Histidine-rich Glycoprotein Binds Fibrin(ogen) with High Affinity and Competes with Thrombin for Binding to the γ'-Chain

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Histidine-rich glycoprotein (HRG) is an abundant protein that binds fibrinogen and other plasma proteins in a Zn2+-dependent fashion but whose function is unclear. HRG has antimicrobial activity, and its incorporation into fibrin clots facilitates bacterial entrapment and killing and promotes inflammation. Although these findings suggest that HRG contributes to innate immunity and inflammation, little is known about the HRG-fibrin(ogen) interaction. By immunoassay, HRG-fibrinogen complexes were detected in Zn2+-supplemented human plasma, a finding consistent with a high affinity interaction. Surface plasmon resonance determinations support this concept and show that in the presence of Zn2+, HRG binds the predominant γA/γA'-fibrinogen and the γ-chain elongated isoform, γA/γ'-fibrinogen, with Kd values of 9 nM. Likewise, 125I-labeled HRG binds γA/γA'- or γA/γ'-fibrin clots with similar Kd values when Zn2+ is present. There are multiple HRG binding sites on fibrin(ogen) because HRG binds immobilized fibrinogen fragment D or E and γ'-peptide, an analog of the COOH terminus of the γ'-chain that mediates the high affinity interaction of thrombin with γA/γ'-fibrin. Thrombin competes with HRG for γ'-peptide binding and displaces 125I-HRG from γA/γ'-fibrin clots and vice versa. Taken together, these data suggest that (a) HRG circulates in complex with fibrinogen and that the complex persists upon fibrin formation, and (b) by competing with thrombin for γA/γ'-fibrin binding, HRG may modulate coagulation. Therefore, the HRG-fibrin interaction may provide a novel link between coagulation, innate immunity, and inflammation.

Fibrinogen is the soluble precursor of fibrin, a critical component of blood clots that endows them with strength and elasticity. Fibrinogen is a glycoprotein composed of three pairs of polypeptide chains, termed Aα, Bβ, and γ, that are connected by disulfide bonds (1). Approximately 10–15% of circulating fibrinogen has a variant γ-chain termed the γ'-chain, which results from differential processing of the γA-chain mRNA transcript (2–4). The γ'-chain is distinguished from the γA-chain by the presence of an acidic 20-residue extension at its COOH terminus (2, 5).

Thrombin catalyzes the conversion of fibrinogen to insoluble fibrin, and during this process some thrombin remains bound to the fibrin network (6). Exosites 1 and 2 are two regulatory domains that flank the active site of thrombin and mediate its binding to fibrin (7, 8). Exosite 1 of thrombin interacts with the central E-domain of fibrin, whereas exosite 2 binds only to the COOH terminus of the γ'-chain. Consequently, thrombin binds γA/γA'-fibrin in a univalent fashion with a Kd value of 2–4 μM. In contrast, both exosites are engaged when thrombin binds to γA/γ'-fibrin, resulting in a higher affinity interaction (Kd value of 0.08–0.18 μM) (5, 9). Fibrin-bound thrombin remains active, and the protease is protected from inhibition by fluid-phase inhibitors, such as antithrombin and heparin cofactor II (6). Because of its bivalent interaction with γA/γ'-fibrin, thrombin bound to γA/γ'-fibrin is more protected from inhibition by fluid-phase inhibitors than thrombin bound to γA/γA'-fibrin (10).

Like thrombin, histidine-rich glycoprotein (HRG)3 binds to fibrinogen and is incorporated into fibrin clots (11). Although the plasma concentration of HRG ranges from 1.6 to 2 μM, the concentration in platelet-rich thrombi may be higher because HRG is stored in the alpha granules of platelets and is released when platelets are activated (12, 13). A 75-kDa glycoprotein, HRG, is composed of two NH2-terminal cystatin-like domains, a central histidine-rich region (HRR) flanked by two proline-rich regions, and a COOH-terminal domain (14). In addition to fibrinogen, HRG also binds plasminogen, heparan sulfate, and divergent cations, such as Zn2+ (12, 15, 16). Therefore, HRG is hypothesized to be an important accessory or adapter protein that brings different ligands together under specific conditions (14).

HRG-deficient mice exhibit a shorter prothrombin time and accelerated fibrinolysis compared with wild-type mice, raising

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the possibility that HRG modulates coagulation and fibrinolysis (17). In addition to its potential role in hemostasis, HRG also has been implicated in innate immunity and inflammation (18). HRG exhibits antifungal and antimicrobial activity in vitro, and these activities are enhanced at low pH or in the presence of Zn\(^{2+}\), conditions that promote ligand binding (19, 20). The antimicrobial activity of HRG has also been demonstrated in vivo and appears to be fibrin-dependent. Thus, compared with wild-type mice, HRG-deficient mice are more susceptible to the lethal effect of Streptococcus pyogenes infection and are rescued with HRG supplementation (21). This phenomenon is fibrin-dependent because fibrin is essential for HRG-mediated bacterial entrapment and killing, processes that prevent bacterial dissemination. In addition, the HRG-fibrin interaction modulates inflammation because HRG-deficient mice exhibit attenuated abscess formation in response to subcutaneous injection of bacteria. Based on these findings, it has been postulated that HRG plays a fibrin-dependent role in both inflammation and innate immunity (21).

