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Factor H binds to washed human platelets

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Summary. Background: Factor H regulates the alternative pathway of complement. The protein has three heparin-binding sites, is synthesized primarily in the liver and copurifies from platelets with thrombospondin-1. Factor H mutations at the C-terminus are associated with atypical hemolytic uremic syndrome, a condition in which platelets are consumed. Objectives The aim of this study was to investigate if factor H interacts with platelets. Methods: Binding of factor H, recombinant C- or N-terminus constructs and a C-terminus mutant to washed (plasma and complement-free) platelets was analyzed by flow cytometry. Binding of factor H and constructs to thrombospondin-1 was measured by surface plasmon resonance. Results: Factor H bound to platelets in a dose-dependent manner. The major binding site was localized to the C-terminus. The interaction was partially blocked by heparin. Inhibition with anti-GPIIb/IIIa, or with fibrinogen, suggested that the platelet GPIIb/IIIa receptor is involved in factor H binding. Factor H binds to thrombospondin-1. Addition of thrombospondin-1 increased factor H binding to platelets. Factor H mutated at the C-terminus also bound to platelets, albeit to a significantly lesser degree. Conclusions: This study reports a novel property of factor H, i.e. binding to platelets, either directly via the GPIIb/IIIa receptor or indirectly via thrombospondin-1, in the absence of complement. Binding to platelets was mostly mediated by the C-terminal region of factor H and factor H mutated at the C-terminus exhibited reduced binding.

Keywords: complement, factor H, hemolytic uremic syndrome, platelets.

Introduction

Factor H (FH) is a glycoprotein known to play a regulatory role in the activation of the alternative pathway of complement.

This plasma protein prevents formation of the C3bBb convertase by competing with factor B and destabilizes the formed convertase by displacing factor B from C3b. It also functions as a cofactor for the proteolytic cleavage of C3b by factor I resulting in the formation of iC3b [1]. FH consists of 20 short consensus repeat (SCR) elements [2]. There are three heparin-binding sites located within SCRs 7, 12–14 and 20 as well as three C3b binding sites at SCRs 1–4, 10–15 and 20 [2–4]. FH circulates in human plasma as a 150-kDa protein, at a concentration of about 500 μg mL⁻¹. The FH-like protein-1 (FHL-1) is a 42-kDa protein which consists of SCRs 1–7, and is derived from the FH transcript by means of alternative splicing. FH and FHL-1 are encoded by a single gene on chromosome 1q32 [5] and both proteins have complement regulatory functions [6]. Homozygous mutations of FH have been found in some patients with membranoproliferative glomerulonephritis and atypical hemolytic uremic syndrome (HUS) (reviewed in [2,7]). Heterozygous FH mutations have been identified in a subgroup of patients with atypical HUS [7–12] and a hot-spot was recognized within SCR 20 [9]. HUS is characterized by a triad of microangiopathic hemolytic anemia, thrombocytopenia and renal failure [13]. The typical form has been associated with infections caused by Shiga toxin producing bacteria in which HUS is usually preceded by a diarrheal prodrome, whereas the atypical form has heterogeneous etiologies [13]. Endothelial cell injury is pivotal to the pathogenesis of HUS [14]. Thrombocytopenia ensues due to platelet consumption in microthrombi, presumably secondary to endothelial cell damage and exposure of the subendothelium. The mechanisms by which FH mutations may lead to HUS are poorly understood. It has been postulated that complement activation may propagate endothelial cell injury [15] and that alterations in FH may thus lead to vascular damage [2,16,17]. A direct interaction of FH with platelets has not been previously studied.

FH is mainly synthesized in the liver, but also in monocytes, fibroblasts, mesangial cells and endothelial cells as summarized by Friese et al. [6]. FH has also been localized to the α-granules of platelets from where it is released upon stimulation with thrombin [18] or upon binding of C3 to the platelet surface [19].
Furthermore, FH was found to copurify from platelets with thrombospondin-1 (TSP-1) [20], a glycoprotein with a well-characterized role in platelet aggregation [21]. Since FH mutations have been associated with HUS, a condition in which platelet activation is a main manifestation [21], and since FH is released from platelets, we postulated that native FH interacts directly with platelets. The aim of this study was therefore to investigate binding of normal and mutated FH to washed platelets in the absence of complement.

