THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 8, Issue of February 28, pp. 5718–5726, 1997
Printed in U.S.A.

Acceleration of Plasminogen Activation by Tissue Plasminogen Activator on Surface-bound Histidine-proline-rich Glycoprotein*

(Received for publication, August 5, 1996, and in revised from, November 8, 1996)

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Histidine-proline-rich glycoprotein (HPRG), also known as histidine-rich glycoprotein, is a major plasminogen-binding protein. In this work we characterized extensively the circumstances under which HPRG accelerates plasminogen activation and the specificity of this effect. Soluble HPRG did not significantly influence plasminogen activation. In contrast, native HPRG bound to hydrazide or nickel chelate surfaces strongly stimulated the activation of plasminogen by tissue plasminogen activator, but not by urokinase or streptokinase. The efficiency of activation on surface-bound HPRG was increased for Glu-plasminogen (41-fold), Lys-plasminogen (17-fold), and cross-linked Glu-plasminogen (11-fold) but not for mini-plasminogen, and was mainly due to a decrease in the apparent K_m. A reduced susceptibility to inhibition by chloride ions contributed to the higher activation rate of Glu-plasminogen on an HPRG surface. The immobilized N- and C-terminal domains, but not the histidine-proline-rich domain of HPRG, also bound plasminogen and stimulated its activation. HPRG-enhanced plasminogen activation was proportional to the quantity of HPRG immobilized and was abolished by anti-HPRG antiserum, by low concentrations of ϵ-aminocaproic acid, by methylation of lysine residues in HPRG, and by treatment of HPRG with carboxypeptidase B. Soluble HPRG and a plasminogen fragment, kringle 1-2-3, acted as competitive inhibitors by binding to plasminogen and immobilized HPRG, respectively. The interaction of the conserved C-terminal lysine of HPRG with the high affinity lysine binding site of plasminogen is necessary and sufficient to accelerate plasminogen activation. Unlike other stimulators of plasminogen activation, the effect of HPRG on fibrinolysis is modulated by factors that influence the equilibrium between solution and surface-bound HPRG.

Plasmin, the primary enzyme of the fibrinolytic system, is formed from plasminogen by the action of plasminogen activators, such as tissue plasminogen activator (t-PA) or urokinase. As a safeguard mechanism to prevent undue plasmin generation under normal physiological conditions, plasminogen is a very poor substrate for its activators, but activation is greatly accelerated by conformational changes or interaction with other proteins (1). Instrumental in this regulation are three of the five kringle domains (K1, K4, and K5), which comprise the lysine binding sites (LBS) of plasminogen and bind lysine analogues or lysine side chains in proteins. In the presence of physiological concentrations of chloride ions, Glu[1]-Pg,[1] the native form of plasminogen, adopts a closed conformation and becomes difficult to activate, but this effect is reversed in the presence of ϵ-aminocaproic acid (ϵ-ACA) (2, 3). In contrast, Lys[78]-Pg, generated by plasmin proteolysis of Glu[1]-Pg, adopts an open conformation and is relatively easier to activate regardless of the presence of chloride ions or lysine analogues. Proteins that interact with the LBS on plasminogen, most notably fibrin (4, 5) but also proteins of the extracellular matrix (6) or cell surface (7), greatly stimulate plasminogen activation, and this effect is competitively inhibited by ϵ-aminocaproic acid (ϵ-ACA) or other lysine analogues. Many of these effectors also bind the plasminogen activator, thereby acting as a template that brings the enzyme and its substrate in close proximity.

HPRG is a relatively abundant protein (~1.5 μM in normal human plasma) whose physiological function is not precisely known. Based on its interactions with plasminogen (8), fibrinogen (9), and heparin (10), HPRG has been suggested to play a role in the regulation of the coagulation and fibrinolytic systems. Its relatively high affinity for plasminogen (K_D approximately 1 μM, in solution) makes HPRG one of the most important plasminogen ligands in plasma, and it has been estimated that about 50% of plasminogen circulates bound to HPRG (8). Previous work has shown that HPRG has a complex influence on the fibrinolytic system. By binding to plasminogen, HPRG reduces the concentration of free plasminogen, competitively inhibits binding of plasminogen to fibrin, and retards fibrinolysis *in vitro* (8). These results suggested that HPRG has an anti-fibrinolytic effect similar to that of lysine analogues, although the physiological relevance of this effect was questioned (11). However, HPRG also exhibits pro-fibrinolytic activities by reducing the rate of reaction between plasmin and α_2-antiplasmin (8, 12). Furthermore, HPRG immobilized onto Sepharose or plastic microtiter plates increases the efficiency of plasminogen activation by t-PA about 30-fold (12), although the authors have not addressed whether surface-adsorbed HPRG was native or denatured, and many denatured proteins stimulate plasmin formation nonspecifically (13). In contrast, HPRG accelerated plasminogen activation by urokinase in solution less

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[1] The abbreviations used are: Glu[1]-Pg, the native form of plasminogen (residues 1–791); t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; HPRG, histidine-proline-rich glycoprotein; Lys[78]-Pg, the plasminogen species obtained by plasmin attack on Glu-Pg (residues 78–791); mini-Pg (Val[442]-Pg), plasminogen derivative containing only the kringle 5 and the protease domain; XGlu-Pg, cross-linked Glu[1]-Pg; ϵ-ACA, ϵ-aminocaproic acid; S-2251, D-valyl-leucyl-lysyl-*p*-nitroanilide; S-2444, glutamyl-glycyl-arginyl-*p*-nitroanilide; CPB, carboxypeptidase B; BSA, bovine serum albumin; LBS, lysine binding sites; Ni-NTA, nickel nitrilotriacetate; RCAM-HPRG, reduced and carboxamido-methylated HPRG; PRCAM-HPRG, partially reduced and carboxamido-methylated HPRG; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitro blue tetrazolium; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; K, kringle.

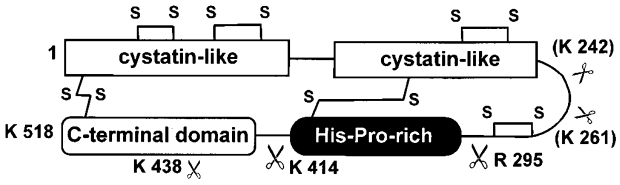

FIG. 1. **Domain structure of HPRG.** Limited plasmin proteolysis initially cleaves HPRG at the sites indicated by *large scissors symbols*, followed by further trimming at the sites indicated by *smaller scissors symbols*. Treatment of plasmin-clipped HPRG with dithiothreitol releases the histidine-proline-rich domain (*dark gray*). The remaining N/C fragment consists of the N-terminal (*white*) and the C-terminal (*light gray*) domains of HPRG, held together by a buried disulfide bond, which is reduced only in the presence of denaturing agents.

than 2-fold (8).

These complex and apparently contradictory effects of HPRG on fibrinolysis prompted us to investigate in more detail the interaction between plasminogen and HPRG, and especially its influence on plasminogen activation. First, we aimed to define the structural features of HPRG required for its interaction with plasminogen by assaying the binding to plasminogen of various HPRG domains and chemical derivatives. Second, we sought to characterize the conditions under which HPRG stimulates plasminogen activation and the specificity of this process by using different plasminogen and plasminogen activator species in the activation assay in the presence of HPRG and HPRG derivatives, either immobilized on various surfaces or in solution. Finally, we investigated how immobilized HPRG accelerates plasminogen activation by comparing the activation rates of different plasminogen forms under various experimental conditions.

