Plasminogen has been implicated in extracellular matrix degradation by invading cells, but few high affinity cell surface receptors for the molecule have been identified. Previous studies have reported that the plasma protein, histidine-rich glycoprotein (HRG), interacts with plasminogen and cell surfaces, raising the possibility that HRG may immobilize plasminogen/plasmin to cell surfaces. Here we show, based on optical biosensor analyses, that immobilized HRG interacts with soluble plasminogen with high affinity and with an extremely slow dissociation rate. Furthermore, the HRG-plasminogen interaction is lysine-dissociable and involves predominantly the amino-terminal domain of HRG, and the fifth kringle domain of plasminogen, but not the carboxyl-terminal lysine of HRG. HRG was also shown to tether plasminogen to cell surfaces, with this interaction being potentiated by elevated Zn\(^{2+}\) levels and low pH, conditions that prevail at sites of tissue injury, tumor growth, and angiogenesis. Based on these data we propose that HRG acts as a soluble adaptor molecule that binds to cells at sites of tissue injury, tumor growth, and angiogenesis, providing a high affinity receptor for tethering plasminogen to the cell surface and thereby enhancing the migratory potential of cells.

Plasminogen is a plasma protein, which, when converted to plasmin, is known to play a pivotal role in fibrinolysis (1, 2). Plasminogen is a 92-kDa modular glycoprotein consisting of a preactivation peptide, five kringle domains, and a catalytic carboxy-terminal serine protease domain (3). Plasminogen has a plasma concentration of 150–200 \(\mu\)g/ml \((-1.5–2 \mu\text{M})\) (4). Plasmin, the active form of plasminogen, is the proteolytic enzyme responsible for degrading fibrin clots and binds to the cell surface through various cell surface receptors (33–35). Significantly, HRG interacts with both plasminogen and cell surfaces, raising the possibility that HRG may immobilize plasminogen/plasmin to the cell surface. In the present study we examined the interaction between HRG and plasminogen using both optical biosensor and cell surface binding assays. Our findings provide the first evidence that HRG can tether plasminogen to the surface of cells, specifically at low pH and in the presence of 20 \(\mu\text{M} Zn^{2+}\), conditions

---

*This work was supported in part by a National Health and Medical Research Council of Australia program grant and a New South Wales Cancer Council program grant. 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.

§ Recipient of a Viertel senior medical research fellowship.

** To whom correspondence should be addressed: Division of Immunology and Genetics, John Curtin School of Medical Research, Australian National University, Canberra, ACT 2601, Australia. Tel: 61-261-252-604; Fax: 61-261-252-595; E-mail: christopher.parish@anu.edu.au.

---

1 The abbreviations used are: HRG, histidine-rich glycoprotein; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); BSA, bovine serum albumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELISA, enzyme-linked immunosorbent assay; Hs27, hexahistidine; HRR, histidine-rich region of HRG; LBS, ligand binding sites; mAb, monoclonal antibody; Ni-NTA, nickel-nitrilotriacetic acid; N1N2, the amino-terminal domain of HRG; PBS, phosphate-buffered saline.
that usually occur at sites of tissue injury, inflammation, tumor growth, and angiogenesis. Based on these findings we propose that under conditions of low pH and elevated free Zn\(^{2+}\), HRG binds to cell surfaces and acts as a high affinity receptor for plasminogen. This would, in theory, enhance the efficiency of conversion of plasminogen to plasmin, protect plasmin from inactivation, and provides a mechanism for polarizing the proteolytic activity of plasmin on the cell surface, resulting in enhanced migratory potential.

EXPERIMENTAL PROCEDURES

Cell Lines—B16F1 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum and incubated at 37 °C in a humidified atmosphere containing 5% CO\(_2\). The Spodoptera frugiperda-derived insect cell line S9 was cultured in SF-900 II serum-free medium (Invitrogen) at 27 °C.

Purification of Human HRG, Plasminogen, and Angiostatin—Native human HRG was purified from fresh human plasma as described previously (36). Briefly, a phosphocellulose column was prepared and equilibrated with loading buffer (0.5 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 6.8) for 24 h. Fresh human plasma was provided by Red Cross House, The Canberra Hospital, Canberra, Australia, and mixed with the protease inhibitors aprotinin (2 \(\mu\)g/ml), phenylmethylsulfonyl fluoride (5 mM) and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (100 \(\mu\)g/ml). The plasma was passed through the equilibrated column, unbound protein was removed by extensive washing with loading buffer, and bound HRG was eluted with 2.0 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 6.8. Native human plasminogen was purified from fresh human plasma, as described previously (37–39), by passage of plasma through a lysine-Sepharose 4B column (Amersham Biosciences). The column was equilibrated with 0.1 M sodium phosphate, pH 7.4, and was washed extensively with 0.3 M sodium phosphate, pH 7.4, before elution of bound plasminogen with 0.1 M e-aminoacaproyc acid, pH 7.4. Purified proteins were stored at −70 °C.

