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Research Article

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Complex Formation of Platelet Thrombospondin with Histidine-rich Glycoprotein

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Abstract. Thrombospondin and histidine-rich glycoprotein are two proteins with diverse biological activities which have been associated with human platelets and other cell systems. Using an enzyme-linked immunosorbent assay, we have demonstrated that purified human platelet thrombospondin formed a complex with purified human plasma histidine-rich glycoprotein. The formation of the thrombospondin-histidine-rich glycoprotein complex was specific, concentration dependent, and saturable. Significant binding was detected when histidine-rich glycoprotein was incubated with thrombospondin immobilized on anti-thrombospondin IgG-coated plates, indicating that the observed complex formation was not due to a thrombospondin interaction with the plastic surface. Sucrose-density-gradient ultracentrifugation of a mixture of thrombospondin and histidine-rich glycoprotein also revealed the formation of fluid-phase complexes, with an estimated stoichiometry of 1 thrombospondin: 3.5 histidine-rich glycoprotein. Fibrinogen, which has been previously shown to bind to adsorbed thrombospondin, did not inhibit the formation of the thrombospondin-histidine-rich glycoprotein complex. Histidine-rich glycoprotein complexed with thrombospondin was capable of binding heparin and neutralizing the anticoagulant activity of heparin in plasma. Specific complex formation between thrombospondin and histidine-rich glycoprotein may play a significant role in influencing platelet blood vessel wall interactions as well as modulating the association of various cells with the extracellular matrix.

Introduction

Thrombospondin (TSP)[1] is a major glycoprotein located in the α-granules of human platelets (1, 2). TSP is a 450-kD protein and is composed of three disulfide-linked subunits (3, 4). It is secreted upon thrombin stimulation and binds to the platelet membrane in the presence of calcium (5). During the process of aggregation, thrombin-stimulated platelets develop a membrane-bound lectin activity that originates from α-granules and appears to play an important role in mediating platelet aggregation by binding to a specific receptor on adjacent platelets (6, 7). Fibrinogen is the receptor for the platelet lectin (8). We have recently found that TSP has hemagglutinating (lectin-like) activity and specifically binds fibrinogen, suggesting that TSP may be similar to the previously identified platelet lectin and may participate in the platelet aggregation process (9, 10).

In addition to platelets, TSP is found in other cell systems. Both cultured human umbilical vein endothelial cells and bovine aortic endothelial cells synthesize and secrete large amounts of TSP (11, 12). Cultured human fibroblasts and aortic smooth muscle cells also synthesize and secrete TSP and incorporate it into extracellular matrix (13, 14). The incorporation by fibroblasts of TSP into extracellular matrix is reminiscent of the role of fibronectin (14). A specific interaction of TSP with fibronectin and collagen has been demonstrated (10, 15). These data suggest that TSP may have multiple functions. In addition to its role in platelet aggregation, TSP may play a role in the formation and organization of the extracellular matrix and in cell-cell and cell-matrix interactions.

A preliminary report of this study was presented at the Annual Meeting of the American Society of Hematology, Washington, DC, 6 December, 1982, and published in abstract form in 1982. *Blood.* 60(Suppl. 1):201a. Dr. Leung is the recipient of Clinical Investigator Award K08 HL00877 from the National Heart, Lung, and Blood Institute, a Clinical Scholarship from the Rockefeller Brothers' Fund, and a Career Scientist Award from the Irma T. Hirschl-Monique Weill-Caulier Trust. Address reprint requests to Dr. Leung.

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1. *Abbreviations used in this paper:* CM, carboxymethyl; C, complement; ELISA, enzyme-linked immunosorbent assay; VIIIR:Ag, Factor VIII Related:Antigen; GP, glycoprotein; HRGP, histidine-rich glycoprotein; TSP, thrombospondin.

Histidine-rich glycoprotein (HRGP) is an α_2-glycoprotein in human plasma (16, 17) of apparently diverse biological functions. HRGP interacts with several divalent metals, suggesting that HRGP may have a role in the transport or homeostasis of metals in vivo (18, 19). HRGP interacts with the high affinity lysine-binding site of plasminogen (20), which mediates the binding of plasmin(ogen) to fibrin and of plasmin(ogen) to α_2-plasmin inhibitor (21). By reducing the binding of plasminogen to fibrin, HRGP may represent a physiological counterpart of antifibrinolytic amino acids, such as 6-aminohexanoic acid, which results in an antifibrinolytic effect. More recently, HRGP was found to interact strongly with heparin, resulting in neutralization of the anticoagulant activity of heparin (22). HRGP, therefore, may play a role in the modulation of the anticoagulant properties of heparin in plasma. HRGP is also immunochemically identical with a serum factor that inhibits autorosette formation between erythrocytes and peripheral lymphocytes; this suggests that HRGP may modulate the immune responses of lymphocytes (23). We have recently found that HRGP is present in human platelets and is released following thrombin stimulation (24). In this study, we present evidence demonstrating specific complex formation between HRGP and TSP. The complex contained heparin-binding and neutralizing activity.

