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Modified C-reactive protein interacts with platelet glycoprotein Ibα

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Abstract:
Herein, we investigated the possible mechanisms by which recombinant modified CRP (m_rCRP) modulates blood platelet function. Modified CRP could activate blood platelets and stimulate their adhesion and aggregation in the absence of any other physiological stimuli. Preincubation of isolated blood platelets with m_rCRP at a concentration as low as 2 μg/ml resulted in significant platelet degranulation (fraction of CD62-positive platelets increased 2-fold, $p < 0.0002$), and at concentrations of 20 μg/ml and 100 μg/ml, increased exposure of the platelet procoagulant surface was observed (expression of annexin V-positive platelets increased to 5.7 ± 1.0% and 10.4 ± 2.2%, respectively, $p < 0.03$, *vs*. 2.9 ± 0.2% in control). Furthermore, m_rCRP (100 μg/ml) strongly augmented spontaneous and ADP-induced fibrinogen binding to platelets ($p < 0.05$), platelet adhesion to fibrinogen and platelet aggregation. Using the Biacore™ surface plasmon resonance technique and glycoprotein Ibα (GPIbα) immobilized on the sensor surface, we demonstrated direct binding between platelet GPIbα and m_rCRP. Binding of m_rCRP to GPIbα and C1q was also observed by ELISA, irrespective of the immobilized ligand. These outcomes strongly support a role of the GPIb-IX-V complex in the interactions of m_rCRP with blood platelets.

Key words:
C-reactive protein, glycoprotein Ibα, platelet activation, procoagulant activity, aggregation, adhesion, surface plasmon resonance

Abbreviations: ADP – adenosine diphosphate, CRP – C-reactive protein, GPIbα – glycoprotein Ibα, mCRP – modified C-reactive protein, nCRP – native C-reactive protein, RGDS – Arg-Gly-Asp-Ser, SPR – surface plasmon resonance, vWf – von Willebrand factor

Introduction

C-reactive protein (CRP) plays a regulatory role in innate immunity and inflammation [15], and it is thought to influence numerous biological processes involving atherogenesis [3, 47]. Under normal physiological conditions, CRP is present in serum at concentrations below 10 mg/l, and its levels can increase 100- to 1000-fold within 24 to 72 h in response to a variety of inflammatory stimuli, such as trauma, tissue necrosis, and infection [20, 38].

It is now apparent that the biological action of CRP is strongly dependent on its structure. The native CRP (nCRP) molecule is a pentamer composed of five identical noncovalently bound subunits, each having a molecular mass of ~23 kDa and consisting of 206

amino acids [37]. By exposing nCRP to heat, urea, or acidic conditions in the absence of calcium ions or by a direct immobilization of nCRP onto polystyrene plates, pentameric CRP may undergo dissociation into free subunits termed monomeric CRP [30, 39, 40]. It needs to be emphasized that the transformation of nCRP into monomeric CRP is a physiological phenomenon that may occur, for instance, when nCRP binds to cellular membranes on a variety of cells [25], including platelets [16]. Modified CRP (mCRP) exhibits unique antigenic, electrophoretic and ligand-binding properties distinct from those of pentameric CRP. In addition to adverse physicochemical properties, mCRP has been reported to possess novel biological activities that significantly differ from those of nCRP. It has been demonstrated that primarily mCRP may interact with various lipoproteins [24], and it can also interact with immunoglobulins, including immune complexes [4, 34]. mCRP was also found to promote neutrophil-endothelial cell adhesion [52] and to delay apoptosis in human neutrophils [26]. Khreiss et al. reported the different activities of CRP isoforms in the regulation of shear-induced platelet adherence to neutrophils and in neutrophil aggregation, pointing to a possible role for mCRP in acute coronary syndromes [28].

Both CRP isoforms have been shown to naturally occur *in vivo*; however, their physiological implications should be considered with respect to their differential distribution. nCRP is known to be synthesized by hepatocytes and represents the circulating form of the protein [21]. However, immunohistochemical studies also demonstrated the possible presence of nCRP and/or mCRP in extrahepatic tissues, such as neuronal cells [51], lung epithelial cells [42], renal cortical tubular epithelial cells [22], and human coronary artery smooth muscle cells [8]. Considering the clones and titers of antibodies utilized in those studies, it remains unclear whether a CRP pentamer was detected in those tissues. If so, it further remains unknown whether an nCRP pentamer was deposited at those sites from a circulating plasma pool or was produced locally by the cells, in addition to what exactly is the contribution of extrahepatic CRP to the serum levels of CRP. mCRP is a poorly soluble isoform that has been found only in tissues or cells [10, 14, 45], thus representing a tissue-based CRP pool.

The regulation of cell function by CRP is widely discussed in the literature. However, in appreciation of the fact that mCRP is a naturally occurring and biologically relevant isoform of CRP, studies using CRP must address how each isoform influences the systems under study. The binding of both CRP isoforms to cells involves, at least in part, Fc receptors for immunoglobulins, including Fc RI (CD64), Fc RIIa (CD32) and Fc RIIIb (CD16) [2, 12, 13, 31]. The first two receptors (CD64 and CD32) are implicated in the interaction of nCRP with cells. The last receptor (CD16) is implicated, although not considered critical, in the interaction of CRP monomers with leukocytes and endothelial cells [26, 27]. With blood platelets, which only express CD32, the PAF receptor and phosphocholine have also been suggested as potential sites of nCRP binding [19]. As the biological activity of mCRP towards blood platelets (which lack CD16) has been reported [16, 18], we herein investigated possible mechanisms underlying the interactions of mCRP with select platelet membrane receptors. Using a recombinant form of mCRP (m_rCRP), we report that mCRP is a very potent platelet activator capable of interacting with blood platelets *via* the GPIb-IX-V receptor.