Despite emerging evidence that the HRG-fibrin(ogen) interaction is physiologically important, little is known about the biochemical foundation of this interaction or its functional consequences. To address these gaps in knowledge, we set out to (a) quantify the binding of HRG to fibrin(ogen), (b) identify the HRG binding domains on fibrin(ogen), and (c) determine whether there is overlap between the HRG and thrombin binding domains on fibrin(ogen).

EXPERIMENTAL PROCEDURES

Materials

Reagents—Human thrombin, prothrombin, and plasminogen-free fibrinogen were from Enzyme Research Laboratories (South Bend, IN). Plasmin and factor XIII were from Haematologic Technologies Inc. (Essex Junction, VT). Prionex was from Pentapharm (Basel, Switzerland). A 20-amino acid analog of the COOH terminus of the \(\gamma^\prime\)-chain of fibrinogen, \(\gamma^\prime\)-peptide (VRPEHPAETYDLSYPEDDL), was prepared by Bachem Bioscience, Inc. (King of Prussia, PA), and a sheep antibody against (VRPEHPAETEYDSLYPEDDL), was prepared by Bachem Biosciences, Inc. (King of Prussia, PA), and a sheep antibody against this peptide was from Affinity Biologicals (Ancaster, ON). The two Tyr residues within the \(\gamma^\prime\)-peptide were modified with phosphate groups in place of sulfate to enhance stability (22).

Preparation of Fibrinogen Fragments—Fragment X was prepared by limited plasmin digestion of fibrinogen (23). Fragments D and E were generated by plasmin digestion of \(\gamma^\alpha /\gamma^\alpha^\prime\)-fibrinogen (25). Digested material was applied to a 12-ml UNO Q-12 ion exchange column (Bio-Rad) using a Bio-Rad Biologic Duoflow system at a flow rate of 5 ml/min. To elute non-specifically bound proteins, the column was washed with 30 ml of 0.02 M sodium phosphate, 0.01 M citric acid, pH 7.6. Fragment D was eluted with 100 ml of 0.1 M sodium phosphate, 0.05 M citric acid, pH 5.0, whereas fragment E was subsequently eluted with 40 ml of the same buffer at pH 4.4 (26). Protein-containing fractions were identified by absorbance at 280 nm and pooled. Purified fragments were dialyzed into 10 mM Hepes-NaOH, 150 mM NaCl, pH 7.4 (HBS) and concentrated. Final concentrations were determined at 280 nm (27). The integrity of the fragments was assessed by SDS-PAGE analysis on 4–15% polyacrylamide gels (Ready-Gel, Bio-Rad) under reducing and non-reducing conditions. Samples were stored in aliquots at −80 °C.

Preparation of \(\gamma^\prime\)-directed IgG Fab Fragments—For some experiments, fragment antibody binding (Fab) regions from the affinity-purified \(\gamma^\prime\)-peptide-directed IgG were generated by papain digestion (28), isolated with a Fab preparation kit (Pierce), assessed for purity by SDS-PAGE analysis, and then concentrated and dialyzed against HBS.

Labeling of Proteins—\(\gamma^\prime\)-Peptide was labeled with fluorescein isothiocyanate as previously described (9). \(\alpha\)-Thrombin was radiolabeled with \(^{125}\text{I}\)-labeled YPRck, and HRG was radiolabeled with Na\(^{125}\text{I}\) (McMaster University Nuclear Reactor, Hamilton, ON) using Iodo-beads (Pierce) as described (29). \(^{125}\text{I}\)-YPRck-thrombin and \(^{125}\text{I}\)-HRG concentrations were 5–6 \(\mu\text{M}\), as determined by absorbance at 280 nm, with radioactivity of 500,000–800,000 cpm/\(\mu\text{g}\) of protein. FPRck-thrombin was prepared as described (30).

Methods

Surface Plasmon Resonance (SPR)—The interaction of HRG with immobilized \(\gamma^\alpha /\gamma^\alpha^\prime\) or \(\gamma^\alpha /\gamma^\alpha^\prime\)-fibrinogen, biotinylated \(\gamma^\prime\)-peptide, or fragments X, D, or E was assessed by SPR using a BIAcore 1000 (GE Healthcare) as previously described (24, 30) but with some modifications. Briefly, proteins were covalently linked to separate flow cells of a carboxymethylated dextran (CM4) biosensor chip at a flow rate of 5 \(\mu\text{l/min}\) using an amine coupling kit (GE Healthcare). Proteins were immobilized using 10 \(\mu\text{M}\) acetate buffer at varying pH values to maximize adsorption. \(\gamma^\alpha /\gamma^\alpha^\prime\) or \(\gamma^\alpha /\gamma^\alpha^\prime\)-fibrinogen or fragments X and D were immobilized at pH 5.5 to ~3000–7000 response units (RU). Fragment E was immobilized at pH 4.5 to ~2000–3000 RU. For fibrin binding studies, immobilized \(\gamma^\alpha /\gamma^\alpha^\prime\) or \(\gamma^\alpha /\gamma^\alpha^\prime\)-fibrinogen was converted to fibrin by three successive 60-min injections of 100 \(\mu\text{M}\) thrombin at 5 \(\mu\text{l/min}\) (30). To prepare streptavidin-conjugated CM4 flow cells, 0.4 mg/ml streptavidin (Sigma) at pH 4.5 was injected. Biotinylated \(\gamma^\prime\)-peptide (30) was adsorbed to the immobilized streptavidin to 200–300 RU. Remaining reactive groups were neutralized with 1 M ethylenediamine, and non-specifically adsorbed proteins were removed by treatment with 0.5 M NaCl. An unmodified flow cell served as the control. All SPR procedures were done in HBS containing 0.005%
HRG Modulates Thrombin Binding to Fibrin

Twin20 and 2 mM CaCl$_2$, and flow cells were regenerated with 250 mM imidazole and 2 mM EDTA between runs.