Methods

Materials. *p*-Nitrophenyl phosphate and type VII calf mucosa alkaline phosphatase were obtained from Sigma Chemical Co. (St. Louis, MO). Protein A was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Microtitration plates and a Titertek multiscan photometer were purchased from Flow Laboratories, Inc. (McLean, VA). All reagents were of analytical grade.

Protein purification. HRGP was purified with minor changes in the method of Haupt and Heimburger (16) as modified by Lijnen et al. (20). Soybean trypsin inhibitor (100 mg/liter final concentration) was added to acid citrate dextrose plasma. 50% polyethylene glycol, 4,000 mol wt, was added to a final concentration of 6% to remove the fibrinogen and the precipitate was removed by centrifugation. The supernatant was processed as detailed by Lijnen et al. (20) by adsorption to carboxymethyl (CM)-cellulose 52, elution with NH_4HCO_3, 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 (ELISA) (10, 25). These were removed by affinity chromatography utilizing the insolubilized IgG fraction of rabbit antisera directed against these contaminants. Purified plasma HRGP, which was kindly supplied by Dr. Norbert Heimburger, Behringwerke AG, served as a standard. When analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, purified HRGP showed a single band with apparent M_r of 60,000 unreduced, and two bands with apparent M_r of 74,000 and 67,000 when reduced with 2% dithiothreitol. This is in agreement with previously published results (16, 18, 20). A 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 M_r = 75,000. (22). Purified human platelet TSP was isolated from thrombin-stimulated platelet releasate by Sepharose 4B gel exclusion chromatography and heparin-Sepharose affinity chromatography by the method of Lawler et al. (3), as previously described (10). The isolation and purification of Factor VIII Related:antigen (VIIIR:Ag), fibronectin, α_2-plasmin inhibitor, antithrombin III, and platelet membrane glycoproteins (GP) IIb and IIIa have been described in detail (25–28). Factor X was a generous gift from Dr. J. Jesty, State University of New York, Stony Brook. Peak I human fibrinogen was kindly supplied by Dr. M. Mosesson, Mt. Sinai Medical Center, Milwaukee, WI. Heparinase (Lot No. EL17403) was purchased from Miles Laboratories Inc. (Elkhart, Ind.). [*N-sulphonate*-^{35}S] heparin (mol wt 16,000–17,000) was purchased from Amersham Corp. (Arlington Heights, IL). Purified human albumin and γ-globulin were purchased from Behring Diagnostics, Inc. (Woodbury, NY). Clinical grade heparin (1,000 USP units/ml) was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ).

Antisera. Antisera to the purified HRGP were raised in rabbits by using methods previously described (25). Insolubilized HRGP-depleted plasma was used to remove traces of contaminating antibodies from the HRGP antisera. HRGP-depleted plasma was prepared by utilizing the fibrinogen-depleted plasma supernatant as detailed above for the purification of HRGP. The supernatant was mixed with CM-cellulose 52 (1 g/40 ml supernatant) for 1 h and the resin removed by centrifugation. This adsorption step was repeated and a portion of the supernatant was coupled to Sepharose beads. The absorbed HRGP antisera produced a single precipitin arc when diffused against human serum and yielded a reaction of immunologic identity between serum and the purified HRGP. γ-globulin fractions of the absorbed anti-HRGP sera and normal rabbit sera were prepared as previously described (25). The absorbed anti-HRGP IgG did not react by ELISA with purified human fibrinogen, VIIIR:Ag, IgG, albumin, plasminogen, fibronectin, or TSP.

The monospecificity of the antisera to TSP, VIIIR:Ag, fibronectin, α_2-plasmin inhibitor, antithrombin III, and platelet membrane GPIIb and GPIIIa have been described in detail previously (10, 25, 28). Monospecific antisera to complement (C) components $C1_q$, C3, C4, and C5 were kindly supplied by Dr. Margaret Polley, Cornell University Medical College, New York. Monospecific anti-Factor X was kindly supplied by Dr. J. Jesty, State University of New York, Stony Brook. Antisera to Factor XII and high molecular weight kininogen were purchased from Miles Laboratories, Inc.