Materials and Methods

Chemicals

m_rCRP was prepared as described previously [26]. Briefly, m_rCRP with both cysteine residues mutated to alanine and with an added N-terminal formylmethionine residue was expressed in *Escherichia coli* and isolated from inclusion bodies to obtain > 95% purity. SDS-PAGE analysis revealed a predominant protein band with a molecular weight of 23 kDa. Heat-inactivated protein was generated by placing a sample in a boiling water bath for 1 h. m_rCRP is a good model protein for biological mCRP (expressed from nCRP) and is suitable for comparative studies [24]. Native human C1q complement component was obtained from Merck KGaA (Darmstadt, Germany). Goat polyclonal antibodies to CRP (conjugated with HRP) and sheep polyclonal antibodies to C1q (conjugated with AP) were obtained from Abcam (Cambridge, MA, USA). Recombinant human glycoprotein Ibα (GPIbα), recombinant human CD16b, monoclonal anti-human GPIbα and monoclonal anti-human CD16b antibodies were obtained from R&D Systems Inc. (Minneapolis,

MN, USA). Insoluble equine tendon collagen type I and human thrombin were obtained from Chrono-Log Corp. (Havertown, PA, USA). Sepharose 2B gel was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Fluorolabeled monoclonal antibodies, including anti-CD61 FITC, anti-CD61 PerCP, anti-CD62 PE, and annexin V-PE, as well as other materials for flow cytometry were obtained from Becton Dickinson (San Diego, CA, USA). Fluorolabeled fibrinogen from human plasma (Oregon Green 488 Conjugate, Fg-OG) was obtained from Molecular Probes (Eugene, OR, USA). Purified human von Willebrand factor (vWf) was obtained from Diagnostica Stago (Asnières-sur-Seine, France). Alkaline phosphatase-conjugated rabbit anti-mouse IgG, bovine serum albumin fraction V (BSA), HSA, Arg-Gly-Asp-Ser (RGDS), adenosine diphosphate (ADP), *p*-nitrophenyl phosphate, TMB, cyanogen bromide, polymyxin B, the Endotoxin Gel formation Assay Kit (E-Toxate®) and all other chemicals were obtained from Sigma (St. Louis, MO, USA), unless otherwise stated.

Blood collection

Blood was obtained from healthy donors (49 individuals: 20 men and 29 women; mean age: 29 ± 9 years) under the guidelines of the Helsinki Declaration for human research, and the studies were approved by the committee on the Ethics of Research in Human Experimentation at the Medical University of Lodz. None of the donors had taken aspirin or other drugs affecting platelet function for at least 10 days prior to blood collection or had a history suggesting the occurrence of underlying hemostatic disorders.

Platelet preparation

Blood was drawn through 11-gauge needles into polypropylene tubes into a one-ninth volume of buffered 0.105 M sodium citrate and immediately centrifuged for 12 min at 190 × g to obtain platelet-rich plasma (PRP). Next, PRP was layered on top of BSA-Sepharose 2B gel column prepared as described previously [48]. When the whole volume of the applied PRP entered the gel, Tyrode's buffer was applied to the column to elute platelets. In the studies of annexin V binding to platelets, we used Tyrode's buffer containing 2 mM $CaCl_2$ and 0.1% BSA. The fibrinogen-binding assay was performed in the isolated platelets suspended in Tyrode's buffer containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$. To estimate platelet counts in the suspensions of isolated cells, a photometric method was used [49].

Flow cytometry measurements

Two-color flow cytometry with the use of anti-CD61 as the gating antibody, as well as anti-CD62/PE antibody, annexin V-PE or fibrinogen-OG for staining permitted the analysis of changes in platelet activation and reactivity in the presence of m_rCRP. Isolated platelets were incubated with m_rCRP at a concentration of 2, 5, 10, 20 or 100 μg/ml for 10 min at RT prior to activation (5 min at RT) with 0.5 U/ml thrombin (to measure annexin V binding) or 20 μM ADP (to measure fibrinogen binding). Alternatively, to eliminate the possible interference from endotoxin in m_rCRP preparations, isolated platelets were either co-incubated with polymyxin B (up to 10 μg/ml) or incubated with heat-inactivated m_rCRP (100°C, 1 h). The possible interactions of m_rCRP with the platelet vWf receptor were evaluated by preincubating (10 min) platelets with 2.5 μg/ml anti-GPIbα or 8 μg/ml vWf. Preparation and staining were performed according to the standard Becton Dickinson Procedures for Flow Cytometric Analysis of Platelets with slight modifications [5, 50].

To analyze the expression of P-selectin (CD62P antigen) and phosphatidylserine (PS) exposure on surface membrane in nonactivated or agonist-stimulated platelets, 10-μl aliquots of platelet suspensions were transferred to a mixture of gating and staining antibodies (CD61/FITC and CD62/PE or annexin V-PE). Labeling was allowed to proceed for 30 min in the dark, and the reaction was subsequently stopped by the addition of CellFix solution at a final concentration of 1% v/v. The reported values are presented as fractions of CD62- or annexin V-positive platelets (%) and referred to as the expression of a given target antigen.