To measure the affinity of HRG for immobilized fibrinogen, fibrin, or fibrinogen fragments, aliquots of HRG (0–1 μM) in buffer containing 20 μM ZnCl$_2$ were injected at a flow rate of 30 μl/min. To quantify Zn$^{2+}$ dependence, binding of 200 nM HRG to immobilized γH/$\gamma$- or γH$'$-fibrinogen was monitored in the presence of varying concentrations of ZnCl$_2$ (0–60 μM) using dual injection mode. The binding of thrombin to immobilized fragment D or E was monitored by injection of FPRck-thrombin (0–15 μM) into flow cells.

The γ$'$-directed IgG was used to assess the contribution of the γ$'$-chain to the interaction of HRG with immobilized γ$'$-peptide or fibrinogen. A saturating amount of γ$'$-peptide-directed IgG or a non-immune IgG (2 μg/ml) was first injected into flow cells containing immobilized γ$'$-peptide or γH/$\gamma$- or γH$'$-fibrinogen. The binding of HRG to fibrinogen or fibrin fragments was then measured as described above, except that flow cells were re-saturated with 0.5 μM γ$'$-directed IgG or control IgG before each HRG injection.

Binding of HRG to immobilized γ$'$-peptide in the absence or presence of FPRck-thrombin was monitored using a BIACore T200. Biotinylated γ$'$-peptide was adsorbed to streptavidin-conjugated CM4 flow cells to 100 RU in the presence of 20 μM ZnCl$_2$ at a flow rate of 5 μl/min. An unmodified flow cell served as a control. Using dual injection mode, 1 μM HRG was injected at 10 μl/min followed by a second injection of FPRck-thrombin or prothrombin at concentrations ranging from 0 to 8 μM. Flow cells were regenerated with 250 mM imidazole, 2 mM EDTA, and 1 M NaCl. All experiments were performed at least twice.

SPR Data Analysis—Kd values were determined by kinetic analysis of on- and off-rates of HRG binding to immobilized ligands using Scrubber2 version 2.0a (Bio-Logic Software Co., Campbell, Australia) as described previously (24, 30). For further assessment of binding, the amount of HRG bound at the equilibrium position (Req) was determined using the Langmuir 1:1 binding model (BIACore Evaluation software Version 3.2) and was plotted against the titrant concentration. Molar stoichiometries were determined as described in the BIACore technology handbook (BIACore 1000). The correction factor to account for the orientation of the immobilized fibrinogen and fibrin was 0.25, which corresponds to 25% of the amount of immobilized fibrin accessible to the γ$'$-peptide-directed IgG as determined in a separate study (10). The correction factor for immobilized γ$'$-peptide was 0.7, which corresponds to 70% correct orientation of peptide accessible to an analyte (BIACore).

Interaction of 125I-HRG with Fibrin Clots—In a series of microcentrifuge tubes, γH/$\gamma$- or γH$'$-fibrinogen, in concentrations ranging from 0 to 1.25 μM, was clotted with 10 nM thrombin in the presence of 40 nM 125I-HRG as previously described (30). Clots were formed in 20 mM Tris-HCl and 150 mM NaCl, pH 7.4 (TBS), containing 0.005% Tween (TBS-Tween) and 2 mM CaCl$_2$ and 20 μM ZnCl$_2$. To prepare a reference curve, HRG-fibrinogen complexes in plasma were detected using a previous described ELISA (24) with some modifications. Briefly, 100 μl of fibrinogen-directed capture antibody (Affinity Biologicals) diluted to 20 μg/ml in 50 mM NaHCO$_3$, pH 9.6, was added to wells of a 96-well Immunon 4 HBX plate (Thermo) and incubated overnight at 4 °C. To block nonspecific binding, 200 μl of 10 mg/ml bovine serum albumin (Sigma) was added to each well and incubated for 1 h at 23 °C. Wells were washed 3 times with 150 μl of phosphate-buffered saline (PBS) containing 10 μM ZnCl$_2$ and 0.1% Tween 20. Normal and HRG-deficient plasma samples were dialyzed against PBS to remove citrate, reconstituted with 18 μM ZnCl$_2$, and then serially diluted up to 1600-fold with HBS containing 1% ovalbumin, 0.1% Tween 20, 2 mM CaCl$_2$, 100 mM hirudin (Behring), and 18 μM ZnCl$_2$. To prepare a reference curve, HRG-fibrinogen complexes were generated by incubating varying concentrations of HRG (0–1.6 μg/ml) with fibrinogen (0–0.8 μg/ml) in a 2:1 molar ratio. 100 μl of each of these mixtures or plasma was added to wells and incubated for 2 h at 23 °C. After three sequential washes with PBS containing ZnCl$_2$, HRG-fibrinogen
complexes were detected in purified and plasma systems using a HRG-directed IgG-horseradish peroxidase (HRP) conjugate, prepared as specified by the manufacturer using a Lightning-link HRP conjugation kit (Cedarlane, Burlington, ON). After incubation for 1 h at 23 °C, bound HRP conjugates were detected as specified by the supplier. Experiments were repeated three times.