ELISA. The assay was performed according to the method of Voller et al. as described previously (10, 29). Briefly, microtitration plates were coated with purified TSP by incubating TSP (0.2 ml) in the bicarbonate-coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6, 0.02% NaN_3) for 3 h at 37°C. The wells were washed three times with Tris-Tween buffer (0.01 M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.4). Purified HRGP diluted in Tris-Tween buffer was added in duplicate to coated wells for 18 h at 4°C. The washing procedure was repeated and alkaline phosphatase-conjugated, immunopurified anti-HRGP IgG (1:800 dilution) was added for 3 h at 37°C. After washing, the substrate *p*-nitrophenyl phosphate was added and color development was followed by repeated readings at 405 nm in a Titertek multiscan photometer. The formation of the TSP-HRGP complex was expressed as the enzymatic activity of the bound alkaline phosphatase (ΔA405 min^{-1}). For the double antibody ELISA, anti-TSP IgG (8 μg/ml) in coating buffer was applied to the plastic wells for 3 h at 37°C. After washing, TSP (4 μg/ml) in Tris-Tween buffer was added for 4 h at 37°C. The washing step was repeated, and HRGP in Tris-Tween buffer was added and the remaining steps were carried out as described above.

Determination of protein-coating efficiency on microtitration plates. Purified human plasma HRGP was labeled with ^{125}I by using the modified chloramine T method (30). Microtitration plates were coated in duplicate

with 0.2-ml portions of labeled HRGP at different concentrations in the bicarbonate-coating buffer for 3 h at 37°C. After washing three times with Tris-Tween buffer, each well was cut out, the radioactivity of the coated protein counted, and the coating efficiency of HRGP at different concentrations determined. The coating efficiency of TSP has been determined in previous studies (10).

Sucrose-density-gradient ultracentrifugation. TSP and HRGP were incubated in 10 mM Tris-HCl, 0.15 M NaCl, pH 7.4, with 1 mM Ca^{++} for 1 h at 37°C. 0.2-ml aliquot of the incubation mixture was layered on top of a 10–40% linear sucrose gradient (5 ml) in the same buffer and centrifuged at 165,000 *g* for 18 h at 4°C in a SW65 rotor (Beckman Instruments Inc., Fullerton, CA). The gradient was fractionated by collecting eight drop-fractions, and an aliquot (0.16 ml) of each fraction was coated onto the microtitration well by incubating at 4°C for 18 h. TSP and HRGP in each fraction were detected and quantified by the corresponding specific ELISA. The following proteins were used for calibration: thyroglobulin, $S_{20}w$ = 19; catalase, $S_{20}w$ = 11.3; human serum albumin, $S_{20}w$ = 4.6. Linearity of the sucrose gradients was confirmed by measuring the refractive indices of alternating fractions.

^{35}S-heparin-binding studies. Purified HRGP, TSP, and albumin were coupled separately to CNBR-activated Sepharose 4B beads as described (31). An aliquot of TSP coupled beads was incubated with HRGP at 1 mg/ml for 1 h at 37°C. The beads were washed extensively with Tris-Tween buffer and then incubated with 100,000 dpm (0.045 μCi) ^{35}S-heparin (sp act 0.015 μCi/μg) in 20 mM Tris-HCl, 0.15 M NaCl, 2 mM $CaCl_2$, pH 7.4, for 1 h at 37°C. The beads were washed five times with Tris-Tween buffer containing 2 mM $CaCl_2$. ^{35}S-heparin was eluted from the beads by boiling in 2% sodium dodecyl sulfate for 10 min and counted in a liquid scintillation counter.

Thrombin time assays. The influence of the TSP-HRGP complex on the neutralization of heparin in plasma was evaluated by determination of thrombin times of normal plasma, plasma depleted in HRGP, and the depleted plasma in the presence of TSP beads preincubated with HRGP. 0.2 ml of plasma (diluted one-half with water), and 0.1 ml of beads (or buffer) were incubated in the presence of clinical grade heparin (0.25 unit/ml final concentration) for 20 min at 37°C. 0.1 ml of thrombin solution (~0.2 NIH units/ml final concentration) was added and the clotting time recorded. Thrombin times reported represent mean values of at least two separate measurements. HRGP-depleted plasma was prepared by repeated absorption of normal plasma with CM-cellulose 52 as described above.

Results

ELISA of purified plasma HRGP. A specific ELISA for the detection and quantification of plasma HRGP was developed. HRGP in increasing concentrations was passively adsorbed to the wells of a microtitration plate. Affinity-purified, monospecific, anti-HRGP IgG which was directly conjugated with alkaline phosphatase (1:800 dilution) was added. The resulting hydrolysis of the substrate *p*-nitrophenyl phosphate was linear with the concentration of HRGP added to the well up to 1,000 ng/ml.

By using ^{125}I-labeled HRGP, the actual amount of HRGP bound to the plastic well was determined at each concentration of protein added and this was correlated with the color generated in the ELISA (Fig. 1). No differences in reactivity with specific antibody were noted when nonlabeled HRGP was compared with ^{125}I-labeled HRGP.

