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Neutrophil Cathepsin G Modulates the Platelet Surface Expression of the Glycoprotein (GP) Ib-IX Complex by Proteolysis of the von Willebrand Factor Binding Site on GPIbα and by a Cytoskeletal-Mediated Redistribution of the Remainder of the Complex

By Charles A. LaRosa, Michael J. Rohrer, Stephen E. Benoit, Marc R. Barnard, and Alan D. Michelson

The effects of neutrophil cathepsin G on the glycoprotein (GP) Ib-IX complex of washed platelets were examined. Cathepsin G resulted in a concentration- and time-dependent decrease in the platelet surface GPIb-IX complex, as determined by flow cytometry, binding of exogenous von Willebrand factor (vWF) in the presence of ristocetin, and ristocetin-induced platelet agglutination. Cathepsin G resulted in proteolysis of the vWF binding site on GPIbα (defined by monoclonal antibody [MoAb] 6D1), as determined by increased supernatant glycocalicin fragment (a proteolytic product of GPIbα); decreased total platelet content of GPIb; and lack of effect of either cytochalasin B (an inhibitor of actin polymerization), prostaglandin I_2 (an inhibitor of platelet activation), or prior fixation of the platelets. However, cathepsin G resulted in minimal decreases in the binding to fixed platelets of MoAbs TM60 (directed against the thrombin binding site on GPIbα) and WM23 (directed against the macroglycopeptide portion of GPIbα). In contrast to its proteolytic effect on GPIbα, the cathepsin G-induced decrease in platelet surface GPIX and the remnant of the GPIb-IX complex (defined by MoAbs FMC25 and AK1) was via a cytoskeletal-mediated redistribution, as determined by lack of change in the total platelet content of GPIX and the GPIb-IX complex; complete inhibition by cytochalasin B, prostaglandin I_2, and prior fixation of platelets. Experiments with *Serratia* protease-treated and Bernard-Soulier platelets showed that neither platelet surface GPIb nor cathepsin G-induced proteolysis of GPIb were required for the cathepsin G-induced redistribution of the remnant of the GPIb-IX complex or the cathepsin G-induced increase in platelet surface P-selectin. In summary, neutrophil cathepsin G modulates the platelet surface expression of the GPIb-IX complex both by proteolysis of the vWF binding site on GPIbα and by a cytoskeletal-mediated redistribution of the remainder of the complex. Prior studies show that, although thrombospondin 1, antiserine proteases, and plasma are all inhibitors of cathepsin G, the effects of cathepsin G on platelets, including an increase in surface GPIIb-IIIa, occur during close contact between neutrophils and platelets in a protective microenvironment (eg, thrombosis and local inflammation). Taken together, the data suggest that, in a protective microenvironment, neutrophils play a role in transforming platelets from a state favoring adhesion to damaged vessel walls (mediated by vWF binding to the GPIb-IX complex) to a state favoring platelet-to-platelet aggregation (mediated by fibrinogen binding to the GPIIb-IIIa complex) and platelet-to-leukocyte adhesion (mediated by P-selectin).

PLATELETS ARE ACTIVATED by cathepsin G, a serine protease released from the azurophilic granules of stimulated neutrophils.[1-6] The potency of cathepsin G as a platelet agonist is similar to that of thrombin,[6] a physiologically important platelet activator.[7-9] Thrombin results in increased platelet surface expression of P-selectin (reflecting α granule secretion)[10] and the glycoprotein (GP) IIb-IIIa complex (a receptor for fibrinogen, von Willebrand factor [vWF], fibronectin, and vitronectin)[11-14] and decreased platelet surface expression of the GPIb-IX complex (a receptor for vWF).[15-20] It has recently been reported that neutrophil cathepsin G, to a similar or greater extent than thrombin, also results in an increased platelet surface expression of P-selectin[6] and the GPIIb-IIIa complex[6,21] and a decreased platelet surface expression of the GPIb-IX complex.[6,21] The effects of cathepsin G are inhibited by plasma,[6] thrombospondin 1,[22] and antiserine proteases.[23] However, recent studies[23,24] have provided evidence that the cathepsin G-mediated effects of neutrophils on platelets can occur during close contact between neutrophils and platelets in a protective microenvironment (eg, thrombosis and local inflammation).