EXPERIMENTAL PROCEDURES

Reagents—Two-chain human recombinant t-PA (specific activity 630,000 units/mg relative to the International Standard), a t-PA mutein (BM06.022) containing only the kringle 2 and the protease domain of human t-PA (K_2P t-PA), t-PA standard, and cyanogen bromide fragments of fibrinogen were generous gifts from Drs. U. Kohnert and S. Fischer (Boehringer, Mannheim, Germany). Two-chain human urokinase and bovine serum albumin (BSA) were from Calbiochem. Streptokinase, carboxypeptidase B (CPB), goat anti-human plasminogen antiserum, D-valyl-leucyl-lysyl-*p*-nitroanilide (S-2251), ϵ-ACA, *p*-nitrophenyl phosphate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and nitroblue tetrazolium (NBT) were from Sigma. Affinity-purified, alkaline phosphatase-conjugated rabbit anti-goat IgG was from Cappel. Lysine-Sepharose and cyanogen bromide-activated Sepharose were from Pharmacia and nickel-NTA-agarose from QIAGEN. Nickel chelate-coated microtiter plates were purchased from Xenopore and hydrazide-coated plates from Costar.

Protein Isolation and Treatments—Human HPRG was isolated from human plasma by metal-chelate affinity chromatography on nickel-NTA-agarose.[2] Briefly, 10 volumes of human plasma containing 5 mM imidazole were applied to one volume of Ni-NTA-agarose, and after washing with 20 mM and 80 mM imidazole buffer, pH 7.5, human HPRG was eluted with 250 mM imidazole, pH 7.5. The procedure for "clipping" human HPRG with plasmin-Sepharose was similar to that described for rabbit HPRG (14), but the reaction time was reduced to 2 h.

Because rabbit HPRG can be obtained in larger quantities than the human counterpart and its domains have been relatively well characterized (14), the HPRG derivatives have been prepared starting from the rabbit protein, unless otherwise indicated. Reduction of plasmin-clipped HPRG with dithiothreitol under non-denaturing conditions releases the 30-kDa histidine-proline-rich domain of HPRG and the remaining N/C fragment can be further reduced under denaturing conditions to yield the 45-kDa N-terminal and the 20-kDa C-terminal domains (see Fig. 1 for a schematic representation of rabbit HPRG domains, including the plasmin cleavage sites). Fully or partially reduced-and-carboxamidomethylated HPRG (RCAM-HPRG and PRCAM-HPRG) were obtained by reduction of disulfide bonds in HPRG with dithiothreitol in the presence or absence of 3 M guanidinium hydrochloride, respectively (14). Reductive methylation of lysine residues in HPRG was carried out using a 10-fold molar excess of formaldehyde and sodium borohydride (15); 85% of surface lysine residues were found to be modified as assessed by reaction with trinitrobenzenesulfonate (16). Removal of the C-terminal lysine residue of HPRG was achieved by treatment with carboxypeptidase B (CPB, 1:25 molar ratio) in 0.1 M Tris buffer, pH 8.8, for 30 min at room temperature, followed by purification of CPB-treated HPRG on heparin-Sepharose. Carbohydrate-oxidized HPRG was obtained by incubation of a 2 mg/ml protein solution in 0.2 mg/ml sodium periodate in 0.1 M sodium acetate, pH 4.5, for 1 h at room temperature, followed by desalting on a gel-filtration column. The preparation of goat antisera against rabbit or human HPRG was described previously (17).

Glu^1-Pg was purified from human plasma by affinity chromatography on lysine-Sepharose (18). Lys^{78}-Pg was prepared by continuously mixing a Glu^1-Pg solution (2 mg/ml in 0.1 M sodium phosphate, pH 8.0) with plasmin-Sepharose (ratio 1:30 v/v) for 3 h at room temperature; after removing the gel by centrifugation, Lys^{78}-Pg was isolated from the supernatant by affinity chromatography on lysine-Sepharose. Val^{442}-plasminogen (mini-Pg) and plasminogen kringle domains (K1-2-3 and K4) were obtained from Glu^1-Pg by digestion with elastase, followed by affinity purification on lysine-Sepharose and gel filtration on Ultrogel AcA54 (19). Cross-linked plasminogen ($XGlu^1$-Pg) was prepared from Glu^1-Pg and 1,5-dinitro-2,4-difluoro-benzene, as described (20).

Concentrated stock solutions of all proteins were dialyzed against 5 mM sodium phosphate, pH 7.2, and stored frozen in aliquots at −20 °C. The concentrations of the HPRG and plasminogen derivatives were determined spectrophotometrically using published values for the extinction coefficients and molecular weights (21), and those of plasminogen activators using the BCA Protein Assay Kit (Pierce).

Solid-phase Binding Assays—HPRG (10 μg/ml in 0.1 M carbonate buffer, pH 9.6) was passively adsorbed to polystyrene microtiter plates (Corning) by overnight incubation at ambient temperature. The unoccupied sites in the wells were blocked by subsequent treatment with 1 mg/ml BSA for at least 3 h. The following steps were carried out at ambient temperature (except the ligand binding step) using 100 μl of solution/well, and between the incubation steps the wells were washed three times for 1 min with TBS (Tris-buffered saline: 15 mM Tris and 150 mM sodium chloride, pH 7.4). All solutions were made in TBS containing 1 mg/ml BSA and 0.05% Tween 20. For the *direct* binding assay, solutions containing various concentrations of Glu-Pg were incubated in the HPRG-coated wells for 1 h at 37 °C. For the *competitive* binding assay, solutions containing a fixed amount of Glu-Pg (0.2 μM) *and* various concentrations of HPRG (or its derivatives) were incubated in the HPRG-coated wells for 3 h at 37 °C. The amount of plasminogen bound to each well was estimated from the rate of hydrolysis of *p*-nitrophenol phosphate (5 mg/ml in 0.1 M glycine, pH 10.5, containing 1 mM magnesium chloride and 0.5 mM zinc chloride) after sequential incubation with primary antibody (1:500 dilution of goat anti-human plasminogen for 1 h) and secondary antibody (1:1000 dilution of alkaline phosphatase-conjugated rabbit anti-goat IgG for 30 min). The absorbance change at 405 nm was recorded with a plate reader (Thermomax, Molecular Devices).

Ligand Blots—HPRG domains were separated under reducing conditions by SDS-PAGE on 14% acrylamide gels and electrotransferred onto a polyvinylidene difluoride membrane (Immobilon P, Millipore) for 1 h at 25 V/cm inter-electrode distance. The membrane was then blocked with 10 mg/ml BSA and 0.05% Tween 20 in TBS buffer (1 h at ambient temperature) and incubated with ligand (0.5 μM plasminogen in blocking buffer overnight at 4 °C on a rocking platform), primary antibody (1:500 dilution of goat anti-human plasminogen antiserum in blocking buffer for 1 h at ambient temperature), and secondary antibody (1:2000 dilution alkaline phosphatase-conjugated rabbit anti-goat IgG in blocking buffer for 1 h at ambient temperature). The blots were visualized with BCIP and NBT (0.2 mg/ml each in 0.1 M Tris buffer, pH 9.5, containing 0.1 M sodium chloride and 5 mM magnesium chloride).

Plasminogen Activation Assay—Plasminogen activation was carried out at 37 °C in microwell plates and monitored by recording the hydrolysis of the chromogenic plasmin substrate S-2251 with a kinetic plate reader (Thermomax, Molecular Devices). Absorbance at 405 nm was recorded every 30 s over a period of 1–2 h, and the plate was shaken before each reading. The reaction was initiated by addition of the plasminogen activator after the other ingredients were preincubated at 37 °C for at least 10 min to achieve thermal equilibration. The plasminogen activator solution was dispensed from a repetitive multi-channel pipettor to insure speed and reproducibility. Less than 30 s passed between the addition of the reagent and the start of the assay, which was negligible compared with the total length of the assay. Typically,

[2] C. T. Saez and W. T. Morgan, submitted for publication.