Materials and methods

**FH, recombinant constructs and mutant**

Wild-type FH was obtained from Calbiochem (La Jolla, CA, USA). Recombinant histidine-tagged deletion constructs SCRs 1–7 (FH-H1), SCR 8–11, SCR 8–20, SCR 15–20 were cloned and expressed in the baculovirus system and purified as described [22]. Recombinant fragments SCRs 1–7, 8–11, 8–20, 15–20 and SCR 15–20mut, histidine-tagged and mutated in SCR 20 at positions R1203E, R1206E, R1210S, K1230S and R1231A, with reduced binding to C3b and heparin, have been previously described [4]. The bioactivity of FH fragments, which are to a certain extent glycosylated [23–25], is comparable to the wild-type [17,23,26]. For certain experiments FH was labeled with Alexa555 (Molecular Probes, Leiden, the Netherlands) or 125I. Purity of the proteins (> 95%) was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and by silver staining and no contamination of FH with TSP-1 was found when immunoblotting was performed with rabbit anti-TSP-1 antibody (1 : 100; Calbiochem).

**Platelet-rich plasma and washed (plasma-free) platelets**

Venous blood was collected from 44 healthy adult volunteers (22 males and 22 females) not using any medications and platelet-rich plasma (PRP) obtained as described [27]. Platelets were washed of plasma components [27] and resuspended in serum-free HBSS without calcium or magnesium. Platelet-rich plasma and washed (plasma-free) platelets (22 males and 22 females) not using any medications and platelet-rich plasma (PRP) obtained as described [27]. Platelets were diluted in 500 µL saline (PBS; 0.140 M NaCl, 0.003 M KCl, 0.01 M phosphate, pH 7.4; Medicago, Uppsala, Sweden) and binding detected by flow cytometry using a FACS Calibur instrument (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Acquisition and processing of data from 5000 cells per sample was carried out with the CELLQuest software. Percent binding was calculated after subtraction of the background fluorescence as determined by the binding of the control antibody. Similarly, Alexa555-labeled FH at 50 µg mL⁻¹ was incubated with thrombin-activated platelets.

In each experiment platelets were identified by positive immunofluorescence using mouse anti-human CD41–phycoerythrin (PE) antibody (Immunotech) by flow cytometry.

Flow cytometry

Binding of FH, recombinant deletion and mutant constructs to platelets was determined by flow cytometry. Washed thrombin-activated platelets were incubated with or without FH (1, 10 or 100 µg mL⁻¹; 10 µg mL⁻¹ = 0.07 µM) for 1 h at 37 °C and gently mixed every 15 min. Cells were washed and incubated with goat anti-human FH antibody (1 : 10; Calbiochem) or goat serum as a negative control (1 : 10; Vector Laboratories, Burlington, MA, USA) for 1 h at 37 °C, both diluted in 1% BSA–HBSS. In separate experiments washed platelets were incubated with mouse anti-human FH antibody (20 µg mL⁻¹; Serotec, Oxford, UK) or mouse IgG1 as an isotype control (Dako, Glostrup, Denmark).

Saturability of binding was tested by incubating increasing concentrations of FH (1, 10, 100, 250, 500, 750, 1000 µg mL⁻¹) with thrombin-activated washed platelets, using the mouse anti-human FH antibody.

