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J Immunol 2013; 190:6457-6467; Prepublished online 15 May 2013;
doi: 10.4049/jimmunol.1300610
http://www.jimmunol.org/content/190/12/6457

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Identification of a Novel Mode of Complement Activation on Stimulated Platelets Mediated by Properdin and C3(H_2O)

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Elevated numbers of activated platelets circulate in patients with chronic inflammatory diseases, including atherosclerosis and coronary disease. Activated platelets can activate the complement system. Although complement activation is essential for immune responses and removal of spent cells from circulation, it also contributes to inflammation and thrombosis, especially in patients with defective complement regulation. Proinflammatory activated leukocytes, which interact directly with platelets in response to vascular injury, are among the main sources of properdin, a positive regulator of the alternative pathway. The role of properdin in complement activation on stimulated platelets is unknown. Our data show that physiological forms of human properdin bind directly to human platelets after activation by strong agonists in the absence of C3, and bind nonproportionally to surface CD62P expression. Activation of the alternative pathway on activated platelets occurs when properdin is on the surface and recruits C3b or C3(H_2O) to form C3b,Bb or a novel cell-bound C3 convertase [C3(H_2O),Bb], which normally is present only in the fluid phase. Alternatively, properdin can be recruited by C3(H_2O) on the platelet surface, promoting complement activation. Inhibition of factor H–mediated cell surface complement regulation significantly increases complement deposition on activated platelets with surface properdin. Finally, properdin released by activated neutrophils binds to activated platelets. Altogether, these data suggest novel molecular mechanisms for alternative pathway activation on stimulated platelets that may contribute to localization of inflammation at sites of vascular injury and thrombosis. ***The Journal of Immunology*****, 2013, 190: 6457–6467.**

Platelets are small and anucleate circulating particles that derive from bone marrow megakaryocytes. These cells play a central role in hemostasis, inflammation, and disease-related thrombosis, because they are the first circulating blood cells that, upon activation, rapidly adhere to tissue, to leukocytes (recruitment), and to one another in response to vascular injury (1, 2). However, excessive or inadvertent platelet activation is common at sites of endothelial damage, underlying many cardiovascular disorders, such as myocardial infarction, unstable angina, and stroke (3). Patients with chronic inflammatory conditions, such as unstable atherosclerosis, hypercholesterolemia, and coronary disease, have a higher number of activated platelets (4, 5) and platelet/leukocyte aggregates (6) circulating in the blood.

Stimulated platelets activate the complement system on or near their surface (7–12). In general, normal inflammatory processes require complement activation for an effective immune response, as well as for efficient removal of spent cells from circulation. In pathological acute and chronic inflammatory diseases (e.g., systemic lupus erythematosus, cancer, atherosclerosis, ischemia/reperfusion injury, neuroinflammation), excessive complement activation contributes to tissue damage and leads to elevated release of proinflammatory by-products (e.g., C5a, C3a) and C5b-9 end product (membrane attack complex [MAC]) (13), which, in turn, participate in leukocyte recruitment, vascular inflammation, platelet activation, and thrombosis (14, 15). Thus, understanding the molecular mechanisms by which complement activates on stimulated platelets becomes essential for understanding its role in platelet-mediated physiology and disease pathogenesis. Both the classical (7) and the alternative pathways (9) were shown to activate on the platelet surface, despite the presence of complement regulatory proteins. In addition, individuals with paroxysmal nocturnal hemoglobinuria or atypical hemolytic uremic syndrome (aHUS), diseases in which the activity of one or more complement regulatory proteins (e.g., factor H, CD59, CD55, CD46) is impaired (16, 17), have exacerbated complement activation on their platelets (18, 19). However, recent studies indicate that complement activates in the microenvironment surrounding the stimulated platelet upon release of chondroitin sulfate A (10), but not on the platelet surface, when complement regulation is intact (11). Therefore, the mechanisms by which complement activates on stimulated platelets remain controversial.

The alternative pathway of complement represents a true safeguard system of the human host and is initiated in the fluid phase by the spontaneous hydrolysis of the thioester bond in C3 to produce C3(H_2O), which is functionally and structurally similar to C3b

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Received for publication March 5, 2013. Accepted for publication April 9, 2013.

This work was supported by National Institutes of Health Grant 1P30HL101317-01, American Heart Association Grant 12GRNT10240003, and the University of Toledo DeArce-Koch Memorial Endowment Fund (all to V.P.F.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: aHUS, atypical hemolytic uremic syndrome; COX, cyclooxygenase; E_SC3b, C3b-coated sheep erythrocyte; GAG, glycosaminoglycan; MAC, membrane attack complex; NHS, normal human serum; P_2, dimeric form of properdin; P_3, trimeric form of properdin; P_4, tetrameric form of properdin; PMN, polymorphonuclear cell; P_n, nonphysiological aggregated form of properdin; rH19-20, recombinant protein consisting of domains 19–20 of factor H; RT, room temperature; Tyrode/PGE/Hep buffer, Tyrode's buffer containing PGE_1 and heparin.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1300610

(20) (tickover theory; reviewed in Ref. 21). Binding of factor B to C3(H_2O) in the presence of factor D allows the generation of an unstable fluid-phase C3 convertase [C3(H_2O),Bb], which has the ability to digest C3 to generate C3b fragments (22). The C3b fragments can bind to any nearby surface with exposed amino or hydroxyl groups (23). Factor B can then bind to this membrane-bound C3b and be cleaved by factor D to generate the membrane-bound alternative pathway C3 convertase (C3b,Bb) (24). Properdin, the only known positive regulator of the alternative pathway, stabilizes the C3b,Bb complex, increasing its $t_{1/2}$ by 5–10-fold (25), making possible the efficient amplification of C3b deposition on target surfaces (24). Del Conde et al. (9) showed that C3b binds directly to activated platelets by using P-selectin (CD62P) as a receptor, and they proposed this as a mechanism by which alternative pathway activation occurs on the surface of activated platelets. In contrast, Hamad et al. (11) detected binding of only C3(H_2O) to stimulated platelets, which was not the result of proteolytic cleavage of C3 or alternative pathway complement activation.

Properdin is found in plasma at a concentration of 4–25 μg/ml (26). It is mainly produced by leukocytes, including monocytes (27), T cells (28), and neutrophils (29). These cells release properdin upon stimulation with TNF-α, PMA, C5a, and fMLP and may significantly increase the local concentration of properdin, especially at sites of inflammation (reviewed in Refs. 30, 31). Shear-stressed endothelial cells can also produce properdin (32). The 53-kDa properdin monomers associate in a head-to-tail manner, generating dimeric (P_2), trimeric (P_3), and tetrameric (P_4) physiological forms of properdin that are present in plasma in a ratio of 26:54:20 (reviewed in Ref. 31; 33). Recently, we and other investigators reported that properdin, in addition to stabilizing the alternative pathway convertases, acts as a highly selective pattern recognition molecule by binding directly to certain surfaces [e.g., apoptotic and necrotic cells (34–37) and *Chlamydia pneumoniae* (38)] and serving as a platform for de novo C3b,Bb assembly (reviewed in Ref. 31). In this study, we investigated the role of properdin in complement activation on platelets, because among the main sources of properdin are activated granulocytes and monocytes that interact with platelets during inflammatory and thrombotic syndromes (forming platelet/leukocyte aggregates) (reviewed in Ref. 39) and are present in increased numbers at sites of physiological and pathological inflammation where platelets and complement play essential roles.

Our data show that alternative pathway complement activation on activated platelets occurs when properdin is bound to stimulated, but not resting, platelets. Properdin binds to activated platelets in a manner that is not proportional to CD62P surface exposure; the level of binding varies, depending on the platelet agonist used, and does not require the presence of C3 fragments on the platelet surface. The platelet-bound properdin recruits C3(H_2O) and/or C3b to the surface of activated platelets and forms a novel cell-bound C3(H_2O) convertase [C3(H_2O),Bb] or C3b,Bb. Moreover, C3(H_2O) on the platelet surface can also initiate complement activation by recruiting properdin and factor B. Finally, properdin freshly secreted by stimulated polymorphonuclear leukocytes binds to activated platelets. Our results define a novel molecular mechanism by which the alternative pathway of complement activates on stimulated platelets that is mediated by the physiological forms of properdin and C3(H_2O).