Angiostatin was purified as described previously (40). Typically, 100 \(\mu\)g/ml plasma-derived or recombinant HRG and/or 150 \(\mu\)g/ml plasma-derived plasminogen/angiostatin was added to 5 \(\times\) 10\(^5\) B16F1 cells in RPMI 1640 supplemented with 10% fetal calf serum, 1% L-glutamine, 100 \(U\) penicillin, and 100 \(\mu\)g/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO\(_2\) and 10% fetal calf serum. The medium was changed every 2 to 3 days. The harvested cells were collected, and cell-bound angiostatin was detected using a rabbit polyclonal plasminogen Ab (DAKO A/S, Glostrup, Denmark) followed by secondary antibody detection with a sheep anti-rabbit Ig horseradish peroxidase conjugate (Amad Biotech, Melbourne, Australia). Plate-bound angiostatin was detected using APTS peroxidase substrate (Kirkegaard and Perry Laboratories Inc. Gaithersburg, MD) by measuring absorbance at 405 nm (PerkinElmer, Boston, MA) using a Ther mastar 1000 spectrophotometer (Beckman Instruments). The data were analyzed using SoftMaxPro software (Molecular Devices Corp., Sunnyvale, CA).

Immunofluorescence Flow Cytometry—Cell lines were analyzed for HRG or plasminogen/angiostatin cell surface binding by flow cytometry. Typically, 100 \(\mu\)g/ml plasma-derived or recombinant HRG and/or 150 \(\mu\)g/ml plasma-derived plasminogen/angiostatin was added to 5 \(\times\) 10\(^5\) B16F1 cells in PBS and 0.1% BSA, pH 7.2, or 20 \(\mu\)M Zn\(^{2+}\) for 60 min at 4 °C and washed three times with PBS and 0.1% BSA. Zinc acetate was used as a source of Zn\(^{2+}\), and Zn\(^{2+}\) was added after BSA to PBS to prevent precipitation. Cell-bound HRG was detected using the HRG-4 mAb followed by secondary detection with sheep anti-mouse Ig fluorescein isothiocyanate (Amad Biotech). Rabbit polyclonal and cell-bound plasminogen or angiostatin was detected using a rabbit polyclonal plasminogen Ab (DAKO A/S, Glostrup, Denmark) followed by secondary detection with sheep anti-rabbit Ig fluorescein isothiocyanate (Amad Biotech). Specific (HRG-4; AGEN, Brisbane, Australia) mAb, and N1 and N1N2 domain were detected using a HRG-specific (HRG-4; AGEN, Brisbane, Australia) mAb, and N1 and N1N2 were detected using a His\(_6\)-specific (Roche Diagnostics) mAb (His\(_6\)). Chemiluminescence was detected using ECL Western blotting detection reagents (Amer sham Biosciences).

Enzyme-linked Immunosorbent Assays (ELISAs)—ELISAs were performed by coating 96-well PVC microtiter plastic plates (Dynax Technologies Inc., Chantilly, VA) overnight at 4 °C with plasminogen or recombinant full-length HRG, C-mutant HRG, N1 fragment, or N1N2 domain (50 \(\mu\)g/ml, 5 \(\mu\)g/ml) diluted in a 0.05 M Na\(_2\)CO\(_3)/NaHCO\(_3\) buffer, pH 9.6 (Sigma). Plates were then washed in PBS and 0.01% Tween 20 (Sigma) and blocked for 120 min at room temperature with 3% (w/v) BSA diluted in PBS. For the plasminogen-pretreated plates, recombinant forms of HRG diluted in PBS and 1% BSA (50 \(\mu\)g/ml, 5 \(\mu\)g/ml) were added to the plates, allowed to bind for 60 min at room temperature, and then washed in PBS and 0.01% Tween 20. HRG and HRG fragment binding to empty blocked plates was used as the negative background control. Bound full-length HRG, C-mutant HRG, and the N1N2 domain were detected using HRG-4, whereas bound N1 fragment was detected using His\(_6\), followed by secondary antibody detection with a sheep anti-mouse Ig horseradish peroxidase conjugate (Amad Biotech, Melbourne, Australia). Plate-bound peroxidase was detected using APTS peroxidase substrate (Kirkegaard and Perry Laboratories Inc. Gaithersburg, MD) by measuring absorbance at 405 nm (PerkinElmer, Boston, MA) using a Ther mastar 1000 spectrophotometer (Beckman Instruments).
were effectively disrupted by injecting 100 μM L-lysine for 1 min at a flow rate of 100 μl/min. Experiments used an automated program to control triplicate injections of each binding protein. Flow-cell 1 was used as a blank reference cell, with the background binding to the dextran matrix detected in flow-cell 1 being subtracted from responses in flow-cells 2, 3, and 4. Binding curves were analyzed using the BIA Evaluation program (Pharmacia Biosensor).

RESULTS

Effect of Zn²⁺ and pH on the Binding of Human HRG to Cell Surfaces—Previous studies have shown that the binding of HRG to surface-immobilized heparin is dependent on both Zn²⁺ and pH, with Borza and Morgan (41) suggesting that Zn²⁺ and pH act synergistically to affect the conformation and thus function of HRG. This observation prompted us to test the effect of Zn²⁺ with immobilized HRG is dependent on the plasminogen concentration (20, 21). Using the optical biosensor, we further characterized immobilized HRG binding studies showed that the interaction of soluble plasminogen (20, 21). Using the optical biosensor, we further characterized the interaction of soluble plasminogen with immobilized HRG. 

