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Plasminogen Carbohydrate Side Chains in Receptor Binding and Enzyme Activation: A Study of C6 Glioma Cells and Primary Cultures of Rat Hepatocytes

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The human [Glu[1]]-plasminogen carbohydrate isozymes, plasminogen type I (Pg 1) and plasminogen type II (Pg 2), were separated by chromatography and studied in cell binding experiments at 4°C with primary cultures of rat hepatocytes and rat C6 glioma cells. In both cell systems, Pg 1 and Pg 2 bound to an equivalent number of receptors, apparently representing the same population of surface molecules. The affinity for Pg 2 was slightly higher. With hepatocytes, the K_D for Pg 1 was 3.2 ± 0.2 μM, and the K_D for Pg 2 was 1.9 ± 0.1 μM, as determined from Scatchard transformations of the binding isotherms. The B_{max} was approximately the same for both isozymes. With C6 cells, the K_D for Pg 1 was 2.2 ± 0.1 μM vs. 1.5 ± 0.2 μM for Pg 2. Again, the B_{max} was similar with both isozymes. ^{125}I-Pg 1 and ^{125}I-Pg 2 were displaced from specific binding sites by either nonradiolabeled isozyme. The K_I for Pg 2 was slightly lower than the K_I for Pg 1 with hepatocytes (0.9 vs. 1.3 μM) and with C6 cells (0.6 vs. 1.1 μM). No displacement was detected with miniplasminogen at concentrations up to 5.0 μM. Activation of Pg 1 and Pg 2 by recombinant two-chain tissue-plasminogen activator (rt-PA) was enhanced by hepatocyte cultures. The enhancing effect was greater with Pg 2. Hepatocyte cultures did not affect the activation of miniplasminogen by rt-PA or the activation of plasminogen by streptokinase. Unlike the hepatocytes, C6 cells did not enhance the activation of plasminogen by rt-PA or streptokinase; however, plasmin generated in the presence of C6 cells reacted less readily with α_2-antiplasmin.

Key words: tissue-plasminogen activator, α_2-antiplasmin, protein glycosylation, miniplasminogen, streptokinase

Cell surfaces play an important role in the regulation of fibrinolytic proteinase activity. Specific receptors that bind plasminogen and/or plasmin have been demonstrated on endothelium [1,2], platelets [3], rat hepatocytes [4], circulating white cells [5], U937 promyeloid leukemia cells [6], HT-1080 fibrosarcoma cells [7], fetal lung fibroblasts [6], SW 1116 carcinoma cells [8], and rat C6 glioma cells [9]. These plasmin(ogen) receptors concentrate fibrinolytic activity near the cell membrane [10]. In tumor cells, plasmin that is bound to the cell surface may help direct extracellular matrix

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degradation, which in turn allows for tumor growth and cell metastasis [11]. Importantly, plasminogen that is bound to some cell surface receptors is activated at an enhanced rate [1,3,4]. In addition, receptor-bound plasmin is protected from physiologic proteinase inhibitors [4,6]. Plasminogen endocytosis or catabolism has not been associated with receptor binding in any cell system.

Native human plasminogen has an N-terminal glutamic acid; however, plasmin efficiently converts [Glu^1]-plasminogen into [Lys^{77}]-plasminogen [12]. The modified form of the zymogen is more readily activated [13]. In addition, [Lys^{77}]-plasminogen binds to at least some cell surface receptors with higher affinity [14,15]. Plasminogen receptors from most cells bind [Glu^1]-plasminogen with a K_D in the low micromolar range [3–6,9]. The density of surface receptors is typically high. Plasminogen binding occurs primarily via the lysine binding site of the kringle 1–3 (K1–3) domain; however, other regions of the molecule, including the kringle 4 (K4) and miniplasmin domains, are involved in the interaction as well [16].

Plasminogen consists of a mixture of two major isozymes termed Pg 1 and Pg 2 [17]. These isozymes are equivalent in amino acid sequence [18], and both species are glycosylated at Thr 345; however, Pg 1 has a second glycosylation site at Asn 288 [19–21]. In many ways, the function of the two isozymes is equivalent; however, the noncovalent binding interaction between plasminogen and α_2-antiplasmin (α_2AP) is stronger with the Pg 2 isozyme [22]. In addition, the enhancing effect of fibrin on the rate of plasminogen activation is different for Pg 1 and Pg 2 [23]. Most plasminogen receptor studies have been performed with mixtures of Pg 1 and Pg 2; however, recently, Gonzalez-Gronow et al. [24] studied the binding of the separated isozymes to U937 cells. These investigators demonstrated that the monocytoid cells bind considerably more Pg 2 than Pg 1. This result seems to reflect a significant difference in available surface binding sites; at saturation, the amount of Pg 2 binding was approximately tenfold the level of Pg 1 binding, and Pg 1 did not displace Pg 2 from receptor sites.

The mechanism of enhanced plasminogen activation on cell surfaces remains unclear. Hajjar et al. [14] demonstrated conversion of receptor-bound [Glu^1]-plasminogen into [Lys^{77}]-plasminogen on endothelium. Ellis et al. [25] demonstrated enhanced activation of plasminogen by urokinase using U937 cells and postulated that this reflects plasmin-mediated conversion of single-chain urokinase into the more active two-chain form at the cell surface.

Workers from this laboratory recently reported the characterization of plasminogen receptors in primary cultures of rat hepatocytes [4] and rat C6 glioma cells [9]. In both studies, mixtures of the two plasminogen isozymes were used. The current investigation was undertaken with two specific aims: 1) to assess quantitatively the binding of Pg 1 and Pg 2 to rat hepatocytes and C6 cells and 2) to assess further the requirements for activation of surface bound plasminogen. Towards the first goal, glutaraldehyde-cross-linked concanavalin A-Sepharose was used to prepare highly purified [Glu^1]-Pg 1 and [Glu^1]-Pg 2. In addition, plasminogen activation was studied in the two cell systems, using Pg 1, Pg 2, and miniplasminogen as substrates and recombinant two-chain tissue plasminogen activator (rt-PA) and streptokinase as enzymes.