Secondary antibodies, rabbit anti-goat IgG:FITC (1 : 400; Calbiochem) or goat F(ab')₂ antimmune IgG:FITC (1 : 20; Dako), in HEPES buffer [27] were applied as appropriate. Platelets were diluted in 500 µL ice-cold phosphate-buffered saline (PBS; 0.140 M NaCl, 0.003 M KCl, 0.01 M phosphate, pH 7.4; Medicago, Uppsala, Sweden) and binding detected by flow cytometry using a FACS Calibur instrument (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Acquisition and processing of data from 5000 cells per sample was carried out with the CELLQuest software. Percent binding was calculated after subtraction of the background fluorescence as determined by the binding of the control antibody. Similarly, Alexa555-labeled FH at 50 µg mL⁻¹ was incubated with thrombin-activated platelets.

A F(ab')₂ fragment was produced from the polyclonal goat anti-human FH antibody by pepsin digestion (Pierce, Rockford, IL, USA). Inhibition experiments were carried out in which FH 100 µg mL⁻¹ was preincubated with this F(ab')₂ fragment (150 µg mL⁻¹) at 37 °C for 1 h before addition to platelets. In these experiments binding was detected with the mouse anti-human FH antibody.

Similarly to experiments with FH, platelets were incubated with 1 and 10 µg mL⁻¹ of SCRs 1–7 (10 µg mL⁻¹ = 0.21 µM), 8–11 (10 µg mL⁻¹ = 0.37 µM), 8–20 (10 µg mL⁻¹ = 0.11 µM), 15–20 (10 µg mL⁻¹ = 0.24 µM) and SCR 15–20mut (10 µg mL⁻¹ = 0.24 µM) and binding detected with the goat anti-human FH antibody.

The importance of the heparin-binding sites was analyzed by preincubation of FH 100 µg mL⁻¹ and SCRs 1–7, 8–11, 8–20, 15–20 as well as SCR 15–20mut (10 µg mL⁻¹) with heparin 1000 IU mL⁻¹ (Leo Pharma, Malmö, Sweden) at 37 °C for 1 h before addition to thrombin-activated washed platelets. Likewise, in separate experiments platelets were first preincubated with heparin before FH was added.

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The role of the platelet receptor GPIIb/IIIa for FH binding to platelets was studied by preincubation of thrombin-activated washed platelets with Reopro® (abciximab 100–200 µg mL⁻¹; Eli Lilly, Indianapolis, IN, USA) [29], at room temperature for 15 min, after which platelets were incubated with FH 10 µg or 100 µg mL⁻¹, alternatively SCRs 1–7, 8–20, 15–20 as well as SCR 15–20mut 10 µg mL⁻¹. Similarly, monoclonal antiplatelet receptor GPIb (CD42b, 100 µg mL⁻¹; Dako) was used as a control. In certain experiments platelets were preincubated with Reopro® and FH preincubated with heparin before these were combined.

We investigated if FH competitively inhibits binding of fibrinogen to the GPIIb/IIIa receptor. Platelets were not thrombin-activated in these experiments. The method used for detection of fibrinogen binding has been previously described [27,30]. Washed platelets were incubated with or without FH 10 µg mL⁻¹ or 100 µg mL⁻¹ at 37 °C for 30 min, washed in PBS and fixed. Cells were then washed and fibrinogen 1 mg mL⁻¹ was added for 5 min at room temperature. Platelets were washed and incubated with FITC-conjugated chicken antihuman fibrinogen antibody (Diapensia, Gothenburg, Sweden) at a final concentration of 100 µg mL⁻¹ or FITC-conjugated chicken antihuman insulin antibody as a negative control (100 µg mL⁻¹; Diapensia), both diluted in HEPES buffer, incubated for 30 min on ice and fluorescence detected.

The possibility that FH binds to platelets via tsp-1 was investigated by preincubation of platelets with rabbit antihuman tsp-1 antibody (1 : 400) for 1 h at 37 °C prior to addition of FH (100 µg mL⁻¹) and detection of FH binding as described above. In addition, in some experiments Reopro® was added to the platelets for a further 15 min as described above. In other experiments FH (100 µg mL⁻¹) was combined with tsp-1 (2 µg mL⁻¹; Sigma) and incubated with thrombin-activated washed platelets. Binding was compared with binding in which FH alone was used. The effect of added tsp-1 was evaluated by 1 h preincubation of tsp-1 with rabbit antihuman tsp-1.