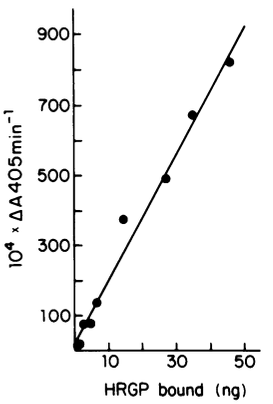

Figure 1. HRGP standard curve by ELISA. The amount of HRGP bound was correlated with the color generated in the ELISA. The radiolabeled purified HRGP varying from 10 ng/ml–1 μg/ml in coating buffer was applied to the plastic wells for 3 h at 37°C. After washing, alkaline phosphatase-conjugated, immunopurified anti-HRGP IgG (1:800 dilution) was added for 3 h at 37°C. After washing, the substrate *p*-nitrophenyl phosphate (1:1,000) was added and color development followed in a Titertek multiscan photometer. The reaction was expressed as the enzymatic activity of the bound alkaline phosphatase ($10^4 \times \Delta A405\ min^{-1}$). The wells were then washed, cut out, and counted, and the amount of protein in each well determined. Y = 18.13x + 13.8, r = 0.991.

Complex formation of HRGP with TSP. An ELISA was used to determine whether HRGP forms a complex with adsorbed TSP. TSP in increasing concentrations was coated directly on the wells of a plastic microtitration plate. Incremental amounts of purified plasma HRGP were added and followed by the sequential addition of alkaline phosphatase-conjugated, immunopurified monospecific, anti-HRGP IgG and the enzyme substrate. The extent of the formation between fluid-phase HRGP and adsorbed TSP was detected and quantified by the enzymatic activity of the bound alkaline phosphatase (Fig. 2). A dose-response relationship of HRGP binding to adsorbed TSP was demonstrated, with maximal binding occurring at about 1.0 μg/ml HRGP. The apparent dissociation constant (K_D) for the complex formation was ~0.42 μg/ml or 7 nM. No inhibition of the complex formation was noted in the presence of 2 mM EDTA.

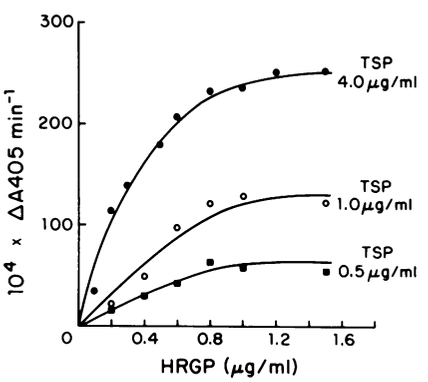

Figure 2. ELISA detection of TSP-HRGP complex formation. TSP at three concentrations in coating buffer was applied to the plastic wells for 3 h at 37°C. After washing, HRGP at various concentrations in Tris-Tween buffer was added for 18 h at 4°C. After washing, alkaline phosphatase-conjugated anti-HRGP IgG and the enzyme substrate were sequentially added as described in Fig. 1.

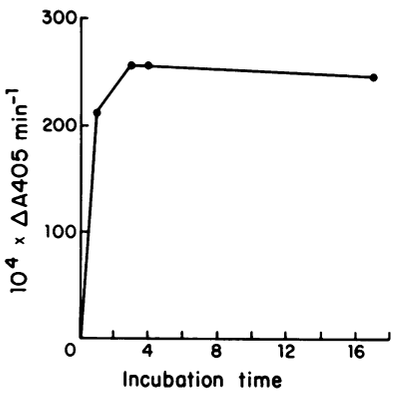

Figure 3. Time course study of TSP-HRGP complex formation. TSP (4 μg/ml) in coating buffer was applied to the plastic wells for 3 h at 37°C. After washing, HRGP (2 μg/ml) in Tris-Tween buffer was added for various periods of incubation time (hours). The remaining steps were carried out as described in Fig. 1.

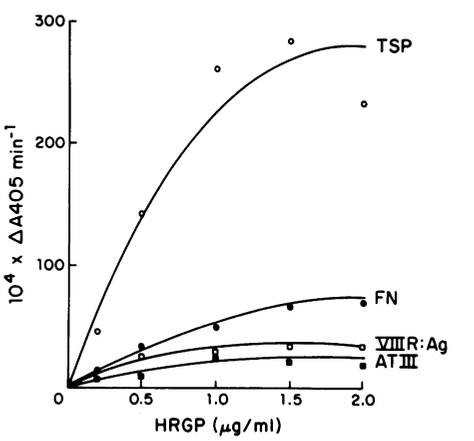

Figure 5. Interaction of HRGP with adsorbed proteins. The proteins (4 μg/ml) in coating buffer were applied to the plastic wells for 3 h at 37°C. After washing, HRGP in Tris-Tween buffer at concentrations indicated was added for 18 h at 4°C. Detection of complex formation of HRGP with the adsorbed proteins were carried out as described in Fig. 1. AT III, antithrombin III; FN, fibronectin.

To study the time course of the HRGP binding to adsorbed TSP, complex formations with different incubation time periods were determined (Fig. 3). Maximum binding occurred at 3 h of incubation at 4°C.