Materials and Methods

Buffers

The buffers used were citrate buffer (9.35 mM Na_3Citrate, 4.75 mM citric acid, 17.35 mM dextrose, 145 mM NaCl [pH 6.5]); Tyrode's buffer (136.9 mM NaCl, 2.7 mM KCl, 983.8 μM $MgCl_2$ $6H_2O$, 3.2 mM Na_2HPO_4, 3.5 mM HEPES, 0.35% BSA, 5.5 mM dextrose, 2 mM $CaCl_2$ [pH 7.4]); Tyrode/PGE/Hep buffer (Tyrode's buffer containing 1 μM PGE_1 and 2 IU/ml heparin); Gelatin veronal buffer (5 mM veronal, 145 mM NaCl, 0.004% NaN_3, 0.1% gelatin); PBS (10 mM sodium phosphate, 140 mM NaCl, 0.02% NaN_3 [pH 7.4]); Mono S buffer A (50 mM sodium phosphate [pH 6]); Mono S buffer B (50 mM sodium phosphate, 0.5 M NaCl [pH 6]); and Mg-EGTA buffer stock (0.1 M $MgCl_2$, 0.1 M EGTA [pH 7.3]).

Abs

The following murine mAbs were used in this study: IgG1 anti-human properdin (#1; Quidel), IgG1 isotype control (eBioscience), IgG1 anti-human C3/C3b (Cedarlane Laboratories), PE-conjugated IgG1 anti-human C3/C3b (Cedarlane Laboratories), allophycocyanin-conjugated IgG1 anti-human CD42b (BioLegend), PE/Cy5-conjugated IgG1 anti-human CD62P (BioLegend), IgG2a anti-human C5b-9 neo-epitope (Dako), IgG2a anti-human factor Bb neoantigen (AbD Serotec), Alexa Fluor 488-conjugated IgG1 anti-human CD11b (BioLegend), and PE-conjugated IgG2a anti-human CD16b (BioLegend). The following polyclonal Abs were used: Alexa Fluor 488-conjugated goat anti-mouse polyclonal IgG (Invitrogen) and F(ab′)$_2$ polyclonal goat anti-C3b IgG (LifeSpan BioSciences).

Serum and complement proteins

Purification of properdin (34) and C3 (40), as well as the generation of C3b (41), was carried out as previously described. Properdin-depleted serum, normal human serum (NHS), factor D, and factor B were purchased from CompTech.

Separation of physiological forms of properdin

Physiological polymeric forms of properdin (P_2–P_4) were separated from nonphysiological aggregated forms of properdin (P_n) by gel-filtration chromatography. The P_n forms are known to accumulate after prolonged storage and freeze/thaw cycles and induce nonspecific complement activation in solution (33) and on certain surfaces (reviewed in Ref. 31; 34). Briefly, pure properdin (5 mg) was loaded onto a Phenomenex Bio Sep-Sec-S4000 column (600 × 7.8 mm) with a guard column (75 × 7.8 mm) and eluted at a flow rate of 0.5 ml/min in PBS. Purified, physiological forms of properdin were stored at 4°C and used within 2 wk of separation, as previously described (33, 34).

Platelet isolation and activation

Human platelets were isolated via venipuncture from the blood of healthy donors. The Institutional Review Board from the University of Toledo College of Medicine and Life Sciences approved the protocols, and written informed consent was obtained from all donors, in accordance with the Declaration of Helsinki. Blood was drawn into ACD tubes (BD Vacutainer), and platelet-rich plasma was separated by centrifugation at 200 × *g* for 15 min at room temperature (RT) with no brake. The platelet-rich plasma was collected, and platelets were washed twice using citrate buffer at 440 × *g* for 10 min at RT. Platelets were resuspended to a final concentration of 1 × 10^8 cells/ml in Tyrode's buffer. Agonists used to activate the platelets included thrombin (Sigma) at 1 IU/ml (or various doses as specified in the figure legends), arachidonic acid (Chrono-log) at 1 mM (or various doses as specified in the figure legends), or ADP (Sigma) at 20 μM for 30 min at 37°C. The platelets were then washed once with Tyrode/PGE/Hep buffer (to prevent further platelet activation and aggregation) by centrifuging at 2000 × *g* for 10 min at RT. To assess platelet activation, expression of CD62P (using PE/Cy5-mouse IgG anti-human CD62P) was detected on $CD42b^+$ (allophycocyanin-mouse IgG anti-human CD42b) platelets. Finally, platelets were washed and fixed with 1% paraformaldehyde for 30 min at 4°C, prior to acquisition using a BD FACSCalibur flow cytometer (BD Biosciences). A minimum of 10,000 events/sample was acquired, and the data were analyzed using FlowJo software version 7.6.5.

Measurement of properdin binding to platelets

Nonactivated, thrombin-activated, arachidonic acid–activated, or ADP-activated platelets (2 × 10^6 platelets/100 μl) were incubated with properdin P_2–P_4 forms (0–25 μg/ml) in Tyrode/PGE/Hep for 1 h at RT. Platelets were washed twice with Tyrode/PGE/Hep by centrifuging at 2000 × *g* for 10 min at RT. Binding of properdin was assessed by flow cytometry using an anti-properdin mAb, followed by an Alexa Fluor 488–conjugated anti-mouse IgG Ab. After washing, platelets were stained with allophycocyanin-mouse anti-human CD42b and with PE/Cy5-mouse anti-human CD62P. Finally, platelets were washed and fixed, and the data were acquired and analyzed as described in the previous section. In some experiments, properdin was added to the activated platelets or to C3b-coated sheep erythrocytes (E_SC3b; control) in the presence of F(ab′)$_2$ polyclonal goat anti-C3b Abs.

Measurement of alternative pathway complement activation on platelets (C3b and C5b-9 deposition)

Nonactivated, thrombin-activated, or arachidonic acid–activated platelets (2×10^6 platelets/100 μl) were incubated in the presence or absence of properdin. Washed platelets were then incubated with properdin-depleted serum (60%) for various lengths of time at 37˚C. In separate experiments, platelets were used at 2×10^6 platelets/100 μl and incubated with the properdin-depleted serum, as described above, but in the presence of 25 μM a recombinant protein consisting of domains 19–20 of factor H (rH19-20), a competitive inhibitor of factor H–mediated cell surface regulation (17, 42–45). In all experiments, platelets were incubated with serum in the presence of 5 mM Mg-EGTA to selectively measure complement activation by the alternative pathway. Negative controls were incubated with serum in the presence of 10 mM EDTA to inhibit complement activation. Complement activation was stopped by washing samples with cold Tyrode's buffer containing 10 mM EDTA. Deposition of C3b was detected using PE-anti C3/C3b or an unlabeled anti-C3b mAb, followed by Alexa Fluor 488–conjugated goat anti-mouse IgG. Similarly, C5b-9 was detected using anti–C5b-9 neo-epitope Ab, followed by Alexa Fluor 488–conjugated goat anti-mouse IgG. The platelets were stained with allophycocyanin-mouse anti-human CD42b and PE/Cy5-mouse anti-human CD62P and analyzed as described above.

Separation of C3 and C3(H_2O)

C3(H_2O) was separated from C3 by cation-exchange chromatography, as described previously (40). Briefly, C3 was incubated at 37˚C for 2 h to convert intermediate/inactive forms of C3 to C3(H_2O). The sample was diluted with Mono S buffer A and loaded onto a 1-ml Mono S column. The column was washed with buffer A and eluted using a 20-ml salt gradient (0–100%) of buffer B at a flow rate of 1 ml/min. Purified C3 and C3(H_2O) were dialyzed against PBS, stored at 4˚C, and used within 2 wk of separation.

Measurement of recruitment to the platelet surface of C3 components by properdin and of properdin by C3(H_2O)

Nonactivated, thrombin-activated, or arachidonic acid–activated platelets (2×10^6/100 μl) were incubated with or without properdin (25 μg/ml) in Tyrode/PGE/Hep for 1 h at RT. Platelets were washed twice with Tyrode/PGE/Hep and then incubated with C3, C3b, or C3(H_2O) (100 μg/ml), or, in the case of thrombin-activated platelets, with various concentrations of C3 (H_2O) (0–100 μg/ml). Binding of C3 components was assessed by flow cytometry using an anti-C3 mAb, followed by an Alexa Fluor 488–conjugated anti-mouse IgG Ab. Alternatively, the activated platelets were incubated with C3(H_2O) (100 μg/ml) first, followed by washing and subsequent incubation with P_3 (0–25 μg/ml). Binding of properdin was assessed by flow cytometry, as described above.

