-stable complex with a molecular weight higher than that of free UK was not detected.

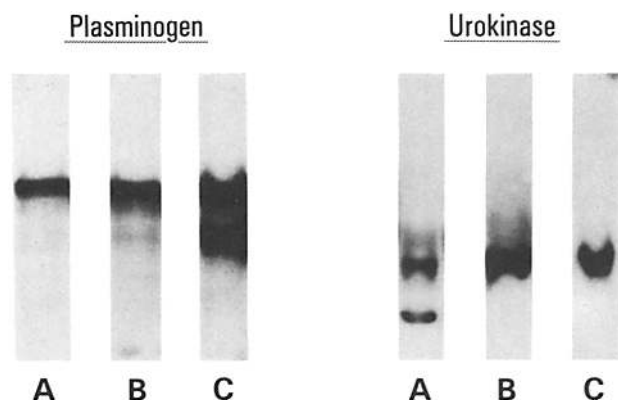


Figure 3. Characterization of the ligands, 125 I-urokinase and 125 I-plasminogen, added and bound to the U937 and GM 1380 cells on SDS PAGE. The ligands bound to the cells for 60 min at 37°C were extracted with 2% SDS with $\geq 95\%$ solubilization of radioactivity. The extracts were subjected to SDS PAGE and compared with the added ligands. 125 I-Plasminogen samples have been run on 7% polyacrylamide gels under reducing conditions, and 125 I-UK samples have been run on 10% polyacrylamide gels under nonreducing conditions. The autoradiograms of the gels are shown. Lanes A, B and C are the added ligand, the ligand bound to the U937 cells, and the ligand bound to the GM 1380 cells, respectively. As estimated from the mobilities of the bands relative to protein standards, the estimated molecular weights of the proteins were, for plasminogen in lane A, 94,000; for plasmin heavy chain in lane C, 62,000; for high and low molecular weight UK in lane A, 54,000 and 34,000, respectively.

Table III. Temperature-dependence of Plasminogen- and Urokinase-binding to U937 and GM1380 Cells

¹²⁵ I-Ligand	Molecules bound/cell			
	U937 Cells		GM1380 Cells	
	37°C	4°C	37°C	4°C
Urokinase	24,500 ± 7,500	21,300 ± 3,500	32,700 ± 4,300	5,000 ± 450
Plasminogen	68,400 ± 3,500	17,000 ± 2,300	75,600 ± 8,100	64,800 ± 600

¹²⁵I-UK or ¹²⁵I-plasminogen was added at 0.5 nM and 50 nM, respectively, to the cells pre-equilibrated at 37° or 4°C. UK binding was measured at 20 min and plasminogen binding at 60 min.

The similarity in the apparent dissociation constants of UK and of plasminogen for the two cell lines suggests that similar mechanisms may be involved in the interaction of these ligands with each cell line. The effect of temperature on binding of ¹²⁵I-UK and ¹²⁵I-plasminogen to U937 and GM 1380 cells is shown in Table III and indicates that the binding sites mediating these interactions are not precisely identical. UK binding to the U937 cells was minimally affected by temperature as the extent of the interaction was similar at 37°C and 4°C. This result is consistent with previously reported data on the temperature sensitivity of UK binding to these cells (29). In contrast, ¹²⁵I-UK binding to the GM 1380 cells exhibited a marked temperature dependence. ¹²⁵I-UK binding to the GM 1380 cells at 4°C was only 15% of the level observed at 37°C. Time course experiments indicated that UK binding to the GM 1380 cells reached apparent equilibrium within 20 min at both temperatures so that the decrease in binding at 4°C was not attributable to a slower rate of ligand binding. Binding of ¹²⁵I-plasminogen to the two cell lines showed an entirely different pattern of temperature sensitivity. Interaction of this ligand with the GM 1380 cells was relatively temperature insensitive, whereas binding to the U937 cells was temperature sensitive. The extent of plasminogen binding to the U937 cells was fourfold higher at 37°C than at 4°C. By Scatchard analyses, it was determined that this difference was a consequence of a decrease in the number of plasminogen binding sites at 4°C, and the affinity of the interaction was the same at the two temperatures.