A similar effect of Zn²⁺ concentration on HRG binding was observed with CHO-K1 cells (data not shown). We also investigated the effect of pH on HRG binding to cell surfaces in the presence of an optimal Zn²⁺ concentration (20 μM) and found that binding was minimal at pH 8.0 but increased steadily as the pH was reduced to 6.0 (Fig. 1, C and D). In fact, there was a 250-fold increase in the level of cell surface-bound HRG across this pH range. Thus, it appears that both Zn²⁺ and pH directly affect the interaction of HRG with cell surfaces, with optimal HRG binding occurring in the presence of ~20 μM Zn²⁺ and at pH 6.0.

Soluble Plasminogen Binds to Immobilized HRG—Previous studies identified plasminogen as an important HRG ligand (20, 21). Using the optical biosensor, we further characterized this interaction by monitoring the binding and dissociation of soluble plasminogen to immobilized HRG (Fig. 2A). These binding studies showed that the interaction of soluble plasminogen with immobilized HRG is dependent on the plasminogen concentration over the range tested of 32–2,000 nM (3–185 μg/ml). The almost flat dissociation curve (Fig. 2A) indicates an extremely slow off-rate or dissociation between HRG and plasminogen, reflecting a high affinity interaction (see analysis below). In fact, even when dissociation was allowed to occur for many hours, plasminogen remained tightly bound to HRG (data not shown). Nevertheless, bound plasminogen could be very effectively displaced by exposure to 100 mM L-lysine, indicating that lysine can disrupt HRG-plasminogen complexes, and hence the interaction is likely to be mediated by lysine residues (Fig. 2B). Alternatively, lysine may disrupt the plasminogen-HRG complex by binding to plasminogen and inducing a conformation change that results in the complex dissociating. Similar association-dissociation results were obtained with plasminogen-I and plasminogen-II (data not shown), isoforms of the molecule that differ by one glycosylation site (43–45).

An analysis of the binding curves for the interaction between soluble plasminogen and immobilized HRG using the BIA Evaluation program showed that the data could be described by both a bivalent analyte model and a two-state conformation model. The bivalent analyte model assumes that each molecule of the analyte (plasminogen) can interact bivalently with one or two immobilized ligand (HRG) molecules(s). Binding to the first HRG molecule is defined by a single set of rate constants (k₁ and kₙ₁), and binding to the second HRG molecule is defined by a second set of rate constants (kₙ₂ and kₙ₂), thus allowing the model to take cooperative effects into account (Table I). Such cooperative effects could well explain the extremely slow off-rates that are observed (Fig. 2). The two-state conformation model, on the other hand, assumes that one molecule of plasminogen binds to one or more molecules of immobilized HRG, and that this is then followed by a conformation change (represented as * in the reactions below) in the complex, which stabilizes the interaction. Again, two sets of kinetic constants are produced (Table 1), involving two-step association and dissociation, shown below.

\[
\text{A} + \text{B} \rightarrow \text{AB} \quad \text{(Step 1)}
\]

\[
\text{AB} \rightarrow \text{AB}* \quad \text{(Step 2)}
\]

\[
\text{AB}* \rightarrow \text{A} + \text{B} \quad \text{(Step 1)}
\]

\[
\text{AB} \rightarrow \text{A} + \text{B} \quad \text{(Step 2)}
\]

Both the bivalent analyte and two-state conformation models estimate an apparent Kᵢ value for the interaction of ~200–300 nM, with the most notable feature of the interaction between immobilized HRG and soluble plasminogen being the extremely slow dissociation/off-rate, indicating a high affinity, highly stable interaction.