The effect of thrombin activation on tsp-1 expression was tested in washed platelets. Platelets were activated with thrombin or not activated, fixed and incubated with a polyclonal rabbit antihuman tsp-1 (1 : 1500) or rabbit serum (1 : 1500) as a negative control for 1 h at 37 °C. Platelets were washed and incubated with swine antirabbit IgG:FITC (1 : 20; Dako).

The possible presence of C3 on washed platelets was tested. Platelets were incubated with rabbit antihuman C3c (1 : 50; Dako), or rabbit immunoglobulin fraction (Dako) as a negative control, for 1 h at 37 °C. Platelets were washed and incubated with swine antirabbit IgG:FITC (1 : 10). As a positive control platelets were incubated for 30 min with 1 mg mL⁻¹ purified C3 [31] at 37 °C.

Equilibrium and kinetics of FH binding to platelets

The $K_d$ of FH binding to platelets was measured by a modified Scatchard analysis. Washed platelets were incubated with a combination of radiolabeled $^{125}$I-FH (10 µg mL⁻¹) and increasing concentrations of cold FH (0–100 µg mL⁻¹) for 1 h at 37 °C. After washing with PBS, the radioactivity counts were measured in a gamma counter and the $K_d$ value determined by a Scatchard equation and graph.

In order to establish the number of FH molecules bound per platelet, a state of equilibrium binding was achieved using increasing concentrations of radiolabeled FH (1, 10, 25, 50, 75 µg mL⁻¹) incubated for 1 h with $10^9$ mL⁻¹ platelets. When equilibrium was attained the kinetics of binding was determined by measuring bound $^{125}$I-FH after 2, 10, 30 and 60 min.

FH, constructs and mutant binding to tsp-1 detected by surface plasmon resonance

Interactions of FH and tsp-1 were analyzed by the surface plasmon resonance technique using a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden) as described [4,32]. Tsp-1 was coupled via a standard amine-coupling procedure to the flowcell of a sensor chip (carboxylated dextran chip CM5; Biacore) until an appropriate level of coupling for the binding experiments (> 4000 resonance units) was reached. A control flowcell was prepared without injecting protein. Native FH and recombinant construct SCRs 1–7, 1–11, 8–20 or 15–20 as well as SCR 15–20mut were dialyzed against the running buffer (PBS, pH 7.4, 75 mM NaCl). Each ligand (1 µM) was injected separately into the flowcell coupled with tsp-1 and into the control flowcell (without tsp-1). Each analysis was done at least twice using independently prepared sensor chips. For kinetic analysis of the interaction of FH with tsp-1, a concentration series of FH (1–320 nM) was run at a flow rate of 30 µL min⁻¹ in three independent experiments on a chip with a lower surface density of tsp-1 (< 1000 resonance units) as described earlier [4]. For the heparin inhibition series, increasing concentrations of heparin (3–300 µg mL⁻¹), low molecular weight; Sigma) were added to the analyte, SCR 15–20 (50 µg mL⁻¹), directly before the injection.

Statistics

Differences between platelets incubated with or without FH, with respect to binding, were assessed by the Mann–Whitney U-test. A P-value of ≤0.05 was considered significant. Statistical analyses were performed using SPSS version 11 (SPSS, Chicago, IL, USA).