The influence of m_rCRP on Oregon Green-labeled human fibrinogen (Fg-OG) binding to blood platelet GPIIb-IIIa was monitored using a previously described procedure [5] with slight modifications. Briefly, isolated platelets were incubated with 2.5 mM RGDS or 100 μg/ml m_rCRP. Cells were then mixed with 0.3 mg/ml Fg-OG and incubated for 10 min (RT) with stirring (300 rpm). To trigger fibrinogen binding, ADP was added at a final concentration of 20 μM, and the platelet suspensions were stirred for an additional

10 min. The specific fibrinogen binding in samples was estimated by subtracting the fibrinogen binding in controls (platelet suspensions incubated with RGDS). The reported values are expressed as the ratios of mean fluorescence intensities in samples and controls ($MFI_{sample}/MFI_{control}$) [5].

Additionally, the fraction of platelet microparticles released upon activation was measured in experiments of P-selectin expression and annexin V binding to platelets. As fibrinogen is involved in the formation of platelet aggregates (acting as a 'bridging molecule' between cells), the fraction of platelet aggregates was also measured in studies of fibrinogen binding. Platelet microparticles and aggregates were distinguished from platelets based on their characteristic flow cytometry profiles of forward light scatter (FSC), corresponding to cell size, *vs.* side light scatter, reflecting cell granularity. The percentages of platelet microparticles and aggregates in the marker-positive platelets were evaluated using a histogram plot analysis. All samples were run as single measurements performed on a flow cytometer. For all samples, 5,000 events were acquired using the LSR II Flow Cytometer (Becton Dickinson).

Platelet adhesion assay

Microplates containing the coating solution of 2 mg/ml fibrinogen or albumin were washed twice with 0.9% NaCl by plate inversion, and the wells were supplemented with ADP (final concentration 10 μM). Then, the suspensions of gel-filtrated platelets in Tyrode's buffer (1.5×10^8 cells/ml) were preincubated with 2, 20 or 100 μg/ml m_rCRP (10 min, RT) and immediately added to the wells (1 h incubation, RT). Platelet adhesion was measured using a microplate reader (Benchmark, BioRad Laboratories GmbH, Muenchen, Germany) according to previously described methods [1, 17].

Platelet aggregation assay

Platelet aggregation in response to 2, 20 or 100 μg/ml m_rCRP or 2 μg/ml collagen was monitored for 10 min in suspensions of isolated platelets adjusted to 3×10^8 cells/ml by using a dual-channel optical aggregometer (Chrono-Log Corp., Hovertown, USA) according to Born's protocol [6]. The readings of maximal aggregation were assessed with the use of the Platelet Aggregation Monitoring and Analysis computer software [35].

Solid Phase Binding Assay

Binding of mCRP to GPIbα

The wells of 96-well microtiter plates were coated overnight at 4°C with GPIbα (10 μg/ml in PBS, 1 μg of protein per well). In parallel, the plates were coated with either HSA or proteins that specifically interact with m_rCRP, i.e., CD16b or C1q (1 μg of protein per well). Unbound aliquots of proteins were washed off the wells, and the nonspecific binding sites were blocked by incubation with 1% BSA in Tris-buffered saline (TBS, pH 7.4) containing 0.05% Tween 20 (TBST) (1 h RT). Direct binding assays were performed by adding increasing concentrations of m_rCRP dissolved in TBST containing 1 mM $CaCl_2$ to the immobilized GPIbα, CD16b, C1q or HSA coated onto plates that were subsequently incubated for 1 h at RT. Unbound m_rCRP was aspirated, and the wells were washed three times with TBST. To detect the bound m_rCRP, the plates were incubated for 1 h (at RT) with polyclonal antibodies to CRP conjugated with horseradish peroxidase and then washed with TBST. The reaction was developed using tetramethylbenzidine (TMB), and the absorbance was monitored at 450 nm. Prior to data analysis, the background unspecific binding to HSA was subtracted from that read for the samples.

In parallel, direct binding of m_rCRP to vWf or anti-GPIbα (1 μg of protein per well) was assessed by adding increasing concentrations of m_rCRP dissolved in TBST containing 1 mM $CaCl_2$ to the vWf- or anti-GPIbα-coated wells.

Binding of GPIbα to mCRP

The wells of 96-well microtiter plates were coated overnight at 4°C with m_rCRP (1 μg of protein per cell). In parallel, to detect nonspecific binding, the plates were coated with HSA (1 μg/cell). Unbound aliquots of proteins were washed off the wells, and nonspecific binding sites were blocked by the incubation with 1% BSA in TBST, ph 7.4 (1 h, RT). Direct binding assays were performed by adding increasing concentrations of GPIbα, CD16b or C1q dissolved in TBST containing 1 mM $CaCl_2$ to the m_rCRP- or HSA-coated wells, and the plates were subsequently incubated for an additional hour at RT. Unbound portions of GPIbα or C1q were aspirated, and the wells were washed three times with TBST. To detect GPIbα or CD16b, the plates were incubated with monoclonal

anti-GPIbα or anti-CD16b, respectively, for 1 h at RT. Furthermore, the wells were washed three times with TBST and incubated for an additional hour at RT with rabbit anti-mouse secondary antibodies conjugated with alkaline phosphatase. The bound C1q was detected using a polyclonal anti-C1q antibody conjugated with alkaline phosphatase. After washing with TBST, *p*-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer, pH 9.8) was added, and color development was analyzed at 405 nm on a microplate reader. Prior to data analysis, the background binding to HSA was subtracted from the readings of samples.