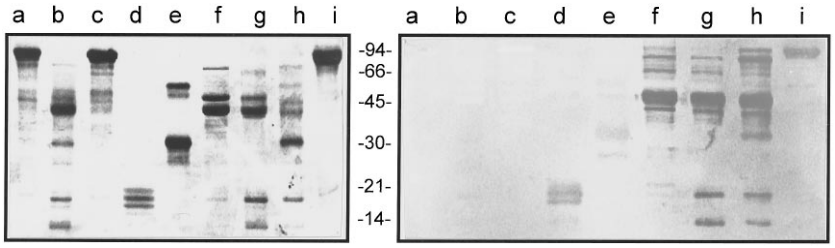

FIG. 2. **Ligand-blot of HPRG and its derivatives with plasminogen.** *Left panel*, reducing SDS-PAGE of HPRG derivatives. The lanes were loaded with 0.1 nmol of protein: *a*, methyl-HPRG; *b*, CPB-treated, plasmin-clipped HPRG; *c*, CPB-treated HPRG; *d*, C-terminal domain; *e*, histidine-proline-rich domain; *f*, N-terminal domain; *g*, N/C fragment; *h*, plasmin-clipped HPRG; *i*, HPRG. After electrophoresis, the proteins were stained with Coomassie Blue. *Right panel*, after proteins were electrotransferred to polyvinylidene difluoride, the membrane was sequentially incubated with plasminogen, anti-plasminogen, and alkaline phosphatase-conjugated secondary antibody, and then developed with BCIP and NBT.

the reaction mixture consisted of 0.2 μM Glu-plasminogen, 0.36 nM t-PA, 0.25 mM S-2251 in 100 μl of "mass buffer" (15 mM sodium phosphate, 150 mM sodium chloride, 0.01% Triton X-100, and 1 mg/ml BSA, pH 7.2). Additional reagents included in the reaction mixture, such as ϵ-ACA or fibrinogen fragments, or different reaction conditions are specified in the figure legends. In some experiments, different species of plasminogen or plasminogen activator were used as noted.

For assaying the rates of plasminogen activation in solution, the microtiter plates used in the assay were blocked by passive adsorption of BSA. This as well as incorporation of BSA and detergent in the mass buffer was necessary to prevent nonspecific adsorption of proteins to the microtiter plates and consequent spuriously high activation rates. Different methods were used to coat HPRG on the surface of the wells: passive adsorption (as described above for plasminogen binding assays), binding of HPRG to nickel-chelate microtiter plates (following the manufacturer's protocol), or coupling of periodate-oxidized HPRG to hydrazide-coated plates (a 10 μg/ml protein solution in 0.1 M sodium acetate, pH 5.2, was incubated in the wells overnight at ambient temperature, followed by washing and blocking for 1 h with a 5 mg/ml BSA solution in PBS). Plates coated passively with HPRG constantly yielded relatively poor results in this assay and were not used further. The other surfaces yielded comparable results, although nickel chelate-coated plates, which bind HPRG derivatives directly without requiring derivatization of the carbohydrate groups, are more convenient.

Assuming a constant rate of plasminogen activation, the concentration of plasmin increases linearly with time and that of the *p*-nitroaniline follows a parabolic curve (5). With the boundary condition that no plasmin is present at time zero, the change of absorbance at 405 nm with time is given by $\Delta A(t) = A(t) - A(0) = k \cdot t^2 = \frac{1}{2} \cdot k_1 \cdot k_2 \cdot t^2$, where k is the apparent plasminogen activation rate in mA/min^2, k_1 is the plasminogen activation rate in nM/min, and k_2 represents plasmin activity toward S-2251 under given experimental conditions, expressed as mA/min·nM. Using active-site titrated plasmin as standard, we determined a value k_2 of 1.15 mA/min·nM plasmin under our assay conditions (0.25 mM S-2251 in 100 μl of mass buffer, 37 °C). A plot of $A(t)$ *versus* t^2 yields a straight line with a slope k. Since the software provided by Molecular Devices did not support this type of analysis, a program was written in Visual Basic 4.0 (Microsoft) to analyze the plate reader data files after export to ASCII (text) format. The activation rate, k, was obtained by least squares regression of $\Delta A(t)$ *versus* t^2 through data points at low substrate conversion. In practice, a limit of 70 mA for ΔA (corresponding to the hydrolysis of ~10% of S-2251) was selected as the cut-off value, providing between 20 and 120 data points/well for analysis. The amount of plasmin generated at the end of the interval used for analysis did not exceed 6 nM, or about 3% of the initial substrate concentration.

RESULTS

Role of the C-terminal Lysine Residue of HPRG in Binding to Plasminogen—Both human HPRG (8) and rabbit HPRG (22) interact with the LBS of human plasminogen with comparable affinity (K_D approximately 1 μM). Since the HPRG-plasminogen interaction does not appear to be species-specific, and since rabbit HPRG domains can be reproducibly prepared in large quantities by limited proteolysis with plasmin (14), we aimed to identify the conserved lysine residues of HPRG involved in the interaction with plasminogen. In the following experiments, rabbit HPRG and its derivatives were used unless otherwise indicated. The ligand blot in Fig. 2 shows that reductive methylation of lysine residues in HPRG or treatment with CPB abolishes plasminogen binding, indicating that the C-terminal lysine of HPRG (Lys^{518} in rabbit) is required for the interaction. Among the HPRG domains generated by limited proteolysis with plasmin, the C-terminal and the N-terminal but not the His-Pro-rich domain of HPRG bound plasminogen. Since CPB treatment also abrogates plasminogen binding to the HPRG domains (*lane b* in Fig. 2), the probable new plasminogen binding sites generated by plasmin digestion are Lys^{242} or Lys^{261} (Fig. 1), the putative C-terminal residue sites of the N-terminal domain (14). Comparable results were obtained by affinity chromatography of the same HPRG species on plasminogen-Sepharose (data not shown), suggesting that binding was not affected by plasminogen immobilization.

To quantify the interaction, the data obtained in a solid-phase binding assay were fitted to a binding isotherm using non-linear least square regression analysis (Fig. 3*A*). As with other plasminogen ligands, Lys-Pg had higher affinity for immobilized rabbit HPRG (K_D = 13.8 ± 1.6 nM) than for Glu-Pg (72.4 ± 5.4 nM), and similar values were obtained using immobilized human HPRG (13.7 and 60.7 nM, respectively). Since the binding of plasminogen to immobilized HPRG could be competitively inhibited by ϵ-ACA (Fig. 3*A*, *inset*), which binds to the LBS of plasminogen, soluble HPRG species that interact with plasminogen should also prevent its binding to immobilized HPRG. Indeed, the results of competitive binding experiments (Fig. 3*B*) agreed with those obtained in ligand blots. The efficiency of the inhibition, reflecting the affinity for plasminogen, was: N-terminal domain ~ N/C fragment ~ clipped HPRG > C-terminal domain ~ rabbit HPRG ~ human HPRG > histidine-proline-rich domain ~ CPB-treated HPRG ~ methylated HPRG.

Effect of HPRG in Solution on Plasminogen Activation—Proteins like HPRG, which bind to the LBS of plasminogen, usually influence plasminogen activation. The influence of HPRG and HPRG derivatives in *solution-phase* on plasminogen activation was measured in BSA-coated wells (Table I). In a series of control experiments carried out in parallel, HPRG derivatives did not influence the amidolytic activity of t-PA toward S-2444 (0.5 mM), and only RCAM-HPRG and the N-terminal domain of HPRG had a significant effect on plasmin activity toward S-2251 (0.25 mM), inhibiting by 50% at 1 μM concentration. Interestingly, the same two HPRG derivatives stimulated plasminogen activation severalfold. However, the preparation of these two derivatives requires denaturation and renaturation, and the near UV-CD spectrum of RCAM-HPRG resembles that of denatured HPRG more than that of native protein (14). Since denatured proteins stimulate plasminogen formation nonspecifically (13), the effect of the two HPRG derivatives may simply reflect partial misfolding upon renatur-