Measurement of C3 convertase [C3(H_2O),Bb and C3b,Bb] formation on platelets

Thrombin-activated or arachidonic acid–activated platelets (2×10^6/100 μl) were incubated with one of the following for 1 h at RT: Tyrode's buffer alone, properdin (25 μg/ml), C3(H_2O) (50 or 100 μg/ml), C3b (50 or 100 μg/ml), or first with properdin, followed by washing and then C3(H_2O) or C3b (1 h each). Alternatively, C3(H_2O) or C3b was added first, followed by washing and then properdin (1 h each). After washing, the ability to form Bb was assessed by resuspending the pellet in 100 μl factor D (2 μg/ml) along with factor B (80 μg/ml) for 30 min at RT. The formation of Bb was assessed by flow cytometry using anti-human complement factor Bb neo-epitope mAb, followed by an Alexa Fluor 488–conjugated anti-mouse IgG Ab. The platelets were then stained with allophycocyanin-mouse anti-human CD42b and PE/Cy5-mouse anti-human CD62P and analyzed as described in the preceding section.

Polymorphonuclear cell isolation and activation and assessment of properdin binding to stimulated platelets

Fresh blood was drawn from healthy volunteers into EDTA tubes (BD Vacutainer). Polymorphonuclear cells (PMNs) were isolated using a Polymorphprep gradient (Axis-Shield PoC AS), following the manufacturer's instructions (>90% pure PMNs). PMNs (2.5×10^7/ml), in $HBSS^{+2}$ (Life Technologies) + 0.2% BSA, were activated using PMA (10 ng/ml; Enzo Life Sciences) for 30 min at 37˚C, as described previously (29). The supernatant was collected after centrifuging the cells at $600 \times g$ for 10 min at 4˚C and centrifuged again at $13{,}000 \times g$ for 10 min to remove cell debris. HALT protease inhibitor (Thermo Scientific) was added (1:100) to the supernatant. Activation of PMNs was verified by flow cytometry by double gating on forward scatter and side scatter for PMNs and on $CD16b^+$ cells and measuring the levels of CD11b on the gated population. Supernatant (50 μl) of PMA-activated neutrophils was incubated with nonactivated or thrombin-activated (1 U/ml) platelets (2×10^6), in a final reaction volume of 100 μl, containing 10 mM EDTA to avoid complement activation in the supernatant. Binding of properdin was assessed by flow cytometry using an anti-properdin mAb or an IgG1 isotype control, followed by an Alexa Fluor 488–conjugated anti-mouse IgG Ab. After washing, platelets were stained with allophycocyanin-conjugated mouse anti-human CD42b to gate on the platelet population, as well as with PE/Cy5-mouse anti-human CD62P to assess platelet activation, and were analyzed as described above.

Statistics

Data were analyzed with GraphPad Prism 4.0 software. Unpaired Student *t* tests were used to assess the statistical significance of the difference between the groups assessed for properdin binding, CD62P expression, or C3b or C5-9 deposition. One-way ANOVA–Dunnett multiple-comparison test was used to compare all of the conditions of resting and thrombin-stimulated platelets incubated with different C3 components [C3, C3 (H_2O), C3b] in the presence or absence of properdin. Unpaired Student *t* tests were used to determine the significance of differences with regard to the binding of C3 components to arachidonic acid–activated platelets (with or without properdin). Two-way ANOVA–Bonferroni posttest was applied to determine the statistical significance of whether properdin recruits C3 (H_2O) to the surface of activated platelets, or vice versa, at different C3 (H_2O) or properdin concentrations, respectively.

Results

The physiological forms of properdin bind to activated, but not resting, platelets

Various studies suggest that properdin selectively recognizes certain surfaces, such as apoptotic or necrotic cells and pathogens (31, 34, 35, 37, 38, 46), leading to de novo convertase assembly and alternative pathway complement activation. Considering the importance of complement system activation in vascular inflammation, thrombosis, and thrombocytopenia, we examined the ability of properdin to bind platelets. Properdin subjected to prolonged storage and/or freeze/thaw cycles is known to accumulate nonphysiological, high m.w. aggregates (P_n) (33, 47) that can consume complement in solution (33) and bind nonspecifically to surfaces (reviewed in Ref. 31; 34, 48). Therefore, the native physiological forms of properdin (P_2, P_3, and P_4) were separated from the nonphysiological forms (P_n) by gel-filtration chromatography, as previously described (34). We tested the binding of the physiological forms of properdin to washed nonactivated (Fig. 1A) and thrombin-activated (Fig. 1B) platelets, and the data indicate that P_2, P_3, and P_4 bind only to activated platelets (Fig. 1B, 1C) in a dose-dependent manner (Fig. 1D). Unseparated properdin, which has P_n forms, bound to both nonactivated and activated platelets (Supplemental Fig. 1), confirming the importance of eliminating the P_n forms from the raw preparation before testing.

The level of properdin binding to platelets depends on the agonist but is not proportional to the level of exposure of P-selectin (CD62P)

The extent to which stimulated platelets activate complement on or near their surface, when exposed to plasma or serum, is higher on platelets stimulated with strong agonists versus weak agonists (7, 8). Therefore, we sought to determine whether platelets activated by different agonists would preserve the ability to bind properdin. Platelets were activated with thrombin or arachidonic acid (strong agonists) or ADP (weak agonist), and their ability to bind properdin was compared. The data in Fig. 1E show that the binding of properdin to platelets stimulated with arachidonic acid is ~4-fold higher than thrombin-stimulated platelets, whereas no binding of properdin to ADP-activated platelets was detected. Thus, the use of thrombin-activated platelets allowed more stringent conditions for assessing the significance of properdin binding, because these

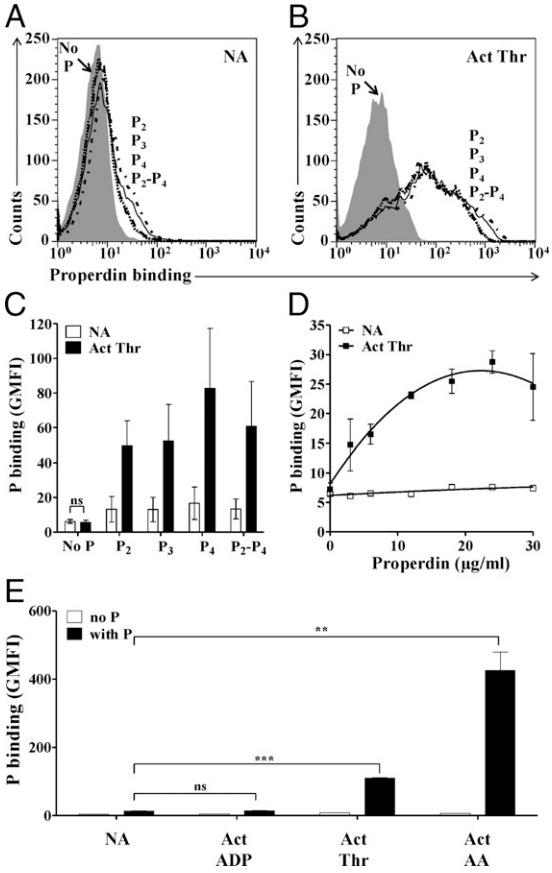

FIGURE 1. Analysis of binding of physiological forms of properdin to activated platelets stimulated by different agonists. Nonactivated (NA) (**A**) or thrombin-activated (Act Thr) (**B**) platelets (2×10^6/100 μl) were incubated in the presence or absence of 25 μg/ml of P_2, P_3, P_4, or P_2–P_4 pool (at a ratio of 1:2:1) for 30 min at RT in Tyrode/PGE/Hep buffer. Platelets were washed, and binding of properdin was assessed by FACS using an anti-properdin mAb, followed by an Alexa Fluor 488–conjugated anti-mouse IgG Ab. (**C**) Graphical representation of properdin binding to Act Thr platelets, as determined in (A) and (B), and expressed as means and SDs of triplicate geoMean fluorescence intensity (GMFI) values. The differences between the ability of properdin to bind to activated platelets versus nonactivated platelets were all significant ($p < 0.05$). (**D**) Dose-dependent binding curve of properdin (0–30 μg/ml) to NA and Act Thr platelets. (**E**) Binding of properdin (25 μg/ml) to NA platelets and to platelets activated with ADP (20 μM), thrombin (1 IU/ml), or arachidonic acid (1 mM). For all experiments, allophycocyanin-labeled anti-CD42b mAb was used to gate on the platelet population. Results are representative of three separate experiments and are shown as mean and SD of triplicate observations. $^{**}p < 0.01$, $^{***}p < 0.001$, unpaired *t* test.

platelets bind notably less properdin than do arachidonic acid–activated platelets.