Surface plasmon resonance (SPR) analysis

SPR analysis of the interaction between m_rCRP and its potential (GP1bα) and known ligand (C1q) was performed using a BIAcore X (Pharmacia Biotech Inc.). Briefly, protein solutions were diluted to concentrations of 20–40 μg/ml in 10 mM acetate buffer (pH 3.5–5) and then covalently attached to the carboxymethylated dextran surface of a CM5 sensor chip (BIAcore AB) using amine coupling chemistry (BIAcore AB amine coupling kit) according to the manufacturer's instructions. For each protein, different levels of baseline SPR signals after binding to the sensor chip were observed (GPIbα = 1500 RU, C1q = 9000 RU). A sensor chip with immobilized BSA (3000 RU) was used as a control for binding specificity. Experiments were performed at 25°C with the use of PBS as a running buffer (pH 7.4) with a flow rate of 10 μl/min and injection volume of 20 μl. Sensorgrams were analyzed with BIAevaluation 3.1 software (BIAcore AB). The association and dissociation constants were determined by global fitting of the data using the Langmuir binding model (one-to-one interaction). Each dissociation constant (K_D) was obtained by calculating the ratio of the dissociation and association rate constants (k_d/k_a): $K_A = K_D^{-1}$.

Statistical analysis

The results are expressed as arithmetic means ± standard error (SE). The normality of the data distribution was verified with Shapiro-Wilk's test. The normally distributed raw data were analyzed with either pairwise or unpaired Student's *t*-test or one-way analysis of variance with the Bonferroni correction for multi-

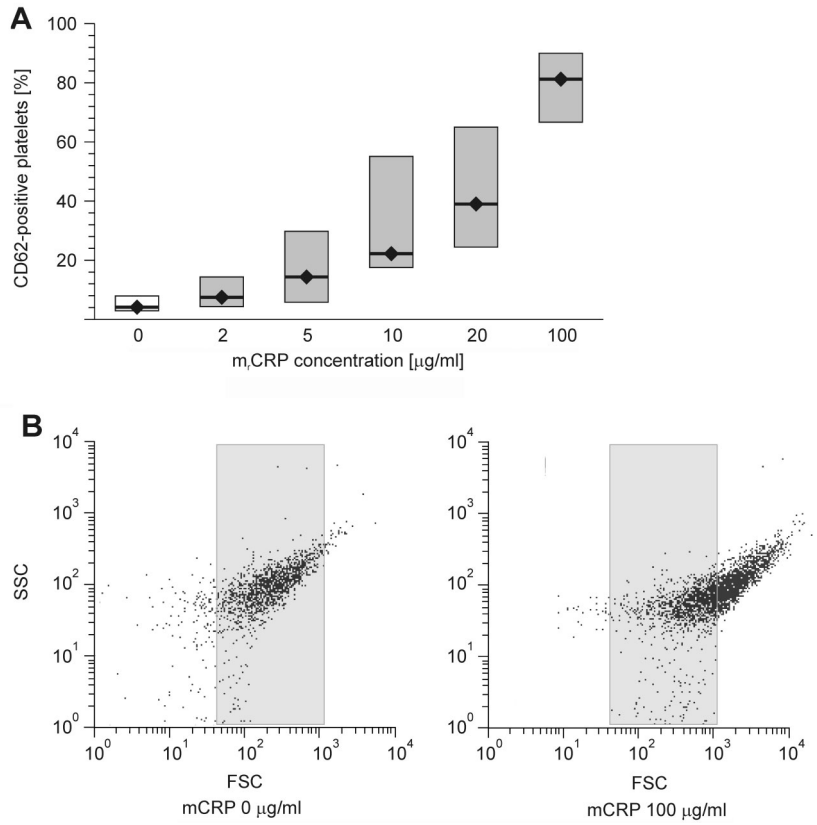

Fig. 1. Effect of mCRP on CD62 expression in resting platelets. (**A**) Data are presented as the means ± SE (n = 17). The isolated platelets were preincubated with 0, 2, 5, 10, 20 or 100 μg/ml m_rCRP for 10 min at RT. The fractions of P-selectin–positive platelets (**A**) and microparticles (**B**) were analyzed in cells fixed in 1% paraformaldehyde and double-labeled with anti-CD61-FITC (gating antibody) and anti-CD62P-PE (30 min, RT). All comparisons between m_rCRP and control were statistically significant at $p < 0.0002$ (lower-tail Wilcoxon's sign rank test with Bonferroni's correction for multiple comparisons). (**B**) Exemplary dot plots of anti-CD61-gated objects (referred to as "blood platelets") prior to and after their incubation with 100 μg/ml m_rCRP (for experimental details, see above). In both dot plots, the framed region refers to the population of 'normoplatelets;' the objects located on the left of this region on the FSC axis were considered platelet microparticles, and those located on the right were considered aggregates

ple comparisons. Some data departing from normality were assessed with the nonparametric Mann-Whitney U test. Pearson's linear correlations or Spearman's rank correlations were used to assess potential associations between parameters.

Results

Changes in platelet activation in the presence of mCRP

Figure 1A shows the effects of the increasing concentrations of m_rCRP on platelet degranulation expressed as the percentage of platelets expressing P-selectin (CD62-positive platelets). Preincubating isolated platelet suspensions with m_rCRP resulted in significant platelet degranulation at all m_rCRP concentrations tested. The observed effect was strongly dose-dependent ($R_P = 0.66$, $p < 0.0001$).

Similar changes were observed when platelet-derived microparticles were analyzed. In the presence of m_rCRP, platelets dose-dependently formed more microparticles than control platelets (absence of m_rCRP) ($R_P = 0.3$, $p < 0.005$), with the greatest changes observed at 100 μg/ml m_rCRP (19.7 ± 3.1% *vs*. 10.4 ± 1.9% in control ($p < 0.001$)) (Fig. 1B). Preincubating platelets with either monoclonal anti-GPIbα (2.5 μg/ml) or anti-vWf (8 μg/ml) had no significant effect on the observed m_rCRP-mediated degranulation.