Plasminogen Binding to the Surface of B16F1 Cells Is Enhanced Dramatically in the Presence of HRG—Although plasminogen has been reported to interact with HRG, there has been no previous indication that HRG can potentiate the interaction of plasminogen with the cell surface. Using flow cytometry, we found that 150 μg/ml plasminogen binding to the surface of B16F1 cells is enhanced greatly (up to 14-fold) when the cells have been either precoated with 100 μg/ml plasma-derived HRG (Fig. 3A, HRG/plasminogen) or when plasminogen is coincubated with HRG (Fig. 3A, HRG + plasminogen). The effect of HRG on plasminogen binding to B16F1 cells was maximal when the cells were coincubated with a mixture of plasminogen and HRG, rather than precoating the cells with HRG prior to plasminogen exposure (p < 0.01, Fig. 3B). HRG also enhanced the binding of plasminogen to cells over a wide range of plasminogen concentrations (0.1–200 μg/ml) (data not shown), although these studies showed that in the presence of physiological concentrations of HRG (100 μg/ml, 1.3 μM), maximal plasminogen binding occurred with 150 μg/ml (1.6 μM) plasminogen, which is the physiological plasma concentration of this molecule. Further studies aimed at characterizing the effect of soluble HRG on plasminogen cell surface binding showed that excess soluble HRG (>5 times molar excess of HRG over plasminogen) (1.6 μM plasminogen, 8 μM HRG) did not inhibit cell surface plasminogen binding (Fig. 3C). Even relatively low concentrations of HRG (0.3 μM) were sufficient to support near maximum plasminogen binding, suggesting that HRG is an efficient adaptor molecule for binding plasminogen to the cell surface. Our initial studies indicated that pH affects HRG cell surface binding (Fig. 1, C and D). Using flow cytometry, we found that the ability of HRG to potentiate the binding of plasminogen to cells was pH-dependent (Fig. 3D). Thus, plasminogen binding steadily increased with decreasing pH, the lowest plasminogen binding occurring at pH 8.0, and the highest at pH 6.0. At all pH values tested,
Fig. 1. Effect of Zn\(^{2+}\) and pH on HRG binding to B16F1 melanoma cells. A, B16F1 cells were incubated with 100 μg/ml plasma-derived HRG in PBS and 0.1% BSA, pH 7.2, in the presence of 0, 20, or 100 μM Zn\(^{2+}\) and then analyzed for HRG binding using the HRG-specific mAb HRG-4 and flow cytometry. Representative flow cytometry histograms show HRG binding at 0, 20, and 100 μM Zn\(^{2+}\). Shaded histograms represent background binding of HRG-4 in the absence of HRG; open histograms represent HRG binding. B, numerical values showing HRG binding to B16F1 cells as a -fold increase in median fluorescence above background at different Zn\(^{2+}\) concentrations (0–100 μM). C, B16F1 cells were incubated with 100 μg/ml plasma-derived HRG in PBS, 0.1% BSA, and 20 μM Zn\(^{2+}\) at various pH values (6.0–8.0) and then analyzed for HRG binding by flow cytometry. Representative flow cytometry histograms show HRG binding at pH 6.0, 6.5, 7.0, 7.5, and 8.0. Shaded histograms represent background binding of HRG-4 in the absence of HRG; open histograms represent HRG binding. D, numerical values showing HRG binding to B16F1 cells between pH 6.0 and 8.0 as a -fold increase in median fluorescence above background. Data are the mean ± S.E. of three determinations.
HRG Acts as a Plasminogen Receptor

plasminogen binding was virtually totally dependent on the presence of HRG (Fig. 3D). These data suggest that pH modulates plasminogen binding by regulating the interaction of HRG with the cell surface.

Analysis of the Regions of HRG and Plasminogen Involved in the HRG-Plasminogen Interaction.—Lysine residues have been predicted to play an important role in the interaction between HRG and plasminogen, the biosensor studies outlined in Fig. 2 supporting this view (46). It has been suggested that the carboxyl-terminal lysine residue on HRG is likely to be a candidate as one of the key plasminogen-binding residues (46). On the other hand, it has been proposed that the amino-terminal region of HRG may interact with plasminogen (46). To test these possibilities, we produced in insect cells using a baculovirus expression system, recombinant full-length HRG, C-mutant HRG, and the first HRG lacking the carboxyl-terminal lysine residue, the N1N2 domain were recognized by HRG-4, whereas N1 was only detected by His$_9$, suggesting that the epitope recognized by HRG-4 mAb is located within the N2, and not the N1 portion of the N1N2 domain. Furthermore, these studies indicate that the HRG-4 mAb interacts with both denatured (Western blotting studies) and native (ELISA data) forms of HRG, C-mutant HRG, and N1N2.

The recombinant full-length HRG and C-mutant HRG were then tested for their ability to bind plasminogen. The two forms of HRG were immobilized onto the surface of a CM5 sensor chip, and the binding of soluble plasminogen was determined by the biosensor. Resultant biosensor sensorgrams (Fig. 5A) showed that the binding of plasminogen to full-length HRG is essentially identical to the binding of plasminogen to C-mutant HRG. Because of high background binding of soluble HRG we were, unfortunately, unable to use the biosensor to examine the binding of soluble HRG to immobilized plasminogen. As an alternative approach, we immobilized plasminogen in the wells of plastic microtiter plates and used an ELISA to measure the binding of both full-length HRG and C-mutant HRG to the immobilized plasminogen. Again, these experiments indicated that plasminogen binds to C-mutant and full-length HRG with similar affinity, and thus the carboxyl-terminal lysine residue on HRG does not appear to be essential for plasminogen binding (Fig. 5B). To investigate further the plasminogen binding domain within HRG, we also tested whether the recombinant N1 fragment or the N1N2 domain could bind to immobilized plasminogen. ELISA studies indicated that the N1N2 domain exhibited significant binding to plasminogen ($p < 0.01$), although binding appears to be somewhat lower than full-length recombinant HRG, whereas the smaller fragment, N1, failed to bind to plasminogen ($p = $ not significant) (Fig. 5C). Biosensor studies with immobilized N1N2 or N1 and soluble plasminogen were unsuccessful because of the poor immobilization of these HRG fragments to the surface of the biosensor chips.