The thrombin-induced decrease in the platelet surface expression of GPIb is not the result of proteolysis[16,25] or a conformational change,[16] but is the result of a cytoskeletal-mediated translocation of the entire GPIb-IX complex to the membranes of the open surface canalicular system.[18] In contrast, a number of proteases (eg, calcium-dependent protease,[26] *Serratia* marcescens protease,[27,28] plasmin,[29-31] and neutrophil elastase[32,33]) decrease the platelet surface expression of GPIb by proteolysis of an α chain fragment that contains the vWF binding site.

In this study, we examined the mechanism of the cathepsin G-induced decrease in the platelet surface expression of the GPIb-IX complex. We determined that cathepsin G modulates the platelet surface expression of the GPIb-IX complex both by proteolysis of the vWF binding site on GPIbα and by a cytoskeletal-mediated redistribution of the remainder of the complex.

MATERIALS AND METHODS

Murine Monoclonal Antibodies (MoAbs)

GPIb-IX–specific MoAbs. MoAbs 6D1 (provided by Dr Barry S. Coller, SUNY, Stony Brook, NY) and AK2 (provided by Dr Michael C. Berndt, Baker Medical Research Institute, Melbourne, Australia) are directed against the vWF binding site on the amino terminal domain of platelet membrane GPIbα.[34-36] TM60 (provided

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by Dr Naomasa Yamamoto, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) is directed against the thrombin binding site on the amino terminal domain of GPIbα.[37-39] WM23 and AK3 (provided by Dr Berndt) are directed against the macroglycopeptide portion of GPIbα.[36,40] FMC25 (provided by Dr Berndt) is directed against platelet membrane GPIX.[41] AK1 (provided by Dr Berndt) is directed against the GPIb-IX complex.[42] AK1 only binds to the intact GPIb-IX complex, not to uncomplexed GPIb or GPIX.[42]

P-selectin–specific MoAbs. MoAb S12 (provided by Dr Rodger P. McEver, University of Oklahoma, Oklahoma City) is directed against P-selectin.[43,44] P-selectin, also referred to as GMP-140,[43] PADGEM protein,[45] and CD62,[46] is a component of the α granule membrane of resting platelets that is only expressed on the platelet surface membrane after platelet degranulation and secretion.[10,43]

Other MoAbs. 6F1 (provided by Dr Coller) is directed against the platelet membrane GPIa-IIa complex.[47] Y2/51 (Dako Corp, Carpinteria, CA) is directed against platelet membrane GPIIIa.[48] 7E3 (provided by Dr Coller) is directed against the platelet membrane GPIIb-IIIA complex.[49] OKM5 (provided by Dr Patricia Rao, Ortho Diagnostic Systems, Raritan, NJ) and F13 (provided by Dr Irwin D. Bernstein, Fred Hutchinson Cancer Research Center, Seattle, WA) are directed against platelet membrane GPIV.[50,51]

Antibodies were biotinylated or conjugated with fluorescein isothiocyanate (FITC), as previously described.[19,52] In some experiments, 7E3 was directly conjugated with phycoerythrin (PE) by Molecular Probes (Eugene, OR).

Flow Cytometric Analysis of Platelet Surface GPs

The method has been previously described.[28,31] The protocol was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts Medical Center. Peripheral blood was drawn from healthy adult volunteers who had not ingested aspirin or other antiplatelet drugs during the previous 10 days. The first 2 mL of blood drawn was discarded and then blood was drawn into a sodium citrate Vacutainer (Becton Dickinson, Rutherford, NJ), which does not result in platelet activation.[53] After addition to platelet-rich plasma of citrate albumin wash buffer (128 mmol/L NaCl, 4.3 mmol/L $NaH_2PO_4 \cdot H_2O$, 7.5 mmol/L Na_2HPO_4, 4.8 mmol/L sodium citrate, 2.4 mmol/L citric acid, 0.35% bovine serum albumin, 11 mmol/L glucose), pH 6.5, with 50 ng/mL prostaglandin (PG) E_1, washed platelets were prepared by centrifugation as previously described.[28] The concentration of washed platelets was adjusted to 150,000/μL in modified Tyrode's buffer (137 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L $MgCl_2$, 12 mmol/L $NaHCO_3$, 0.4 mmol/L Na_2HPO_4, 0.35% bovine serum albumin, 10 mmol/L HEPES, 5.5 mmol/L glucose), pH 7.4. The washed platelets (150,000/μL) in modified Tyrode's buffer, pH 7.4, were incubated at 22°C for 20 minutes with various concentrations of purified human neutrophil cathepsin G (Calbiochem, La Jolla, CA), 1 U/mL purified human α-thrombin (provided by Dr John W. Fenton II, New York Department of Health, Albany, NY), or control buffer. The samples were then fixed in 1% formaldehyde for 30 minutes at 22°C. In time course experiments, the platelets were incubated at 22°C with 10 μg/mL cathepsin G for various time points up to 20 minutes, and aliquots were then fixed immediately with 1% formaldehyde as above. After fixation, all samples were diluted 10-fold in modified Tyrode's buffer, pH 7.4. Samples were then incubated (22°C for 20 minutes) with saturating concentrations of FITC-conjugated Y2/51 (GPIIIa-specific) and a biotinylated MoAb (6D1, TM60, WM23, FMC25, AK1, or S12), followed by incubation (22°C for 20 minutes) with 30 μg/mL PE-streptavidin (Jackson ImmunoResearch, West Grove, PA). Samples were then diluted 10-fold in modified Tyrode's buffer, pH 7.4, and stored at 4°C until flow cytometric analysis was performed (within 24 hours).