Tab. 1. The influence of m_rCRP on annexin V binding to blood platelets. Data are presented as the means ± SE (n = 6). Isolated platelets suspended in Tyrode's buffer supplemented with 2 mM $CaCl_2$ and 0,1% BSA were incubated with 2, 20 or 100 μg/ml m_rCRP for 10 min at RT prior to their activation with thrombin (0.5 U/ml, 5 min, RT). The fractions of annexin-positive platelets and microparticles were analyzed in cells double-labeled with anti-CD61-FITC (gating antibody) and annexin V-PE (30 min, fixed in 1% CellFix). Statistically significant differences between m_rCRP and control (pairwise Student's *t*-test) are presented in the table

	Annexin-positive platelets (%)	
	Resting platelets	Activated platelets
Control (no m_rCRP)	2.9 ± 0.8	10.6 ± 3.5
m_rCRP 2 μg/ml	2.9 ± 0.6	8.8 ± 2.3
m_rCRP 20 μg/ml	5.7 ± 1.0 ($p < 0.015$)	11.1 ± 2.7
m_rCRP 100 μg/ml	10.4 ± 2.2 ($p < 0.035$)	11.1 ± 2.8

To eliminate the possibility that the observed m_rCRP effects might be due to endotoxin contamination, m_rCRP was admixed with polymyxin B (10 μg/ml) prior to its addition to platelet suspensions. Polymyxin B, which binds and inactivates endotoxin in protein preparations, did not significantly alter m_rCRP-induced platelet degranulation (data not shown). The specific activity of m_rCRP was further evaluated by strong heat denaturation of the protein in a 100°C water bath for 1 h. All noted m_rCRP effects were abolished after heat inactivation (data not shown).

The effect of mCRP on platelet procoagulant activity

Platelet procoagulant activity in the presence of m_rCRP was quantified and expressed as the fraction of annexin V-positive cells. Preincubation of isolated platelets with 20 or 100 μg/ml m_rCRP led to a considerably increased fraction of annexin V-positive platelets (Tab. 1). The effect of m_rCRP on annexin V binding was not detectable when platelets were activated with thrombin (0.5 U/ml; 10 min, RT).

Influence of mCRP on fibrinogen binding to platelets

We compared the role of m_rCRP in mediating the interaction of exogenously added fibrinogen with blood platelets. The effect of m_rCRP on fibrinogen binding to blood platelets was measured at a high m_rCRP concentration (100 μg/ml) in both resting and ADP-activated platelets. Spontaneous fibrinogen binding was strongly augmented in platelets preincubated with m_rCRP (mean fluorescence intensity of Fg-OG-positive cells in the presence of 100 μg/ml m_rCRP was 159.7 ± 44.8 compared to 3.5 ± 0.8 in control, $p < 0.02$, $n = 7$). Incubation of ADP-stimulated platelets with m_rCRP again led to a significant increase in fibrinogen binding from 35.6 ± 9.4 in control to 187.4 ± 56.2 in the presence of 100 μg/ml m_rCRP ($p < 0.05$). Changes in fibrinogen binding to the platelet surface were accompanied by parallel changes in the proportion of platelet aggregates: the fractions of aggregates in control and m_rCRP-stimulated platelets were 0.15 ± 0.02% *vs*. 2.25 ± 0.6% ($p < 0.02$) for nonactivated platelets and 0.24 ± 0.06% *vs*. 2.47 ± 0.50% ($p < 0.01$) for ADP-stimulated platelets ($n = 7$).

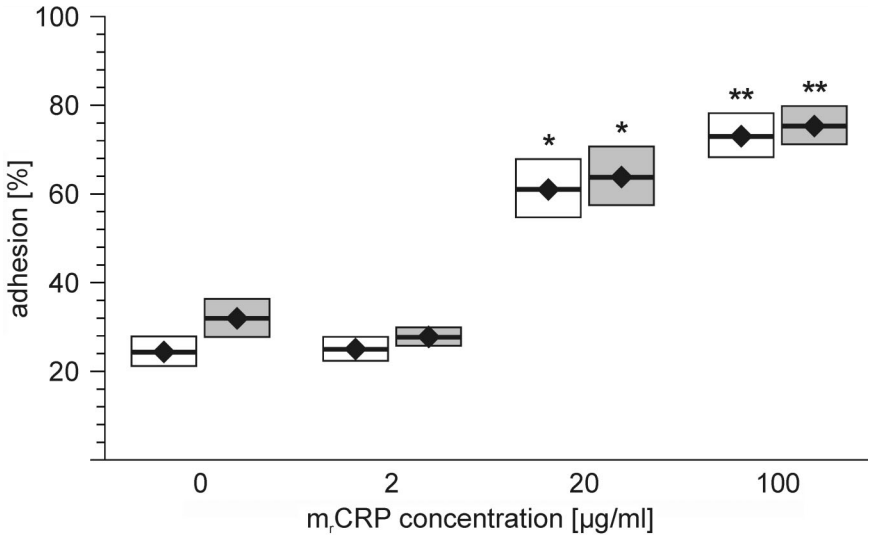

Fig. 2. Effects of mCRP on spontaneous and ADP-stimulated platelet adhesion. Data are presented as the means ± SE (n = 5). The adhesion of resting (white bars) or ADP-stimulated platelets (grey bars) in the presence of 0, 2, 20 or 100 µg/ml m_rCRP was measured on fibrinogen-coated plates. Statistically significant differences (paired Student's *t*-test with Bonferroni's correction) are indicated by asterisks (* $p < 0.05$, ** $p < 0.005$)

