Histidine-Rich Glycoprotein Is Present in Human Platelets and Is Released Following Thrombin Stimulation

By Lawrence L. K. Leung, Peter C. Harpel, Ralph L. Nachman, and Enrique M. Rabellino

Histidine-rich glycoprotein, an α2-glycoprotein in human plasma, has been shown to interact with heparin, with the high-affinity lysine-binding site of plasminogen, with divalent cations, and is associated with the rosette formation between erythrocytes and lymphocytes. A specific enzyme-linked immunosorbent assay for histidine-rich glycoprotein has been developed and used to demonstrate that histidine-rich glycoprotein is present in human platelets. Histidine-rich glycoprotein was detected and quantified in detergent extracts of washed human platelets, with a mean level of 371 ng/10^9 platelets. Plasma histidine-rich glycoprotein, either in the platelet suspending medium or on the surface of the platelets, accounted for less than 3.4% of the detectable platelet histidine-rich glycoprotein.

Histidine-rich glycoprotein was also demonstrated in human bone marrow megakaryocytes by immunofluorescence. The extent of histidine-rich glycoprotein release from platelets was dependent on the thrombin dose and correlated directly with the extent of serotonin release. The platelet and plasma histidine-rich glycoprotein were similar by immunochemical analysis. Anti-histidine-rich glycoprotein IgG did not inhibit platelet aggregation. Histidine-rich glycoprotein released by platelets following thrombin stimulation may play a significant role in modulating inflammatory events in the microenvironment of the platelet plug.

MATERIALS AND METHODS

Histidine-Rich Glycoprotein (HRGP), an α2-glycoprotein in human plasma, was first isolated in 1972. HRGP interacts with several divalent metals, suggesting that HRGP may have a role in the transport or homeostasis of metals in vivo. It has been shown that HRGP interacts with the high-affinity lysine-binding site of plasminogen. The high-affinity lysine-binding site of plasminogen appears to play an important role in the regulation of physiologic fibrinolysis, by mediating the interaction between plasminogen and fibrin and between plasminogen and α2-plasmin inhibitor. By reducing the binding of plasminogen to fibrin, HRGP may represent a physiologic counterpart of antifibrinolytic amino acids, such as 6-aminohexanoic acid, resulting in an antifibrinolytic effect. More recently, HRGP was found to interact strongly with heparin, resulting in neutralization of the anticoagulant activity of heparin. HRGP may therefore play a role in the modulation of the anticoagulant properties of heparin in plasma. α2-Plasmin inhibitor and α2-macroglobulin, the plasma inhibitors of plasmin, and α1-antitrypsin, the leukocyte elastase inhibitor, have been identified as platelet constituents. In this study, we present evidence demonstrating the presence of HRGP in human platelets and its release following thrombin stimulation.

Isolation and Purification of Plasma HRGP

HRGP was purified with minor changes in the method of Haupt and Heimburger, as modified by Lijnen et al. Soybean trypsin inhibitor (100 mg/liter final concentration) was added to acid citrate dextrose (ACD) plasma. Fifty percent polyethylene glycol, 4,000 mol wt, was added to a final concentration of 6% to remove the fibrinogen and the precipitate removed by centrifugation. The supernatant was processed, as detailed by Lijnen, by adsorption to CM-cellulose 52, elution with NH4HCO3, and affinity chromatography of the eluate on a column containing the high-affinity lysine-binding site of plasminogen. Traces of IgG, fibrinogen, and plasminogen were detected in the HRGP preparation by enzyme-linked immunosorbent assays. These were removed by affinity chromatography utilizing the insolubilized IgG fraction of rabbit antiserum directed against these contaminants. Purified plasma HRGP, kindly supplied by Dr. Norbert Heimburger, served as a standard. When analyzed on SDS-PAGE, purified HRGP showed a single band with apparent mol wt of 60,000 unreduded, and two bands with apparent mol wt of 74,000 and 67,000 when reduced with 2% dithiothreitol. This is in agreement with previously published results.