Results

FH binds to washed platelets

FH bound to non-activated platelets and to platelets activated with either thrombin or ADP. Thrombin activation increased FH (10 µg mL⁻¹) binding by 22% (from 9 to 11% binding to the platelet population, median of three flow cytometry experiments) in comparison with non-activated platelets, and was also documented using radiolabeled FH (Fig. 1A).
number of radiolabeled FH molecules bound to each thrombino-activated platelet was $3.8 \times 10^5$, whereas the number of molecules bound to each resting platelet was $2.7 \times 10^5$. ADP activation increased FH (10 $\mu$g mL$^{-1}$) binding by 89% (from 9 to 17%, median of four flow cytometry experiments). Results pertaining to thrombin-activated platelets are shown. Binding was dose dependent as shown in Fig. 1B. A final concentration of 100 $\mu$g mL$^{-1}$ FH was incubated with platelets at $1 \times 10^7$ mL$^{-1}$ in an attempt to correspond to the ratio of FH to platelets in the human circulation (FH $500 \mu$g mL$^{-1}$; platelets $1.4-4 \times 10^8$ mL$^{-1}$). At this concentration a median binding of 24% (polyclonal antibody) and 18% (monoclonal antibody) was found (Fig. 1B). FH binding as detected by fluorescence intensity using polyclonal and monoclonal
antibodies is shown in Fig. 1C and D, respectively. Alexa555-labeled FH bound to 19.5% of platelets.

Saturability of FH binding to platelets ($1 \times 10^7 \text{mL}^{-1}$) was obtained at a FH concentration of approximately $500 \mu \text{g mL}^{-1}$ (Fig. 1E). Maximal binding was noted after 10 min incubation (Fig. 1F).

The $K_d$ of FH binding to platelets, tested by combining radiolabeled FH with cold FH and analyzed by a Scatchard plot, was 2.39 $\mu\text{M}$ (data not shown).

Inhibition experiments using a F(ab')$_2$ fragment (goat antihuman FH) reduced binding by a mean of 42% (from 9.7 to 5.6%, two experiments).

Mechanism by which FH binds to platelets

In order to identify the mechanisms by which FH binds to platelets we localized the binding domain(s) within FH and tried to identify the receptor(s) on platelets that mediate binding.

FH binds to platelets mainly via the C-terminus domain

Recombinant constructs representing SCR 1–7, SCR 8–20 and SCR 15–20 of FH bound to platelets in a dose-related manner and all constructs that contained the C-terminus of FH (SCR 8–20 and SCR 15–20) showed strong binding (Table 1).

Table 1: Binding of factor H (FH) constructs and mutant to platelets as determined by flow cytometry

<table>
<thead>
<tr>
<th>FH construct / Concentration (µg mL$^{-1}$)</th>
<th>Binding (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets not incubated with FH constructs</td>
<td>6.5 (0.0–16.0)a</td>
<td>0.13 NS</td>
</tr>
<tr>
<td>SCR 1–7</td>
<td>10.3 (9.7–13.6)b</td>
<td>0.13 NS</td>
</tr>
<tr>
<td>SCR 1–7mut</td>
<td>10.3 (9.8–13.6)</td>
<td>0.13 NS</td>
</tr>
<tr>
<td>SCR 8–11</td>
<td>17.7 (16.4–33.3)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>SCR 8–11mut</td>
<td>17.7 (16.4–33.3)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>SCR 8–20</td>
<td>8.7 (4.2–22.6)d</td>
<td>0.30 NS</td>
</tr>
<tr>
<td>SCR 8–20mut</td>
<td>8.7 (4.2–22.6)</td>
<td>0.30 NS</td>
</tr>
<tr>
<td>SCR 8–20</td>
<td>8.5 (3.8–23.5)</td>
<td>0.37 NS</td>
</tr>
<tr>
<td>SCR 8–20mut</td>
<td>8.5 (3.8–23.5)</td>
<td>0.37 NS</td>
</tr>
<tr>
<td>SCR 15–20</td>
<td>20.2 (8.9–30.5)b</td>
<td>0.03</td>
</tr>
<tr>
<td>SCR 15–20mut</td>
<td>20.2 (8.9–30.5)</td>
<td>0.03</td>
</tr>
<tr>
<td>SCR 15–20</td>
<td>30.6 (21.7–34.9)b</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>SCR 15–20mut</td>
<td>30.6 (21.7–34.9)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>SCR 15–20</td>
<td>20.3 (11.6–32.9)b</td>
<td>0.011</td>
</tr>
<tr>
<td>SCR 15–20mut</td>
<td>20.3 (11.6–32.9)</td>
<td>0.011</td>
</tr>
<tr>
<td>SCR 15–20</td>
<td>30.0 (15.2–54.3)d</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>SCR 15–20mut</td>
<td>30.0 (15.2–54.3)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>SCR 15–20</td>
<td>15.7 (4.1–27.24)d</td>
<td>0.057 NS</td>
</tr>
<tr>
<td>SCR 15–20mut</td>
<td>15.7 (4.1–27.24)</td>
<td>0.057 NS</td>
</tr>
</tbody>
</table>