The effect of mCRP on platelet adhesion

mCRP itself significantly intensified the adhesion of isolated platelets to fibrinogen in a dose-dependent manner. As a positive control, ADP (10 µM) induced significantly increased platelet adhesion to fibrinogen (from 24.4 ± 3.2% in nonstimulated platelets to 32.0 ± 4.3% in ADP-induced platelets, $p < 0.0025$). As a negative control, the extent of adhesion of isolated platelets to albumin was found to be in the range of 4.3 to 14.2% (n = 5) and remained higher in comparison to values of adhesion to albumin measured in PRP (2.1 ± 0.7%, n = 3). Preincubating platelets with m_rCRP at concentrations of 20 and 100 µg/ml resulted in over a 2-fold, statistically significant increase in spontaneous platelet adhesion to fibrinogen ($p < 0.05$ and $p < 0.005$ for 20 and 100 µg/ml m_rCRP, respectively) (Fig. 2). A similar intensifying effect of m_rCRP on platelet adhesion to fibrinogen was also revealed in the presence of ADP (adhesion increased by 32% ($p < 0.05$) and 43% ($p < 0.005$) in the presence of 20 and 100 µg/ml m_rCRP, respectively).

The effect of mCRP on platelet aggregation

When monitoring platelet aggregation, m_rCRP was found to act as a very potent platelet agonist. Upon direct stimulation of isolated platelets with m_rCRP, we observed vastly increased maximal platelet aggregation up to 41.5 ± 6.3 AU (NS, n = 6), 68.1 ± 4.4 AU (NS, n = 7) and 71.7 ± 2.7 AU ($p = 0.01$, n = 8) for 2,

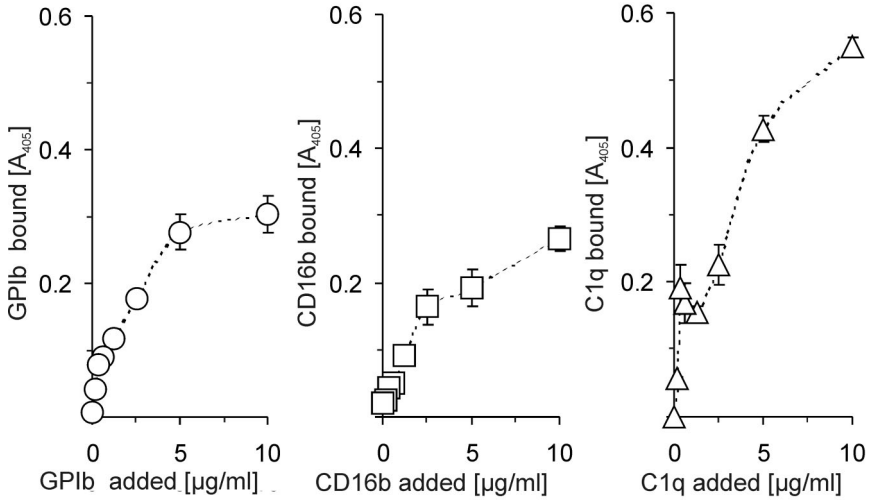

Fig. 3. Binding of GPIbα, CD16b and C1q to immobilized m_rCRP. Data are presented as the means ± SE of three separate experiments. mCRP was coated onto ELISA plates and incubated with increasing concentrations of GPIbα (O), CD16b (□) and C1q (Δ). The bound proteins were evaluated by ELISA using specific antibodies. The significance of differences, as estimated with the Kruskal-Wallis nonparametric test and the *post-hoc* all pairwise comparisons Conover-Inman test, was as follows: GPIbα (O): $\mu_{mrCRP\ 0} \neq \mu_{mrCRP \geq 0.3\ \mu g/ml}$, $p < 0.0001$; CD16b (□): $\mu_{mrCRP\ 0} \quad \mu_{mrCRP \geq 0.3\ \mu g/ml}$, $p < 0.01$; C1q (Δ): $\mu_{mrCRP\ 0} \quad \mu_{mrCRP \geq 0.3\ \mu g/ml}$, $p < 0.0001$.