The sensitivity of plasminogen and UK binding to trypsin treatment of the cells is summarized in Table IV. The cells were treated with 500 µg/ml trypsin for 30 min at 37°C, and the enzyme was neutralized with excess soybean trypsin inhibitor before addition of ¹²⁵I-UK or ¹²⁵I-plasminogen. Trypsin treatment decreased UK binding to both cell lines by >81%. This decrease was not a result of cytolysis, cytotoxicity, or proteolysis of the ligand (as measured by changes in the precipitability of the ligand in TCA). Plasminogen

binding to both cell lines exhibited a partial sensitivity to trypsinization. Trypsin treatment decreased plasminogen binding by 40 and 33% to the GM 1380 and U937 cells, respectively. This partial sensitivity may reflect incomplete proteolysis of the plasminogen binding sites or their micro-environment at the time and/or concentration of trypsin selected or a decrease in the affinity of the receptors due to proteolysis. Treatment of the GM 1380 cells with trypsin caused their release from the culture wells, and the results shown in Table IV were obtained by measuring the binding of the ligands to the cells in suspension. In view of the decrease in the capacity of the suspended GM 1380 cells to bind both ligands, the contribution of the subcellular matrix to the binding of plasminogen and urokinase to these cells was assessed. ¹²⁵I-Plasminogen or ¹²⁵I-UK were bound to the attached GM 1380 cells for 60 min at 37°C. Rather than extracting the bound ligands with SDS, 1% deoxycholate was used, under the conditions described by McKeown-Longo and Mosher (18), to selectively extract cell surface-associated ¹²⁵I-plasminogen or ¹²⁵I-UK. Treatment with deoxycholate extracted >90% of the ligands specifically bound to the GM 1380 cell monolayers, excluding a significant contribution of the extracellular matrix to the observed binding of plasminogen and UK to these cells.

Plasminogen binding to platelets is inhibited by ω-amino-carboxylic acids such as 6-AHA (19). These compounds interact with the lysine-binding sites associated with the kringle structures of plasminogen (28). As shown in Fig. 4, 6-AHA also inhibited the binding of plasminogen to U937 and GM 1380 cells. This inhibition was dose dependent, and occurred in a similar concentration range. 50% inhibition was obtained at 2.5 and 10 µM 6-AHA for the GM 1380 and U937 cells, respectively. As the *K_d* for the high affinity lysine-binding site of plasminogen is ~9 µM compared to 5 mM for the low affinity lysine-binding sites (15), the data suggest that the high affinity site must be unoccupied for interaction of plasminogen with the cells to occur. The binding

Table IV. Effect of Trypsin Treatment of U937 and GM1380 Cells on Their Capacity to Bind Urokinase and Plasminogen

Treatment	Molecules bound/cell			
	Plasminogen		Urokinase	
	U937	GM1380	U937	GM1380
Untreated	55,400 ± 6,200	86,200 ± 4,500	36,600 ± 800	44,800 ± 3,500
Trypsin	37,100 ± 1,400	51,700 ± 5,800	3,200 ± 800	3,800 ± 800

The cells were treated with 500 µg trypsin in 0.5 mM EDTA for 30 min at 37°C. This treatment resulted in detachment of the GM1380 cells. Soybean trypsin inhibitor at 0.5 mg/ml was added; and after 10 min, the cells were washed three times by centrifugation. Binding was measured by addition of 100 nM ¹²⁵I-plasminogen or 0.5 nM ¹²⁵I-UK to the cell suspensions at 37°C. UK binding was measured at 20 min and plasminogen binding at 60 min. Treatment of the cells with EDTA alone caused minimal changes in the binding of the two ligands to either cell line.