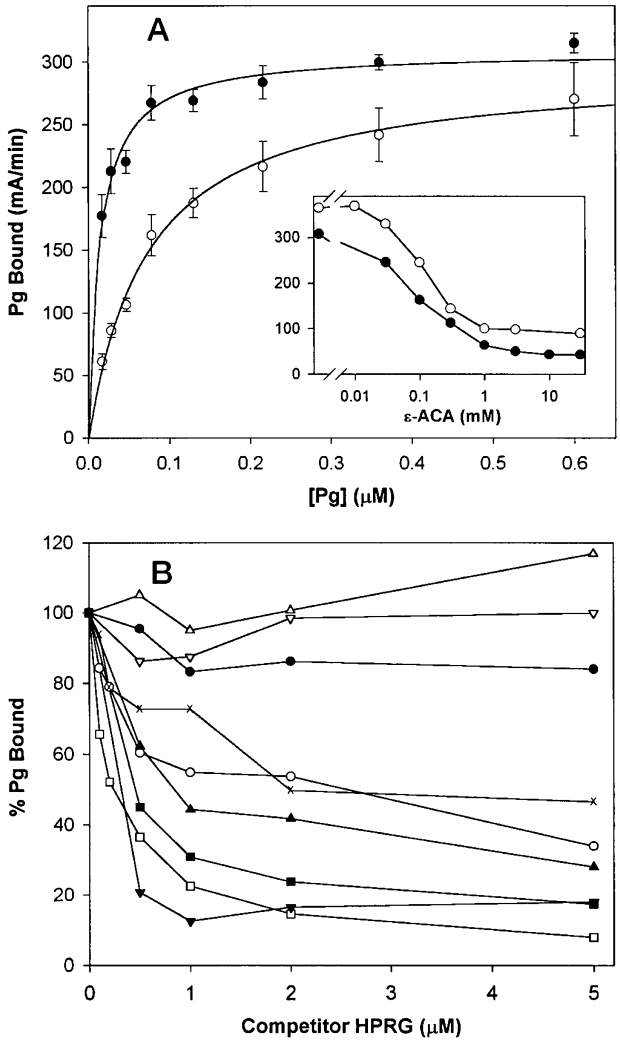

FIG. 3. **Plasminogen binding to immobilized HPRG in direct and competition enzyme-linked immunosorbent assay.** *Panel A*, binding was assessed by incubating Glu-Pg (○) or Lys-Pg (●) at various concentrations in plastic wells coated with rabbit HPRG. After sequential incubations with anti-plasminogen antiserum and alkaline phosphatase-conjugated secondary antibody, the bound plasminogen was assayed by measuring the rate of hydrolysis of *p*-nitrophenol phosphate. *Inset*, the same experiment was performed at constant concentrations of Glu-Pg (0.2 μM) or Lys-Pg (0.1 μM) and various concentrations of ε-ACA. *Panel B*, inhibition of Glu-Pg binding to plastic-immobilized rabbit HPRG by HPRG derivatives in solution. The following competitors were used: human HPRG (×), rabbit HPRG (○), plasmin-clipped HPRG (□), N/C fragment (■), N-terminal domain (▼), histidine-proline-rich domain (●), C-terminal domain (▲), methylated HPRG (▽), and CPB-treated HPRG (△). Bound plasminogen was assayed as described above.

ation. In these cases, the *A versus* t^2 plots were not linear but curved upward (data not shown), indicating a biphasic activation with an initial slow step ("lag phase") similar to that observed with fibrin or fibrinogen fragments (23). This implies that *in situ* plasmin digestion of the HPRG derivatives increases their stimulatory potency by generating new plasminogen binding sites, a phenomenon facilitated by the absence of disulfide bonds and potential misfolding. Inhibition of plasmin activity in the presence of these two derivatives supports this interpretation, since good plasmin substrates would compete with the chromogenic substrate.

Plasminogen Activation on an HPRG Surface: the Effect of Plasminogen Conformation and Plasminogen Activator—Plastic-immobilized HPRG has been reported to stimulate the activation of Glu-Pg by t-PA (12). To address the mechanism of this phenomenon and characterize its specificity, we carried out plasminogen activation in the presence of immobilized HPRG, using various plasminogen or plasminogen activator species, and the results were compared with those obtained with other known stimulators of plasminogen activation. Several plasminogen species were used to assess the role of plasminogen conformation in this effect: Glu-Pg is flexible and its conformation is influenced by chloride ions and ε-ACA, Lys-Pg prefers an open conformation, XGlu-Pg is locked into a closed one (20), and mini-Pg lacks kringles 1–4 of the regulatory region.

Of the three plasminogen activators tested, t-PA was the most sensitive to the effect of surface-bound HPRG. In the presence of immobilized HPRG, the activation of Glu-Pg, Lys-Pg, and XGlu-Pg by t-PA was accelerated 15-, 5-, and 2-fold, respectively (Table II). The activation of mini-Pg was not affected, suggesting that at least one of the plasminogen kringles 1–4 is required for the effect. It is remarkable that HPRG stimulated the activation of Lys-Pg and XGlu-Pg, derivatives whose conformation and activability is not influenced by ε-ACA. However, immobilized HPRG did not stimulate plasminogen activation by urokinase, and it had only a limited effect when streptokinase or a K_2P t-PA mutant (containing the t-PA kringle 2 and the protease domain) were used, indicating that the effect of immobilized HPRG on plasminogen activation is specific for t-PA and involves one of the fibronectin, epidermal growth factor, or kringle 1 domains of t-PA. As effector, immobilized HPRG more closely resembled the fibrinogen fragments. Apart from the stronger effect of the fibrinogen fragments, which may be attributed in part to the larger amount of protein used in the assay, the most important differences between the two effectors were the shape of the $A(t)$ *versus* t^2 plots (linear for the immobilized HPRG, but curved up for the fibrinogen fragments) and the activation of mini-Pg by t-PA (stimulated by fibrinogen fragments, but not by the immobilized HPRG).

The kinetic parameters for the activation of Lys-, Glu-, and XGlu-Pg by t-PA were determined in the presence or absence of immobilized HPRG (Table III). These apparent values facilitate a comparison between the activation processes in solution and on a surface, although the first order reaction rates for plasminogen activation in the presence of immobilized HPRG are likely to be underestimated due to the concurrent, inefficient activation in solution. The catalytic efficiency of Glu-Pg activation was enhanced significantly more (41-fold) than that of either Lys-Pg (17-fold) or XGlu-Pg (11-fold), but with all derivatives a decrease in the apparent K_m was the main factor responsible for the acceleration effect. To explore whether Glu-Pg was more sensitive to the surface effect because of its conformational flexibility, the three plasminogen species were activated at different concentrations of chloride ions in solution or on the HPRG surface (Fig. 4). Only the activation of Glu-Pg was inhibited by chloride ions, but interestingly, this effect was diminished in the presence of immobilized HPRG, particularly at low concentrations of chloride ions.

Plasminogen Activation on an HPRG Surface: the Effect of HPRG and the Surface—Next we examined how the enhancement of plasminogen activation depends on the quantity and nature of the immobilized HPRG. When increasing amounts of periodate-oxidized HPRG were immobilized onto hydrazide plates, the accelerating effect of surface-bound HPRG was initially proportional to the amount of protein immobilized, but a plateau was reached around 150 ng (2 pmol) of rabbit HPRG/well, presumably because the surface became saturated. Since 100 μl of solution/well wets an area of 0.94 cm^2, and assuming that all HPRG added to the wells was immobilized, a surface concentration of about 2.2 pmol/cm^2 HPRG can be computed, in

TABLE I

The influence of HPRG derivatives in solution on plasminogen activation and plasmin amidolytic activity

Plasminogen (0.2 μM) was activated with t-PA (0.36 nM) in 96-well microtiter plates coated with BSA as described under "Experimental Procedures." The HPRG species indicated in the table were added to the reaction mixture at a final concentration of 1 μM. Plasmin amidolytic activity was determined by replacing plasminogen and its activator by plasmin (22 nM) in the plasminogen activation assay. Averages and standard deviations of triplicates are shown.