$P$-values evaluated by comparison of platelets incubated with or without FH constructs. Platelets that were not incubated with FH constructs were washed and thrombin-activated, similarly to those incubated with FH constructs. Median of: a21; b3; c5; d6; e12 experiments. NS, Not significant.

Involvement of heparin-binding sites in FH binding to platelets

Preincubation of FH with heparin reduced binding of FH to platelets by 36% (median of percentage reduction, from 32 to 18% binding, eight experiments). Preincubation of SCR 1–7, 8–20 and 15–20 with heparin reduced binding by 14, 23 and 22%, respectively. Preincubation of platelets with heparin before addition of FH had no effect on FH binding, indicating that the heparin-binding sites on FH and not on platelets are essential for this interaction. These results were further confirmed by incubating platelets with SCR 8–11, a construct lacking heparin-binding sites [33]. As shown in Table 1, binding of this construct was clearly reduced and no dose-response was demonstrated. Furthermore, preincubation with heparin did not reduce binding of this construct.

Platelet GPIIb/IIIa receptor mediates FH binding

The platelet GPIIb/IIIa receptor is essential for platelet interactions with fibrinogen, von Willebrand factor and other known platelet-activating peptides [34]. We therefore investigated if this receptor was involved in FH binding to platelets. This was achieved by blocking the receptor using an anti-GPIIb/IIIa antibody (Reopro®) and by competitive inhibition with fibrinogen.

Preincubation of platelets with Reopro® 100 µg mL$^{-1}$ reduced binding of FH 100 µg mL$^{-1}$ to platelets by 34% (median of percentage reduction) from 32 to 21% binding (seven experiments) ($P < 0.02$). Preincubation of platelets with Reopro® reduced binding of FH 10 µg mL$^{-1}$ to platelets by 45% (median) from 19 to 11% binding (six experiments) ($P < 0.03$). As a control for platelet receptors, FH was preincubated with anti-GPIIb antibody, which did not reduce binding.

Similar experiments were carried out using FH constructs to determine binding via the GPIIIa receptor. Reopro® 100 µg mL$^{-1}$ reduced binding of SCR 1–7 by 36% (from 21 to 14% binding, three experiments), of SCR 8–20 by 32% (from 24 to 17%, three experiments) and of SCR 15–20 by 23% (from 25 to 5%, six experiments).

Washed platelets (not thrombin-activated) were incubated with or without FH, after which fibrinogen was added. Fibrinogen binding was detected on platelets not exposed to FH at a median of 44% (four experiments). When FH 10 µg mL$^{-1}$ was present binding of fibrinogen was reduced to a median of 39% (11% reduction, four experiments). When FH 100 µg mL$^{-1}$ was present binding of fibrinogen was reduced to a median of 35% (21% reduction, four experiments). These results indicate that the presence of the higher concentration of FH partially inhibited fibrinogen binding to the GPIIb/IIIa receptor and together with the above-mentioned Reopro® experiments suggest that GPIIb/IIIa is, to a certain extent, involved in FH binding to platelets via both the C- and N-termini.

A combination of Reopro® and heparin reduced FH binding to platelets from 32 to 13% (58% reduction, three experiments).