MATERIALS AND METHODS

Materials

Minimum essential medium with Earle's salts (MEM), Earle's balanced salts (EBSS) and bovine crystalline insulin were purchased from Gibco Laboratories (Grand

Island, NY). H-D-Val-L-Leu-L-Lys-p-nitroanilide HCl (S-2251) and H-D-Ile-L-Pro-L-Arg-p-nitroanilide (S-2288) were from Helena Laboratories. Type IV collagenase (*Clostridium*), fetal calf serum, acid-soluble rat tail collagen, bovine serum albumin (BSA), and concanavalin A-Sepharose were from Sigma. $Na^{125}I$ was from Amersham, and Iodobeads were from Pierce. All other chemicals were reagent-grade.

Proteins

[Glu^1]-plasminogen (mixed Pg 1 and Pg 2) was purified from human plasma according to the procedure of Deutsch and Mertz [26] as previously modified [4]. These mixed preparations typically contained 60–70% Pg 2 as determined by acid gel electrophoresis [27]. α_2AP was purified according to the procedure of Wiman [28]. rt-PA was a generous gift from Genentech Corporation. The initial mixture of single-chain rt-PA and two-chain rt-PA was converted into a homogeneous preparation of the two-chain species using plasmin-Sepharose [29]. In the plasminogen activation experiments reported here, only two-chain rt-PA was studied. The concentration of active two-chain rt-PA was determined based on amidase activity with different concentrations of S-2288, using the kinetic constants (k_{cat} and K_M) reported by Urano et al. [30]. Streptokinase was purified from Kabikinase (Kabi, Stockholm, Sweden) by the method of Castellino et al. [31]. Miniplasminogen was prepared by digesting plasminogen with porcine elastase and purified by chromatography on lysine-Sepharose [18].

Separation of Pg 1 and Pg 2

Plasminogen carbohydrate variants were resolved by either of two methods at 4°C. The first procedure was to elute the plasminogen from lysine-Sepharose with a gradient of ϵ-aminocaproic acid (EACA) (0–25 mM) as described by Brockway and Castellino [17]. A significant fraction of the protein was discarded to avoid cross-contamination of the isozyme preparations. In the second method, the isozymes were separated with concanavalin A-Sepharose (con A-Sepharose) using a modification of the procedure of Gonzalez-Gronow and Robbins [32]. The commercial preparation of con A-Sepharose required modification, since small amounts of the con A continuously leached from the matrix, despite extensive washing with high-salt buffers (1.5–2.0 M NaCl) and α-D-methylmannoside (1 M). The loss of con A most likely reflected an equilibrium between dimeric and tetrameric states of the lectin [33]. To avoid con A leaching, the resin was treated with 0.25% glutaraldehyde according to the procedure of Kowal and Parsons [34]. A combination of Pg 1 and Pg 2 was obtained by lysine-Sepharose chromatography. The mixture was pooled; dialyzed against 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS); and applied to the con A-Sepharose column equilibrated in the same buffer. Pg 1 bound to the resin; Pg 2 was recovered in the initial wash. The Pg 1 was eluted with 0.5 M α-D-methylmannoside in PBS. Pooled fractions of Pg 1 and Pg 2 were concentrated with an Amicon Ultrafiltration Cell (Ym-10 Diaflo membrane) and extensively dialyzed against PBS. The final preparations were subjected to sodium dodecyl sulfate (SDS)-gel electrophoresis using the Hepes/Imidazole pH 7.4 buffer system of McLellan [35]. As is shown in Figure 1, [Glu^1]-Pg 1 was resolved from [Glu^1]-Pg 2 in this system. [Lys^{77}]-plasminogen was also resolved from [Glu^1]-plasminogen when preparations of either individual isozyme were analyzed. There was no [Lys^{77}]-plasminogen in the Pg 1 and Pg 2 preparations shown in Figure 1 or in any other preparations used for the binding studies reported here.

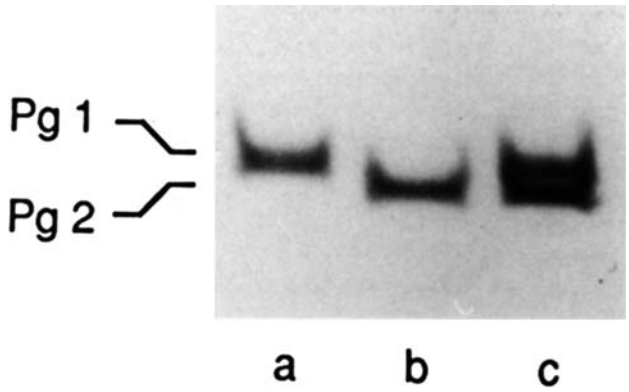

Fig. 1. Polyacrylamide gel electrophoresis of purified [Glu^1]-plasminogen type I (Pg 1) and [Glu^1]-plasminogen type II (Pg 2). **Lane a.** Pg 1. **Lane b.** Pg 2. **Lane c.** Mixture of Pg 1 and Pg 2.

Radioiodination

Plasminogen was iodinated using Iodobeads as described by the manufacturer. Desalting was performed on Sephadex G-25 (Pharmacia). Specific activities varied from 0.5 to 1.5 μCi/μg protein.

Hepatocyte and C6 Glioma Cell Culture

Hepatocytes were obtained from female Sprague-Dawley rats using the two-step collagenase perfusion technique [36,37] as modified by Gonias et al. [4]. The cells were cultured in 35 mm collagen-coated plates (30 μg collagen/well) and incubated for 2–3 h at 37°C in 5% CO_2. Each well was washed twice with EBSS/10 mM HEPES, pH 7.4 (buffer A). Experiments were performed immediately. Rat C6 glioma cells were cultured in 24 well tissue culture plates as described by Hall et al. [9]. Experiments with C6 cells were performed using cultures that were brought to near confluence in MEM with 10% fetal calf serum in about 4–5 days without medium exchange.