Angiostatin, which consists of the first four kringle domains and part of the fifth kringle domain of plasminogen, is a fragment of plasminogen which exhibits antiangiogenic activity. Because the present study has shown that plasminogen binds with high affinity to HRG, it was of interest to determine whether this truncated form of plasminogen also exhibited HRG binding. Based on optical biosensor studies, it was found that angiostatin did not bind with high affinity to immobilized HRG compared with plasminogen, 2 μM angiostatin exhibiting rapid association and dissociation, a sensorgram typical of a low affinity interaction. In fact, a component of the intact plasminogen sensorgram involves rapid binding and dissociation (Fig. 6A, upper panel), this aspect of the plasminogen-HRG interaction being retained by angiostatin, i.e. very rapid association and dissociation rates (Fig. 6A). Similarly, immunofluorescence flow cytometry studies indicated that the presence of physiological concentrations of HRG (100 μg/ml) could dramatically enhance the binding of plasminogen to the surface of B16F1 melanoma cells, whereas HRG did not promote the binding of angiostatin to the surface of B16F1 cells (Fig. 6B). It should be noted that ELISA studies, using immobilized angiostatin, demonstrated that the polyclonal anti-plasminogen antibody used in the cell binding studies reacted strongly with angiostatin (data not shown).
**DISCUSSION**

For extracellular matrix-degrading enzymes, such as plasmin, to aid cell invasion optimally they need to be tethered to the surface of invading cells. Many cell types express specific receptors for urokinase plasminogen activator, but to date, few if any high affinity cell surface receptors for plasminogen have been described, although low affinity ($K_d \sim 1 \mu M$) receptors including annexin-II (17, 18), $\alpha$-enolase (15), and gangliosides (16) have been identified. Here we show for the first time that the soluble plasma protein HRG can act as an adaptor protein that tethers plasminogen to cell surfaces in a highly stable manner. Interestingly, the binding of HRG (and thus plasminogen binding to the surface of cells is enhanced dramatically in the presence of HRG. A, B16F1 cells were preincubated with or without 100 $\mu g/ml$ plasma-derived HRG in PBS and 0.1% BSA (pH 7.2, 20 $\mu M Zn^{2+}$), washed with PBS and 0.1% BSA, incubated with 150 $\mu g/ml$ plasminogen in PBS and 0.1% BSA, pH 7.2, and then analyzed for plasminogen binding by flow cytometry (upper panel, HRG/plasminogen). Alternatively, B16F1 cells were incubated simultaneously with 100 $\mu g/ml$ plasma-derived HRG and 150 $\mu g/ml$ plasminogen (HRG + plasminogen) on plasminogen binding to cells. Plasminogen binding is expressed as a -fold increase in median fluorescence relative to background fluorescence, with error bars being S.E. (n = 3). For each treatment condition, white histograms represent plasminogen binding in the absence of HRG, and black histograms represent plasminogen binding in the presence of HRG, and shaded histograms representing background binding of the polyclonal plasminogen antibody in the absence of plasminogen; open histograms represent plasminogen binding.

**FIG. 3.** Plasminogen binding to the surface of cells is enhanced dramatically in the presence of HRG. A, B16F1 cells were preincubated with or without 100 $\mu g/ml$ plasma-derived HRG in PBS and 0.1% BSA (pH 7.2, 20 $\mu M Zn^{2+}$), washed with PBS and 0.1% BSA, incubated with 150 $\mu g/ml$ plasminogen in PBS and 0.1% BSA, pH 7.2, and then analyzed for plasminogen binding by flow cytometry (upper panel, HRG/plasminogen). Alternatively, B16F1 cells were incubated simultaneously with 100 $\mu g/ml$ plasma-derived HRG and 150 $\mu g/ml$ plasminogen (HRG + plasminogen) on plasminogen binding to cells. Plasminogen binding is expressed as a -fold increase in median fluorescence relative to background fluorescence, with error bars being S.E. (n = 3). For each treatment condition, white histograms represent plasminogen binding in the absence of HRG, and black histograms represent plasminogen binding in the presence of HRG, and shaded histograms representing background binding of the polyclonal plasminogen antibody in the absence of plasminogen; open histograms represent plasminogen binding. B, quantitative comparison, based on data from A, of the effect of preincubating cells with HRG (HRG/plasminogen) versus the simultaneous incubation of cells with HRG and plasminogen (HRG + plasminogen) on plasminogen binding to cells. Plasminogen binding is expressed as a -fold increase in median fluorescence relative to background fluorescence, with error bars being S.E. (n = 3). For each treatment condition, white histograms represent plasminogen binding in the absence of HRG, and black histograms represent plasminogen binding in the presence of HRG. C, B16F1 cells were incubated with 150 $\mu g/ml$ plasminogen (1.6 $\mu M$) and 0, 0.3, 1.3, or 8 $\mu M$ plasma-derived HRG in PBS and 0.1% BSA (pH 7.2, 20 $\mu M Zn^{2+}$) and then analyzed for plasminogen binding by flow cytometry. Numerical values show plasminogen binding as a -fold increase in median fluorescence above background binding of plasminogen-specific antibody to cells. D, B16F1 cells were incubated with 150 $\mu g/ml$ plasminogen and either with (black histogram) or without (white histogram) 100 $\mu g/ml$ plasma-derived HRG in PBS, 0.1% BSA, 20 $\mu M Zn^{2+}$ at pH 6.0, 6.5, 7.0, 7.5, and 8.0, and then analyzed for plasminogen binding by flow cytometry. Plasminogen binding is shown as a -fold increase in median fluorescence above background binding of plasminogen-specific antibody to cells.