Others have shown that alternative pathway complement activation on platelets requires CD62P (9). The maximum CD62P levels on thrombin- and arachidonic acid–activated platelets were achieved with <1 U/ml or <1 mM, respectively (Fig. 2A, 2B, arrows). As expected, platelet activation, as measured by exposure of CD62P, varied between the different stimuli (thrombin > arachidonic acid; Fig. 2A–C). Interestingly, although the maximum level of CD62P expression is significantly higher (~1.5–2-fold) on platelets stimulated with thrombin (Fig. 2A, 2C) compared with arachidonic acid (Fig. 2B, 2C), the binding of properdin to the arachidonic acid–activated platelets (Fig. 2E, 2F) is significantly higher (~3-fold) than to the thrombin-activated group (Fig. 2D, 2F).

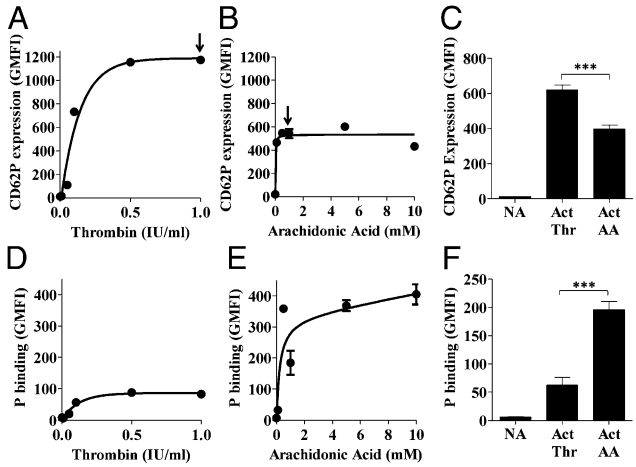

FIGURE 2. The binding of properdin to activated platelets is not proportional to CD62P levels on the platelet surface. Platelets were activated using thrombin (Thr) [0–1 IU/ml (**A**, **D**); 1 IU/ml (**C**, **F**)] or arachidonic acid (AA) [0–10 mM in (**B**) and (**E**); 1 mM in (C) and (F)]. Nonactivated (NA) or activated (Act) platelets (2×10^6/100 μl) were incubated in the presence of 25 μg/ml of P_3 for 60 min at RT. (D–F) Binding of properdin was assessed by FACS using an anti-properdin mAb, followed by an Alexa Fluor 488–conjugated anti-mouse IgG Ab. (A–C) Platelet activation was assessed using PE/Cy5-labeled anti-CD62P mAb. The arrow in (A) and (B) represents the dose of activator, used in (C) and (F), at which maximal CD62P expression was achieved. An allophycocyanin-labeled anti-CD42b mAb was used to gate on platelets. The data are representative of two independent experiments shown as mean and SD. $^{***}p < 0.001$, unpaired *t* test.

Altogether, these data suggest that the binding of properdin to activated platelets depends on the mode of stimulation, but it is not directly proportional to the level of CD62P exposed on activated platelets.

We also determined whether native properdin can directly activate platelets. Incubating the resting platelets with properdin during the activation step does not result in an increase in the expression of CD62P or gpIIbIIIa or annexin V binding on the platelet surface (Supplemental Fig. 2). Properdin cannot act as a costimulator either, because when the resting platelets were incubated with submaximum doses of thrombin or arachidonic acid in the presence of properdin, there was no increase in expression of CD62P or gpIIbIIIa or annexin V binding beyond the level achieved using thrombin or arachidonic acid alone (data not shown).

Properdin binding to activated platelets does not require previous C3 fragment deposition on the platelets

Complement C3 is present in platelets (9), and its spontaneously hydrolyzed form [$C3(H_2O)$], as well as its C3b fragment, was shown to bind to platelets (9, 11) and to properdin (49, 50). To determine whether properdin was binding through C3 components on the platelets, we assessed whether the binding of properdin could be inhibited by polyclonal $F(ab')_2$ anti-C3 Abs. Fig. 3A shows that 10 μg/ml of the Ab completely inhibited the binding of properdin to C3b-opsonized sheep erythrocytes. On the contrary, not even 10-fold more (100 μg/ml) of the same Ab was able to inhibit the binding of $P_{2–4}$ to thrombin- or arachidonic acid–activated platelets (Fig. 3B, 3C). In addition, neither C3 components nor properdin was detected on the surface of washed thrombin- or arachidonic acid–activated platelets by flow cytometry (Supplemental Fig. 3). These data indicate that the physiological forms of properdin bind selectively to activated platelets independently from C3.

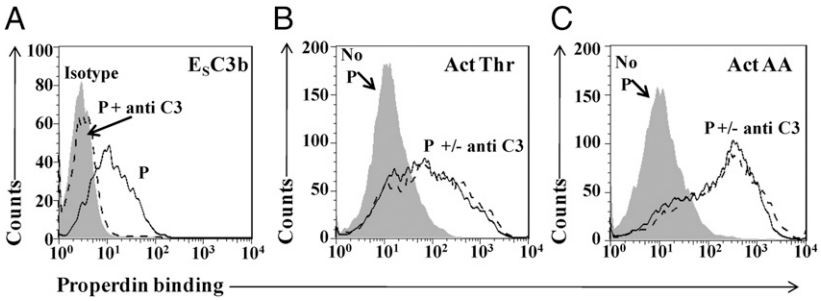

FIGURE 3. The binding of properdin to platelets is not mediated by C3. (**A**) E_SC3b (5×10^6/100 μl) in gelatin veronal buffer without calcium and magnesium were incubated with or without 10 μg/ml anti-C3 polyclonal Ab for 15 min at 4°C. Without washing, P_2–P_4 (10 μg/ml) was added to the cells and incubated for 1 h at 4°C. Cells were washed, and properdin binding was detected using an anti-properdin mAb or an IgG1-isotype control, followed by an Alexa Fluor 488–conjugated anti-mouse IgG Ab. Thrombin-activated (Act Thr) (**B**) or arachidonic acid–activated (Act AA) (**C**) platelets (2×10^6/100 μl) were incubated or not with 100 μg/ml anti-C3 polyclonal Ab for 30 min at RT. Without washing, P_2–P_4 (10 μg/ml) was added to the cells and incubated for 30 min at RT. Platelets incubated without anti-C3 and without P_2–P_4 were used as a negative control. Platelets were washed, and binding of properdin was assessed using an anti-properdin mAb, followed by an Alexa Fluor 488–conjugated anti-mouse IgG Ab. An allophycocyanin-labeled anti-CD42b mAb was used to gate on the platelet population.

Properdin bound to stimulated platelets promotes complement activation on the platelet surface

To determine the functional consequences of the interaction between properdin and stimulated platelets, we investigated whether physiological forms of properdin that are bound to activated platelets have the ability to promote complement activation. Washed, nonactivated and thrombin- or arachidonic acid–activated platelets were incubated in the presence or absence of purified properdin (P_3 forms). Platelets were then washed and exposed to properdin-depleted serum to study alternative pathway activation mediated only by properdin bound to the activated platelets. Fig. 4A shows that rapid C3b deposition occurs on activated platelets preincubated with properdin in as soon as 5 min compared with activated platelets alone, nonactivated platelets, or nonactivated platelets preincubated with properdin. Maximum C3b deposition on properdin-bound activated platelets was observed after 20 min. Activated platelets with properdin on their surface induced ~2.2-fold increase in C3b deposition versus activated platelets alone at 30 min (Fig. 4B). Only the group of thrombin-activated platelets with properdin on their surface induced significant C3b deposition when exposed to P-depleted serum for 30 min versus 0 min (Fig. 4B). Moreover, we observed that C3b deposition was ~10-fold higher on arachidonic acid (1 mM)-stimulated platelets with properdin on their surface (Fig. 4C) compared with the thrombin-activated platelets with surface-bound properdin (Fig. 4B; right bars). Arachidonic acid–activated platelets without properdin on their surface induced C3b deposition, although it was at a level that was ~4-fold less than platelets with properdin on their surface (Fig. 4C). These data indicate that alternative pathway activation on stimulated platelets is greatly enhanced when properdin is bound to the platelet surface, even when complement-regulatory functions on the platelets are normal.