Plasminogen Binding Studies

Binding studies with hepatocytes and C6 cells were performed at 4°C as previously described [4,9]. Briefly, different concentrations of ^{125}I-Pg 1 or ^{125}I-Pg 2 were incubated with cells in buffer A with 10 mg/ml BSA for 5 h (the time required for binding to reach apparent equilibrium). In some wells, the binding buffer was supplemented with 20 mM lysine as well. The lysine inhibits specific plasminogen binding to cell surface receptors by associating with the plasminogen domains that mediate the receptor interaction [1,3,4,9,10,15]. Specific binding was defined as the difference between total binding (in the absence of lysine) and binding observed in the presence of 20 mM lysine [4,9]. This procedure for determining specific binding differs from the classic approach, which involves the use of a ligand that competes for receptor binding sites. In previous experiments with the cell types under consideration here, we demonstrated that levels of ^{125}I-plasminogen specific binding are comparable when determined using lysine or a 50-fold molar excess of unlabeled plasminogen [4,9].

After incubating ^{125}I-plasminogen with cells in culture, the medium was aspirated, and the wells were washed three times with buffer A (no lysine). Lysis solution (0.1 M

NaOH, 2.5% SDS) was added to each well and incubated overnight. Radioactivity was determined in an LKB minigamma gamma counter (counting efficiency >75%). Protein content was determined by the method of Lowry as reviewed by Peterson [38]. The number of cells per microgram of hepatocyte protein was 340 ± 60 [4]; for C6 cells, it was 4,350 ± 150 [9]. These values were used to calculate the molecules of ligand bound per cell.

In control experiments, the binding of ^{125}I-plasminogen to collagen-coated cell culture plates without cells was studied under the conditions used in the cell binding experiments. When the plasminogen concentration was less than 3.0 μM, specific plasminogen binding to the collagen represented no more than 2–8% of the binding observed with cultures of hepatocytes.

In radioligand displacement experiments, a standard concentration of radioiodinated Pg 1 or Pg 2 (0.05 μM for hepatocytes, 0.1 μM for C6 cells) was incubated with different concentrations of the nonradiolabeled isozymes for 5 h at 4°C. The wells were washed, and cell-bound radioactivity was determined as described above.

All plasminogen binding studies were performed at least in triplicate using different cell preparations for each experiment. Each data point for a given experiment represented a duplicate determination. Saturation isotherms were fitted to the equation for a rectangular hyperbola using the nonlinear regression method of Marquardt. The same data were plotted using the Scatchard transformation. The K_D and B_{max} were then determined by linear regression. The values presented represent the average ± standard error of the mean (SEM).

Radioligand displacement data were analyzed by plotting specific ^{125}I-plasminogen binding against the log of the unlabeled protein concentration. The displacement curves and the IC_{50} values were determined by nonlinear regression to fit a four parameter logistic equation

$$Y = A + \frac{B - A}{1 + \frac{(10^x)^D}{(10^C)^D}}$$

where A is the residual specific binding, B is the total specific binding, C is the log IC_{50}, and D is the Hill coefficient. Goodness of fit was determined by the method of Marquardt. Since the concentration of radioiodinated ligand was well below the K_D, and bound ^{125}I-plasminogen represented less than 4% of total radioligand, the K_I was determined from the linear relationship with the IC_{50}.

Plasminogen Activation Studies

Plasminogen, Pg 1, Pg 2, or miniplasminogen (1.0 μM) and S-2251 (0.5 mM) were preincubated with hepatocyte cultures or C6 cells in buffer A with 10 mg/ml BSA for 30 min at 22°C. The buffer for C6 cells also included myoinositol (5.5 mM), glucose (28 mM), sodium pyruvate (5 mM), serine (0.75 mM), glycine (0.4 mM), sodium selenite (30 nM), $CuSO_4$ (0.2 μM), $ZnSO_4$ (0.1 μM), $FeSO_4$ (3 μM), and L-ascorbic acid (1.4 μM). Various concentrations of rt-PA or streptokinase were added to the wells, and the plates were incubated for an additional 95 min. Substrate hydrolysis was terminated with glacial acetic acid. Absorbances were determined at 406 nm. When hepatocytes were prepared and washed as described here, intrinsic inhibitors of plasmin

were not detectable [4]. Equivalent experiments were performed in empty cell culture plates (no cells). The compounds used in the C6 cell culture system did not affect plasminogen activation. The addition of collagen to cell culture plates does not affect the extent of plasminogen activation by rt-PA [4].

Inhibition of Plasmin by α_2AP

Plasmin inhibition experiments with C6 cells were performed as previously described for hepatocytes [4]. Briefly, plasminogen (1.0 μM) and rt-PA (0.8 nM) were added to washed C6 cells in buffer A with 10 mg/ml BSA (volume, 0.75 ml). After 90 min, the buffer was separated from the cells and saved (solution 1). The wells were then immediately reconstituted with 0.75 ml of fresh buffer A and BSA (solution 2). No additional rt-PA or plasminogen was included in solution 2. In some wells, the buffer was not separated from the cells (solution 3). α_2AP and S-2251, or 2251 alone, were added simultaneously to all three solutions. The final concentrations of inhibitor and substrate were 0.4 μM and 0.5 mM, respectively. Plasmin activity was determined by substrate hydrolysis (15 min). Equivalent experiments were performed in wells that had no cells.