**Interaction of HRG with Fluorescein-labeled γ'-Peptide in the Absence or Presence of ZnCl₂**—The binding of 1.1 μM HRG to 0.05 μM fluorescein-γ'-peptide was monitored by fluorescence in the absence or presence of Zn²⁺ using a PerkinElmer Life Sciences LS 50B luminescence spectrometer (9). Briefly, the base-line fluorescence (Iₒ) was determined at excitation and emission wavelengths (slit widths) of 492 (5 nm) and 532 nm (2.5 nm), respectively, and an emission filter at 515 nm. The mixture was then titrated with aliquots of ZnCl₂ up to 20 μM, and fluorescence intensity (I) was monitored after each addition. I/Iₒ values were plotted against the concentration of ZnCl₂, and the data were subjected to nonlinear regression analysis as previously described (9).

**Statistical Analyses**—Results are presented as the mean ± S.D., and the significance of differences in the means was determined using t tests. For these analyses, p < 0.05 was considered statistically significant.

**RESULTS**

**Interactions of HRG with γₐ/γₐ- or γₐ/γ'/Fibrinogen**—Although HRG has previously been shown to bind fibrinogen (11), the distinction between γₐ/γₐ- and γₐ/γ'/fibrinogen binding has not been investigated. SPR was used to characterize the interaction between HRG and the two isoforms of fibrinogen. γₐ/γₐ- or γₐ/γ'/fibrinogen was immobilized on separate flow cells of a CM4 sensor chip, and an unmodified flow cell served as the control. HRG did not bind either form of fibrinogen in the presence of Ca²⁺ alone (data not shown). Because Zn²⁺ facilitates the binding of ligands to HRG (14), we examined the effect of Zn²⁺ on the HRG-fibrinogen interaction. The Req increased as a function of the ZnCl₂ concentration and saturated at ~30 μM ZnCl₂ (Fig. 1). At each ZnCl₂ concentration, more HRG bound to γₐ/γ'-fibrinogen than to γₐ/γₐ-fibrinogen. The apparent Kₐ values of ZnCl₂ necessary to promote HRG binding to γₐ/γₐ- or γₐ/γ'-fibrinogen were 4.9 ± 0.2 and 1.2 ± 0.8 μM, respectively. The Zn²⁺ dependence of the interaction of HRG with fibrinogen(ogen) is in apparent contradiction to previous work demonstrating HRG binding to fibrin without Zn²⁺ addition (11). Because no binding was detected in the absence of Zn²⁺ in our study, it is likely that the HRG preparation used in the previous report contained sufficient amounts of Zn²⁺ to enable the interaction. For the remainder of the study, ZnCl₂ was used at a concentration of 20 μM. This concentration was chosen because the physiological concentration of Zn²⁺ in plasma ranges from 10 to 20 μM (15, 31). As evidenced from the similarity in saturation profiles illustrated in Fig. 1, more than 80% of HRG is bound to both forms of fibrinogen at 20 μM ZnCl₂.

To determine the affinity of HRG for fibrinogen, increasing concentrations of HRG were sequentially injected into flow cells containing immobilized fibrinogen in the presence of 20 μM ZnCl₂. The sensograms reveal slow association and dissociation phases for HRG binding to both isoforms of fibrinogen (Fig. 2, A and B). Kₐ values were obtained by kinetic analysis of the on- and off-rates by globally fitting the binding data. In the presence of Zn²⁺, HRG binds γₐ/γₐ- and γₐ/γ'/fibrinogen with similar affinity, Kₐ values of 8.8 ± 0.9 and 8.9 ± 3.9 nM, respectively (Table 1). These values agree with the previously reported Kₐ of 6.7 nM determined by immunoassay (11). Plots of calculated Req values for each HRG concentration revealed saturable binding and demonstrated that binding of HRG to γₐ/γ'/fibrinogen was significantly (p < 0.005) higher than that for γₐ/γₐ-fibrinogen (Fig. 3), suggesting that 2-fold more HRG is bound to the γₐ/γ'/fibrinogen isoform. The molar stoichiometries for the interaction of HRG with γₐ/γₐ-fibrinogen and γₐ/γ'/fibrinogen are 1.7 ± 0.3 and 3.2 ± 0.5, respectively (Table 1). Because the extended COOH terminus of the γ'-chain is the feature that distinguishes γₐ/γ'-fibrinogen from γₐ/γₐ-fibrinogen, the increased binding of HRG to γₐ/γ'/fibrinogen suggests that the γ'-chain provides the additional HRG binding site.