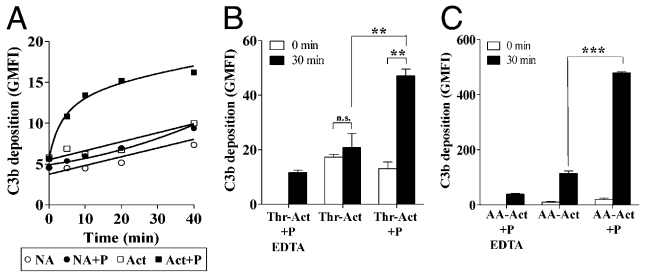

FIGURE 4. Properdin promotes complement activation on the surface of activated platelets. Nonactivated (NA), thrombin-activated (Thr-Act), or arachidonic acid–activated (AA-Act) platelets (2×10^6/100 μl) were incubated in the presence or absence of P_3 or P_{2-4} (25 μg/ml) for 60 min at RT. Platelets were then washed and incubated with 60% properdin-depleted serum in the presence of Mg-EGTA at 37°C for various lengths of time (**A**) or for 30 min (**B**, **C**). Deposition of C3b on the surface of platelets was assessed using a PE-labeled anti-C3 mAb (A) or unlabeled anti-C3/C3b mAb, followed by Alexa Fluor 488–conjugated anti-mouse IgG (B, C). An allophycocyanin-labeled anti-CD42b mAb was used to gate on the platelet population. As controls, platelets (in the presence or absence of properdin) were incubated with 60% properdin-depleted serum in EDTA (10 mM). In (A), the EDTA controls gave an average GMFI of 5.3 (± 0.69) at 40 min (data not shown). The graphs show one representative experiment of three independent experiments. Each bar in (B) and (C) represents the mean and SD of triplicate observations. $^{**}p < 0.01$, $^{***}p < 0.001$, unpaired *t* test.

Inhibition of factor H–mediated cell surface protection enhances properdin-mediated complement activation on platelets

To determine whether impairing complement regulation on platelets enhances properdin-mediated complement activation, we used a competitive inhibitor of factor H cell surface complement regulation known as rH19-20 (17, 42–45). This inhibitor is a recombinant protein consisting of domains 19–20 of factor H that competes with full-length factor H for binding to cell surface C3b and polyanions, inhibiting the ability of factor H to protect cell surfaces, without affecting fluid-phase complement regulation (42). Factor H can bind activated platelets (51, 52). Mutations in the C terminus of factor H impair the efficient binding of factor H to cell surfaces and were associated with deposition of complement on the platelet surface in patients with aHUS, which can result in thrombosis and thrombocytopenia (18). Thus, we examined the effect of inhibiting factor H regulation on the platelet surface during properdin-mediated complement activation on platelets. The data in Fig. 5 indicate that inhibiting factor H complement regulation using rH19-20 on thrombin- or arachidonic acid–activated platelets with properdin increases C3b deposition by ~4-fold (thrombin activated; Fig. 5A) and ~8-fold (arachidonic acid activated; Fig. 5B) compared with activated platelets with properdin but without rH19-20. Inhibiting factor H complement regulation in the absence of properdin induces significant C3b deposition compared with the EDTA control. This increase is similar to the C3b deposition that occurs when the platelets have properdin on their surface and factor H regulation has not been inhibited.

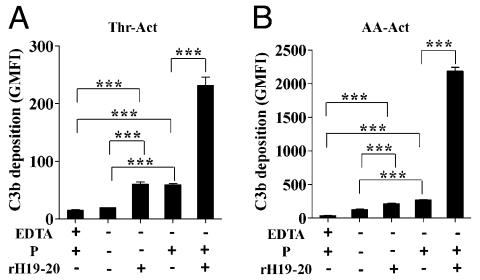

FIGURE 5. Properdin-mediated complement activation is exacerbated when cell surface protection by factor H is inhibited. Thrombin-activated [Thr-Act (**A**)] or arachidonic acid-activated [AA-Act (**B**)] platelets (2×10^6/100 μl) were incubated in the presence or absence of P_2–P_4 (25 μg/ml) for 60 min at RT. Platelets were then washed and incubated with 60% properdin-depleted serum in the presence of Mg-EGTA, with or without rH19-20 (25 μM), at 37°C for 30 min. Deposition of C3b on the surface of platelets was assessed using an unlabeled anti-C3/C3b mAb, followed by an Alexa Fluor 488–conjugated anti-mouse IgG. An allophycocyanin-labeled anti-CD42b mAb was used to gate on the platelet population. As controls, platelets (in the presence of properdin) were incubated with 60% properdin-depleted serum in EDTA (10 mM). The graphs show one representative experiment of two independent experiments. Each bar represents the mean and SD of triplicate observations. ***$p < 0.001$, unpaired t test.

Properdin recruits C3 components to the surface of activated platelets, leading to functional C3 convertase formation

We next sought to define the molecular mechanisms involved in properdin-mediated complement activation on stimulated platelets. Properdin can bind to C3b (25) and C3(H_2O) (49, 50), which are structurally similar. Therefore, we analyzed the ability of the properdin that is bound to the activated platelet to recruit C3b and C3(H_2O) to the platelet surface as a potential first step in convertase formation (Figs. 6, 7). It was proposed that C3b binds directly to activated platelets via CD62P (9). Nevertheless, by using Abs against a neo-epitope found on C3(H_2O), Hamad et al. (11) detected binding of C3(H_2O), but not of C3b or nonactivated C3, to activated platelets. Fig. 6A shows the separation of C3 (H_2O) from C3 by ion-exchange chromatography. The data in Fig. 6B, using thrombin-activated platelets, confirm that C3 and C3b do not significantly bind directly to resting or thrombin-activated platelets and that C3 and C3b do not bind even when properdin is present on the platelet surface. In contrast, C3(H_2O) binds significantly to activated platelets without ($p < 0.05$) and with ($p < 0.001$) properdin on their surface (Fig. 6B). Moreover, properdin that is bound directly to activated platelets recruits ~1.5-fold more C3(H_2O) to the platelet surface compared with activated platelets alone (Fig. 6C). On the contrary, the C3(H_2O) that is bound directly to activated platelets (Fig. 6B) is not able to recruit more properdin than the activated platelets without C3(H_2O) (Fig. 6D).

To determine whether the platelet-bound properdin and C3(H_2O) (recruited by properdin) can interact with factors B and D to generate a C3 convertase, thrombin-activated platelets were incubated with buffer or properdin or C3(H_2O), or properdin followed by washing and then C3(H_2O). A group with C3(H_2O) first, followed by properdin, was not included, because C3(H_2O) does not recruit properdin to the platelet surface. After washing the cells, factors B and D were added to the groups of platelets mentioned above, and the formation of Bb, due to proteolytic cleavage by factor D, was assessed by flow cytometry using an anti-Bb neo-epitope Ab (Fig. 6E). Although platelets bind C3 (H_2O) directly (Fig. 6B), formation of Bb was detected only on the surface of thrombin-activated platelets where C3(H_2O) was additionally recruited to the surface by properdin (Fig. 6E).

When analyzing arachidonic acid–activated platelets (Fig. 7), both C3(H_2O) and C3b bind significantly to activated platelets without and with properdin on their surface (Fig. 7A). Properdin bound to activated platelets recruits ~3-fold more C3b ($p < 0.01$) and ~2-fold more C3(H_2O) ($p < 0.05$) to the platelet surface compared with activated platelets alone. We next assessed the ability of platelet-bound properdin, C3b, and C3(H_2O) to generate C3 convertases. Formation of Bb was detected on the surface of arachidonic acid–activated platelets if C3(H_2O) (Fig. 7B, solid line) or C3b (Fig. 7C, solid line) was recruited to the surface by properdin. When platelets first received C3(H_2O) or C3b, convertase formation occurred only after properdin had been recruited (Fig. 7B, 7C, dotted lines). Altogether, these data indicate that on thrombin-activated platelets, only the recruitment of C3(H_2O) by properdin leads to de novo formation of novel cell-bound [C3 (H_2O),Bb] convertases, whereas in the case of arachidonic acid–activated platelets, recruitment of both C3(H_2O) and C3b by properdin leads to convertase formation. Moreover, C3(H_2O) that is bound directly to arachidonic acid–activated platelets can form, albeit to a lesser extent, novel convertases that require the recruitment of properdin for convertase stabilization, facilitating convertase detection (Fig. 7B, dotted line).