**TABLE I**

| Model                | $k_{a1}$ | $k_{d1}$ | $k_{a2}$ | $k_{d2}$  |
|----------------------|----------|----------|----------|----------|
| Bivalent             | 4.6 x 10^4 ± 1.42 x 10^3 ms⁻¹ | 0.19 ± 0.018 s⁻¹ | 1.8 x 10⁻⁵ ± 1.8 x 10⁻⁶ RU⁻¹ | 1.2 x 10⁻⁵ ± 6.3 x 10⁻⁶ s⁻¹ |
| Two-state            | 3.1 x 10⁶ ± 1.9 x 10⁻⁷ ms⁻¹ | 0.19 ± 0.057 s⁻¹ | 5.2 x 10⁻³ ± 2.9 x 10⁻⁴ s⁻¹ | 2.5 x 10⁻⁵ ± 1.3 x 10⁻⁴ s⁻¹ |

* RU, response units.
HRG Acts as a Plasminogen Receptor

Metal divalent cations, in particular, Zn$^{2+}$, are known to interact with the HRR of HRG (23, 24, 49). The high concentration of histidine residues located within the HRR also results in HRG having an ionizing charge that is sensitive to pH in the range between pH 6 and 7 as the histidine residues become protonated (41). Using optical biosensor studies, Borza and Morgan (41) found that HRG binding to immobilized heparin was strikingly pH-sensitive, with maximum binding occurring at pH <6.0. Poor HRG binding was observed at physiological pH in the absence of Zn$^{2+}$, although the interaction was promoted by the addition of free Zn$^{2+}$, and the pH dependence was shifted toward alkaline pH by Zn$^{2+}$ (41). The HRR was suggested to act like a pH sensor, whereby Zn$^{2+}$ and pH act synergistically in regulating HRG function. Consistent with these findings, the present study shows that pH can profoundly alter HRG cell surface binding, whereby HRG binding to B16F1 cells is potentiated greatly at pH 6.0 but reduced at pH 8.0 (Fig. 1, C and D). Of particular relevance here is our recent finding that heparan sulfate is the predominant cell surface ligand for HRG, with Zn$^{2+}$ regulating this interaction and the amino-terminal N1N2 domain of HRG interacting with this ligand (50). Hence, we propose that under conditions of local low pH and free Zn$^{2+}$, the HRR of HRG binds Zn$^{2+}$ and enhances HRG cell surface binding to heparan sulfate via its N1N2 domain. Under such conditions, the modular domain structure of HRG would allow cell surface-bound HRG to immobilize other molecules, such as plasminogen, to the cell surface.

Previous studies based on chemical modification of lysines suggest that HRG binds to plasminogen via HRG lysine residues (46). The biosensor studies described herein support the notion that the plasminogen-HRG interaction is dependent on the well described lysine binding sites (LBS) (8) of plasminogen, as free l-lysine reversed the interaction. Because carboxy-terminal lysine residues of many proteins are often involved in plasminogen binding, recombinant HRG lacking the carboxy-terminal lysine residue was produced. Surprisingly, our data show that there is no difference in the binding of plasminogen to C-mutant HRG and full-length HRG (Fig. 5, A and B), implying that this residue does not play a role in the interaction. On the other hand, the amino-terminal N1N2 domain of HRG, but not a N1 fragment, interacted with plasminogen (Fig. 3C), although it is possible that lysine residues within other regions of HRG may participate in plasminogen binding.

The heavy chain of plasminogen contains five triple loop structures termed “kringles” which are held in a loop structure by three disulfide bridges (4) and contain one high affinity LBS ($K_d = 9 \mu M$) and four or five low affinity LBS ($K_d = 5 \mu M$) that play a crucial role in the regulation of fibrinolysis by interacting specifically with lysine residues on fibrin, $\alpha_2$-antiplasmin, and cell surfaces during the physiological lysis of fibrin. Previous studies suggest that HRG binds to the high affinity LBS (20). Analysis of our own optical biosensor data suggests that immobilized HRG interacts bivalently with plasminogen, the interaction being found to have extremely slow off-rates (Fig. 2A), suggesting that cooperative binding and/or a change in
conformation contributes to the high affinity interaction. It seems highly likely that the two putative HRG binding sites located within plasminogen consist of a high affinity and a low affinity LBS. Our finding that excess soluble HRG does not interfere with the interaction of plasminogen with cell-bound HRG strongly suggests that plasminogen contains at least two HRG binding sites, whereas HRG contains only one site. Thus, a multivalent array of HRG displayed on a cell surface would be able to interact cooperatively with soluble plasminogen to form a highly stable complex. In fact, the demonstration that angiostatin binds with only low affinity to HRG (Fig. 6A) suggests that one of the HRG binding sites is LBS-5, because angiostatin lacks a portion of the fifth kringle domain of plasminogen, which contains LBS-5.