RESULTS

Cell Binding Experiments With Pg 1 and Pg 2

Pg 1 and Pg 2 bound to hepatocytes in primary culture (Fig. 2). Greater than 85% of the binding was specific. Although complete saturation was not obtained with either isozyme in the concentration range studied (0.1–3.0 μM), the computer-fitted curves of ^{125}I-Pg 1 binding and ^{125}I-Pg 2 binding indicated a saturable process. As was reported previously [4], the presence of a low-affinity, high-capacity plasminogen binding site on the collagen used to coat the culture plates precluded the use of higher concentrations of plasminogen. The saturation isotherms and Scatchard analyses shown in Figure 2 were derived by averaging the results of four different experiments with separate preparations of hepatocytes. In each individual experiment, the affinity for Pg 2 was slightly higher than the affinity for Pg 1. The number of binding sites per cell was comparable for the two isozymes. The average K_D for Pg 1 was 3.2 ± 0.2 μM, and the B_{max} was 9.4 ± 0.8 $\times 10^7$ molecules per cell. The average K_D for Pg 2 was 1.9 ± 0.1 μM, and the B_{max} was 9.8 ± 1.3 $\times 10^7$ molecules per cell. The K_D and B_{max} values are slightly higher than those previously reported for the mixture of Pg 1 and Pg 2 [4]; however, the previous studies were performed using a more complete medium that included a low concentration of lysine.

^{125}I-Pg 2 was displaced from hepatocyte-specific binding sites by both nonradiolabeled Pg 1 and Pg 2 (Fig. 3). When high concentrations of the two isozymes were compared, the difference in ^{125}I-Pg 2 binding displacement was minimal. Therefore, it is highly unlikely that hepatocytes express a specific binding site that recognizes only Pg 2 and not Pg 1. Dissociation constants were determined from the displacement curves. The K_I for nonradiolabeled Pg 1 was 1.3 μM, and the K_I for nonradiolabeled Pg 2 was 0.9 μM. Since the nonradiolabeled plasminogen (>50-fold molar excess) decreased ^{125}I-Pg 2 binding by approximately the same amount as 20 mM lysine, these experiments confirm that lysine may be used to determine specific binding with isolated plasminogen isozymes, as has been previously reported for mixtures of Pg 1 and Pg 2 [4,9]. ^{125}I-Pg 2 displacement was not observed with miniplasminogen at concentrations up to 5 μM (data not shown).

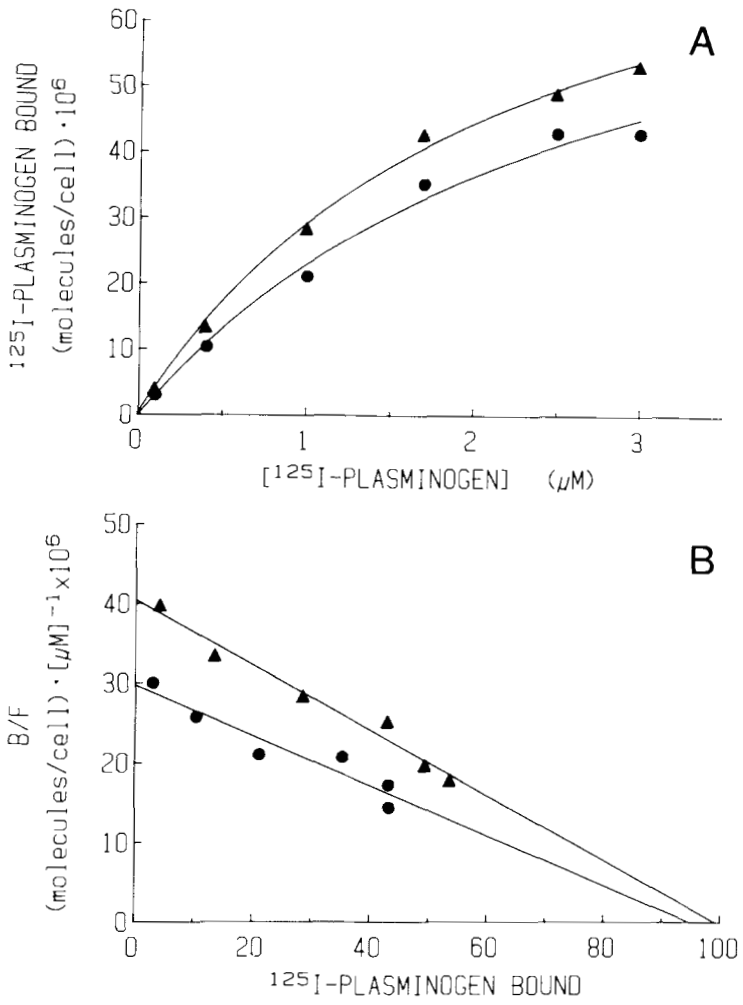

Fig. 2. Concentration dependence of binding of Pg 1 and Pg 2 to rat hepatocytes in primary culture. ^{125}I-Pg 1 (●) and ^{125}I-Pg 2 (▲) were incubated with hepatocytes at 4°C for 5 h. Results from four separate experiments were averaged. **A.** Specific binding as a function of ligand concentration. **B.** Scatchard transformation of the same data.

Near-confluent cultures of C6 cells bound ^{125}I-Pg 1 and ^{125}I-Pg 2. Greater than 65% of the binding was specific. Specific binding was apparently saturable. The saturation isotherms and Scatchard analyses for specific plasminogen binding are shown in Figure 4. The K_D for Pg 1 was 2.2 ± 0.1 μM, and the maximum binding capacity was 2.8 ± 0.6 × 10^6 sites per cell. The affinity for Pg 2 was slightly higher (K_D 1.5 ± 0.2 μM); however, the B_{max} was similar (3.7 ± 0.9 × 10^6 molecules per cell). These values are in good agreement with those determined previously using a mixture of plasminogen isozymes [9].