Properdin bound to activated platelets leads to C9 deposition

Deposition of C3b on host cell surfaces may not necessarily cause complement to progress to its terminal stage (formation of MAC; C5b-9), because host cell surfaces are protected by membrane-bound and fluid-phase complement-regulatory proteins. Platelets have surface-bound CD55, CD46, CD35, and CD59 (53, 54) and are able to bind factor H (51). Thus, we investigated whether properdin-mediated complement activation on the surface of platelets leads to formation of MAC (Fig. 8), despite complement regulation. Washed nonactivated, thrombin-activated, or arachidonic acid–activated platelets were incubated in the presence or absence of properdin and exposed to properdin-depleted serum for 60 min. C5b-9 deposition on the platelets was measured using an Ab specific for a neo-epitope on C9 that is exposed only when C9 is incorporated into MAC. Only the thrombin-activated platelets that had been preincubated with properdin showed an increase in C5b-9 deposition (~2.9-fold increase at 60 min) compared with activated platelets alone, nonactivated platelets, or nonactivated platelets preincubated with properdin (Fig. 8A). In the case of arachidonic acid–activated platelets, MAC deposition was observed on activated platelets without properdin on their surface, and an ~1.6-fold increase was observed on platelets with properdin on their surface (Fig. 8B).

Properdin produced by activated neutrophils binds to activated platelets

It was shown that serum inhibits binding of properdin to surfaces (34, 35). In agreement with this, properdin binding to platelets was inhibited in a dose-dependent manner by NHS (Supplemental Fig. 4). This suggests that properdin binding to activated platelets may occur only when properdin is readily available to activated platelets in the microenvironment and that properdin-mediated complement activation is tightly controlled.

Neutrophils release properdin upon activation by various inflammatory stimuli, such as PMA, fMLP, C5a, and TNF-α (29), and unfractionated properdin also interacts directly with platelets (platelet/leukocyte aggregates) (55), which may increase the local concentration of properdin in a proinflammatory microenvironment. To determine whether properdin derived from the activated neutrophil supernatants binds to platelets, supernatants from PMA-activated neutrophils were incubated with nonactivated and

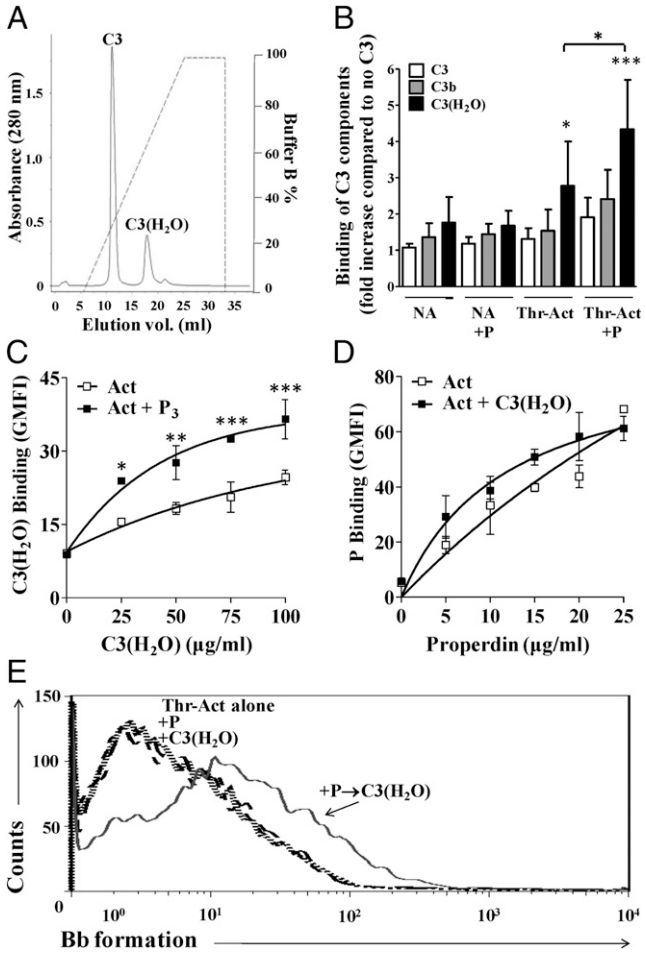

FIGURE 6. Analysis of binding of C3, C3b, and C3(H_2O) to thrombin-activated platelets, with or without properdin on their surface, and the ability to form a functional convertase. (**A**) C3 was separated from C3(H_2O) by cation-exchange chromatography, as described in *Materials and Methods*. (**B**) Nonactivated (NA) or thrombin-activated (Thr-Act) platelets (2×10^6/100 μl) were incubated in the presence or absence of P_3 (25 μg/ml) for 30 min at RT, washed, and then incubated or not with C3, C3(H_2O), or C3b (100 μg/ml) at RT for 1 h. Binding of C3 forms was assessed by FACS using an anti-C3/C3b mAb, followed by an Alexa Fluor 488–conjugated anti-mouse IgG. Allophycocyanin-labeled anti-CD42b Ab was used to gate on platelets. Binding of C3, C3b, and C3(H_2O) to each group of platelets (NA, NA+P, Thr-Act, Thr-Act+P) was normalized to the geoMean fluorescence intensity (GMFI) of each respective group incubated without any C3 components. The results represent the mean and SD of seven independent experiments. Statistical analysis was carried out comparing all columns of NA conditions with the NA + C3 group, as well as all columns of Act conditions with the Act + C3 group. $*p < 0.05$, $***p < 0.001$, one-way ANOVA–Dunnett multiple-comparison test. $*p < 0.05$, binding of C3 (H_2O) to the Act group versus the Act-P group, unpaired Student *t* test. (**C**) Properdin recruits C3(H_2O) to the surface of activated platelets. Thrombin-activated (Act) platelets (2×10^6/100 μl) were incubated in the presence or absence of P_3 (25 μg/ml) for 30 min at RT. Platelets were then washed and incubated with increasing doses of C3(H_2O) (0–100 μg/ml) at RT for 1 h. Binding of C3(H_2O) was assessed by FACS, as described in (B). (**D**) C3 (H_2O) does not recruit properdin to the surface of activated platelets. Thrombin-activated (Act) platelets (2×10^6/100 μl) were incubated in the presence or absence of C3(H_2O) (100 μg/ml) at RT for 1 h. Platelets were then washed and incubated with increasing doses of properdin (0–25 μg/ml) at RT for 1 h. Properdin binding was assessed using an anti-properdin mAb, followed by Alexa Fluor 488–conjugated anti-mouse IgG. Allophycocyanin-labeled anti-CD42b Ab was used to gate on the platelet population. (C and D) Each graph is representative of two independent experiments; data are shown as mean and SD. All data without a *p* value were nonsignificant. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, two-way ANOVA–Bonferroni posttest. (**E**) Properdin bound to activated platelets promotes the formation of C3(H_2O),Bb convertase on the surface of platelets. Thrombin-activated (Thr-Act) platelets were incubated in the presence of P_3 (25 μg/ml) or C3(H_2O) (100 μg/ml) or sequentially with both [P_3→C3(H_2O)] or with neither for 1 h each at RT in Tyrode's buffer. Platelets were then washed and incubated in the presence of factor B (80 μg/ml) and factor D (2 μg/ml) for 30 min at RT in Tyrode's buffer. Formation of Bb on activated platelets was assessed using an anti-Bb neoepitope mAb, followed by an Alexa Fluor 488 $F(ab')_2$ goat-conjugated anti-mouse IgG Ab. Allophycocyanin-labeled anti-CD42b Ab was used to gate on platelets. One representative experiment of three separately performed experiments is shown.

thrombin-activated platelets (that bind significantly less pure properdin than do arachidonic acid–activated platelets). The activated platelets themselves do not have properdin on their surface (Supplemental Fig. 3). As shown in Fig. 9A and 9B, the properdin in the neutrophil supernatant bound only to activated platelets. C3 from activated neutrophil supernatants did not bind to the platelets (data not shown), indicating that the binding of neutrophil-derived properdin to the platelets occurred independently from C3.

Discussion

Our studies reveal that the physiological forms of properdin bind to stimulated platelets, but not to resting platelets, in a manner that is not proportional to P-selectin exposure, leading to the formation of a novel C3 convertase [P-C3(H_2O),Bb] on its surface and allowing the activation of the alternative pathway of the complement system. In addition, C3(H_2O) can initiate activation of complement, as long as properdin is present to stabilize the convertases. The data also show that properdin released by neutrophils binds activated platelets and collectively suggest that properdin is essential for alternative pathway activation to proceed on activated platelets.