In blood, plasminogen circulates in a globular (closed) conformation, probably as a safeguard mechanism to prevent uncontrolled plasmin generation. When bound to a cell or fibrin surface through its LBS, it adopts an extended "open" conformation that is activated more rapidly to form plasmin (3, 4, 51, 52). Borza and Morgan (51) showed previously, using in vitro

![Graphs and plots from Figure 5](https://example.com/fig5.png)
HRG Acts as a Plasminogen Receptor

A

FIG. 6. Angiostatin, a fragment of plasminogen, exhibits low affinity binding to HRG. A, plasma-derived HRG was immobilized onto the surface of a CM5 sensor chip, and either human angiostatin or plasminogen (2 μg) diluted in PBS (pH 7.2) was then injected into the biosensor flow-cell, with binding and dissociation each being monitored for 3 min. A flow-cell within the chip with no immobilized HRG served as a control. B, B16F1 cells were incubated simultaneously with angiostatin or plasminogen (150 μg/ml) and either with or without 100 μg/ml plasma-derived HRG in PBS, 0.1% BSA (pH 7.2, 20 μM Zn2+), and then analyzed for angiostatin/plasminogen binding by immunofluorescence flow cytometry. Angiostatin/plasminogen binding is expressed as a fold increase in median fluorescence relative to background Ab binding, with error bars being the S.E. (n = 3). For each treatment condition, white histograms represent plasminogen binding in the absence of HRG, and black histograms represent plasminogen binding in the presence of HRG.

teolysis during cellular migration (10–14, 53). Processes including tumor cell invasion, angiogenesis, embryogenesis, and leukocyte migration to a site of inflammation require cells to invade and penetrate neighboring tissues (17). Directed cell migration requires localized proteolysis, with urokinase plasminogen activator receptors being polarized rapidly to the leading edge of migrating cells to focus plasmin mediated extracellular matrix degradation (13, 14), and indeed, increased urokinase plasminogen activator receptor expression correlates with a poor prognosis for many invasive human cancers (54–56). Through the generation of plasmin, plasminogen activators catalyze the degradation of most proteins of the extracellular space, including laminin, thrombospondin, fibronectin, and fibrinogen (14). Plasmin can also activate other proteases such as matrix metalloproteinases (17). Expression of the powerful degradative potential of plasminogen heavily depends on plasminogen being associated with the cell surface via a high affinity receptor. In this regard it is interesting to note that tumor cells precoated with HRG are 6-fold more metastatic than their untreated counterparts (data not shown), with the cell bound HRG presumably allowing the recruitment of endogenous plasminogen (or other degradative enzymes) to the tumor cell surface. Thus potentially, HRG may provide the means for tumor cells to “hijack” the degradative potential of plasmin and use its proteolytic ability to aid tumor cell metastasis and tumor angiogenesis.

Acknowledgments—We acknowledge the expert technical assistance of Peng Jian for mouse injections, Eloisa Pagler in the preparation of recombinant HRG, and Geoff Osborne and Sabine Gruninger for flow cytometry advice.

REFERENCES
1. Lijnse, H. B. (2001) Annu. N. Y. Acad. Sci. 936, 226–236
2. Collen, D. (1999) Thromb. Haemostasis 82, 259–270
3. Parry, M. A., Zhang, X. C., and Bode, I. (2000) Trends Biochem. Sci. 25, 53–59
4. Miyashita, C., Wenzel, E., and Heiden, M. (1988) Haemostasis 18, 7–13
5. Collen, D. (2001) Hematology (Am. Soc. Hematol. Educ. Program) 1–9
6. Dans, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L. S., and Skriver, L. (1985) Adv. Cancer Res. 44, 139–296
7. Vassalli, J. D., Sappino, A. P., and Belin, D. (1991) J. Clin. Invest. 88, 1067–1072
8. Flow, E. F., Herren, T., Redlitz, A., Miles, L. A., and Hoover-Plow, J. L. (1995) FASEB J. 9, 938–945
9. Chapman, H. A., Jr., Vavrin, Z., and Hibbs, J. B., Jr. (1982) Cell 28, 653–662
10. Chapman, H. A. (1997) Curr. Opin. Cell Biol. 9, 714–724
11. Labeeunamaki, K., Kousela, P., and Korhonen, T. K. (2000) Methods 21, 125–132
12. Brummer, G., Reimbold, K., Meissauer, A., Schirrmacher, V., and Erkell, L. J. (1990) Exp. Cell Res. 239, 301–310
13. Ploplis, V. A., and Castellino, F. J. (2000) Methods 31, 103–110
14. Estreicher, A., Muhlhauser, J., Carpenter, J. L., Orci, L., and Vassalli, J. D. (1990) J. Cell Biol. 111, 785–792
15. Miles, L. A., Dahlberg, C. M., Plescia, J., Felez, J., Kato, K., and Flow, E. F. (1991) Biochemistry 30, 1882–1891
16. Miles, L. A., Dahlberg, C. M., Levin, E. G., and Flow, E. F. (1989) Biochemistry 28, 9537–9543
17. Brownstein, C., Falcone, D. J., Jacovina, A., and Hajjar, K. A. (2001) Annu. N. Y. Acad. Sci. 947, 143–156
18. Ling, Q., Jacovina, A. T., Deora, A., Fehrman, M., Simantov, R., Silverstein, R. L., Hempstead, B., Mark, W. H., and Hajjar, K. A. (2004) J. Clin. Invest. 113, 38–48
19. Kinge, T., Foster, D., Yashitake, S., and Dave, E. W. (1986) Biochemistry 25, 2220–2225
20. Lijnse, H. R., Hoylaerts, M., and Collen, D. (1980) J. Biol. Chem. 255, 10214–10222
21. Ichinose, A., Mimuro, J., Koide, T., and Aoki, N. (1984) J. Biol. Chem. 259, 939–945
22. Leung, L. L. (1986) J. Biol. Chem. 261, 16905–16910
23. Morgan, W. T. (1981) Arch. Biochem. Biophys. 210, 202–207
24. Morgan, W. T. (1981) Biochemistry 20, 1054–1061
25. Morgan, W. T. (1985) Biochemistry 24, 1496–1501
26. Lifschitz, I. L. (1986) J. Clin. Invest. 77, 1305–1311
27. Waly, D. A., Bacon-Bageley, T., Kendra-Franzuk, S., and DePoli, P. (1987) Semin. Thromb. Hemostasis 13, 371–375
28. Gergani, N. N., Paris, C. R., and Altin, J. G. (1999) J. Biol. Chem. 274, 29633–29640
29. Gergani, N. N., Paris, C. R., Easterbrook Smith, S. B., and Altin, J. G. (1997) Biochemistry 36, 6653–6662
30. Ploplis, V. A., Morgan, W. T., and Blackburn, M. N. (1987) J. Biol. Chem.
38276  