Since HRGP binds heparin (17, 22), and TSP isolation involves heparin-Sepharose affinity chromatography, the possibility was raised whether the observed TSP-HRGP interaction might be due to HRGP binding to a trace amount of heparin in the adsorbed TSP sample. Adsorbed TSP was incubated with enzymatically active heparinase (0.1 U/ml) prior to the addition of HRGP. There was no decrease in the binding of HRGP to the heparinase-treated, adsorbed TSP. Alternatively, TSP was preincubated with heparinase before adsorption to the plastic plate. No decrease in the HRGP binding was noted. The activity of heparinase was demonstrated by its ability to abolish the prolongation of the plasma thrombin time by 0.1 U/ml heparin.

Specificity of the TSP-HRGP complex formation. To demonstrate the specificity of the TSP-HRGP interaction, competitive inhibition studies of the TSP-HRGP complex formation by excess fluid-phase TSP were carried out. Following the coating of TSP (4 μg/ml) on the microtitration plate, HRGP (1 μg/ml and 2 μg/ml) was incubated in Tris-Tween buffer in the presence of increasing concentrations of TSP. 50% inhibition of the complex formation was achieved by 1.5 μg/ml TSP (3.6 nM) (Fig. 4). The small amount of HRGP which complexed to adsorbed TSP in the presence of excess soluble TSP (~15%) was considered "nonspecific" binding. No significant inhibition of HRGP complex formation with TSP was detected by excess fluid-phase albumin or fibrinogen.

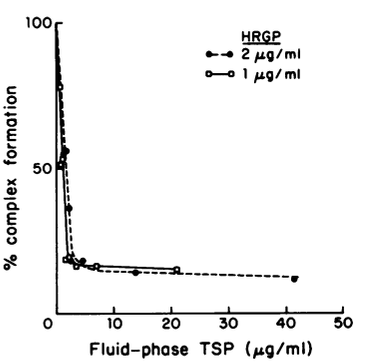

Figure 4. Competitive inhibition of TSP-HRGP complex formation by fluid-phase TSP. TSP (4 μg/ml) in coating buffer was applied to the plastic wells for 3 h at 37°C. After washing, HRGP (1 and 2 μg/ml) in Tris-Tween buffer was added alone or in the presence of increasing concentrations of TSP for 18 h at 4°C. The remaining steps were carried out as described in Fig. 1. TSP-HRGP complex formation in the absence of fluid-phase TSP was considered 100% complex formation.

The specificity of the HRGP interaction with adsorbed TSP was studied further by incubating fluid-phase HRGP with other adsorbed proteins (Fig. 5). Minimal complex formation was detected with purified VIIIR:Ag, fibronectin, and antithrombin III. Previous studies using ^{125}I-radiolabeled proteins had shown that the coating efficiency for VIIIR:Ag and fibronectin on the microtitration plate was comparable with that of TSP (10).

We have previously shown that adsorbed TSP did not bind fluid-phase albumin, γ-globulin, VIIIR:Ag, α_2-plasmin inhibitor, antithrombin III, and platelet membrane GPIIb and GPIIIa (10). In addition, there was no significant complex formation of adsorbed TSP with complement components $C1_q$, C3, C4, C5, high molecular weight kininogen, or Factor X and XII (data not shown).

Demonstration of TSP-HRGP complex formation by double antibody ELISA. To demonstrate that HRGP binding to adsorbed TSP was not due to denatured TSP molecules following interaction with the plastic surface, a double-antibody ELISA-binding system was used. Plastic microtitration plates were coated with monospecific anti-TSP IgG. TSP was added in Tris-Tween buffer and immobilized on the surface by anti-TSP. This was followed by the addition of fluid-phase HRGP, and detection of complex formation of HRGP with the antibody-immobilized

TSP was carried out as previously described. Significant complex formation between HRGP and TSP was demonstrated by using this double-antibody ELISA system (Table I). The binding of HRGP to the antibody-immobilized TSP was also concentration dependent with maximal binding occurring at 1 μg/ml.

The amount of complex formation demonstrated by using the double-antibody ELISA was less than that detected when HRGP was added to TSP coated directly on the microtitration plate (Fig. 2). By using ^{125}I-radiolabeled TSP, the amount of TSP immobilized on the anti-TSP coated plate was determined to be 32.8 ng, which was significantly less than the amount of TSP coated directly on the plate (262.7 ng).