Purified HRGP was radiolabeled to a specific activity of 1.51 x 10⁶ cpm/μg with carrier-free Na¹²⁵I by a modified chloramine-T procedure. Recent study suggests that HRGP purified by this procedure, in the absence of aprotinin, may represent a proteolytic derivative of a native molecule with apparent mol wt of 75,000.
Preparation of Monospecific Anti-HRGP

The final HRGP preparation was used to produce antisera by intradermal injection of the protein in complete Freund's adjuvant into New Zealand white rabbits. Insolubilized HRGP-depleted plasma was used to remove traces of contaminating antibodies from the HRGP antisera. HRGP-depleted plasma was used to prepare purified IgG fractions of the absorbed anti-HRGP sera and normal rabbit sera were prepared as previously described. The absorbed anti-HRGP IgG did not react by ELISA with purified human γ-globulin fractions of the absorbed anti-HRGP sera and normal rabbit sera were prepared as previously described. The absorbed anti-HRGP IgG did not react by ELISA with purified human fibrinogen, factor VIIIr:Ag, IgG, albumin, plasminogen, fibronectin, or thrombospondin. The preparation and purification of these proteins have been described previously.

Enzyme-Linked Immunosorbent Assay (ELISA) for HRGP

The methods used to develop this ELISA are essentially those detailed by Voller et al. Microtitration plates were coated with the IgG fraction of rabbit anti-HRGP IgG. Two-tenth milliliter portions of the IgG (5 μg/ml) in bicarbonate coating buffer (15 mM Na2CO3, 35 mM NaHCO3, pH 9.6, with 0.02% NaN3) were incubated in a humid chamber overnight at 4°C. After washing the wells 3 times for 3 min each with phosphate-buffered saline containing 0.05% Tween 20 (PBS-TWEEN), serial dilutions of HRGP (10–120 ng/ml) in PBS-TWEEN containing bovine albumin (1 mg/ml) were added in duplicate to the coated wells. The plates were incubated for 3 hr at room temperature, the wells washed 3 times, and alkaline phosphatase-conjugated immunofinity-purified rabbit anti-HRGP IgG was added for 3 hr at 37°C. The wash step was repeated, and the substrate (0.2 ml) p-nitrophenyl phosphate (1 mg/ml in 10% diethanolamine buffer, pH 9.8) added. Color development was followed by repeated readings at 405 nm in a Titertek Multiscan photometer. The change in absorbance per minute (∆A405 min⁻¹) was calculated by subtracting the 5-min value from the 25-min reading and dividing by 20.

Preparation of Washed Platelets

Venous blood was obtained in plastic syringes using sodium citrate as anticoagulant from normal volunteers who had had no medication during the previous 2 wk. Platelets were collected and washed once with 120 mM KCl, 75 mM Tris-HCl, 12 mM sodium citrate, pH 6.3, and twice with 150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, as previously described. The washed platelets were resuspended in this EDTA Tris-saline buffer, with the final EDTA concentration adjusted to 5 mM. More than 99.9% of the cells in these samples were platelets, as determined by phase-contrast microscopy. For determination of HRGP content in washed platelets, the platelet pellet was lysed by incubation with 1% Triton X-100 for 30 min at 22°C. Complete lysis was established by microscopic examination, and cell debris was removed by centrifugation in a Beckman Microfuge B for 2 min.

In studies of the release reaction, platelets were labeled with 3H-serotonin (22 μCi/μmole) in platelet-rich plasma by incubation at 37°C for 30 min and then isolated as described above. The labeled cells were suspended in 1 ml of the EDTA Tris-saline buffer, incubated with 2 μM imipramine at 37°C for 5 min, then stimulated with thrombin for 3 min at 22°C. After centrifugation, the radioactivity in the supernatant was measured, and the percent serotonin release was calculated relative to the counts solubilized by addition of 1% Triton X-100 to unstimulated platelets.

Binding of Antibody Against HRGP to Platelets

Affinity-purified, monospecific anti-HRGP IgG and nonimmune rabbit IgG were radio labeled with 125I by the modified chloramine-T method, with a specific radioactivity of 1.9 × 10⁷ cpm/μg for 125I-anti-HRGP and 1.32 × 10⁷ cpm/μg for 125I-nonimmune rabbit IgG. When 125I-anti-HRGP (specific radioactivity 7.1 × 10⁶ cpm/μg) was incubated with increasing amounts of adsorbed HRGP on a microtitration plate, there was a direct correlation between the amount of 125I-anti-HRGP bound and the amount of HRGP on the plate, indicating the functional integrity of anti-HRGP following radiolabeling. Control experiments using 125I-nonimmune rabbit IgG (specific radioactivity 8.8 × 10⁶ cpm/μg) did not show binding.