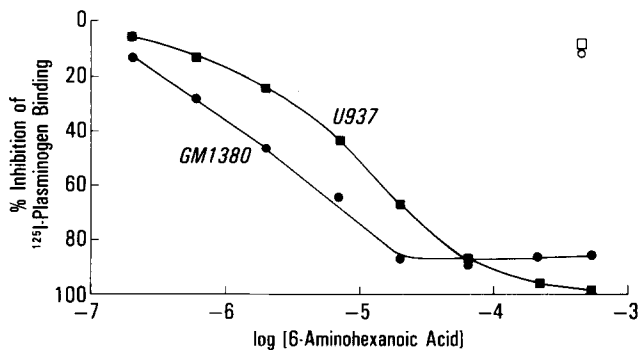


Figure 4. The effect of 6-AHA on the binding of ^{125}I -plasminogen to the U937 (solid square) and GM 1380 (solid circle) cells. ^{125}I -Plasminogen (0.1 μM) and varying concentrations of 6-AHA were added to the cells, and binding was measured after 60 min at 37°C . Percent inhibition was calculated relative to plasminogen binding in the absence of the lysine analogue. At the highest concentration used (50 μM), 6-AHA was not cytotoxic, cytolytic, did not cause detachment of the GM 1380 cells, and did not affect binding of ^{125}I -UK to the GM 1380 (open circle) or U937 cells (open square) at 20 minutes at 37°C . The experiments were performed in Hanks' balanced salt solution to exclude lysine from the medium.

of UK, which also contains kringle structures (II), was not affected by 6-AHA.

The influence of one fibrinolytic component on the binding of the other to the cell lines was assessed. Experiments were performed in which ^{125}I -DIP-UK (0.5 nM) and ^{125}I -plasminogen (0.1 μM) were added to the cells in the presence of a high concentration of nonlabeled DIP-UK (1 μM) or plasminogen (10 μM). As summarized in Table V, plasminogen had only minimal effects on UK binding to the cells. No effect of plasminogen was observed on UK binding to the GM 1380 cells, whereas a slight inhibition of binding (31%) was observed on UK binding to the U937 cells. In contrast, UK exerted a marked effect upon ^{125}I -plasminogen binding to the cells. With both cell lines, UK was found to enhance plasminogen binding. UK at 1 μM enhanced plasminogen binding to the U937 cells by 1.4-fold and to the GM 1380 cells by 3.3-fold. As the enhancement was more extensive with the GM 1380 cells, this effect was further explored using these cells. The UK-induced increase in plasminogen binding to the GM 1380 cells was consistently observed in nine consecutive experiments. The extent of the increase was somewhat variable and ranged from 1.7- to 6.3-fold (compared to binding in the absence of UK). The enhancement was also observed with both active and DIP-UK. As shown in Fig. 5, the enhancement of ^{125}I -plasminogen binding to the GM 1380 cells was dependent upon the concentration of UK added. Enhanced plasminogen binding was observed at UK concentrations >1 nM and was maximal at 500 nM UK. As noted in Fig. 5, at a 10-nM concentration DIP-UK and active UK were equally effective in enhancing plasminogen binding. This was also observed at a higher concentration of UK; at 1 μM , the increase in plasminogen binding induced by active UK was 3.7 ± 1.7 -fold and by DIP-UK was 3.0 ± 2.0 -fold.

Potential mechanisms for the augmented binding of plasminogen in the presence of UK were considered. As a first possibility, we considered whether the enhanced binding was related to plasmin formation or binding to the cells. No en-

Table V. Effect of Nonlabeled Plasminogen and Urokinase on the Binding of ^{125}I -Urokinase and ^{125}I -Plasminogen to U937 and GM1380 Cells

Nonlabeled protein	Relative binding			
	^{125}I -plasminogen		^{125}I -urokinase	
	U937	GM1380	U937	GM1380
Plasminogen (10 μM)	19.6	14.4	69.0	94.3
DIP-UK (1 μM)	140.3	334.8	8.3	9.7

Binding of ^{125}I -plasminogen (100 nM) and ^{125}I -DIP-UK (0.5 nM) to the cells was measured at 60 and 20 min, respectively, in the presence or absence of nonlabeled plasminogen and DIP-UK. Results are calculated relative to samples lacking the nonlabeled proteins which have been assigned values of 100.

zymatic activity was detected in the DFP-treated UK preparations, but low levels of residual activity could form plasmin which could be preferentially bound to the cells. To test this possibility, ^{125}I -plasminogen was bound to the GM 1380 cells in the presence of 100 or 500 nM UK or DIP-UK. Plasminogen binding was enhanced 1.9–2.5-fold at 60 min under these conditions. The radiolabeled ligand bound to the cells was then extracted and analyzed on SDS PAGE. The gels shown in Fig. 6 indicate that the DFP-treated UK did not cause an increase in the proportion of plasmin associated with the cells. The ratio of plasminogen to plasmin heavy chain bands bound to the GM 1380 cells was the same in the presence or absence of DIP-UK. Thus, UK-induced enhancement of plasminogen binding is not dependent upon plasmin formation. When active UK was added, all of the bound ligand was plasmin. This latter result indicates that plasmin can be directly expressed on the cell surface.