Sucrose-density-gradient ultracentrifugation of TSP-HRGP complexes. Sucrose-density-gradient ultracentrifugation was used to assess whether HRGP and TSP formed a complex in the fluid phase. Purified plasma HRGP showed a single peak of reactivity with a sedimentation coefficient of 3.2 S, which is in good agreement with the published value (17)(Fig. 6). When a mixture of HRGP and TSP (at a molar ratio of 3:1) was analyzed, there was a significant shift of the HRGP peak from 3.2S to 11.8S, demonstrating the formation of TSP-HRGP complexes in the fluid phase. Purified TSP showed a major peak with a sedimentation coefficient of 9S, which is also in good agreement with the published sedimentation coefficient for TSP (4, 32)(Fig. 7). In addition, a minor peak with a higher sedimentation coefficient was noted, which possibly represents multimeric forms of TSP. When a mixture of HRGP and TSP was analyzed, there was a significant shift of the TSP peak from 9S to 11.8S. A relatively minor peak with a higher sedimentation coefficient was also noted, suggesting a heterogeneity of the TSP-HRGP complexes. Assuming that the partial specific volumes for TSP and the TSP-HRGP complex are similar, an approximate estimation of the molecular weight of the complex was obtained from the sedimentation coefficients.[2] The TSP-HRGP complex has a mol wt of ~630,000, suggesting a stoichiometry of TSP to HRGP of 1:3.5

Influence on TSP-HRGP complex formation by fibrinogen. We have previously demonstrated that fibrinogen forms a specific complex with adsorbed TSP (10). To test whether fibrinogen interfered with the binding of HRGP to adsorbed TSP, fibrinogen was added together with HRGP in the fluid phase to TSP. The binding of HRGP to adsorbed TSP in the presence of fibrinogen was similar to the binding obtained in the absence of fibrinogen (Table II). Similarly, the binding of fibrinogen to adsorbed TSP was not affected by the presence or absence of HRGP.

Table I. Detection of TSP-HRGP Complex Formation by Double-Antibody ELISA

Fluid-phase proteins	$10^4 \times \Delta A405\ min^{-1}$
None	6±2
TSP	11±2
HRGP	14±3
TSP plus HRGP	100±5

Anti-TSP IgG (8 μg/ml) in coating buffer was applied to the plastic wells for 3 h at 37°C. After washing, TSP (4 μg/ml) in Tris-Tween buffer was added for 4 h at 37°C. After washing, HRGP (1 μg/ml) in Tris-Tween buffer was added for 18 h at 4°C. In control studies, TSP (4 μg/ml) and HRGP (1 μg/ml) in Tris-Tween buffer were added separately to the antibody-coated well. The remaining steps were carried out as described in Fig. 1.

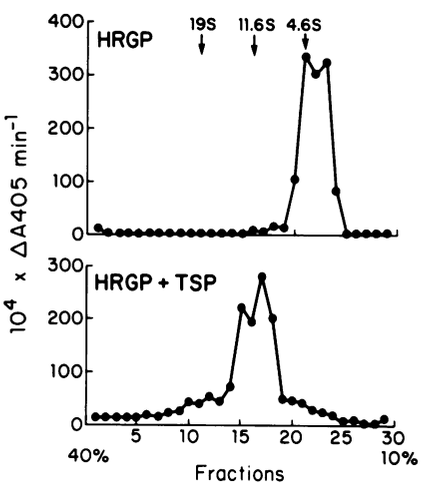

Figure 6. Sucrose-density-gradient ultracentrifugation of HRGP and TSP. HRGP, either alone or together with TSP (molar ratio of 3 HRGP:1 TSP), was incubated in 10 mM Tris-HCl, 0.15 M NaCL, pH 7.4, with 1 mM Ca^{++} for 1 h at 37°C. 0.2-ml aliquots of the incubation mixtures were layered on separate 10–40% linear sucrose gradients (5 ml each) in the same buffer and sedimented at 165,000 *g* for 18 h at 4°C. The gradients were fractionated by collecting 8-drop fractions and an aliquot (0.16 ml) of each fraction was coated onto the microtitration well by incubating at 4°C for 18 h. HRPG in each fraction was quantified by the specific ELISA for HRGP. The arrows in the top panel corresponded to the three proteins used for calibration: thyroglobulin $S_{20}w$ = 19; catalase, $S_{20}w$ = 11.3; and human serum albumin, $S_{20}w$ = 4.6.

2. Assuming that the partial specific volumes for TSP and the TSP-HRGP complex are similar, an approximate estimation of the molecular weight of the complex can be obtained from the sedimentation coefficients, by using the equation (Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J. Biol. Chem.* 236:1372–1379):

$$\frac{S_1}{S_2} = \left(\frac{\text{mol wt}_1}{\text{mol wt}_2}\right)^{2/3}.$$