Radioligand displacement studies were performed with C6 cells, as described for hepatocytes, except that ^{125}I-Pg 1 was used instead of ^{125}I-Pg 2 (Fig. 5). Once again, the radiolabeled ligand was displaced by both isozymes, and Pg 2 was slightly more effective.

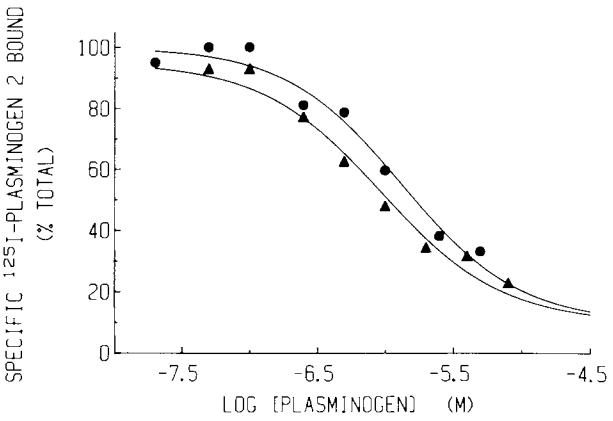

Fig. 3. Inhibition of ^{125}I-Pg 2 binding to hepatocytes by Pg 1 and Pg 2. ^{125}I-Pg 2 (0.05 μM) and increasing concentrations of unlabeled Pg 1 (●) or Pg 2 (▲) were incubated with hepatocytes at 4°C for 4 h. Specific radioligand binding was plotted as a percentage of that demonstrated in the absence of competing ligand.

A 50-fold molar excess of nonradiolabeled Pg 1 and 20 mM lysine displaced ^{125}I-Pg 1 from cellular binding sites similarly, confirming the appropriate use of lysine to determine specific binding. The K_I for Pg 1 was 1.1 μM, and the K_I for Pg 2 was 0.6 μM. The binding constants for Pg 1 and Pg 2 with hepatocytes and C6 cells are summarized in Table I.

Plasminogen Activation in the Presence of Hepatocytes

In a previous investigation, workers from this laboratory demonstrated enhanced activation of a mixture of Pg 1 and Pg 2 by two-chain rt-PA in the presence of hepatocyte primary cultures [4]. This result was somewhat surprising in that hepatocytes in primary culture rapidly catabolize t-PA [39]. We postulated that enhanced activation required plasminogen binding to the cell surface. The difference in receptor affinity for Pg 1 and Pg 2 provided an opportunity to test this hypothesis. Figure 6 shows the activation of equal concentrations of Pg 1 and Pg 2 (1.0 μM) by rt-PA in the presence and absence of hepatocytes. In the absence of cells, plasminogen activation was minimal, and there was almost no detectable difference between Pg 1 and Pg 2. Hepatocytes enhanced the activation of both isozymes; however, the extent of enhancement was greater with Pg 2. Under equivalent conditions, the activation of miniplasminogen by rt-PA was not enhanced by the hepatocytes.

To determine if the enhanced plasminogen activation was caused by cell-mediated conversion of receptor-bound [Glu^1]-plasminogen into [Lys^{77}]-plasminogen [14,15], ^{125}I-[Glu^1]-Pg 1 and ^{125}I-[Glu^1]-Pg 2 were incubated with hepatocytes for 5 h at 37°C. Cell-associated radioactivity was separated from the medium and analyzed by electrophoresis. Conversion of [Glu^1]-Pg to [Lys^{77}]-Pg was not observed with either carbohydrate isozyme (data not shown).

Streptokinase activation of plasminogen and miniplasminogen was studied in the presence of hepatocytes. The concentration of streptokinase was varied from 1.0 to 200 pM. The concentration of plasminogen or miniplasminogen was constant (1.0 μM). In two separate experiments, no enhancement was observed relative to culture wells that did not have hepatocytes (data not shown).

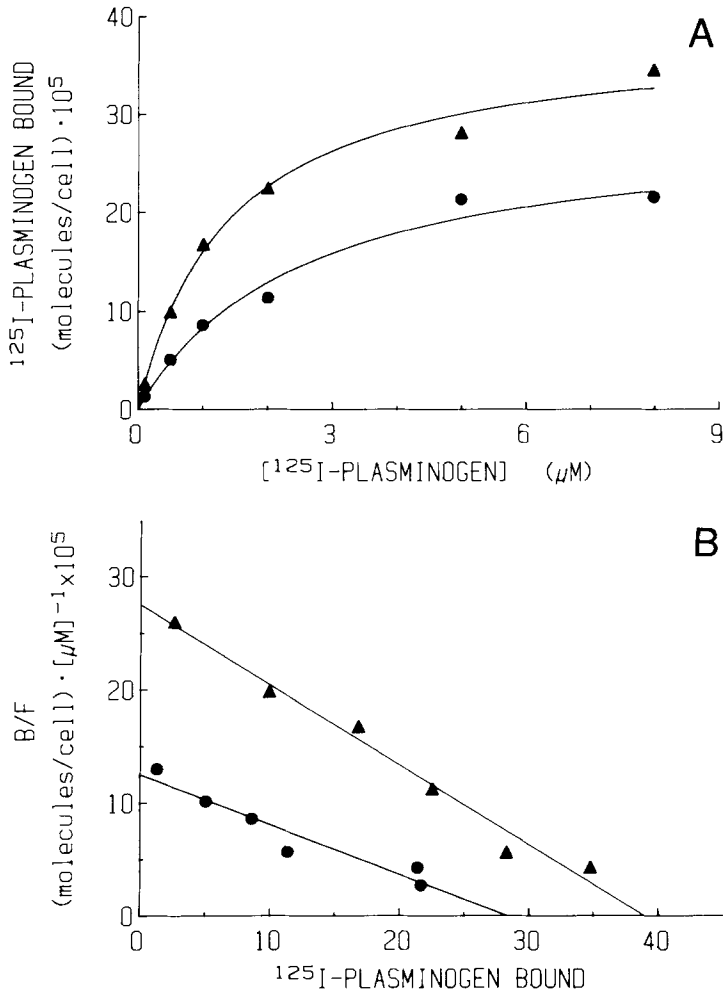

Fig. 4. Concentration dependence of binding of Pg 1 and Pg 2 to rat C6 glioma cells. ^{125}I-Pg 1 (●) and ^{125}I-Pg 2 (▲) were incubated with C6 cells at 4°C for 5 h. **A.** Specific binding as a function of ligand concentration. **B.** Scatchard transformation of the same data.