Our results show that physiological forms of properdin bind to platelets that have been activated by strong agonists (Fig. 1E), but not to nonactivated platelets (Fig. 1A–C), in a dose-dependent manner (Fig. 1D). Thrombin activates platelets via PAR receptors by pathways dependent on phospholipase C and/or phospholipase A_2, the latter of which includes the arachidonic acid–transformation pathway. The arachidonic acid pathway bypasses the need for agonist receptors and activates platelets via the cyclooxygenase (COX) pathway by using thromboxane synthase to produce platelet agonist thromboxane A2 (56). In addition to platelets, neighboring activated cells, such as leukocytes and endothelial cells, express COX isoenzymes (57), which could further contribute to platelet activation. It was shown that inhibitors of COX lead to the inhibition of complement-enhanced platelet aggregation and release of serotonin (58–63). Platelets activated by weak agonists, such as ADP and epinephrine, support less complement activation than do platelets activated by thrombin or arachidonic acid (8). The capacity of platelets to activate complement on their surface when exposed to plasma or serum is proportional to the extent of platelet activation (8), and alternative pathway activation was shown by others to occur as a result of the binding of C3b to activated platelets via P-selectin (CD62P) (9). In this study, our data show that arachidonic acid–stimulated platelets induced significantly lower overall exposure of P-selectin compared with thrombin-activated platelets (Fig. 2A–C). Nevertheless, properdin binding was ~3–4-fold higher (Fig. 2D–F) and C3b deposition was ~10-fold higher (Fig. 4B, 4C) on the arachidonic acid–stimulated platelets compared with thrombin-activated platelets at maximal platelet P-selectin expression for both agonists. Our data show that, unlike the C3b/P-selectin mechanism for alternative pathway complement activation described by Del

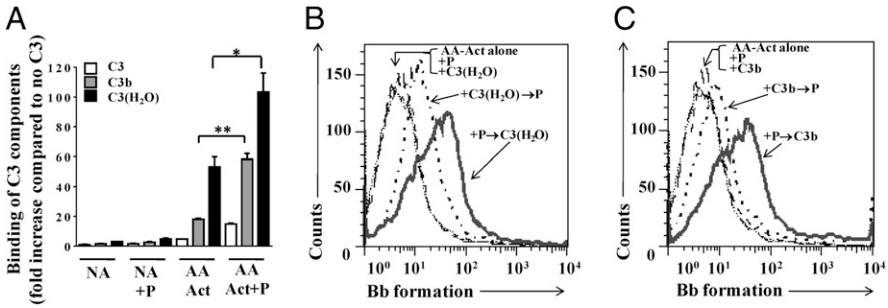

FIGURE 7. Analysis of binding of C3, C3b, and C3(H_2O) to arachidonic acid–activated platelets with or without properdin on their surface and the ability to form a functional convertase. (**A**) Nonactivated (NA) or arachidonic acid–activated (AA Act) platelets (2×10^6/100 μl) were incubated in the presence or absence of P_{2-4} (25 μg/ml) for 30 min at RT, washed, and then incubated with C3, C3(H_2O), or C3b (50 μg/ml) at RT for 1 h. Binding of C3 forms was assessed by FACS using an anti-C3/C3b mAb followed by an Alexa Fluor 488–conjugated anti-mouse IgG. Allophycocyanin-labeled anti-CD42b Ab was used to gate on platelets. Binding of C3, C3b, and C3(H_2O) to platelets was normalized to the geoMean fluorescence intensity (GMFI) observed for each group incubated without any C3 components. The results show one representative experiment of two independent experiments. $*p < 0.05$, $**p < 0.01$, unpaired Student *t* test. (**B**) Arachidonic acid–activated platelets were incubated in the presence of P_2–P_4 (25 μg/ml), C3(H_2O) (100 μg/ml), or sequentially with both [P→C3(H_2O) or C3(H_2O)→P] or neither for 1 h at RT in Tyrode's buffer. (**C**) Arachidonic acid–activated platelets were incubated in the presence of P_2–P_4 (25 μg/ml), C3b (100 μg/ml), or sequentially with both (P→C3b or C3b→P) or neither for 1 h each at RT in Tyrode's buffer. Platelets were then washed and incubated in the presence of factor B (80 μg/ml) and factor D (2 μg/ml) for 30 min at RT in Tyrode's buffer. Formation of Bb on activated platelets was assessed using an anti-Bb neo-epitope mAb, followed by an Alexa Fluor 488 F(ab′)$_2$ goat-conjugated anti-mouse IgG Ab. Allophycocyanin-labeled anti-CD42b Ab was used to gate on platelets. One representative experiment of two separately performed experiments is shown.

Conde et al. (9), properdin binding to activated platelets and C3b deposition are not proportional to the expression of CD62P. Thus, properdin binding may depend on agonist-specific varying exposure of cell surface marker(s) on the platelets. In agreement with this notion, proteomic expression on platelets was shown to vary depending on the platelet agonist used (64).

Importantly, we show that properdin does not rely on C3 fragment deposition on the platelet for binding (Fig. 3). Properdin is a highly positively charged protein (isoelectric point > 9.5) that can interact with certain surfaces directly by recognizing glycosaminoglycan (GAG) chains of surface proteoglycans (35, 65). Candidate sulfated GAGs shown to interact with properdin include heparin (66), heparan sulfate (35, 65), dextran sulfate (67), fucoidan (67), and chondroitin sulfate (35). Interestingly, chondroitin sulfate A, which is released by platelets and found on the platelet surface upon activation (68, 69), enhances the binding of properdin to the activated platelets (data not shown). Other ligands for properdin on cells include DNA on late apoptotic and necrotic cells (37) and bacterial LPS and lipo-oligosaccharide (70). Thus, all cell surface molecules, shown to interact directly with properdin on cells, are negatively charged. Efforts to define the GAGs involved in properdin binding to the platelet surface are underway in our laboratory (University of Toledo College of Medicine).

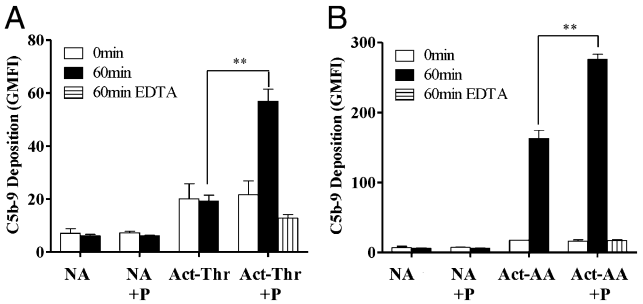

FIGURE 8. Properdin promotes formation of C5b-9 complexes on the surface of activated platelets. Nonactivated (NA), thrombin-activated (Act-Thr) (**A**), or arachidonic acid-activated (Act-AA) (**B**) platelets (2×10^6/100 μl) were incubated in the presence or absence of P_{2-4} or P_3 (25 μg/ml) for 60 min at RT. Platelets were then washed and incubated with 60% properdin-depleted serum in the presence of Mg-EGTA at 37°C for 0 or 60 min (or with EDTA at 37°C for 60 min only as a control). Deposition of C5b-9 complexes was assessed using an anti–C5b-9 neo-epitope mAb, followed by Alexa Fluor 488–conjugated anti-mouse IgG. An allophycocyanin-labeled anti-CD42b mAb was used to gate on platelets. The results are representative of two separate experiments; data shown are mean and SD of triplicate observations. $**p < 0.005$, unpaired *t* test.

Recently, Hamad et al. (11) showed, using specific Abs, that the C3 that binds to platelets consists mainly of C3(H_2O), with no C3b present. Because C3(H_2O) is generated in the fluid phase of blood by the spontaneous hydrolysis of the thioester bond in C3, the C3(H_2O) that was bound to the platelet was nonproteolitically activated (11). In agreement with these results, our data for thrombin-activated platelets show that purified C3(H_2O) indeed binds to activated platelets, whereas C3b binding cannot be detected (Fig. 6B). Platelet-bound C3(H_2O) (Fig. 6) does not lead to the formation of a C3 convertase on the thrombin-activated platelet surface when exposed to factors B and D (Fig. 6E). In contrast, properdin, by recruiting C3(H_2O) (Fig. 6C) to the surface of activated platelets, allows the formation of a novel cell-bound C3(H_2O),Bb convertase in the presence of factors B and D (Fig. 6E), which can lead to activation of the alternative pathway, as measured by C3b and C9 deposition (Figs. 4, 8). It is likely that platelet-bound properdin, in addition to acting as an initiating point for alternative pathway activation on the platelets, is stabilizing the newly formed convertase, facilitating detection of the convertase in our experimental system by making it more resistant to decay compared with the platelets without properdin on their surface.