FH binds to tsp-1 mainly via its C-terminus

Since FH is secreted from platelets together with tsp-1, and FH binding to platelets was not totally abrogated by the
anti-GPIIb/IIIa antibody, we studied interactions between FH and tsp-1 and their importance for FH binding to platelets. We used the surface plasmon resonance technique to show binding of FH to immobilized tsp-1 and to determine the binding affinity and kinetics (Fig. 2A). The $K_d$ value for binding of FH to tsp-1 was 49 (± 4.4) nM at 25 °C. We localized the regions involved in binding using recombinant deletion constructs SCRs 1–7, 8–11, 8–20 and 15–20 (Fig. 2B). SCR 8–20 and SCR 15–20 bound to tsp-1. In contrast, construct SCR 1–7, containing the N-terminus, and SCR 8–11, did not bind to tsp-1.

The effect of heparin on the interaction between SCR 15–20 and tsp-1 was studied. The addition of heparin inhibited the interaction of SCR 15–20 with tsp-1 dose-dependently (Fig. 2C). This indicates that binding of SCR 15–20 to tsp-1 occurs at the heparin-binding site (SCR 20).

**Tsp-1 and FH expression on thrombin-activated platelets and FH binding to platelets via tsp-1**

The above-mentioned experiments indicated that FH and tsp-1 interact with each other. We therefore investigated if these proteins are expressed on the surface of thrombin-activated platelets. Thrombin activation increased expression of tsp-1 4-fold (from 4 to 18% binding of the anti-tsp-1 antibody) and of FH by 0.5-fold (from 4 to 6% binding of the anti-FH antibody). This result suggests that FH may bind to activated platelets via surface expression of tsp-1.

Preincubation of platelets with anti-tsp-1 before addition of FH reduced FH binding by 36% (from 14 to 9%). Combination of tsp-1 with FH increased FH binding to platelets by 28% (median of percentage increase). This increase of FH binding was totally inhibited by preincubation of tsp-1 with anti-tsp-1. An attempt was made to inhibit FH binding by preincubation of FH with anti-tsp-1 and Reopro®, but these experiments could not be interpreted due to very high background fluorescence.

**Lack of C3 on washed platelets**

No C3 was identified on the surface of washed platelets. This indicates that FH does not bind to washed platelets via C3. When C3 was added to washed platelets (as the positive control) the anti-C3c antibody bound to 36% of the population (three experiments, data not shown).

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**Fig. 2.** Binding of factor H (FH), FH constructs and mutant to thrombospondin-1 (tsp-1) detected by surface plasmon resonance. (A) Strong binding of FH to tsp-1 is shown. The inserted figure shows equilibrium binding of FH to tsp-1 expressed in resonance units at increasing concentrations of the analyte (FH concentrations from 1 to 320 nM). The controls (binding to a flowcell without immobilized protein) were subtracted from the binding curves. (B) FH constructs SCR 8–20 and SCR 15–20 bound to tsp-1 whereas SCR 8–11 and SCR 1–7 did not bind. (C) Heparin inhibited binding of SCR 15–20 to tsp-1 in a dose-dependent manner. (D) SCR 15–20mut exhibited weaker binding to tsp-1 than SCR 15–20.
Mutant FH binding to platelets and tsp-1
SCR 15–20mut bound to platelets significantly less than SCR 15–20 (P < 0.0001, Table 1). Binding was not inhibited by heparin but was reduced by Reopro® 100 µg mL⁻¹ by 50% (from 16 to 8%, six experiments) and by Reopro® 200 µg mL⁻¹ by 71% (from 16 to 5%, six experiments). Furthermore, SCR 15–20mut exhibited considerably lower binding to tsp-1 than SCR 15–20 as studied by surface plasmon resonance (Fig. 2D).

Discussion
A novel property of FH is presented in this study that shows binding of FH to washed platelets. FH mutated at the C-terminus exhibited significantly lower binding. This interaction occurred in the absence of complement and other plasma factors. FH mutations have been identified in a subset of patients with atypical HUS [7–12], a condition in which platelets are activated and consumed, leading to thrombocytopenia [13]. In some of these patients the alternative complement pathway is activated [35]. Although this study did not address the mechanisms by which platelets are activated in HUS, we have shown that, in a plasma-free environment, FH can interact with platelets and that mutated FH displays lesser binding.