Plasminogen Activation in the Presence of C6 Cells

Activation of plasminogen by rt-PA was not enhanced by C6 cells.This result was confirmed in experiments with 1.0, 2.0, and 4.0 μM plasminogen (Fig. 7). Enhanced activation was observed with each of these plasminogen concentrations when hepatocytes were substituted for the C6 cells. The activation of miniplasminogen by rt-PA and the activation of plasminogen by streptokinase were also not affected by C6 cells.

Control experiments were performed to determine whether these plasminogen activation experiments were affected by C6 cell-associated proteinase inhibitors. First, preactivated plasmin was added to cultures of C6 cells or to empty culture wells. No difference in the rate of S-2251 hydrolysis was observed. In the second set of control experiments, rt-PA was incubated with C6 cells for 30 min. The medium was then

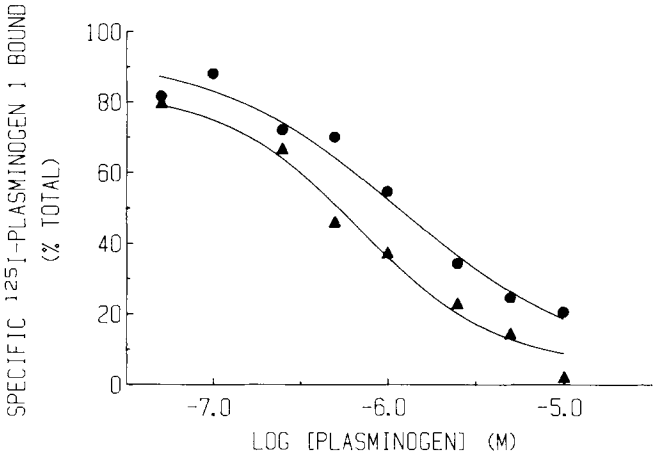

Fig. 5. Inhibition of ^{125}I-Pg 1 binding to C6 cells by Pg 1 and Pg 2. ^{125}I-Pg 1 (0.1 μM) and increasing concentrations of unlabeled Pg 1 (●) or Pg 2 (▲) were incubated with C6 cells at 4°C for 4 h. Specific radioligand binding was plotted as a percentage of that demonstrated in the absence of competing ligand.

separated from the cultures, and plasminogen activation was examined in the absence of cells. No loss of rt-PA activity was demonstrated.

Plasmin Inhibition by α_2AP

Hepatocyte receptors partially protect plasmin from inhibition by α_2AP [4]. Since hepatocytes and C6 cells differ in ability to enhance plasminogen activation by rt-PA, plasmin inhibition experiments were performed with C6 cells (Fig. 8) using the procedure described previously for hepatocytes [4]. Plasminogen was activated in the presence and absence of C6 cells. A premixed solution of S-2251 and α_2AP (hatched bars) or S-2251 alone (open bars) was then added to each well. When no cells were present (labeled "control" in Fig. 8), plasmin inhibition by α_2AP was essentially immediate, since substrate hydrolysis in the presence of the inhibitor was reduced by 85–90%. In the culture wells with C6 cells, significantly more substrate hydrolysis occurred in the presence of α_2AP (40% of that seen in the absence of inhibitor). These studies indicate that at least a fraction of the plasmin was protected from inhibition by the C6 cells.

To examine this effect further, plasminogen was activated in the presence of C6 cells as described above. After 90 min, the media were separated from the cells. About 70% of the plasmin activity (based on S-2251 hydrolysis) was recovered in the cell-associated fraction (labeled "surfaces" in Fig. 8). This percentage was determined assuming equivalent kinetic parameters (k_{cat} and K_M) for the hydrolysis of S-2251 by

Table 1. Binding Constants for Pg 1 and Pg 2 With Rat Hepatocytes and C6 Glioma Cells

	Hepatocytes		C6 cells	
Isozyme	K_D (μM)[a]	B_{max} (sites/cell)	K_D (μM)	B_{max} (sites/cell)
Pg 1	3.2 ± 0.2	9.4 ± 0.8 × 10^7	2.2 ± 0.1	2.8 ± 0.6 × 10^6
Pg 2	1.9 ± 0.1	9.8 ± 1.3 × 10^7	1.5 ± 0.2	3.7 ± 0.9 × 10^6

[a]Values derived from direct binding studies.

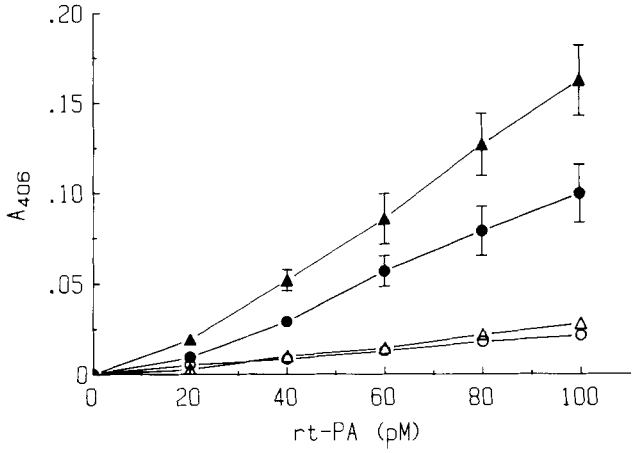

Fig. 6. Activation of Pg 1 and Pg 2 by rt-PA in the presence of hepatocytes. Pg 1 (●), Pg 2 (▲), and S-2251 were added to hepatocyte cultures and incubated for 30 min at room temperature. rt-PA was then added, and incubation was continued for an additional 95 min. Absorbances were determined at 406 nm. The same experiment was performed with Pg 1 (○) and Pg 2 (△) in wells without cells. Each experiment was performed three times (avg. ± SEM).