**Effect of the γ'-Peptide-directed IgG on the Binding of HRG to γₐ/γₐ- or γₐ/γ'/Fibrinogen**—To confirm that HRG binds specifically to the γ'-chain of γₐ/γ'/fibrinogen, we examined the effect of an affinity-purified IgG directed against this region. As an initial control, we demonstrated specific binding of the antibody to immobilized γₐ/γ'-fibrinogen but not to γₐ/γₐ-fibrinogen (data not shown). We next examined the effect of the antibody on HRG binding to γₐ/γ'- or γₐ/γₐ-fibrinogen. The γ'-chain-directed antibody reduced HRG binding to γₐ/γ'-fibrinogen to that observed with γₐ/γₐ-fibrinogen (Fig. 3). As a control, a sheep non-immune IgG was used; the control IgG had no effect on the binding of HRG to fibrinogen (data not shown). Because the interaction of HRG with γₐ/γₐ-fibrinogen is already of high affinity, the addition of the γ'-peptide-di-
Dissociation constants and stoichiometries for the binding of HRG to \( \gamma_A/\gamma_A\)-fibrinogen, \( \gamma_A/\gamma'-\)fibrinogen, or \( \gamma'-\)peptide

| Ligand          | \( K_d \) (nM) | Stoichiometry |
|-----------------|----------------|--------------|
| \( \gamma_A/\gamma_A\)-Fibrinogen | 8.8 ± 0.9 | 1.7 ± 0.3 |
| \( \gamma_A/\gamma'-\)Fibrinogen | 8.9 ± 3.9 | 3.2 ± 0.5 |
| \( \gamma_A/\gamma'-\)Fibrinogen | 19.3 ± 2.6 | 1.8 ± 0.3 |
| \( \gamma_A/\gamma'-\)Fibrinogen | 10.9 ± 0.9 | 2.9 ± 0.6 |
| \( \gamma'-\)Peptide | 0.8 ± 0.1 | 0.7 ± 0.02 |

HRG Binding to the \( \gamma'-\)Peptide—To confirm that the \( \gamma'-\)chain affords HRG an additional binding site, binding of HRG to synthetic \( \gamma'-\)peptide was examined. First, the interaction of HRG with fluorescein-\( \gamma'-\)peptide was examined by fluorescence. Neither HRG nor \( Zn^{2+} \) alone altered the fluorescence intensity of the \( \gamma'-\)peptide, suggesting that in the absence of \( Zn^{2+} \), there is no interaction. However, when \( Zn^{2+} \) was titrated in the presence of HRG, the fluorescence intensity of fluorescein-\( \gamma'-\)peptide decreased in a dose-dependent and saturable manner (Fig. 4A), suggesting that \( Zn^{2+} \) facilitates the binding of HRG to the peptide. Nonlinear regression analysis of the data revealed that the apparent \( K_d \) for \( Zn^{2+} \) required to promote the HRG-fluorescein-\( \gamma'-\)peptide interaction was 9.1 ± 4.5 \( \mu M \). This value is similar to the apparent \( K_d \) of 1.2 \( \mu M \) for \( Zn^{2+} \)-mediated promotion of HRG binding to \( \gamma_A/\gamma'-\)fibrinogen (Fig. 1).

To confirm these results, the interaction of HRG with \( \gamma'-\)peptide was examined by SPR. Biotinylated \( \gamma'-\)peptide was adsorbed to a streptavidin-modified flow cell, and HRG binding was monitored in the presence of 20 \( \mu M \) \( Zn^{2+} \). HRG bound immobilized \( \gamma'-\)peptide in a concentration-dependent and saturable manner, and binding was blocked by the \( \gamma'-\)peptide-directed IgG (Fig. 4B). Based on kinetic analysis, HRG binds the \( \gamma'-\)peptide with a \( K_d \) value of 0.79 ± 0.01 nM in the presence of \( Zn^{2+} \); there is no detectable binding in the absence of \( Zn^{2+} \) (data not shown). These data offer independent confirmation that HRG binds to the COOH terminus of the \( \gamma'-\)chain of \( \gamma_A/\gamma'-\)fibrinogen in a \( Zn^{2+} \)-dependent fashion.

Effect of the \( \gamma'-\)Peptide-directed IgG on the Binding of HRG to \( \gamma_A/\gamma'-\)fibrinogen—Having shown that HRG binds fibrinogen with high affinity in a \( Zn^{2+} \)-dependent fashion, we next used SPR to determine the affinity of HRG for fibrin. To convert immobilized fibrinogen to fibrin, flow cells were treated with thrombin (30). HRG bound to both isoforms of fibrin with affinities (Table 1) and \( R eq \) values similar to those for fibrinogen, suggesting that HRG binding is unaltered when fibrinogen is converted to fibrin. To complement the SPR studies, we also assessed the binding of \( ^{125}I \)-HRG to fibrin clots. Clots contain-

**FIGURE 2. Determination of the affinity of HRG for \( \gamma_A/\gamma'_A\)- or \( \gamma_A/\gamma'-\)fibrinogen in the presence of \( ZnCl_2 \). \( \gamma_A/\gamma'_A\)-Fibrinogen (A) or \( \gamma_A/\gamma'-\)fibrinogen (B) was adsorbed to separate flow cells on a CM4 chip. HRG (0–700 nM) was injected into flow cells for 300 s in the presence of 20 \( \mu M \) \( ZnCl_2 \) and the cells were then washed with HBS buffer containing 2 \( \mu M \) CaCl\(_2\) and 20 \( \mu M \) \( ZnCl_2 \) for 500 s to monitor dissociation. HRG concentrations in nM are indicated adjacent to each sensogram tracing. These data are from a single experiment, which was performed three times.**
when fibrinogen is converted to fibrin. The results suggest that HRG binds fibrinogen and remains bound with fibrin formed from unfractionated fibrinogen, which consists of both isoforms of fibrinogen (11). Taken together, these findings indicate that HRG binds to fibrinogen and remains bound with fibrin formed from unfractionated fibrinogen, which consists of both isoforms of fibrinogen (11).