Electrophoretic Blotting

This technique was performed as described by Burnette. The anti-HRGP sera and preimmune sera were used at 1:50 dilution.

Study of Megakaryocytes by Immunofluorescence

Suspension of human marrow cells enriched for megakaryocytes were prepared using density centrifugation gradients in Percoll as described previously. Isolated megakaryocytes were smeared and fixed in methanol for 10 min, followed by 3 washes in phosphate-buffered saline. Fluorescence staining was performed using the indirect method by incubating smeared cells for 30 min first with rabbit monospecific anti-HRGP and subsequently with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC). Cells were examined with a Leitz Ortholux microscope equipped with a Planfilter illuminator and phase-contrast optics.

Platelet Aggregation Studies

These were performed using Sepharose CL-2B gel filtered platelets as previously described.

RESULTS

ELISA of HRGP

A specific ELISA for the quantification of HRGP was developed. Monospecific, nonaffinity-purified anti-HRGP IgG was passively adsorbed to the wells of a plastic microtitration plate. Purified HRGP was added at increasing concentrations. The amount of HRGP bound to the adsorbed anti-HRGP was monitored by incubation with alkaline phosphatase-conjugated affinity-purified anti-HRGP. The resulting hydrolysis of the alkaline phosphatase substrate p-
Detection and Quantification of HRGP in Platelets

HRGP was detected and quantified in detergent extracts of washed platelets. Table 1 summarizes the results from 7 normal individual donors. HRGP was detected in all 7 extracts, and the range of HRGP was 231–486 ng/10⁹ platelets, with a mean level of 371 ng HRGP/10⁹ platelets.

Three approaches were used to determine the potential contribution of contaminating plasma HRGP to the level of HRGP measured in the platelet detergent extracts. First, after centrifugation to remove the washed platelets for HRGP determination, the supernatant was assayed for the presence of HRGP by ELISA. No HRGP was detected in the supernatant. Second, ¹²⁵I-HRGP was added to the platelet-rich plasma, and the recovery of radioactivity in the platelet lysates was measured. Based on this analysis, plasma HRGP accounted for only 11.5 ng/10⁹ platelets. To assess the presence of tightly bound plasma HRGP on the platelet surface, washed platelets were incubated with monospecific, affinity-purified ¹²⁵I-anti-HRGP IgG and the platelet-associated immunoglobulin was measured. 37.3 Ng of the monospecific anti-HRGP IgG was bound per 10⁹ platelets compared to 5.9 ng nonimmune IgG/10⁹ platelets. Assuming that one monospecific anti-HRGP molecule bound one HRGP molecule, 12.6 ng HRGP resided on the surface of 10⁹ platelets and was accessible to the antibody. Thus, plasma HRGP, either in the platelet suspending medium or on the surface of the platelets, accounted for less than 3.4% of the detectable platelet HRGP.

Release of HRGP From Platelets

The release of HRGP from platelets following thrombin stimulation was assessed. Washed platelets were resuspended in EDTA Tris-saline buffer and stimulated with thrombin for 3 min at 22°C. After incubation, the platelets were centrifuged, and HRGP in the supernatant was measured. In parallel experiments, platelets were labeled with ¹⁴C-serotonin, and its release was measured under identical conditions (Table 2). The extent of HRGP release from platelets was directly related to the dose of thrombin added and correlated with the extent of serotonin release. Thrombin stimulation at the concentrations employed in this study did not cause platelet lysis.

Table 1. HRGP in the Detergent-Soluble Extract of Washed Human Platelets

<table>
<thead>
<tr>
<th>Donor</th>
<th>HRGP (ng/10⁹ Platelets)</th>
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<tbody>
<tr>
<td>1</td>
<td>447</td>
</tr>
<tr>
<td>2</td>
<td>231</td>
</tr>
<tr>
<td>3</td>
<td>486</td>
</tr>
<tr>
<td>4</td>
<td>410</td>
</tr>
<tr>
<td>5</td>
<td>304</td>
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<tr>
<td>6</td>
<td>364</td>
</tr>
<tr>
<td>7</td>
<td>356</td>
</tr>
</tbody>
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Mean ± SD 371.1 ± 80

*Platelets were lysed with 1% Triton X-100 for 30 min at 22°C.