**HRG Acts as a Plasminogen Receptor**

31. Lijnen, H. R., Hoylaerts, M., and Collen, D. (1983) *J. Biol. Chem.* **258**, 3803–3808
32. Burch, M. K., Blackburn, M. N., and Morgan, W. T. (1987) *Biochemistry* **26**, 7477–7482
33. Gorgani, N. N., Smith, B. A., Kono, D. H., and Theofilopoulos, A. N. (2002) *J. Immunol.* **169**, 4745–4751
34. Gorgani, N. N., Altin, J. G., and Parish, C. R. (1999) *Int. Immunol.* **11**, 1275–1282
35. Saigo, K., Shatsky, M., Levitt, L. J., and Leung, L. K. (1989) *J. Biol. Chem.* **264**, 8249–8253
36. Rylatt, D. B., Sia, D. Y., Mundy, J. P., and Parish, C. R. (1981) *Eur. J. Biochem.* **119**, 641–646
37. Rickli, E. E., and Otavsky, W. I. (1975) *Eur. J. Biochem.* **59**, 441–447
38. Wiman, B. (1980) *Biochem. J.* **191**, 229–232
39. Moroi, M., and Aoki, N. (1976) *J. Biol. Chem.* **251**, 5956–5965
40. Stathakis, P., Lay, A. J., Fitzgerald, M., Schleker, C., Matthias, L. J., and Hogg, P. J. (1990) *J. Biol. Chem.* **274**, 8910–8916
41. Borza, D. B., and Morgan, W. T. (1998) *J. Biol. Chem.* **273**, 5493–5499
42. Brown, K. H. (1998) *Am. J. Clin. Nutr.* **68**, 425S–429S
43. Hayes, M. L., and Castellino, J. F. (1979) *J. Biol. Chem.* **254**, 8768–8771
44. Hayes, M. L., and Castellino, F. J. (1979) *J. Biol. Chem.* **254**, 8772–8776
45. Hayes, M. L., and Castellino, F. J. (1979) *J. Biol. Chem.* **254**, 8772–8776
46. Saiz, C. T., Jansen, G. J., Smith, A., and Morgan, W. T. (1995) *Biochemistry* **34**, 2496–2503
47. Aktulga, A., and Ulutin, O. N. (1974) in *Recent Advances in Basic Research and Clinical Aspects* (Ulutin, O. N., and Jones, J. V., ed) pp. 185–191, Excerpta Medica, Amsterdam
48. Gorodetsky, R., Mou, X., Blankenfeld, A., and Marx, G. (1993) *Am. J. Hematol.* **42**, 278–283
49. Morgan, W. T. (1978) *Biochim. Biophys. Acta* **535**, 319–333
50. Jones, A. L., Hulett, M. D., and Parish, C. R. (2004) *J. Biol. Chem.* **279**, 30114–30122
51. Borza, D. B., and Morgan, W. T. (1997) *J. Biol. Chem.* **272**, 5718–5726
52. Walther, P. J., Hill, R. L., and McKee, P. A. (1975) *J. Biol. Chem.* **250**, 5926–5933
53. Reich, E. (1978) in *Biological Markers of Neoplasia: Basic and Applied Aspects* (R. W. Ruddon, ed) pp. 491–500, Elsevier, New York
54. Blasi, F. (1999) *Thromb. Haemostasis* **82**, 298–304
55. Sappino, A. P., Belin, D., Huarte, J., Hirschel-Scholz, S., Saurat, J. H., and Vassalli, J. D. (1991) *J. Clin. Invest.* **88**, 1073–1079
56. Scherrer, A., Wohlwend, A., Kruithof, E. K., Vassalli, J. D., and Sappino, A. P. (1999) *Br. J. Haematol.* **105**, 920–927
Plasminogen Is Tethered with High Affinity to the Cell Surface by the Plasma Protein, Histidine-rich Glycoprotein
Allison L. Jones, Mark D. Hulett, Joseph G. Altin, Phillip Hogg and Christopher R. Parish

J. Biol. Chem. 2004, 279:38267-38276.
doi: 10.1074/jbc.M406027200 originally published online June 25, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406027200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 19 of which can be accessed free at
http://www.jbc.org/content/279/37/38267.full.html#ref-list-1