Next, we examined the effect of varying concentrations of Fab fragments derived from the γ'-peptide-directed IgG on 125I-HRG binding to γA/γA- or γA/γ'-fibrin clots. Fab fragments had minimal effects on HRG binding to γA/γA-fibrin clots (Fig. 5B). In contrast, at 4 μM, the Fab fragments reduced HRG binding to γA-γ'-fibrin clots by 50%, providing further evidence that HRG binds to the γ'-chain of γA/γ'-fibrin. A non-immune sheep IgG was used as a control and demonstrated no effect.

HRG Binding to Fibrinogen Fragments—To localize the HRG binding domains on fibrinogen, binding of HRG to immobilized fibrinogen fragments was examined by SPR (data not shown). To avoid potential contribution of the γ'-chain to HRG binding, fragments X, D, and E were prepared from γA/γA-fibrin clots. Fab fragments derived from the γ'-peptide-directed IgG were added to microcentrifuge tubes, and the binding of 125I-HRG (40 nM) to clots was assessed after thrombin addition. B, the binding of 125I-HRG (10 nM) to 0.25 μM γA/γA- or γA/γ'-fibrin clots (circles) was assessed in the presence of 0–4 μM γE- or γ'-peptide-directed Fab fragments (closed symbols) or a control sheep IgG (open symbols).

Experiments were performed in TBS-Tween containing 20 μM ZnCl2 and 2 mM CaCl2, and clots were generated with 10 nM thrombin. After incubation at 37 °C for 45 min, fibrin clots were pelleted by centrifugation, and the amount of free 125I-HRG in the supernatant was used to calculate the fraction bound. The percent of HRG bound to the clots is plotted versus fibrin or antibody concentrations. Symbols represent the mean ± S.D. of two experiments, each performed in duplicate, whereas the lines represent nonlinear regression analyses of the data.
fibrinogen. In the presence of Zn$^{2+}$, HRG bound fragment X with a $K_d$ value of 63.5 ± 11.8 nm, suggesting that the αC-domain of fibrinogen does not represent the primary HRG binding site. HRG also bound fragments D and E with high affinity in a Zn$^{2+}$-dependent manner, with $K_d$ values of 8.0 ± 1.3 and 23.3 ± 2.2 nm, respectively. As a negative control, we demonstrated that HRG did not bind immobilized FPRck-thrombin in the absence or presence of Zn$^{2+}$. As a positive control, we showed that FPRck-thrombin bound fragment E, with a $K_d$ value of 5.0 ± 0.4 μM, but did not bind fragment D, findings in agreement with previously published results (25). Therefore, these data suggest that, in addition to its interaction with the $\gamma'$-chain, HRG binds to other unique sites on fibrinogen.

**Diffusion of HRG from Fibrin Clots**—To identify differences in the binding of HRG to $\gamma_A/\gamma_A^*$ and $\gamma_A/\gamma'$-fibrin clots, we monitored the dissociation of $^{125}$I-HRG from preformed fibrin clots (Fig. 6). Diffusion in the presence of 2 M NaCl and 2 mM EDTA served as the base-line control because ionic and divalent cation-dependent interactions are abrogated (10, 30). In the presence of Zn$^{2+}$, the rates of diffusion of $^{125}$I-HRG from $\gamma_A/\gamma_A^*$- and $\gamma_A/\gamma'$-fibrin clots were significantly ($p < 0.05$) slowed by 3- and 11-fold, respectively, compared with those determined in the presence of diethylthiocarbamate trihydrate, a specific Zn$^{2+}$ chelator (32). Consistent with the concept that $\gamma_A/\gamma'$-fibrin affords HRG an additional binding site, the rate of $^{125}$I-HRG diffusion from $\gamma_A/\gamma'$-fibrin was 3-fold slower than that from $\gamma_A/\gamma_A^*$-fibrin ($p < 0.05$). Collectively, our results offer independent confirmation that the HRG-fibrinogen interaction is Zn$^{2+}$-dependent and that there is an additional HRG binding site on $\gamma_A/\gamma'$-fibrin.

**Effect of FPRck-thrombin on HRG Binding to the $\gamma'$-Chain**—In addition to binding HRG, the $\gamma'$-chain COOH extension binds thrombin (5, 9). SPR was used to determine whether the two proteins compete for binding to this region. This approach exploits the fact that the dissociation rate of HRG from immobilized $\gamma'$-peptide is much slower than that of thrombin. HRG (1 μM) was injected into flow cells containing immobilized biotinylated $\gamma'$-peptide, and the subsequent dissociation phase was monitored in the absence or presence of FPRck-thrombin or prothrombin in concentrations up to 8.0 μM. Prothrombin was used as a negative control because it does not bind to the $\gamma'$-peptide (33). Whereas FPRck-thrombin displaced HRG from the $\gamma'$-peptide in a concentration-dependent manner, prothrombin did not (Fig. 7). These data confirm that thrombin and HRG compete for binding to the $\gamma'$-peptide.