To determine if plasmin can interact preferentially with the cells, the binding properties of radioiodinated DIP-plasmin were analyzed. As summarized in Table VI, at input concentrations of 0.1 μM , DIP-plasmin and plasminogen bound to the U937 cells to a similar extent. At a 10-fold excess, nonlabeled plasmin and plasminogen inhibited the binding of both ligands to a similar degree, indicating that plasmin bound specifically to the cells and that the ligands competed for the same set of binding sites. DIP-plasmin binding was also inhibited by 6-AHA, implicating the lysine-binding sites in the

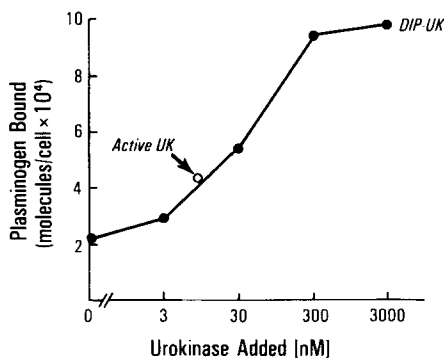


Figure 5. Enhancement of plasminogen binding to the GM 1380 cells by varying concentrations of DIP-UK. ^{125}I -Plasminogen at 0.1 μM and varying concentrations of DIP-UK were added to the GM 1380 cells at 37°C , and specific plasminogen binding was measured after 60 min. The single point (open circle) was obtained by addition of active UK.

interaction of this ligand with the U937 cells. Similar results were also obtained with the GM 1380 cells. Thus, plasmin can bind directly to the cells via the sites which also recognize plasminogen.

To consider whether the basis for enhanced plasminogen binding to the cells in the presence of UK was due to an increase in the affinity or number of plasminogen binding sites, the binding of varying concentrations of plasminogen to the GM 1380 cells was measured in the presence or absence of 1 μ M DIP-UK. The specific binding isotherms derived are shown in Fig. 7. The differences in the extent of plasminogen binding in the presence or absence of UK were greater at the lower concentrations of added plasminogen and became similar at high concentrations of added ligand. These data suggest that the total number of plasminogen-binding sites was very similar in the presence or absence of UK but the apparent affinity of plasminogen for the cells increased slightly in the presence of UK. Scatchard analyses of these results were consistent with this interpretation; namely, the affinity of the cells for plasminogen increased by approximately threefold in the presence of UK.

The above analyses suggest that plasmin can be formed on cell surfaces and remain cell surface associated. Plasmin (and plasminogen) binds to α_2 -antiplasmin, the primary plasmin inhibitor, via its high affinity lysine-binding site before interacting with the active site of the enzyme (4). As the data shown in Fig. 4 suggest that the binding of plasminogen to the GM 1380 and U937 cells is dependent upon the lysine-binding sites, protection of cell-bound plasmin from α_2 -antiplasmin was examined. 125 I-Plasminogen (50 nM) was