HPRG	Activation rate, mA/min^2 × 10^3 (relative rate)		Relative amidolytic plasmin activity[a]
	Glu-Pg	Lys-Pg	
None	3.53 ± 0.27 *(1.00)*	49.0 ± 1.1 *(1.00)*	1.00 ± 0.04
Human HPRG	3.46 ± 0.05 *(0.98)*	65.7 ± 12.6 *(1.34)*	1.00 ± 0.07
Human "clipped" HPRG	4.18 ± 0.29 *(1.18)*	95.8 ± 2.3 *(1.96)*	0.93 ± 0.04
HPRG[b]	3.93 ± 0.63 *(1.11)*	52.4 ± 1.6 *(1.07)*	1.02 ± 0.05
Clipped HPRG	4.51 ± 0.33 *(1.28)*	62.7 ± 3.5 *(1.28)*	1.03 ± 0.06
N/C fragment	4.31 ± 0.05 *(1.22)*	61.2 ± 2.9 *(1.25)*	1.01 ± 0.08
N-terminal domain	22.00 ± 1.04 *(6.23)*	368.0 ± 12.2 *(7.51)*	0.53 ± 0.03
His-Pro-rich domain	3.08 ± 0.19 *(0.87)*	60.2 ± 6.7 *(1.23)*	1.06 ± 0.06
C-terminal domain	3.94 ± 0.41 *(1.12)*	56.9 ± 1.7 *(1.16)*	0.99 ± 0.04
PRCAM-HPRG	3.73 ± 0.25 *(1.06)*	55.9 ± 2.8 *(1.14)*	0.95 ± 0.07
RCAM-HPRG	8.04 ± 0.44 *(2.28)*	199.0 ± 4.1 *(4.06)*	0.60 ± 0.02
CPB-treated HPRG	5.78 ± 0.29 *(1.64)*	55.9 ± 2.8 *(1.14)*	0.95 ± 0.06
Methyl HPRG	3.38 ± 0.26 *(0.96)*	52.0 ± 1.4 *(1.06)*	1.03 ± 0.08

[a] The relative amidolytic activities are reported to facilitate comparison. A relative unit represents 25.4 mA/min.
[b] Rabbit HPRG and its derivatives were used unless otherwise indicated.

TABLE II

The effect of immobilized HPRG on plasminogen activation by various activators

The indicated plasminogen species (0.25 μM) were activated with various plasminogen activators (0.5 nM, except streptokinase, which was used at 0.1 nM) in hydrazide plates with wells coated with periodate-oxidized HPRG or blocked with BSA (control), as described under "Experimental Procedures." To allow comparison with other stimulators of plasmin formation, the activation rates were also determined in the presence of ε-ACA (20 mM) or cyanogen bromide fragments of fibrinogen (FBG/BrCN; 20 μg/ml). The results are the average of duplicate determinations, which differed by less than 10%.

Activator	Plasminogen	Activation rate			
		Control	Immobilized HPRG	ε-ACA (20 mM)	FBG/BrCN (20 mg/ml)
		mA/min^2 × 10^3			
t-PA	Glu-Pg	4.2	64.6	53.3	381
	XGlu-Pg	3.1	6.0	4.0	41.1
	Lys-Pg	73.7	366	54.2	756
	Mini-Pg	98.3	82.6	64.7	162
K_2P t-PA	Glu-Pg	3.4	10.6	69.9	122
	Lys-Pg	77.7	149	80.8	324
u-PA	Glu-Pg	37.8	38.7	244	58.3
	Lys-Pg	658	666	250	586
Streptokinase	Glu-Pg	137	243	61.0	602

TABLE III

Kinetic parameters for plasminogen activation in solution and on an HPRG surface

Plasminogen was activated with t-PA (0.36 nM) in solution or in the presence of periodate-oxidized HPRG adsorbed to hydrazide plates. Duplicate determinations were made at eight plasminogen concentrations, ranging from 0.015 to 1.5 μM for the activation in solution and from 0.006 to 0.6 μM for that on the HPRG surface. The plasminogen activation rates, *v*, were derived from the slope of *A(t) versus t^2* plots, as described under "Experimental Procedures." The kinetic parameters and standard deviations were obtained by fitting the experimental data points to the Michaelis-Menten equation, $v = k_{cat} \cdot [\text{t-PA}] \cdot [\text{Pg}]/(K_m + [\text{Pg}])$, using non-linear least square regression.

Pg	K_m	k_{cat}	k_{cat}/K_m
	μM	*min^{-1}*	*min^{-1} · μM^{-1}*
Activation in solution			
Lys-Pg	0.182 ± 0.044	1.054 ± 0.080	5.79 ± 1.83
Glu-Pg	2.356 ± 0.467	0.225 ± 0.054	0.095 ± 0.032
XGlu-Pg	>5[a]	—[a]	0.025 ± 0.001
Activation on the HPRG surface			
Lys-Pg	0.015 ± 0.002	1.490 ± 0.041	99.3 ± 15.9
Glu-Pg	0.123 ± 0.010	0.480 ± 0.015	3.90 ± 0.44
XGlu-Pg	0.306 ± 0.063	0.0836 ± 0.0087	0.273 ± 0.082

[a] Since the K_m value for the activation of cross-linked Glu-Pg in solution appears to be much larger than the range of substrate concentrations used in this experiment, data were fitted to the linear equation $v = (V_{max}/K_m) \cdot [S]$.

good agreement with the value of 2.5 pmol/cm^2 expected for a monolayer of protein of this size.[3]

To rule out the possibility that the effect of immobilized HPRG on plasminogen activation is due to denaturation upon adsorption, we coupled HPRG to the surface of microtiter plates by three distinct methods and assayed plasminogen activation. The overall results indicate that stimulation of plasminogen activation by surface-bound HPRG is in fact specific and not due to surface denaturation (*vide infra*). The strongest stimulation, 30–50% of the effect of fibrinogen fragments, was obtained in the presence of nickel chelate-bound HPRG. This method of immobilization relies on the binding of histidine residues of the protein to tetradentate nickel chelates (24) and is mild and directional, since the histidine-proline-rich domain of HPRG contains almost all metal-binding sites (25). HPRG bound to hydrazide-coated plates via periodate-oxidized sugar residues was half as efficient as HPRG bound to nickel chelate, presumably because there are several carbohydrate moieties in HPRG (14) that can be used for immobilization and thus several possible orientations. HPRG adsorbed to polystyrene, the most likely to be denatured, had the weakest effect and stimulated plasminogen activation only 2-fold relative to the control.

Interestingly, immobilized native HPRG was twice as active as RCAM-HPRG, opposite to the activation efficiency in solution, further indicating that the effect of surface-bound HPRG was not caused by denaturation (Fig. 5). Further supporting this assertion, anti-rabbit HPRG antiserum specifically inhibited the effect of native, but had little effect on denatured, HPRG. The antibodies appeared to inhibit by blocking the binding of plasminogen to the immobilized protein, since the effects were both protein- and species-specific. Anti-HPRG antisera were effective only against the cognate protein, but not against immobilized fibrinogen fragments, and anti-fibrinogen antibodies did not inhibit the activation effect of surface-bound HPRG from either species.

A series of chemical derivatives and isolated domains of HPRG were used to further characterize the relationship between HPRG structure and the acceleration of plasminogen

[3] Assuming a hydrated radius of 4 nm, typical for a protein of about 70 kDa, one molecule would have a cross-sectional area of 50 nm^2, and closely packed, 1.5 × 10^{12} molecules would cover 1 cm^2, yielding a surface density of 2.5 pmol/cm^2.