Thus, from Fig. 7,

$$\frac{11.8S}{9S} = \left(\frac{\text{mol wt}_{TSP\text{-}HRGP}}{\text{mol wt}_{TSP}}\right)^{2/3}$$

$$\text{Mol wt}_{TSP\text{-}HRGP} = \text{mol wt}_{TSP} \times (1.31)^{3/2}$$

$$= 420{,}000 \times 1.5$$

$$= 630{,}000.$$

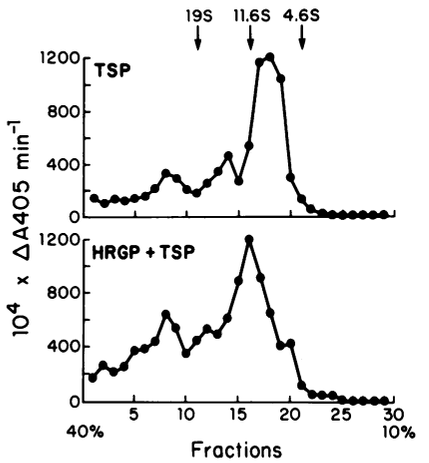

Figure 7. Sucrose-density-gradient ultracentrifugation of HRGP and TSP. TSP, either alone or together with HRGP (molar ratio of 7 HRGP:1 TSP), was analyzed as described in Fig. 7 except that a specific ELISA for TSP was used for detection of TSP in each fraction of the gradients.

Heparin binding by TSP-HRGP complex. Recent studies demonstrate that HRGP interacts strongly with heparin both in purified systems and in plasma, resulting in neutralization of the anticoagulant activity of heparin (22). To determine whether HRGP complexed to TSP binds heparin, purified TSP was coupled to cyanogen bromide-activated Sepharose 4B beads. The TSP coupled beads were incubated with purified plasma HRGP. ^{35}S-heparin binding to the TSP-HRGP complex on the beads was determined (Table III). The amount of heparin bound to TSP beads preincubated with HRGP was significantly greater than that bound to TSP beads alone, indicating that HRGP complexed with TSP retains the heparin-binding property. By using the specific ELISA for HRGP, the amount of HRGP complexed to TSP on the beads was determined to be 50 ng HRGP/50 μl beads. Therefore, 50 ng HRGP specifically bound 20.55 ng heparin (Table III). Assuming a M_r of 16,500 for heparin, the data suggests a stoichiometry of HRGP-heparin complex formation of 1:1.5, which is in good agreement with recent published data (22). Control studies using HRGP-coupled beads showed significant heparin binding while albumin-coupled beads did not, demonstrating the specificity of heparin binding.

To determine whether HRGP complexed with TSP neutralized the anticoagulant activity of heparin in plasma, thrombin time assays were performed (Table IV). HRGP-depleted plasma had a normal thrombin time in the absence of heparin but showed a markedly prolonged thrombin time in the presence

Table II. Influence on TSP-HRGP Complex Formation by Fibrinogen

Protein coat	Fluid-phase	Antibody probe	$10^4 \times \Delta A405$ min^{-1}
TSP	HRGP	Anti-HRGP	189±10
	HRGP plus fibrinogen	Anti-HRGP	178±8
	Fibrinogen	Antifibrinogen	47±4
	HRGP plus fibrinogen	Antifibrinogen	44±3

TSP (4 μg/ml) in washing buffer was applied to the plastic wells for 3 h at 37°C. After washing, HRGP (2 μg/ml) and fibrinogen (4 μg/ml) in Tris-Tween buffer were added either alone or together for 18 h at 4°C. The remaining steps were carried out as described in Fig. 1. Antifibrinogen was used in 1:1,500 dilution.

Table III. Binding of ^{35}S-Heparin by TSP-HRGP Complex

	^{35}S-Heparin Bound
	ng
Albumin beads	1.7±0.2
HRGP beads	178.8±2.6
TSP beads	17.8±0.85*
TSP beads preincubated with HRGP	38.35±0.15*

50 μl of the protein-coupled Sepharose 4B beads were incubated with ^{35}S-heparin (sp act 0.015 μCi/μg) in 20 mM Tris-HCl, 0.145 M NaCl, pH 7.4, containing 2 mM $CaCl_2$ for 1 h at 37°C. The beads were washed five times with Tris-Tween buffer containing 2 mM $CaCl_2$. Proteins were eluted from the beads by boiling in 2% SDS for 10 min. Results are the mean (±SD) of two separate experiments with duplicate determinations in each experiment.
* *P*, 0.0009.

Table IV. Influence of TSP-HRGP Complex on the Thrombin Time in the Presence of Heparin

Plasma	Thrombin time in the presence of 0.25 units/ml heparin
	s
Normal plasma	18
HRGP-depleted plasma	>180
HRGP-depleted plasma plus HRGP beads	89.3
HRGP-depleted plasma plus TSP beads	>180
HRGP-depleted plasma plus TSP beads preincubated with HRGP	107
HRGP-depleted plasma plus albumin beads preincubated with HRGP	>180

The protein-coupled beads were incubated with HRGP-depleted plasma, in the presence of 0.25 units/ml heparin, for 20 min at 37°C, before the addition of thrombin. 100 μl of beads were used in each experiment except for HRGP-coupled beads which were used in 50 μl. Thrombin times reported represent mean values of two separate measurements.

of heparin, which was partially corrected by the addition of HRGP-coupled beads, in agreement with recent published data (22). TSP-coupled beads that were preincubated with purified HRGP also partially corrected the prolonged thrombin time while control studies using albumin beads preincubated with HRGP or TSP beads alone had no effect.