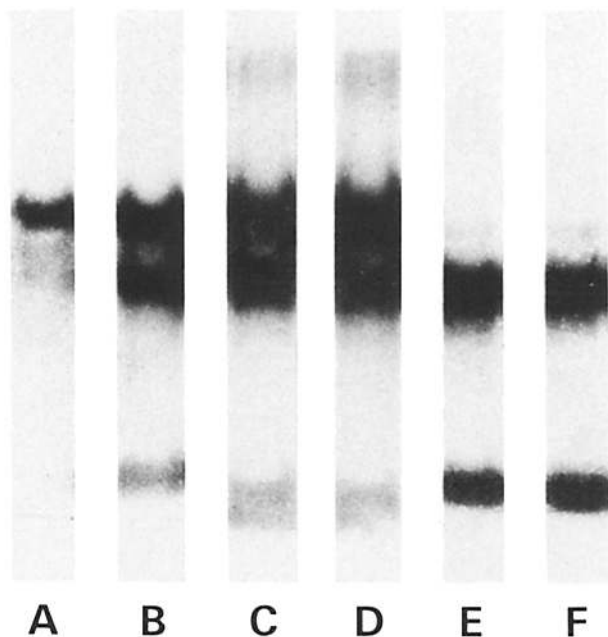


Figure 6. Characterization by SDS PAGE of 125 I-plasminogen bound to the GM 1380 cells in the presence or absence of UK and DIP-UK. 125 I-Plasminogen was bound to the cells for 60 min at 37°C and then bound ligand was extracted and analyzed by SDS PAGE as indicated in Fig. 3. Lanes are as follows: (A) added plasminogen; (B) bound ligand in absence of added UK; (C) bound ligand with 0.5 μ M DIP-UK added; (D) bound ligand with 1 μ M DIP-UK added; (E) bound ligand with 0.5 μ M UK added; (F) bound ligand with 1 μ M UK added.

Table VI. Comparison of Plasminogen and DIP-Plasmin Binding to U937 Cells

Inhibitor	125 I-Ligand bound (molecules/cell)	
	DIP-Plasmin	Plasminogen
None	330,800 \pm 27,500	390,000 \pm 32,300
Plasminogen (1 μ M)	46,500 \pm 8,700	64,200 \pm 6,500
DIP-Plasmin (1 μ M)	48,200 \pm 10,600	82,500 \pm 6,800
6-AHA (0.2 M)	6,400 \pm 800	7,700 \pm 700

125 I-plasminogen or 125 I-DIP plasmin were added to the U937 cells at 100 nM in the presence or absence of the indicated competitors. Binding was measured after a 60-min incubation at 37°C.

bound to the GM 1380 cells for 60 min at 37°C and then a high concentration of streptokinase (75 U/ml) was added to fully convert bound and free plasminogen to plasmin. After an additional 30 min at 37°C, α_2 -antiplasmin was added at final concentrations of 100 or 25 nM. After 30 min, the cell-bound and unbound ligands were analyzed by SDS PAGE on 6% polyacrylamide gels under nonreducing conditions. Autoradiograms of the gels are shown in Fig. 8. At both concentrations of α_2 -antiplasmin, formation of a plasmin/ α_2 -antiplasmin complex (M_r 150,000) was apparent in the supernatant. In contrast, the plasmin/ α_2 -antiplasmin complex was not observed with cell-bound material; only the single band of plasmin (M_r 89,000) was detected. In control experiments, it was determined that the addition of α_2 -antiplasmin did not alter the amount of cell-associated radioactivity; i.e., the complex did not selectively dissociate from the cells. Thus, cell-associated plasmin was protected from its primary plasma inhibitor, α_2 -antiplasmin.

Discussion

In this study, we have demonstrated that two established cell lines interact with two central components of the fibrinolytic system, the plasminogen activator, urokinase, and the zymogen, plasminogen. Binding of UK to U937 cells has been previously demonstrated (29, 34), and the binding param-

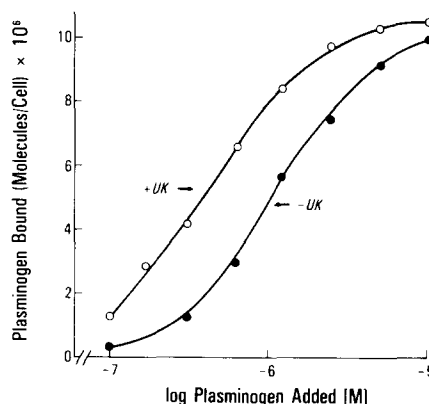


Figure 7. Binding of plasminogen to the GM 1380 cells in the presence and absence of urokinase. Varying concentrations of non-labeled plasminogen with a constant amount of 125 I-plasminogen tracer were added to the cells with or without 1 μ M DIP-UK present. Binding was measured after 60 min at 37°C. The data was analyzed in the Ligand computer program to estimate nonspecific binding and to construct the specific binding isotherms shown.