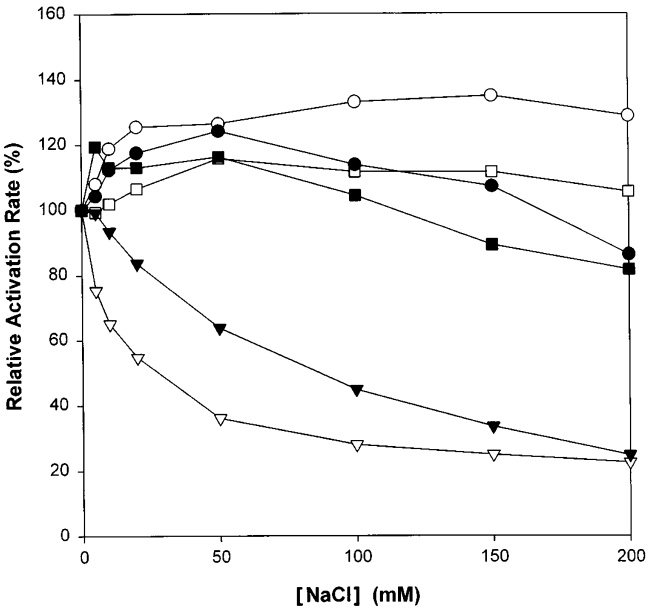

FIG. 4. **The influence of chloride ions on plasminogen activation in solution or on an HPRG surface.** Lys-Pg (0.1 μM, *circles*), Glu-Pg (0.2 μM, *triangles*), or cross-linked Glu-Pg (0.5 μM, *squares*) were activated with t-PA (0.36 nM) in solution (*open symbols*) or in the presence of HPRG immobilized onto hydrazide plates (*closed symbols*). The buffer was 10 mM HEPES, pH 7.3, containing 1 mg/ml BSA, 0.01% Triton X-100, various concentrations of sodium chloride and sodium acetate as required to bring the total concentration of salts to 200 mM. The reaction rates in the absence of chloride ions were used as a reference.

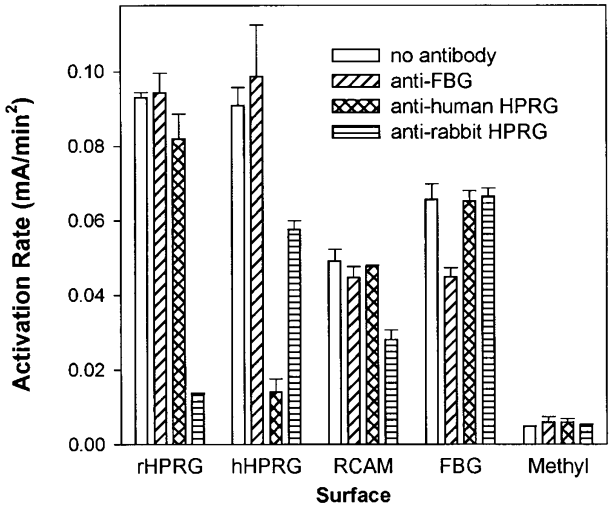

FIG. 5. **Inhibition of plasminogen activation on an HPRG surface by anti-HPRG antisera.** After immobilization of rabbit HPRG (*rHPRG*), human HPRG (*hHPRG*), RCAM-HPRG (*RCAM*), fibrinogen fragments (*FBG*), or methylated HPRG (*Methyl*) on nickel chelate plates, some wells were treated for 2 h with 100 μl of a 1:100 dilution of the indicated antisera, after which the activation assay was carried out with Glu-Pg (0.2 μM) and t-PA (0.36 nM) as described under "Experimental Procedures."

activation. As shown in Table IV, there is a strong positive correlation between plasminogen binding by immobilized HPRG species and plasminogen activation, suggesting that plasminogen bound to HPRG is activated more efficiently than in solution phase. Human HPRG was about 40% more efficient than rabbit HPRG, perhaps due to a smaller area occupied per molecule and thus, higher density on the plate surface. Although the number of amino acid residues is similar in human and rabbit HPRG, the former is less glycosylated and has an

TABLE IV
Plasminogen activation in the presence of various immobilized HPRG species

Plasminogen (0.2 μM) was activated with t-PA (0.36 nM) in nickel chelate-coated microtiter plates to which the indicated HPRG species had been adsorbed as described under "Experimental Procedures." Averages and standard deviations of triplicate determinations are shown. The quantity and "quality" (epitope preservation) of the immobilized HPRG derivatives were estimated by enzyme immunoassay using goat anti-rabbit HPRG antiserum as primary antibody. After blank subtraction, the results were normalized relative to intact rabbit HPRG.

Immobilized HPRG species	Activation rate (relative rate)	Relative immunoreactivity
	mA/min² × 10³	
None	2.7 ± 0.19 *(1.0)*	0
Human[a] HPRG	76.3 ± 3.48 *(28.3)*	
Human clipped HPRG	100.0 ± 3.1 *(37.1)*	
HPRG[b]	42.8 ± 1.76 *(15.9)*	1.00
Clipped HPRG	67.1 ± 2.46 *(25.0)*	1.09
N/C fragment	35.1 ± 1.13 *(13.1)*	1.02
N-terminal domain	32.8 ± 1.83 *(12.2)*	0.61
His-Pro-rich domain	9.8 ± 0.65 *(3.6)*	0.66
C-terminal domain	33.8 ± 0.98 *(12.6)*	0.32
PRCAM-HPRG	40.9 ± 2.31 *(15.2)*	0.87
RCAM-HPRG	13.6 ± 0.43 *(5.0)*	0.59
CPB-treated HPRG	2.1 ± 0.12 *(0.8)*	0.98
Methylated HPRG	2.1 ± 0.05 *(0.8)*	1.05

[a] The values for human HPRG were obtained in a separate experiment in which immobilized rabbit HPRG yielded a value of 53.3 ± 3.1 mA/min.

[b] Rabbit HPRG and derivatives were used unless otherwise indicated.

apparent M_r of 70,000 by SDS-PAGE compared with 90,000 of the latter. Plasmin-clipped forms of rabbit or human HPRG were 30–50% more active than the native counterparts, likely due to the presence of additional binding sites for plasminogen (*vide supra*). Stimulation by the N- and C-terminal HPRG domains was much greater than that by histidine-proline-rich domain and represented about 75% of the native HPRG effect, despite the lower amounts of immobilized protein (61 and 32%, respectively) assessed by immunoassay.

Finally, neither immobilized CPB-treated HPRG nor methyl-HPRG had any effect on plasminogen activation, in good agreement with the lack of plasminogen binding ability. Treatment of immobilized HPRG or plasmin-clipped HPRG with CPB reduced the surface activation effect gradually (data not shown). Sensitivity to CPB treatment of intact HPRG indicates that the original COOH-terminal lysine of HPRG, and not internal lysine residues exposed by plasmin digestion, is responsible for enhanced plasminogen activation, opposite to the behavior observed when fibrin is used as stimulator. In a separate experiment (data not shown), plasminogen activation was carried out in the presence of a mixture of immobilized native and methylated HPRG, and the stimulation was roughly proportional to the percentage of native protein on the surface, showing that the stimulatory effect was not saturated with regard to the immobilized HPRG and the amount of protein that can be bound onto the surface was the limiting factor.

Inhibition of Plasminogen Activation on an HPRG Surface by Competition for Plasminogen or HPRG—To confirm that the stimulation of plasminogen activation in the presence of immobilized HPRG requires binding of plasminogen to the HPRG surface, the inhibition by ligands of either plasminogen or HPRG was tested. As shown in Fig. 6*A*, the effect of ϵ-ACA in the activation assay was closely similar to that in the plasminogen binding assay (Fig. 3*A*, *inset*), providing further evidence that plasminogen binding to immobilized HPRG via LBS is required for activation. The low concentration of ϵ-ACA required to achieve 50% inhibition, near 0.2 mM for both Glu and Lys-Pg, suggests that only the high affinity LBS located on kringle 1 is involved in the interaction. The increase in the