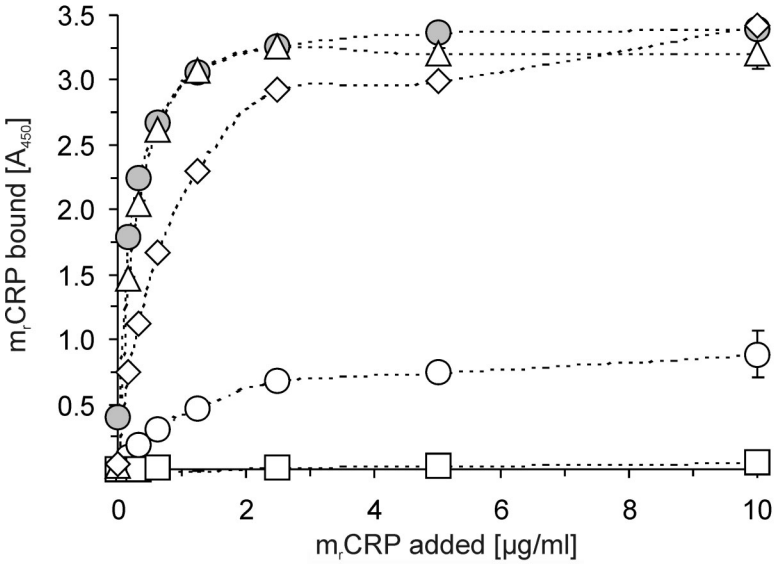

Fig. 4. Binding of m_rCRP to immobilized GPIbα, vWf, anti-GPIb , CD16b and C1q. Data are presented as the means ± SE of three separate experiments. GPIbα (O), vWf (◇), anti-GPIbα (Δ), CD16b (□) and C1q (◎) were coated onto ELISA plates and incubated with increasing concentrations of m_rCRP. The bound m_rCRP was evaluated by ELISA using polyclonal antibodies to CRP. The significance of differences, as estimated with the Kruskal-Wallis nonparametric test and the *post-hoc* all pairwise comparisons Conover-Inman test, was as follows: GPIbα (O): $\mu_{mrCRP\ 0} \neq \mu_{mrCRP \geq 0.3\ \mu g/ml}$, $p < 0.002$; vWf (◇): $\mu_{mrCRP\ 0} \quad \mu_{mrCRP \geq 0.15\ \mu g/ml}$, $p < 0.0001$; anti-GPIbα (Δ): $\mu_{mrCRP\ 0} \quad \mu_{mrCRP \geq 0.15\ \mu g/ml}$, $p < 0.0001$; CD16b (□): $\mu_{mrCRP\ 0} \quad \mu_{mrCRP \geq 2.5\ \mu g/ml}$, $p < 0.0001$; C1q (◎): $\mu_{mrCRP\ 0} \quad \mu_{mrCRP \geq 0.3\ \mu g/ml}$, $p < 0.005$

20 and 100 μg/ml m_rCRP, respectively, compared to 40.1 ± 5.1 AU in 2 μg/ml collagen-activated platelets.

Interactions between GPIbα and mCRP

Enzyme-linked immunosorbent assay (ELISA) experiments and SPR analysis

We evaluated the binding of GPIbα, CD16b and C1q complement component to mCRP-coated microplate wells. All proteins tested in the concentration range of 0.15–10 μg/ml (0.015–1 μg of protein per well) bound reproducibly to m_rCRP. Significant binding of GPIbα to m_rCRP was observed with 0.15 μg/ml GPIbα, whereas significant binding of CD16b and C1q was observed with 0.3 μg/ml protein (Fig. 3).

A parallel complementary analysis was performed for m_rCRP binding to GPIbα, CD16b and C1q coated onto the plates. m_rCRP significantly bound to immobilized GPIbα and C1q even at the lowest m_rCRP concentration tested (0.15 μg/ml). With immobilized CD16b, however, m_rCRP binding was very low and was only significant at concentrations exceeding 5 μg/ml (Fig. 4). Of note, the binding of m_rCRP to C1q significantly exceeded that observed for GPIbα; with 100 μg/ml m_rCRP, there was almost 4-fold higher binding of m_rCRP to C1q compared to its binding with GPIbα ($p = 0.0002$) (Fig. 4). Using the same technique, we demonstrated the concentration-dependent binding of m_rCRP to vWf (up to 2.5 μg/ml; $p < 0.0001$) and anti-GPIbα (up to 1.25 μg/ml; $p < 0.0001$) (Fig. 4).

We also confirmed the ability of m_rCRP to bind to GPIbα and C1q by using an alternate technique, SPR, applying the matrix-immobilized GPIbα and m_rCRP as a soluble ligand. To determine the kinetic parameters of the interaction between GPIbα or C1q and m_rCRP, the SPR sensorgrams were recorded at varying m_rCRP concentrations (data not shown). The values of k_a, k_d and K_D were calculated in accordance with the Langmuir adsorption model and are listed in Table 2. The K_D of 1.44×10^{-7} M for GPIbα suggests a stable interaction between the immobilized GPIbα and the soluble m_rCRP.

Tab. 2. Kinetic parameters of the interaction between soluble m_rCRP and immobilized GPIbα or C1q monitored by surface plasmon resonance (SPR). K_D was calculated as the ratio (k_d/k_a) of dissociation and association rate constants as $K_A = K_D^{-1}$. Sensorgrams were analyzed with BIAevaluation 3.1 software, and the constants were determined by global fitting of the data using the Langmuir binding model (for details, see Materials and Methods)

m_rCRP binding to	k_a(1/Ms)	k_d(1/s)	K_A(1/M)	K_D(M)	χ^2
GP1bα	4.25E + 02	6.10E-05	6.96E + 06	1.44E-07	0.12
C1q	9.24E + 04	3.51E-07	2.63E + 11	3.80E-12	11.3

Discussion

Our present paper investigates the possible mechanisms underlying the interactions between mCRP and blood platelets. We reveal for the first time that one of the major platelet receptors, the GPIb-V-IX complex, the receptor for vWf and thrombin, may be an important mediator of such interactions.