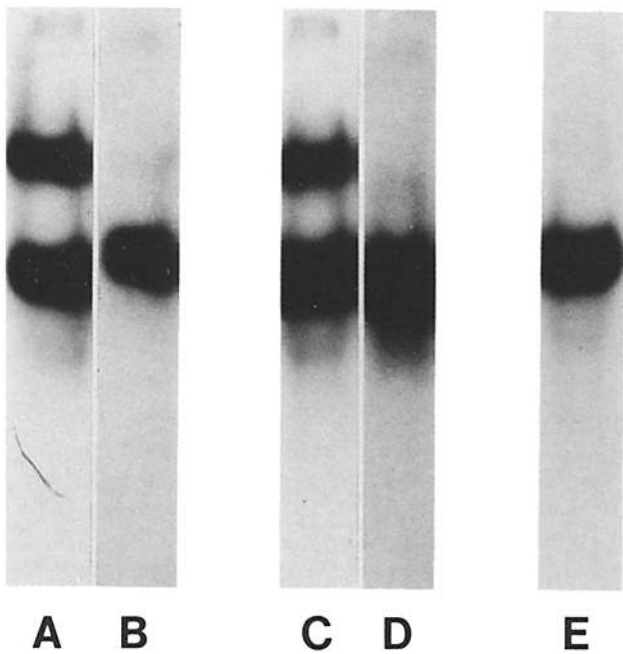


Figure 8. Protection of cell-bound plasmin from α_2 -antiplasmin. ^{125}I -Plasminogen at 50 nM was incubated with the GM 1380 cells for 60 min at 37°C. Streptokinase (75 U/ml) was added; and, after 30 min at 37°C, α_2 -antiplasmin was added. After a 30-min incubation, the supernatant was removed, the cells were washed once, and were extracted with SDS. Supernatants and cell extracts were boiled at the same time and analyzed by SDS PAGE (6% gels under non-reducing conditions). Lanes *A* and *C* are the supernatants obtained with the α_2 -antiplasmin at 100 and 25 nM, respectively. Lanes *B* and *D* are the corresponding cell extracts. Lane *E* is plasmin formed from ^{125}I -plasminogen with the dose of streptokinase used. The estimated molecular weight of plasmin was 89,000 and of the plasmin α_2 -antiplasmin complex, 150,000.

eters that we derived were similar to those reported by Vassalli et al. (34) and Stopelli et al. (29). Our observation that UK also specifically binds to GM 1380 cells demonstrates that a distinct cell type also possesses UK receptors. Binding of UK to both U937 and GM 1380 cells did not require an active catalytic site within the enzyme, consistent with the observations of Vassalli et al. (34) and Stopelli et al. (29) and distinct from the interactions described by Del Rosso et al. (6). Moreover, the affinity of UK for the two cell lines was similar. Thus, the possibility of widely distributed binding sites for UK which interact with this PA with similarly high affinities may be considered. The fact that ^{125}I -UK, which retained 23–34% of its plasmin-activating activity, and DIP-UK, which had no detectable residual activity (<5%), bound to both cell types in an identical manner suggests that the affinity of the receptor system for active and inactive UK cannot be significantly different.

The two cell lines exhibiting UK binding properties were also capable of specifically interacting with plasminogen. The affinity and capacity of the two cell lines for plasminogen were quite similar. The similarity in binding parameters was observed although the methods used to separate bound from free ligand were quite different for the two cell types, suggesting that relatively low affinity interactions can be analyzed by these approaches. The low affinity of these interactions is also similar to that determined for the binding of

plasminogen to platelets (19). It should be noted, that if the existence of plasminogen-binding sites on U937 cells is reflective of their monocyte-like properties (indeed, we have obtained preliminary evidence to indicate that monocytes can bind plasminogen [19, and our unpublished results]), monocytes are exposed to a plasma environment containing $\sim 2 \mu\text{M}$ plasminogen (5). The capacity of both cell lines for plasminogen was very high and is in a range similar to that reported for the number of elastase- and lactoferrin-binding sites on macrophages (2). 6-AHA inhibited the binding of plasminogen to the U937 and GM 1380 cells. This inhibition was observed in a concentration range consistent with occupancy of the high affinity lysine-binding site (15) which is associated with the first kringle of plasminogen (13). The failure of fibrinogen at 5 μM to inhibit plasminogen binding to the cells presumably reflects the low affinity of fibrinogen for the high affinity lysine-binding site of plasminogen (14). Similar results with respect to the involvement of the high affinity lysine-binding site and the failure of fibrinogen to inhibit binding have also been obtained for the interaction of plasminogen with platelets (19). Thus, as suggested for the UK-binding sites, plasminogen-binding sites may also be widely distributed. Moreover, these binding sites appear to require an unoccupied high affinity lysine-binding site for recognition of plasminogen. Consistent with this hypothesis is our preliminary data indicating that a variety of peripheral blood cells may bind plasminogen (19), and the presence of plasmin-binding sites on endothelial cells (1).