Table 2. Release of HRGP and Serotonin From Thrombin-Stimulated Platelets

<table>
<thead>
<tr>
<th>Thrombin* (U/ml)</th>
<th>HRGP ¹⁴C-Serotonin</th>
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<tbody>
<tr>
<td></td>
<td>Percent Release</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>36 ± 12</td>
</tr>
<tr>
<td>1.0</td>
<td>37.1 ± 0.3</td>
</tr>
<tr>
<td>3.0</td>
<td>79.7 ± 13</td>
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</tbody>
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*Washed platelets were stimulated for 3 min at 22°C. ¹⁴C-serotonin and HRGP present in the supernatant after centrifugation at 11,750 rpm in a Beckman microfuge for 2 min were quantified. Percent release was calculated relative to constituents solubilized by addition of 1% Triton X-100 to unstimulated platelets. Serotonin release was measured in the presence of 2 μM imipramine. Results are the mean (± SD) of 3 separate experiments, with duplicate determinations in each experiment.
Analysis of Plasma HRGP and Thrombin-Stimulated Platelet Releasate by Monospecific Anti-HRGP on Electrophoretic Blot

To investigate the immunochemical characteristics of the platelet HRGP, purified plasma HRGP and thrombin-stimulated platelet releasate were analyzed using the monospecific anti-HRGP on electrophoretic blots. Anti-HRGP reacted with two major bands in the reduced plasma HRGP sample with mol wt of 74,000 and 67,000, in agreement with previously published results. Similarly, two major bands were identified in the reduced platelet releasate sample (Fig. 2). It is of note that the relative intensity of the two bands was different in the two samples. The reason for these differences remains to be determined. Other minor bands of lower molecular weight range were also identified in both reduced samples, possibly representing fragments produced by proteolysis. Control studies using the preimmune rabbit sera were negative.

Detection of HRGP in Marrow Megakaryocytes

Human megakaryocytes isolated from normal marrow tissue were studied for the presence of HRGP using the immunofluorescence technique. Using the monospecific anti-HRGP, HRGP was detected in virtually all morphologically recognizable megakaryocytes. Homogeneous staining of variable intensity was observed in all cell areas, except the nucleus (Fig. 3). This pattern of fluorescence staining was similar to that of intracellular factor VIIIIR:Ag, as previously described. The megakaryocytic morphology of the fluorescent stained cells was confirmed by concomitant examination of the cells under phase-contrast microscopy. There was no staining of erythroid and myeloid precursor cells with anti-HRGP. The specificity of the megakaryocyte staining was demonstrated by complete inhibition of cell staining after absorption of anti-HRGP with purified HRGP but not with bovine serum albumin. Control studies using preimmune sera did not show any cell staining.

Platelet Aggregation Studies

Anti-HRGP IgG at 0.5 mg/ml did not inhibit aggregation of gel-filtered platelets induced by thrombin, arachidonic acid, or collagen. Anti-whole-platelet membrane IgG inhibited platelet aggregation under similar conditions.

DISCUSSION

These studies demonstrate that HRGP is present in human platelets. The platelet HRGP does not appear to represent a trapped plasma contaminant in the washed platelet pellet, as suggested by three separate lines of evidence: (1) platelets isolated from plasma containing trace labeled HRGP did not contain significant amounts of plasma protein; (2) binding studies using radiolabeled anti-HRGP revealed only minor traces of HRGP on the washed platelet surface; (3) supernates of washed platelets contained no detectable HRGP prior to thrombin-induced release of significant amounts of the protein. The fact that bone marrow megakaryocytes stained for intracellular HRGP (Fig. 3) is further evidence that HRGP is an intrinsic platelet protein. The extent of HRGP release was dependent on the thrombin dose and correlated directly with the extent of serotonin release (Table 2). Preliminary studies (Leung LLK, Harpel PC, Nachman RR: unpublished observations) demonstrate that the distribution of HRGP in platelet subcellular fractions is similar to that of thrombospondin, a known α-granule protein.

By immunoblot analysis, the platelet releasate sam-
ple showed a similar pattern with the plasma HRGP sample (Fig. 2), indicating that the platelet and plasma HRGP are similar by immunological analysis. It is of interest that the relative intensity of the two bands in the platelet releasate sample was different from that of the plasma HRGP sample. Recent studies suggest that HRGP is susceptible to proteolysis.\(^3\)\(^2\)\(^9\) It remains to be determined whether the 67,000-dalton polypeptide is derived from the 74,000-dalton polypeptide by proteolysis and whether the difference in the ratio of the two polypeptides in the platelet and plasma HRGP samples is related to different extent of proteolysis during the isolation procedures.