Interestingly, the results with arachidonic acid–activated platelets (Fig. 7), which were not assessed in the previous study (11), indicate that activated platelets bind detectable levels of C3b in addition to C3(H_2O) (Fig. 7A). In addition to the C3(H_2O) that is available as a result of C3 tickover (reviewed in Ref. 21), Nilsson and Nilsson Ekdahl (71) hypothesized that the rate of hydrolysis of C3 to C3(H_2O) may be accelerated by the interaction of C3 with certain biological surfaces, such as platelets, and that this C3(H_2O) may serve as an initiating molecule of the alternative pathway. As mentioned, C3(H_2O) no longer has a reactive thioester for interacting covalently with cell surfaces and is normally found forming part of fluid-phase C3 convertases for initiating the alternative pathway. However, Fig. 7B shows that C3(H_2O) bound to arachidonic acid–activated platelets can, in fact, lead to the

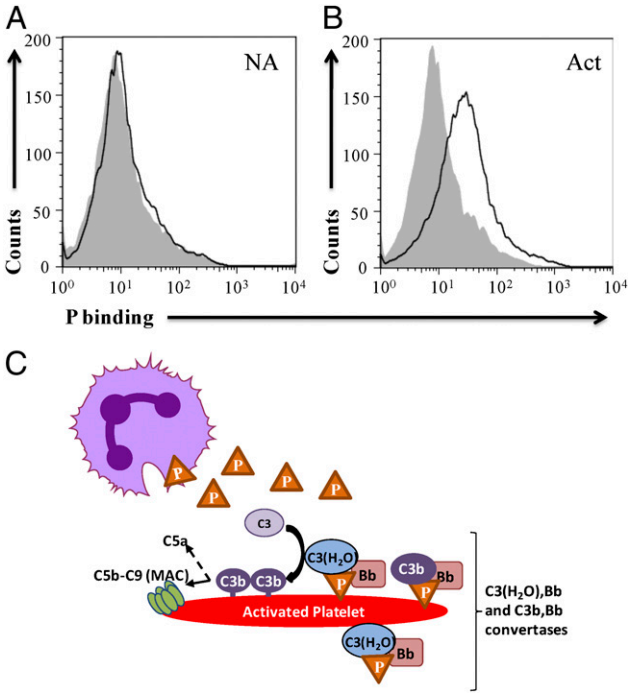

FIGURE 9. Properdin released by activated neutrophils binds to activated platelets. Nonactivated (NA) (**A**) or thrombin-activated (Act) (**B**) platelets were incubated with supernatant from PMA-activated neutrophils for 60 min at RT in Tyrode's buffer with 10 mM EDTA. Platelets were washed, and binding of properdin was assessed by FACS using anti-properdin Ab (open graph), followed by an Alexa Fluor 488–conjugated anti-mouse IgG Ab. IgG1 mAb was used as isotype control (filled graph). Allophycocyanin-labeled anti-CD42b Ab was used to gate on platelets. One representative experiment of two separately performed experiments is shown. (**C**) Model: properdin-mediated complement activation on activated platelets. Properdin (orange triangles) released by PMNs binds to activated platelets and can recruit $C3(H_2O)$ (thrombin-activated platelets) or both $C3(H_2O)$ and C3b (arachidonic acid–activated platelets) to stimulated platelets, allowing the formation of a novel cell-bound $C3(H_2O)$,Bb or a C3b,Bb convertase. $C3(H_2O)$ can also bind to stimulated platelets and in the presence of properdin can promote $C3(H_2O)$,Bb convertase formation. These events can then lead to alternative pathway-complement activation (as shown by C3b and MAC deposition) on the platelet surface.

formation of a novel cell-bound convertase, which can only be detected if properdin is present to stabilize the convertase (Fig. 7C, dotted line). Although we did not detect direct binding of whole C3 to thrombin-activated platelets, and only minimal binding of C3 to arachidonic acid–activated platelets, increased availability of $C3(H_2O)$ may be triggered by contact activation of C3 on gas bubbles, such as those that occur in cardiopulmonary devices and in decompression sickness (reviewed in Ref. 71), potentially contributing to complement activation on platelets.

It is not known why $C3(H_2O)$ binds more than C3b to the activated platelets and to properdin on the platelets (Figs. 6, 7), despite being components that are structurally and functionally similar (20). CR2 (CD21; C3d/iC3b receptor) may be a receptor for $C3(H_2O)$ on B lymphocytes (50), and CR2 was identified on the surface of platelets (72). It is also possible that the C3a region that is present in $C3(H_2O)$, but not in C3b, may contain a site important for interacting with activated platelets. The interacting region between C3 (1402–1435 aa) and properdin (TSR-5) was determined previously (73). To our knowledge, our data indicate for the first time that additional recruitment of $C3(H_2O)$ by properdin, on activated platelets, leads to de novo formation of cell-bound [$C3(H_2O)$,Bb] convertases. These results suggest that properdin on the platelet surface acts as a second contact point for C3 (H_2O), increasing its avidity for activated platelets and allowing $C3(H_2O)$ to form a novel functional C3 convertase [$C3(H_2O)$,Bb].

Our data show that alternative pathway activation proceeds when properdin is bound to the surface of activated platelets, as measured by C3b (Fig. 4) and C5b-9 deposition (Fig. 8) after exposing the platelets to properdin-depleted serum. NHS was used in separate experiments as a serum control that has undergone less postextraction processing than has the depleted sera, and the results were similar (data not shown). This complement activation occurs even when complement regulation on the platelets is normal (i.e., normal membrane-bound and fluid-phase complement-regulatory proteins). However, C3b deposition is significantly enhanced when cell surface protection by factor H is blocked, especially on activated platelets that have properdin on their surface (Fig. 5). Clinical data suggest that enhanced platelet-associated complement activation correlates with increased thrombotic events in patients with aHUS (18, 74), as a result of mutations in the C terminus of factor H that impair cell surface protection (75); properdin-mediated complement activation may contribute to these phenomena.

Properdin derived from PMNs binds to activated platelets (Fig. 9A, 9B). Under physiological inflammatory conditions and normal complement regulation, properdin may direct low-level complement activation on activated platelets and contribute to opsonizing spent platelets for removal. Serum inhibits the ability of properdin to bind to activated platelets in a dose-dependent manner (Supplemental Fig. 4). It is possible that properdin that is freshly secreted by different cell types (27, 29, 32), and does not quickly encounter a nearby platelet surface to bind, eventually loses its ability to bind to surfaces directly once it comes in contact with serum. This regulation would prevent unwanted properdin-mediated complement damage at more distant/bystander cell surfaces while keeping the conventional function of stabilizing the C3 and C5 convertases of the alternative pathway intact. As mentioned, properdin binds DNA and sulfated glucoconjugates. Thus, fluid-phase forms of DNA (76) or glycoproteins might serve as regulators of properdin/surface interactions once properdin has left the microenvironment of the cells producing it (e.g., neutrophils).

Complement also plays a role in tissue damage in many inflammatory diseases that are associated with increased platelet activation and coagulopathies that are not directly associated with defects in complement regulation (e.g., cardiovascular disease, certain cancers, sepsis) (13). Thus, it is possible that, in vascular injury, the local inflammatory leukocytes may readily produce properdin (27–30, 32, 77). This properdin would be available to platelets at high concentrations in the local microenvironment as they become activated by the damaged endothelium and interact with leukocytes [forming platelet/leukocyte aggregates (55)], potentially contributing to complement-mediated disease pathogenesis. In agreement with this notion, Ruef et al. (78) showed that pure, unfractionated properdin (with nonphysiological polymers), when added to whole blood, increases the formation of platelet/leukocyte aggregates. In addition, properdin-mediated complement activation (by inducing formation of MAC and release of C3a and C5a) may stimulate degranulation and activation of other resting platelets (14, 15). Moreover, the properdin-induced complement activation on platelets significantly increases when cell surface protection by factor H is inhibited using a competitive inhibitor (Fig. 5). Thus, this properdin-mediated mechanism may be exacerbated in diseases in which complement regulation is compromised (e.g., paroxysmal nocturnal hemoglobinuria, aHUS) (18, 19).

The data collectively indicate a new mechanism of alternative pathway activation on stimulated platelets that is not proportional to the expression of CD62P, is initiated by properdin, and requires the recruitment of C3(H_2O) or C3b for the formation of a novel initiating cell-bound C3 convertase [P-C3(H_2O),Bb] or of P-C3b, Bb (Fig. 9C). C3(H_2O) can also initiate convertase formation, but it requires properdin for stabilization. These mechanisms may depend on the availability of properdin in the local proinflammatory microenvironment and contribute to complement activation in physiological inflammation, as well as to complement-mediated tissue damage in inflammatory diseases.

Acknowledgments

We thank Laci Bloomfield for excellent technical support and Dr. Michael Pangburn and Dr. Ellinor Peerschke for helpful discussions.

Disclosures

The authors have no financial conflicts of interest.

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