Initially, we demonstrated that m_rCRP has the potential to effectively stimulate platelet function under *in vitro* conditions. To exclude any possible interactions between plasma proteins and mCRP in the observed mCRP-platelet effects, most platelet studies have been performed in suspensions of isolated platelets. Because mCRP has been reported to be a powerful platelet agonist when used at 100 μg/ml [41] and because CRP acute-phase levels during inflammation are generally reported to be between 2 and 100 μg/ml [20, 38], we used m_rCRP in a wide range of concentrations. At the lowest concentration, considerable effects of m_rCRP were observed in the experiments of platelet degranulation and aggregation, whereas platelet adhesion was significantly affected only by higher m_rCRP concentrations. It should be noted that mCRP evoked significant platelet function even without any further supplementation with other subthreshold stimuli, which corroborates the recent findings reported by Molins et al., who observed mCRP-induced thrombosis by promoting platelet deposition and thrombus growth on the collagen surface [33]. The outcomes of the present study are consistent with earlier reports evaluating the ability of biologically derived mCRP (i.e., mCRP produced from nCRP by short-term heating to 70°C) to stimulate platelet aggregation and secretion [18, 46]. Regardless of whether prepared from nCRP by heating, urea chelation, acid treatment, or by recombinant expression, mCRP has been shown to exert stimulatory effects on the regulation of shear-induced platelet adherence to neutrophils and neutrophil aggregation [28], promote neutrophil-endothelial cell adhesion [52] and a proinflammatory endothelium phenotype [27] and exhibit anti-cancer effects in a mouse model of mammary adenocarcinoma [29]. Interestingly, in the work by Khreiss et al. [26], mCRP prevented annexin binding to neutrophils and attenuated neutrophil DNA fragmentation, leading to the repression of neutrophil apoptosis and thereby amplifying the acute inflammatory response. Our results indicated that m_rCRP enhanced the binding of annexin to blood platelets and the formation of platelet-derived microparticles, thus pointing to the role of mCRP as an agonist in platelet activation. We also demonstrated the enhanced exposure of procoagulant surface and release of intraplatelet granules by m_rCRP, two processes required for normal hemostasis and important in antimicrobial host defense and the induction of inflammation and tissue repair. Furthermore, our findings on the stimulatory effect of m_rCRP on the binding of fibrinogen to GPIIb-IIIa complex on platelet surface remain in line with our own earlier report in which we postulated that the modulation of platelet function by pentameric nCRP might be mediated by GPIIb-IIIa [5]. Our observation was later confirmed in a study by Brennan et al.; however, considering the experimental protocol used by the authors, it appears that they might have observed the interplay between GPIIb-IIIa and mCRP rather than nCRP [7].

Recently, Eisenhardt et al. reported an interesting mechanism of dissociation of nCRP to mCRP by cell membrane components on activated platelets or other cells [16]. Although the paper emphasizes the need to use nonactivated cells when studying nCRP, it also validates and extends the importance of our findings presented herein. The significant (patho)physiological implication emerging from our study is that the presence of mCRP, whether added exogenously or generated *in situ* from nCRP, is an important activator of circulating blood platelets. Its levels and activities may become intensified *via* self-perpetuating positive feedback mechanisms, thus contributing to the development of prothrombotic states.

Knowledge of the mechanisms responsible for the interactions of mCRP with platelets appears fundamental in defining the role of mCRP as an augmenting protein that boosts the activated natural immune responses of different cell types on the one hand, and on the other hand, functions as a physiologically meaningful mediator of platelet function and inflammatory reaction [44]. Therefore, in parallel to platelet functional studies, we searched for the potential target(s) of mCRP on the platelet surface. We focused on investigating the ability of mCRP to bind to GPIbα, the major ligand-binding subunit of the platelet GPIb-IX-V complex. Using two-directional ELISA analysis (with immobilization of either mCRP or GPIbα) and recombinant human GPIbα, we demonstrated the direct binding of mCRP to GPIbα. Immo-

bilized mCRP bound to GPIbα as efficiently as it did to CD16b, the receptor known to mediate some neutrophil and endothelial cell interactions of mCRP [26, 27]. Surprisingly, when the receptors were immobilized onto the plate, GPIbα, but not CD16b, bound mCRP, which may be explained by possible structural changes induced in the CD16b molecule because of immobilization onto a hydrophobic plastic surface. Nonreciprocal binding reactions are widely reported in the literature and reflect changes in protein structure (and by inference, binding sites) when adsorbed to a plastic surface [9, 43]. To validate our assays and to underscore that mCRP can indeed bind CD16 expressed on the cell surface, we confirmed earlier functional studies on the CD16-dependent action of m_rCRP on leukocytes [26, 28].

Importantly, regardless of whether mCRP or GPIbα was immobilized, the extent of the binding of mCRP with GPIbα was significantly lower compared to its binding with C1q, which indicates the higher affinity of mCRP to the complement pathway over the cell membrane receptor GPIbα. Conversely, our SPR analysis indicates that a very stable interaction can occur between soluble mCRP and immobilized GPIbα, the part of the vWf receptor that plays a pivotal role in regulating platelet adhesion to damaged blood vessels at high shear stress [11]. Taken together, our data suggest platelet GPIb-IX-V complex as a new ligand for mCRP.

Although GPIbα and the GPIb-IX-V complex are involved in mCRP-mediated platelet activation, our findings indicate that neither the binding of monoclonal anti-GPIbα nor the binding of a vWf – a natural ligand for the GPIb-IX-V complex – result in inhibition of the mCRP-mediated degranulation of platelet α-granules. Such an outcome may be a simple implication of the fact that m_rCRP is likely to interact with domains of the GPIbα subunit other than anti-GPIb or vWf. As mCRP has been shown to bind cholesterol and to enter into lipid raft domains in the cell membrane [23], it is possible that mCRP interacts with and stimulates GPIbα activities within the intramembrane zone of this cell-bound receptor. Monoclonal anti-GPIb and vWf binding would be expected to occur at sites far removed from the cell membrane zone and more accessible to the extracellular aqueous environment. This possibility is even more intriguing when one considers that the GPIb-IX-V complex typically becomes internalized in the course of an ongoing platelet activation, exhibiting lower surface membrane expression with increased platelet activation [32, 36]. mCRP may bind to and initiate GPIb cell internalization, which in turn triggers the inside-out expression of α-granule contents (CD62) and phosphatidylserine (annexin positivity). Hence, the role of GPIb in the mCRP-mediated platelet response might be restricted to the initial steps of activation when the expression of GPIb-IX-V in platelet surface membranes remains substantial. Taken together, the interaction of mCRP with a protein receptor such as GPIbα may not be an absolute requirement for activating platelets, but merely one of several alternate mechanisms of blood platelet activation by mCRP. Further investigations are certainly needed to localize and characterize the binding sites for mCRP on the GPIbα subunit of the vWf receptor.

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