Discussion

TSP and HRGP are two proteins with diverse biological properties. TSP has been identified in human platelet, endothelial cells, fibroblasts, and smooth muscle cells (1, 2, 11–14). TSP interacts with heparin, fibrinogen, fibronectin, and collagen, suggesting that it may be important in cell-cell and cell-substratum interactions (3, 10, 15). HRGP, an α_2-glycoprotein in plasma, has specific interactions with heme, plasminogen, and heparin (17–20, 22). It may also play a role in modulating the immune responses of lymphocytes (23). HRGP is present in human platelets and is released following thrombin stimulation (24). In this study, we have demonstrated that purified human plasma HRGP forms a complex with purified platelet TSP. This was supported by three different lines of evidence: (*a*) There was specific and saturable binding of HRGP to TSP adsorbed on microtitration plates. (*b*) Significant complex formation was detected when HRGP was incubated with TSP immobilized on anti-TSP-coated plates, indicating that the observed TSP-HRGP complex formation was not due to a TSP interaction with the plastic surface. (*c*) Sucrose-density-gradient ultracentrifugation of a mixture of HRGP and TSP revealed the presence of fluid-phase TSP-HRGP complexes.

The sucrose-density-gradient ultracentrifugation analyses revealed the presence of TSP-HRGP complexes with an apparent stoichiometry of 3.5 HRGP:1 TSP (Fig. 7). Our recent finding that TSP has hemagglutinating activity is consistent with the concept that TSP is multivalent (9). Electron microscopic studies have demonstrated that TSP has three to four well-defined nodular domains connected by flexible regions (32). It remains to be determined whether the HRGP-binding sites are located on these nodular domains of the TSP molecule.

TSP-HRGP-binding studies demonstrate an apparent K_D of 7 nM (Fig. 2). It is possible that TSP adsorbed on the plastic surface has an altered affinity for HRGP and this may not represent an accurate estimation of K_D. However, it should be noted that the competitive inhibition studies by fluid-phase TSP (Fig. 4), as well as the distribution of the free and bound HRGP in the sucrose-gradient ultracentrifugation analysis (Fig. 6), suggest that the native TSP molecule has a high affinity for HRGP in the fluid phase.

We have previously demonstrated that adsorbed TSP formed a complex with fibrinogen and to a lesser extent with fibronectin (10), suggesting that TSP may play an important role in the platelet aggregation process. In this study, fibrinogen did not inhibit complex formation of HRGP with TSP and conversely, HRGP did not interfere with fibrinogen binding to adsorbed TSP (Table II). This suggests that the binding sites of TSP for HRGP are different from those for fibrinogen. The molecular structure of TSP may be similar to that of fibronectin (33), another major platelet α-granule protein of similar molecular size, with different structural domains associated with independent functional activities.

Recent studies demonstrate that HRGP interacts strongly with heparin and neutralizes the anticoagulant activity of heparin in plasma, suggesting that HRGP may play a role in the modulation of the anticoagulant properties of heparin (22). TSP also binds heparin (3). In this study, HRGP complexed with TSP was capable of binding heparin and neutralizing the anticoagulant activity of heparin in plasma, with an estimated stoichiometry of 1 HRGP:1.5 heparin, which is in agreement with recent published data (22). This suggests that the TSP-binding site and the heparin-binding site are two separate functional domains on the HRGP molecule. It will be of interest to determine whether the TSP-HRGP complex interacts with heparan sulfate proteoglycans on vascular surfaces or extracellular matrix and modulates vessel wall reactivity.

The biological significance of the TSP-HRGP interaction remains to be fully defined. Thrombin-stimulated platelets release TSP from the α-granule, which binds to the activated platelet surface in the presence of Ca^{++}. HRGP is also present in human platelets and is released following thrombin stimulation (24). Our studies suggest the possibility that plasma HRGP, as well as platelet HRGP, if both proteins have the same functional capabilities, may interact specifically with TSP on the activated platelet surface and regulate heparin action. In addition, since HRGP interacts with the high affinity lysine-binding site of plasminogen (20), the specific binding of HRGP to TSP on the platelet surface may also serve to localize plasminogen onto the activated platelet surface and modulate fibrinolysis in the microenvironment of the platelet plug. Thus, the TSP-HRGP complex in a focal area of active hemostasis and thrombosis could serve as a prothrombotic mediator leading to an increased deposition of fibrin.

It is of great interest that in addition to platelets, TSP has been demonstrated in human endothelial cells, fibroblasts, and smooth muscle cells (11–14). Recent studies suggest that a serum factor that inhibits autorosette formation of murine thymocytes and peripheral lymphocytes is immunochemically similar to HRGP (23). Whether specific TSP-HRGP complex formation plays a role in the cell-cell interaction between these various cellular systems is a subject for further study.

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