Compared to a plasma level of 100 \(\mu\)g/ml, 4 \(\times\) 10\(^6\) platelets in 1 ml of blood contain 148 ng HRGP (based on a mean level of 371 ng/10\(^8\) platelets, Table 1). Thus, platelet HRGP constitutes 0.14% of the blood level on a volume basis. Platelets from 1 ml of platelet-rich plasma contain approximately 330 \(\mu\)g of protein,\(^3\)\(^0\) thus, platelet HRGP accounts for 0.045% platelet proteins. In plasma with 70 mg of protein/ml, plasma HRGP constitutes 0.15% of plasma proteins. Therefore, there is no relative concentration of HRGP in platelets on a protein basis. Similar relationships have been described for platelet \(\alpha\)-macroglobulin, \(\alpha\)\(_1\)-antitrypsin,\(^5\) and \(\alpha\)\(_2\)-plasmin inhibitor,\(^1\)\(^4\) as contrasted to other proteins, such as factor VIIIR:Ag and fibrinogen, which are concentrated in the platelets relative to plasma. Since platelets are concentrated in the fibrin clot, HRGP released by platelets following thrombin stimulation may achieve a high local concentration and thus play a significant role in modulating fibrinolysis in the microenvironment of the platelet plug.\(^*\)

The biologic importance of plasma as well as platelet HRGP remains to be fully clarified. Recent studies suggest that in addition to interaction with the high-affinity lysine-binding site of plasminogen,\(^3\)\(^3\) with divalent cations,\(^3\)\(^4\) plasma HRGP binds heparin with high affinity.\(^1\)\(^3\) HRGP is therefore an efficient competitor of antithrombin-III for the binding of heparin and may modulate the anticoagulant properties of heparin in plasma. HRGP is also immunologically identical with a serum factor that inhibits autotransfusion formation between erythrocytes and peripheral lymphocytes, suggesting that HRGP may play a role in modulating the immune responses of human lymphocytes.\(^3\)\(^1\)\(^3\)\(^2\) Whether platelet HRGP has the same functional capabilities as plasma HRGP remains to be determined.

It is of interest that recent studies show that a human hepatoma cell line synthesized and secreted all the major components of the fibrinolytic system, including fibrinogen, plasminogen, and \(\alpha\)\(_2\)-plasmin inhibitor. However, HRGP was not detected.\(^3\)\(^3\) Whether bone marrow megakaryocytes, which stained for intracellular HRGP (Fig. 3), represent a site of HRGP synthesis remains to be determined.

ACKNOWLEDGMENT

We thank Dr. Norbert Heimburger for providing us with a sample of purified plasma histidine-rich glycoprotein for electrophoretic blot analysis. The invaluable technical assistance of Barbara Ferris and T. S. Chang is gratefully acknowledged.

REFERENCES

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*It is possible to estimate the platelet mass in a platelet plug by making the following assumptions: (1) The platelet has a mean volume of 6.6 \(\mu\)m.\(^1\)\(^4\) (2) Approximately 50% by volume of a typical platelet plug is made up of aggregated platelets.\(^3\)\(^3\) Thus, there are approximately 7.6 \(\times\) 10\(^8\) platelets/1 ml of platelet plug (0.5 ml = 0.5 \(\times\) 10\(^6\) \(\mu\)m, which equals 3.6 \(\times\) 10\(^6\) platelets). When compared with a platelet concentration of 4 \(\times\) 10\(^8\) platelets/ml of blood, there is a 190-fold increase in platelet concentration in the platelet plug. Based on a mean level of 371 ng HRGP/10\(^8\) platelets, and assuming a complete release of intracellular HRGP, the local concentration of platelet released HRGP in the microenvironment of the platelet plug will be 56.4 \(\mu\)g/ml, or 0.94 \(\mu\)M. Since the \(K_d\) of HRGP binding to plasminogen is 1 \(\mu\)M, it is likely that platelet-released HRGP can reach a sufficiently high local concentration to interfere with fibrinolysis in a platelet plug.
Histidine-rich glycoprotein is present in human platelets and is released following thrombin stimulation

LL Leung, PC Harpel, RL Nachman and EM Rabellino