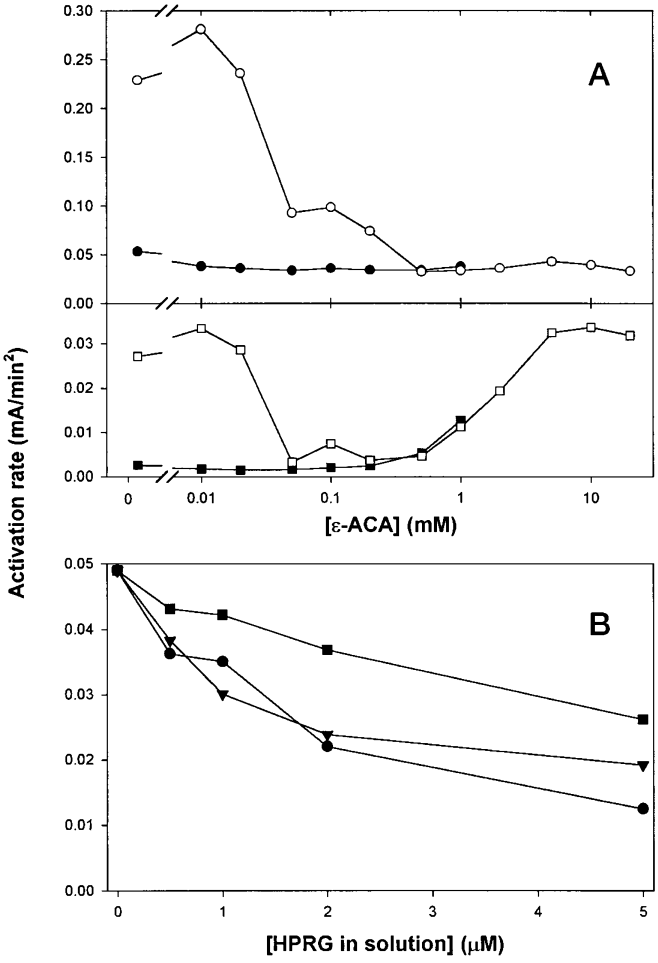

FIG. 6. **Inhibition of plasminogen activation on an HPRG surface by competition for lysine binding sites of plasminogen.** *Panel A*, Lys-Pg (0.2 μM, *top*) or Glu-Pg (0.2 μM, *bottom*) were activated with t-PA (0.36 nM) in solution (●, ■) or on an HPRG surface (○, □) in the presence of increasing concentrations of ϵ-ACA. HPRG was immobilized to hydrazide-coated plates via periodate-oxidized sugar moieties. *Panel B*, Glu-Pg (0.2 μM) was activated with t-PA (0.36 nM) on HPRG surface (nickel chelate), in the presence of soluble rabbit HPRG (●), human HPRG (■), or plasmin-clipped rabbit HPRG (▼).

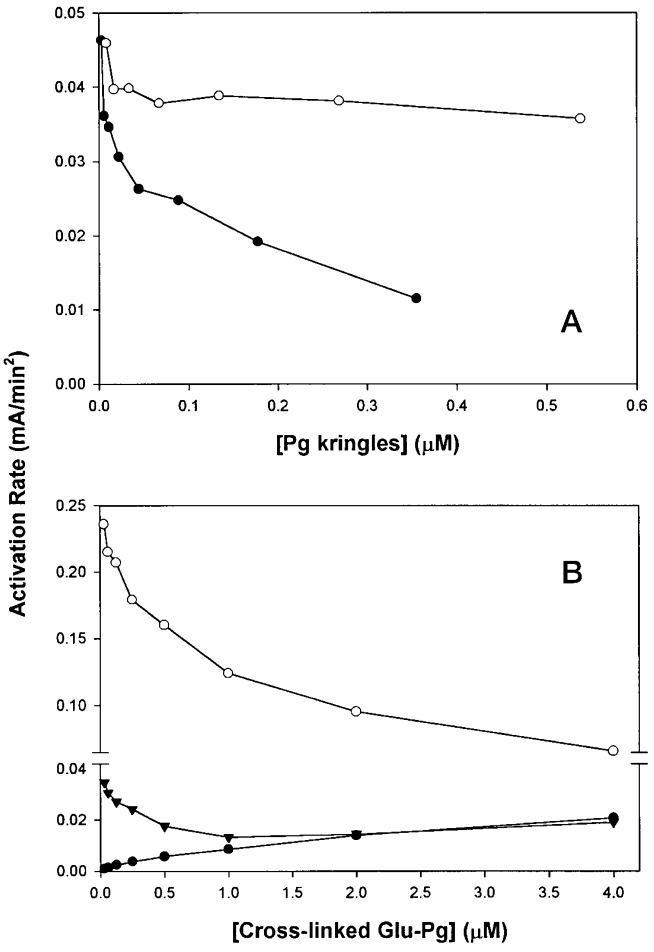

FIG. 7. **Inhibition of plasminogen activation on an HPRG surface by competition for HPRG.** *Panel A*, Glu-Pg (0.2 μM) was activated with t-PA (0.36 nM) on HPRG surface (hydrazide), in the presence of plasminogen kringle 4 (○) or kringle 1-2-3 fragment (●). *Panel B*, Lys-Pg (0.1 μM, ○) or Glu-Pg (0.2 μM, ▼) were activated with t-PA (0.36 nM) in the presence of increasing concentrations of cross-linked Glu-Pg. The activation of cross-linked Glu-Pg (●) alone is shown for comparison.

activation rate of Glu-Pg at higher concentrations of ϵ-ACA (above 2 mM) is due to occupancy of the lower affinity LBS in plasminogen, causing Glu-Pg to assume a more open conformation and abrogating the inhibitory effect of chloride ions (2, 26).

Binding of plasminogen to immobilized HPRG could also be prevented by soluble HPRG. The overall effect was an inhibition of plasminogen activation (Fig. 6*B*), since the interaction between HPRG and plasminogen *in solution* does not increase plasminogen activability but does prevent plasminogen binding to the stimulatory, *immobilized* HPRG, causing an *apparent* inhibition of plasminogen activation. This inhibition effect was relatively weak due to the low K_m of plasminogen activation on a surface (see Table III). Thus, a *2-fold* reduction of plasminogen concentration from $2K_m$ (approximately the concentration of Glu-Pg used in the experiment shown in Fig. 6*B*) to K_m will reduce the activation rate from $0.66V_{max}$ to $0.5V_{max}$, only a 25% decrease. It has been previously shown that soluble HPRG weakly inhibits fibrinolysis (8) or plasminogen activation in the presence of fibrin (11).

The activation assay was carried out also in the presence of plasminogen kringles, to identify which LBS of plasminogen bind to the immobilized HPRG. The data in Fig. 7*A* show that plasminogen kringle 1-2-3 inhibited stimulation and hence bound to immobilized HPRG, while the kringle fragment 4 did not have any effect, providing additional evidence that HPRG interacts with the high affinity LBS on kringle 1. The effect of plasminogen kringle 5 could not be assessed in this inhibition assay, since mini-Pg was efficiently activated in solution. However, kringle 5 does not appear to be involved in HPRG-enhanced plasminogen activation, since the activation of mini-Pg by t-PA was stimulated by fibrinogen fragments only and kringles 1–5 but not kringles 1–3 inhibited plasminogen activation in the presence of fibrin (21).

When XGlu-Pg was used in the activation assay together with fixed amounts of Glu-Pg or Lys-Pg, the activation effect was reduced in a concentration-dependent manner (Fig. 7*B*). XGlu-Pg is activated very slowly since it is locked in the closed conformation (20), but it can compete with the other plasminogen forms for binding to the stimulatory HPRG surface. Glu-Pg and Lys-Pg activation were inhibited by 50% at about 0.5 μM and 1.0 μM XGlu-Pg, respectively, denoting that Lys-Pg has a higher affinity than Glu-Pg for the HPRG surface.[4] XGlu-Pg minimally affected the activation rate of Glu-Pg or Lys-Pg in solution (data not shown), indicating that it inhibits plasminogen activation in the presence of immobilized HPRG by binding to HPRG and not to t-PA.

[4] It can be easily shown that 50% inhibition is achieved at a concentration of competitor $[X_{0.5}]$ given by the formula $[X_{0.5}]/K_{m,X} = [S]/K_{m,S} + 1$, where $K_{m,i}$ is the Michaelis-Menten constant for substrate i.