solution-phase and membrane-bound plasmin. Plasmin inhibition experiments with S-2251 and α_2AP were then performed using the two separated fractions. In the aspirated media, the α_2AP inhibited the plasmin efficiently. The amidase activity was 17% of that demonstrated in the absence of inhibitor. By contrast, α_2AP functioned poorly as an inhibitor of plasmin in the reconstituted wells of C6 cells. Substrate hydrolysis remained near 45% of that determined in the absence of inhibitor. Although these studies are qualitative, the results strongly suggest that C6 cell-receptor-bound plasmin is less reactive with α_2AP. The results previously demonstrated with hepatocytes were similar [4].

DISCUSSION

The two major plasminogen isozymes differ in carbohydrate content [19–21]. The distinguishing structure is a glucosamine-based oligosaccharide substituted at Asn-288 in Pg 1 but not in Pg 2. In U937 cells, this difference in structure has a major effect on plasminogen receptor binding [24]. At saturation, Pg 2 binding greatly exceeds the level of Pg 1 binding, suggesting that some U937-cell receptors may be available to only the former isozyme [24]. Miles et al. [16] demonstrated the importance of the K1–3 plasminogen domain in receptor interactions, and this domain includes Asn-288. The preactivation peptide (residues 1–77) also affects plasminogen–receptor interactions [14,15], most likely by interfering with the critical K1–3 binding site.

In this investigation, the binding of human Pg 1 and Pg 2 to cell surface receptors was studied using two rat cell types (hepatocytes and C6 cells). The species differences are probably not significant since plasminogens purified from bovine, ovine, porcine, canine, and rat plasma compete with human plasminogen for specific cell-surface binding sites in rat hepatocytes and C6 cells [40]. The receptor binding affinities of the various plasminogens are similar.

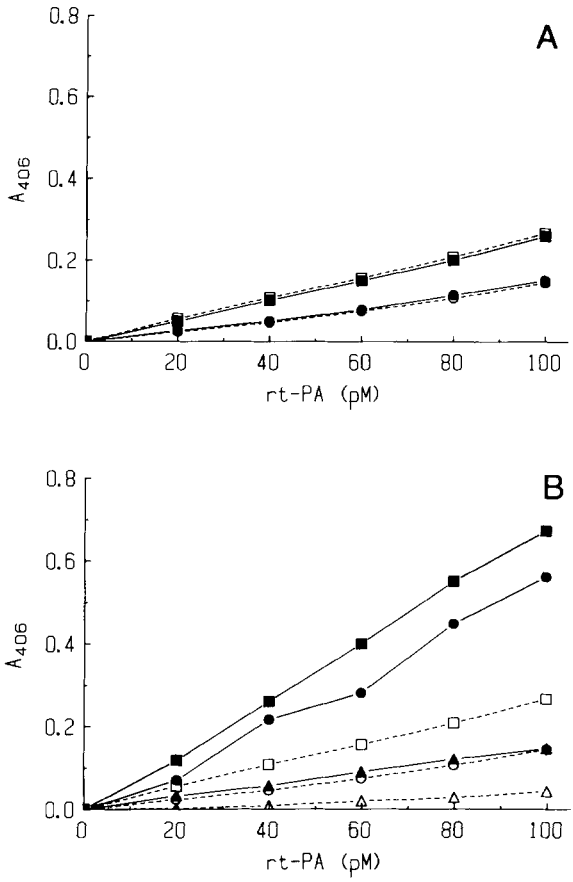

Fig. 7. Plasminogen activation by rt-PA in the presence of C6 glioma cells and hepatocytes. **A.** A mixture of Pg 1 and Pg 2 was activated in the presence of C6 cell cultures. The plasminogen concentration was 4 μM (■) or 2 μM (●). Equivalent concentrations of plasminogen [2 μM (○) and 4 μM (□)] were activated in wells without cells. **B.** The activation of three different concentrations of plasminogen in hepatocyte cultures: 4 μM (■), 2 μM (●), and 0.2 μM (▲) and in wells without cells 4 μM (□), 2 μM (○), and 0.2 μM (△).

The studies presented here demonstrate that a comparable number of cell surface sites are available for Pg 1 and Pg 2 binding to primary cultures of rat hepatocytes and C6 glioma cells. The radioligand displacement experiments demonstrate that at least a large fraction and perhaps all of the sites for Pg 1 and Pg 2 are the same. With C6 cells, displacement of ^{125}I-Pg 1 was essentially complete with 10 μM Pg 2. Somewhat lower concentrations of nonradiolabeled plasminogen were studied in the hepatocyte experiments due to the large amounts of protein required in the culture system; however, Pg 1 displaced ^{125}I-Pg 2 nearly as well as nonradiolabeled Pg 2.

A small but consistent difference in receptor affinity for Pg 1 and Pg 2 was demonstrated in both cell systems. With hepatocytes, the difference in receptor affinity for Pg 1 ($K_D = 3.2$ μM) and Pg 2 ($K_D = 1.9$ μM) was statistically significant ($P = 0.0005$), as determined with an unpaired, two-tailed t test. With C6 cells, the difference in affinity for Pg 1 and Pg 2 was only marginally significant based on an analysis of the saturation isotherm data; however, a higher affinity for Pg 2 was also demonstrated in the radioligand displacement studies.