To determine whether the same was true with fibrin clots, we next examined the effect of increasing concentrations of FPRck-thrombin on $^{125}$I-HRG binding to $\gamma_A/\gamma_A^*$ or $\gamma_A/\gamma'$-fibrin clots. At 10 μM, FPRck-thrombin reduced the amount of HRG bound to $\gamma_A/\gamma'$-fibrin clots by 90% but only reduced HRG bound to $\gamma_A/\gamma_A^*$-fibrin clots by 15% (Fig. 8A). Similar results were obtained in the reciprocal competition experiments using varying concentrations of HRG and a fixed concentration of $^{125}$I-YPRck-thrombin (Fig. 8B). Collectively, these data confirm that HRG and thrombin compete for binding to the $\gamma'$-chain on $\gamma_A/\gamma'$-fibrinogen in a mutually exclusive fashion.

**Detection of HRG-Fibrinogen Complexes in Plasma**—Because HRG binds fibrinogen with high affinity, it was of interest to determine whether HRG-fibrinogen complexes can be detected in plasma by immunoassay. Plasma was first dialyzed to remove citrate and then reconstituted with 18 μM ZnCl$_2$. The concentration of HRG-fibrinogen complexes detected in normal plasma was 1 μM, whereas no complexes were detected in HRG-deficient plasma. Because the plasma concentration of HRG ranges from 1.6 to 2 μM (12, 13, 24), our findings suggest that in the presence of Zn$^{2+}$ ~50–60% of HRG in plasma circulates in complex with fibrinogen.

**DISCUSSION**

Despite increasing evidence that the interaction of HRG with fibrin(ogen) plays an important role in innate immunity, inflammation, and coagulation, little is known about this inter-
action. To address this gap, we characterized the binding of HRG to \(\alpha/\gamma\)-peptide. Biotinylated \(\gamma\)-peptide was immobilized on a streptavidin-modified flow cell to 200 RU, and an unmodified flow cell served as a control. Arrows indicate injection of 1 \(\mu\)M HRG for 200 s followed by injections of FPRck-thrombin (panel A) or prothrombin (panel B) at the concentrations (\(\mu\)M) indicated. Injections were carried out in the presence of 20 \(\mu\)M Zn\(^{2+}\). These are data from a single experiment, which was performed three times.

FIGURE 7. Effect of FPRck-thrombin or prothrombin on the interaction of HRG with \(\gamma\)-peptide. Biotinylated \(\gamma\)-peptide was immobilized on a streptavidin-modified flow cell to 200 RU, and an unmodified flow cell served as a control. Arrows indicate injection of 1 \(\mu\)M HRG for 200 s followed by injections of FPRck-thrombin (panel A) or prothrombin (panel B) at the concentrations indicated. Injections were carried out in the presence of 20 \(\mu\)M Zn\(^{2+}\). These are data from a single experiment, which was performed three times.

FIGURE 8. Effect of competitors on the binding of \(125^I\)-HRG or \(125^I\)-YPRck-thrombin to \(\gamma\)/\(\alpha\)- or \(\gamma\)/\(\gamma\)-fibrin clots. A. \(125^I\)-HRG (20 nM) was added to microcentrifuge tubes containing 2 \(\mu\)M \(\gamma\)/\(\alpha\)-fibrinogen (triangles) or \(\gamma\)/\(\gamma\)-fibrinogen (circles) in the presence of FPRck-thrombin (0–10 \(\mu\)M). B. \(125^I\)-YPRck-thrombin (20 nM) was added to microcentrifuge tubes containing 2 \(\mu\)M \(\gamma\)/\(\alpha\)-fibrinogen (triangles) or 0.25 \(\mu\)M \(\gamma\)/\(\gamma\)-fibrinogen (circles) in the presence of HRG (0–2 \(\mu\)M). In both experiments 2 mM CaCl\(_2\) plus 20 \(\mu\)M ZnCl\(_2\) were present and clotting was initiated with 10 nm thrombin. After incubation at 23 °C for 45 min, fibrin was pelleted by centrifugation, and free \(125^I\)-HRG or \(125^I\)-YPRck-thrombin in the supernatant was used to calculate the bound fraction. The percent of fibrin-bound \(125^I\)-HRG or \(125^I\)-YPRck-thrombin is plotted versus the FPRck-thrombin or HRG concentration, respectively. The symbols represent the mean ± S.D. of two experiments, each performed in duplicate, whereas the lines represent nonlinear regression analyses of the data.

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more acidic conditions. Consequently, the decrease in pH that occurs with reduced tissue perfusion may also enhance the affinity of HRG for both Zn$^{2+}$ and its ligands (14). Therefore, the current data extend the concept that Zn$^{2+}$ serves as a dynamic switch that regulates HRG activity and directs it to various pathways involved in hemostasis (14, 34).