DISCUSSION

HPRG is arguably the most abundant plasminogen ligand in plasma, and as much as 50% of plasminogen is estimated to circulate as a complex with HPRG. The proposed anti-fibrinolytic role of this interaction was recently questioned (11), since HPRG only retards fibrinolysis by 15–30%. Indeed, since the concentration of plasminogen in normal human plasma is about 2 μM, halving the concentration of free plasminogen cannot have a large effect on the fibrin-stimulated plasminogen activation, whose K_m is at least 1 order of magnitude lower. The data we present in this paper support the earlier proposal that HPRG is pro-fibrinolytic rather than anti-fibrinolytic (12). Apparently contradictory results have been reported earlier regarding the effect of HPRG on plasminogen activation; almost no effect was seen when soluble HPRG and u-PA were used (8), but a strong acceleration was noticed with immobilized HPRG and t-PA (12). This discrepancy is easily accounted for in view of our observations that HPRG-enhanced plasminogen activation is activator-specific and also depends on the physical state of HPRG. Since t-PA is associated with intravascular fibrinolysis and urokinase with plasmin generation in the extravascular milieu, the HPRG effect is relevant only in the former setting. In this paper, we characterized in detail the features of plasminogen activation in the presence of HPRG.

First, there is a marked difference between the effects of HPRG in solution and on a surface. In solution, native HPRG alone has little effect on plasminogen activation, but inhibits the acceleration effect of strong stimulators. However, immobilized HPRG has a clear pro-fibrinolytic activity, strongly enhancing plasminogen activation. The differences between surface-bound and soluble HPRG are probably caused by the much higher local concentration of the former and not by a conformational change induced upon binding to the surface, because similar results were obtained with HPRG immobilized by distinct, mild methods.

Second, the active species on the surface is native, not denatured HPRG. This was demonstrated by specific inhibition by antisera to HPRG, by the higher rate of activation in the presence of immobilized native HPRG as compared with RCAM-HPRG, and by the correspondence between the activity of surface-bound HPRG and the mildness of the immobilization method used. In contrast, only non-native HPRG species stimulate plasminogen activation in solution, and since denatured proteins are often aggregated, it is tempting to speculate that micro-heterogeneity plays a role in this effect.

Third, only plasminogen bound to immobilized HPRG is activated efficiently, as shown by the correlation between the HPRG species active in plasminogen binding and activation assays, as well as by the decrease in stimulation upon competition with ligands for either plasminogen or HPRG. The interaction occurs between the C-terminal lysine residue of native HPRG and sites newly created by plasmin in the N-terminal domain on one hand, and the high affinity LBS of plasminogen situated on kringle 1-2-3 on the other. Indeed, the C-terminal lysine residue preceded by a proline is conserved in human (27) and bovine (28) HPRG. However, the weak effect of immobilized histidine-proline-rich domain or RCAM-HPRG on plasminogen activation illustrates that a C-terminal lysine residue alone, in the absence of an appropriate structural context, does not confer stimulatory properties. Notably, immobilized HPRG exerts its effect via plasminogen kringle 1-2-3, whereas with fibrin, binding to kringle 5 (21) is important for enhanced plasminogen activation.

Fourth, immobilized native HPRG has an intrinsic, immediate stimulatory activity on plasminogen activation (showed by linear $A(t)$ *versus* t^2 plots) since it possesses the requisite C-terminal lysine residue and its effect is abolished by prior treatment with CPB. In contrast, other effectors such as fibrin or fibrinogen fragments exhibit a lag period during which the internal lysine residues are exposed by plasmin to form new plasminogen binding sites that accelerate the activation process. Although clipping HPRG with plasmin *prior to* the assay increased the stimulatory activity, the difference was small (about 50%) and immobilized HPRG is only slowly digested by plasmin.

Finally, the high specificity for the plasminogen activator species but not for plasminogen offers clues to the mechanism of plasminogen activation on an HPRG surface. A mere conformational change in plasminogen, such as that induced in Glu-Pg by ϵ-ACA in the presence of chloride ions, cannot explain why the activation of Lys-Pg or XGlu-Pg (found in the open and closed conformation, respectively) is enhanced, nor can it explain why urokinase is not stimulated by immobilized HPRG. For the same reason we can rule out a conversion of Glu-Pg to Lys-Pg on the HPRG surface as the sole accelerating factor. The latter two mechanisms may be responsible in part for the stronger stimulation observed with Glu-Pg, especially since the inhibition by chloride ions was reduced in the presence of immobilized HPRG.

Simultaneous binding of plasminogen and activator, as shown for fibrin (4), extracellular matrix proteins (29), or cell surfaces (30), greatly accelerates plasminogen activation. However, HPRG does not appear to bind t-PA directly (12), and, in addition, we were not able to detect binding of HPRG to t-PA in ligand blot experiments (data not shown). We cannot rule out that t-PA and HPRG interact with low affinity, below our detection limit. In fact, a comparison among t-PA, the K_2P t-PA mutant, and u-PA strongly suggests that, in addition to the interaction between the active site of the activator and the scissile bond of the immobilized plasminogen, t-PA also binds to one of the proteins on the surface (either HPRG or another plasminogen molecule) via one of its N-terminal domains. Even a low affinity interaction would increase the avidity of binding to the surface, where the local concentration of immobilized molecules is very high, but would have a limited effect in solution due to the translation and rotation entropy. A tetramolecular activation complex (HPRG . . . Pg . . . t-PA . . . HPRG or Pg) could explain why the activation is accelerated by immobilized but not soluble HPRG, and also the reduced K_m value on the HPRG surface. Yet another effect arising from the high local concentration of protein on the surface can also contribute to the enhanced activation: "trapping" of t-PA by the plasminogen layer bound to the HPRG-coated surface. Diffusion of a ligand from a compartment containing a high concentration of protein, [P], to the bulk solution is slowed down by a factor of $1 + [P]/K_d$ (where K_d is the dissociation constant of the protein-ligand complex) when the protein is in excess over the ligand (31). These conditions are met for plasminogen bound to the HPRG surface, which achieves a high local concentration and is in excess over t-PA. Thus, the activator molecules near the surface are likely to bind repeatedly to new substrate molecules and turn over many times before they diffuse back into the bulk solution. Such an effect was described for phospholipase A, an enzyme that hydrolyzes its substrate slowly in solution, but whose activity is greatly increased efficiently at interfaces (32).

At this point, we can only speculate on the physiological significance of our findings. Although in this work, HPRG was immobilized by non-physiological and quasi-irreversible methods, one or more of the several HPRG ligands, *e.g.* thrombospondin (33) or vitronectin (34), may act as an anchor to immobilize HPRG *in vivo*. Particularly attractive for this role are negatively charged glycosaminoglycans found on cell sur-

faces. HPRG has been shown to interact with heparin (10, 35), heparan sulfate (36, 37), and dermatan sulfate (38). Binding of heparin to HPRG requires histidine residues (39), is sensitive to pH changes in the physiological range (40), and appears to involve the histidine-proline-rich domain (14). Interestingly, in our experiments, the maximal stimulation effect was obtained with HPRG immobilized onto a nickel chelate surface, and divalent metal ions bind also to the histidine-proline-domain of HPRG (25). It is also possible for HPRG to become immobilized on the cell surface of activated platelets (41) or T-cells (42).

Although many surface-bound proteins (*e.g.* cell surface or extracellular matrix proteins) accelerate plasminogen activation, they are invariably immobilized. In contrast, HPRG is a plasma protein found in solution phase and only accelerates plasminogen activation when immobilized. Thus, its activity can be modulated by factors that affect the HPRG equilibrium between solution and surfaces, for example an enhanced binding of HPRG to glycosaminoglycans upon a decrease in the local pH. This hypothesis can account for the modular structure of HPRG in terms of its function and bears a striking resemblance to the structure-function relationships of kininogen, another member of the cystatin superfamily. When immobilized to negative surfaces via its lysine-histidine-rich domain (somewhat reminiscent of the histidine-proline-rich domain of HPRG), kininogen optimally positions kallikrein and factor XI, thereby facilitating their activation by surface-bound factor XIIa, and hence triggers the intrinsic coagulation pathway (43, 44). Further experiments are needed to characterize the binding of HPRG to surfaces under physiological and pathophysiological conditions.

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