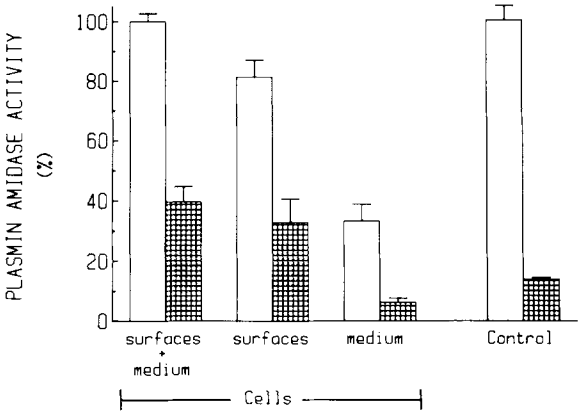

Fig. 8. Inhibition of plasmin by α_2AP in the presence of C6 cells. Plasminogen was activated at room temperature with rt-PA in cultures of C6 cells ("Cells") and in wells without cells ("Control"). After 90 min, the medium ("medium") was separated from the cells ("surfaces") and studied separately. In these experiments, the C6 cells were immediately reconstituted with new buffer (no additional Pg was added). In some wells, the initial medium and cells were studied together without separation ("surfaces + medium"). Each well received S-2251 alone (open bars) or α_2AP and S-2251 simultaneously (hatched bars). Plasmin activity was determined by substrate hydrolysis for 15 min. Plasmin amidase activity in the separated fractions ("medium" or "surfaces") was expressed as a percentage of the activity observed when S-2251 was added to the unfractionated wells ("surfaces + medium").

Indirect evidence that carbohydrate side chains affect the interaction of plasminogen with cell surface binding sites has been derived from plasma clearance experiments performed in mice [41]. In this system, ^{125}I-Pg 2 cleared slightly more rapidly than ^{125}I-Pg 1, probably reflecting differences in binding to endothelium and other cells exposed to the blood such as hepatocytes. A markedly increased rate of plasma clearance was demonstrated with recombinant plasminogen that contained no carbohydrate. Receptor binding studies have not been reported with the recombinant protein. Unpublished experiments performed in our laboratory have demonstrated that Pg 1 is insensitive to enzymes that dissociate N-linked carbohydrate chains under nondenaturing conditions (endoglycosidase F, up to 10 units/ml for 36 h at 37°C, and N-glycosidase F, up to 10 units/ml for 36 h at 37°C). Therefore, it has not been possible to compare the receptor interactions of Pg 2 and deglycosylated Pg 1.

Additional support for a difference in Pg 1 and Pg 2 binding affinity with hepatocytes was obtained in plasminogen activation experiments. The activation of Pg 2 was enhanced by the cells to a greater degree than Pg 1. The average enhancement factor for Pg 2 (hepatocytes vs. no cells) was 600% ± 30%, as determined by averaging data obtained with different concentrations of rt-PA in Figure 6. The enhancement factor for Pg 1 was 370% ± 50%. When interpreting the plasminogen activation data shown in Figure 6, it is important to consider that only 1.0–2.0% of the plasminogen is bound to the surface at any given time. At least four separate reactions with different kinetic parameters may be occurring simultaneously: 1) surface-associated plasminogen activated by surface associated rt-PA, 2) surface-associated plasminogen activated by solution-phase rt-PA, 3) solution-phase plasminogen activated by surface-bound rt-PA, and 4) solution-phase plasminogen activated by solution-phase rt-PA. Since the activation studies correlate with receptor binding affinity, the data support the requirement of

plasminogen receptor binding for enhanced activation in the hepatocyte system (mechanisms 1 or 2 as opposed to mechanism 3).

Miniplasminogen, like plasminogen, is a substrate for rt-PA. Miniplasminogen may interact with plasminogen receptors; however, the affinity is greatly reduced compared with plasminogen [16]. In the hepatocyte system, both the interaction of miniplasminogen with the plasminogen receptor and the enhanced activation of miniplasminogen by rt-PA were not demonstrated. These data suggest that the enhanced activation of plasminogen by rt-PA in the hepatocyte cultures does not simply reflect a substrate-independent change in the catalytic efficiency of the enzyme.

[Glu^1]-plasminogen exists in two distinct conformations, one of which is more readily activated by all plasminogen activators, including urokinase, streptokinase, and rt-PA [42–44]. The easily activated conformation is favored only when anions such as chloride are removed from the buffer or when ϵ-amino caproic acid is added. It is possible that the plasminogen receptor enhances activation merely by altering the preferred conformation of the bound plasminogen; however, if this was true, enhanced plasminogen activation by streptokinase would be expected as well. The data presented here suggest that a more complex mechanism is involved. The most likely explanation is that a ternary complex involving receptor, plasminogen, and rt-PA is required for enhanced plasminogen activation. Confirmation of this hypothesis will require more intensive experimentation with different activator species.

The inability of C6 cells to promote plasminogen activation by rt-PA may indicate that the plasminogen receptor in this cell type is different from the binding site(s) present on hepatocytes. An alternative explanation might involve the equivalent plasminogen-receptor interaction coupled with differences in the ability of rt-PA to bind and function at the surfaces of the two cell types. The latter hypothesis is supported by our data demonstrating similar plasminogen receptor binding parameters (K_D) and plasmin inhibition results (Fig. 8) with the two cell systems. The difference in B_{max} for C6 cells and hepatocytes is accounted for by the difference in cell size; the receptor density in the membrane may be nearly equivalent. The nature of the plasminogen binding site(s) on various cell types remains incompletely understood. In comparison with U937 cells [24], both hepatocytes and C6 cells show different behavior with respect to the separated plasminogen isozymes. A counterpart to the U937 plasminogen binding site that recognizes only Pg 2 was not demonstrated in either cell type studied here. In all three cell systems, however, carbohydrate structure is important. These studies demonstrate the significance of carbohydrate heterogeneity in plasminogen binding to cell surfaces in general.

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