Although we show that Zn$^{2+}$ is essential for the interaction of HRG with fibrin(ogen), the mechanism by which Zn$^{2+}$ mediates this binding is unknown. Both HRG and fibrin(ogen) bind Zn$^{2+}$ (37, 38). Therefore, Zn$^{2+}$ may act as a cofactor that simultaneously binds HRG and fibrin(ogen) in a coordinated fashion (39). Alternatively, Zn$^{2+}$ binding to HRG may induce conformational changes that facilitate its interaction with fibrin(ogen), a concept supported by the observation that Zn$^{2+}$ alters the conformation of a synthetic His-Pro-rich peptide (16). Furthermore, the intrinsic fluorescence of HRG decreases upon Zn$^{2+}$ titration (data not shown), providing additional support for the notion that Zn$^{2+}$ alters HRG conformation. Binding of Zn$^{2+}$ to the HRR domain of HRG indirectly promotes the interaction of heparan sulfate or plasminogen with the NH$_2$-terminal cystatin domains of HRG, suggesting that Zn$^{2+}$ binding to the HRR domain modulates other domains (15, 40). These data point to a mechanism whereby Zn$^{2+}$ modulates the structure and function of HRG.

Because HRG binds to the γ' chain of γ$_A$/γ' fibrin(ogen), novel roles of HRG can be envisioned. In addition to binding HRG, the COOH terminus of the γ' chain also binds thrombin and factor XIIIa and, by so doing, may modulate coagulation and fibrinolysis (10, 41). The importance of fibrin as a reservoir of thrombin is highlighted by the observation that thrombi harvested at autopsy contain abundant amounts of active thrombin (42). Fibrin-bound thrombin has been postulated to be an important mediator of thrombus expansion because of its capacity to locally activate platelets and to promote its own generation through activation of factor V and factor VIII (43, 44). The procoagulant activity of thrombin bound to γ$_A$/γ' fibrin appears to be greater than that of thrombin bound to γ$_A$/γ$_A$-fibrin because the γ' chain mediates the high affinity interaction of thrombin with fibrin, and γ$_A$/γ' fibrin affords bound thrombin more protection from inhibition by the anti-thrombin-heparin complex than γ$_A$/γ$_A$-fibrin (10). Further support for this concept comes from epidemiological studies that suggest that higher circulating levels of γ$_A$/γ' fibrinogen are associated with an increased risk of cardiovascular disease (45, 46). HRG competes with thrombin for binding to the γ' chain as evidenced by its capacity to displace FPRck-thrombin from γ' peptide or from γ$_A$/γ' fibrin clots. Consequently, by displacing fibrin-bound thrombin, HRG may have anti-thrombotic properties. HRG may also compete with factor XIII, which is proposed to bind the γ' chain (47), thereby attenuating fibrin cross-linking and ending HRG with pro-fibrinolytic activity. Studies in HRG-deficient mice support the concept that HRG affects hemostasis. HRG-deficient mice have a shorter prothrombin time and a longer bleeding time than their wild-type counterparts. In addition, thrombi formed in HRG-deficient mice are more susceptible to fibrinolysis than those generated in control mice (17). The contribution of the HRG interaction with the γ' chain to the anticoagulant and anti-fibrinolytic activities of HRG remains to be determined.

In addition to its role in hemostasis, fibrinogen appears to be an important mediator of inflammation and innate immunity because fibrinogen and fibrin stimulate peripheral blood mononuclear cells and vascular smooth muscle cells to synthesize proinflammatory cytokines (48–50). Furthermore, bacteria trapped within fibrin clots are protected from host defenses and the action of antibiotics (51). However, because HRG has antimicrobial properties, the HRG-fibrin interaction promotes bacterial entrapment and killing (21). The capacity of HRG to enhance bacterial killing has been localized to its NH$_2$-terminal and HRR domains. Thus, in the presence of Zn$^{2+}$ or when the pH is low, HRG induces lysis of the bacterial cell wall (19, 20). These conditions can occur at sites of injury or wound-healing where activated platelets release Zn$^{2+}$ and local ischemia lowers pH (13, 52). Activated platelets also release HRG (13) and, by so doing, may amplify the antimicrobial effect. Our observation that HRG binds fibrinogen and fibrin provides a regulatory mechanism by which HRG may mediate bacterial killing within a clot. The importance of HRG in modulating the inflammatory response has been confirmed in mouse models. Thus, compared with wild-type mice, HRG-deficient mice given subcutaneous injections of *S. pyogenes* exhibit attenuated abscess formation and reduced recruitment of neutrophils and macrophages to the site of infection (21), suggesting that HRG modulates the inflammatory response. In support of this concept, a synthetic peptide analog of the HRR of HRG attenuated the secretion of interleukin-8 from lipopolysaccharide-stimulated, CD14-transfected monocytes (53). These observations raise the possibility that HRG plays a part in the inflammatory response to infection.

Although HRG is an abundant plasma protein with multiple ligands, its physiological role remains unknown (for review, see Ref. 18). HRG is hypothesized to be an important effector of hemostasis and immunity. The ability of HRG to bind fibrinogen and displace thrombin from fibrin may provide an important link between these two systems and reveals a potential mechanism by which this could occur. Additional regulation may result from variations in the local pH and/or Zn$^{2+}$ concentration.

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