UK increased plasminogen binding to both U937 and GM 1380 cells. The extent of this increase was 1.4-fold for the U937 cells and 3.4-fold for the GM 1380 cells. Enzymatically active UK was not required for this augmentation, suggesting that conversion of plasminogen to plasmin was not essential for inducing increased ligand binding. SDS PAGE and direct analyses of the binding of plasmin to the cells verified this conclusion. The increase in plasminogen binding in the presence of UK may reflect an increase in the apparent affinity or the number of binding sites for the ligand on the cells. The data support the former possibility by suggesting that there is a slight increase in the affinity of plasminogen for the GM 1380 cells in the presence of UK. Such a change in affinity could arise either from an interaction of UK with plasminogen resulting in enhanced binding of such a complex to the cells or from an alteration of the plasminogen-binding sites due to occupancy of the UK receptor. The effect of UK on plasminogen binding was saturable with respect to UK concentration, and the increase in plasminogen binding was observed in a range consistent with occupancy of the UK-binding site. Thus, the data is consistent with communication between the UK- and plasminogen-binding sites.

Analyses of the ligand bound to the GM 1380 cells indicated that plasmin as well as plasminogen can become directly associated with the cells. Plasmin could be formed in the soluble phase and then associate with the cell surface. If this is the mechanism for plasmin association with the cell, then the interaction of both plasmin and plasminogen with the cells must occur with similar affinity to account for the linearity that the Scatchard plots obtained. Consistent with this interpretation is the data demonstrating that plasmin and plasminogen bind to the same set of sites (Table VI). An alternative mechanism for detection of plasmin on the cell surface is that bound plasminogen is activated by the ex-

ogenously added or the endogenous PA activity of the cells. In either case, it is clear that plasmin can remain cell surface bound. Indeed, when active UK was added to the GM 1380 cells, virtually all of the cell-associated ligand bound at 60 min was plasmin. Thus, the presence of plasmin(ogen) binding sites provides a means for cells to convert their intrinsic PA activity, with restricted substrate specificity, to the broad proteolytic activity of the enzyme plasmin. Once plasmin is formed or binds on the cell surface, it is protected from its primary inhibitor, α_2 -antiplasmin, relative to free plasmin. On this basis, local proteolysis could be achieved in an environment rich in plasmin inhibitors. While further studies will be necessary to establish the accessibility of the active site of plasmin bound to the cellular receptor to low and high molecular weight substrates, the binding of DIP-plasmin to the cells indicates that the active site of the enzyme is not directly involved in the interaction.

We have shown that plasminogen binds to platelets through two distinct mechanisms (20). One pathway is dependent upon platelet fibrin which is expressed on the cell surface as a consequence of thrombin stimulation. The U937 and GM 1380 cells did not express fibrinogen-related antigens on their cell surfaces as detected with radiolabeled F(ab')₂ fragments of an antiserum reactive with the D and E regions and carboxy-terminal region of the A α chain of bovine fibrinogen. The second pathway of plasminogen binding to platelets is dependent on membrane glycoprotein GPIIb/IIIa. We have identified molecules immunologically related to GPIIb/IIIa on U937 and GM 1380 cells (24). GPIIb/IIIa-related molecules have also been demonstrated on endothelial cells (10, 31). These molecules appear to be similar, but are not precisely identical. As noted in this study, the UK- and plasminogen-binding sites on the two cell lines studied are similar to one another with respect to their affinity for the ligands, their sensitivity to trypsinization, and, in the case of plasminogen, with respect to the requirement for an unoccupied high affinity lysine-binding site for the ligand. Nevertheless, the binding sites for each ligand on the two cell lines are not precisely identical based upon the differential sensitivities of the interactions to temperature. Thus, it becomes an interesting avenue of investigation to explore the relationship between this family of related, but not necessarily identical, cell surface molecules and the plasminogen- and urokinase-binding sites on these cells.

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