FH binding to platelets is multivalent and may engage more than one site of the FH protein as well as various binding sites and different proteins on the platelet surface (Fig. 3). Binding seems to involve mainly the C-terminus of FH. The C-terminus contains C3b and heparin-binding sites. Recent studies provide evidence that this region is involved in binding to cell surfaces containing glycosaminoglycans such as sialic acids [3,4] and enables FH to differentiate between activating (foreign) and non-activating (host) surfaces [36]. FH binds to platelets directly via the GPIIb/IIIa receptor, which is exposed after platelet activation, or indirectly via tsp-1, which is expressed on the surface of activated platelets. Thus FH may bind via more specific mechanisms such as receptor-mediated binding, which may be inhibited by antibodies and competitive inhibition with fibrinogen, as well as less-specific mechanisms involving the heparin-binding sites of FH and glycosaminoglycans on cells. The complexity of FH binding and ligand specificity were previously documented for binding to C3b on erythrocytes [37,38].

Binding of FH to platelets involves the heparin-binding sites. The heparin-binding site at the C-terminus appears to be more accessible than the other two heparin-binding sites since heparin binding of the full protein can be inhibited by an antibody directed to the C-terminus [39]. This may explain why the N-terminus construct (SCR 1–7) binds to a lesser degree than the C-terminus construct (SCR 15–20) though both have one heparin-binding site. The SCR 8–11 construct, which lacks a heparin-binding site, has a binding site for C-reactive protein and binds to microbial ligands such as the pneumococcal Hic protein [33,40]. This construct bind platelets weakly and no binding to tsp-1 was detected. This further indicates that the heparin-binding sites are involved in binding of FH to platelets. In addition, our results suggest that binding of FH to GPIIb/IIIa occurs both at the C- and N-termini, whereas binding via tsp-1 occurs mostly via the C-terminus.

FH mutations in HUS patients have been mostly identified at the C-terminus of the protein. Using purified mutant FH from a HUS patient and a recombinant SCR 20-mutated construct, a recent study showed reduced binding to endothelial cells in comparison with the wild-type protein, suggesting that the mutated protein may not be capable of protecting the endothelial cell layer [17]. Mutated FH was capable of binding

Fig. 3. Schematic diagram of mechanisms by which factor H (FH) binds to thrombin-activated washed platelets. FH binds to platelets mostly via the C-terminus and heparin-binding sites. On washed platelets it may bind both to the GPIIb/IIIa receptor and to surface-bound thrombospondin-1 (tsp-1). % refers to percent binding of whole FH or constructs to platelets.

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to platelets, although to a lesser degree. The reason for this loss of function may be related to the fact that SCR 15–20mut does not contain a heparin-binding site and does not bind to tsp-1. Thus binding of the C-terminus mutant to platelets is primarily mediated by GPIIb/IIIa and this binding could be almost completely abrogated by anti-GPIIb/IIIa (Reopro®).

Tsp-1 is a glycoprotein stored in and secreted from the α-granules of platelets and known to induce platelet aggregation via integrin-associated protein (IAP, CD47) and fibrinogen [41]. It copurifies from platelets with FH [20] and we have shown that these two proteins interact with each other and localized the binding site to the C-terminus. Binding at the C-terminus was diminished by addition of heparin and the SCR 15–20mut exhibited reduced binding, indicating that the C-terminus heparin-binding site in SCR 20 of FH is important for the interaction. As the two proteins are secreted simultaneously from activated platelets we suggest that they may interact in vivo.

Current theories regarding the pathogenesis of HUS have focused on endothelial cell damage, with exposure of the subendothelium leading to deposition and consumption of platelets. We show that FH binds to platelets and suggest that this interaction may regulate complement activation on the platelet membrane. Reduced binding of mutated FH to platelets may promote uninhibited complement activation. Further studies are ongoing to address the interactions of FH with platelets in the presence of complement.

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