Samples were analyzed in an EPICS Profile II flow cytometer (Coulter Cytometry, Hialeah, FL) equipped with a 500 mW argon laser (Cyonics, San Jose, CA) operated at 15 mW and a wavelength of 488 nm. The fluorescence of FITC and PE were detected using 525 nm and 575 nm band pass filters, respectively. After identification of platelets by gating on both FITC positivity and their characteristic light scatter, binding of the biotinylated MoAb was determined by analyzing 5,000 individual platelets for PE fluorescence. Background binding, obtained from parallel samples run with FITC-Y2/51 and biotinylated mouse IgG (Boehringer Mannheim, Indianapolis, IN), was subtracted from each test sample.

In some experiments, before the addition of cathepsin G, the platelets were incubated at 22°C with or without (1) 10 μmol/L PGI_2 (Sigma, St Louis, MO) (an inhibitor of platelet activation) for 30 minutes, (2) 6 μmol/L cytochalasin B (Sigma) (an inhibitor of actin polymerization[54]) for 15 minutes, or (3) 2.5 μg/mL *Serratia marcescens* metalloprotease (provided by Dr G.A. Jamieson, American Red Cross, Rockville, MD) for 30 minutes. The degree of proteolysis of platelet surface GPIb by *Serratia* protease was assessed by flow cytometry with MoAb 6D1, as previously described.[28]

In other experiments, washed platelets from normal donors were compared with washed platelets from a patient with Bernard-Soulier syndrome (see below).

In some experiments, washed platelets were fixed with 1% formaldehyde and diluted in modified Tyrode's buffer, pH 7.4, before incubation with or without cathepsin G.

Flow Cytometric Analysis of Total Platelet GPIb-IX

The total platelet content of GPIb-IX was determined by flow cytometric analysis of permeabilized platelets, as previously described.[31] Washed platelets (final concentration, 75,000/μL) were incubated at 22°C for 20 minutes with either 10 μg/mL cathepsin G, 1 U/mL thrombin, or no agonist. After fixation with 1% formaldehyde and a 10-fold dilution in modified Tyrode's buffer, pH 7.4, the platelets were incubated at 22°C for 20 minutes with an FITC-conjugated MoAb (6D1, FMC25, or AK1; in 7-fold excess of the saturating concentration for platelet surface GPIb-IX) and 0.1% Triton X-100 (Sigma) in modified Tyrode's buffer, pH 7.4. After a further 10-fold dilution, platelet fluorescence was analyzed by flow cytometry. Nonpermeabilized controls were prepared identically, except that Triton X-100 was omitted.

Ristocetin-Induced Binding of vWF to Platelets

The ristocetin-induced binding of vWF to platelets was determined by a slight modification of a previously described method.[53] Washed platelets were incubated with 10 μg/mL cathepsin G or control buffer. At various time points up to 20 minutes, aliquots were fixed with 1% formaldehyde for 30 minutes at 22°C, diluted 40-fold with modified Tyrode's buffer, pH 7.4, and incubated (22°C for 15 minutes) with pooled platelet-poor plasma from normal donors (as a source of vWF) and ristocetin (BioData, Horsham, PA; final concentration, 1.4 mg/mL). The mixture was then incubated (22°C for 15 minutes) with 28 μg/mL of either polyclonal FITC-conjugated anti-vWF goat IgG antibody (Atlantic Antibodies, Stillwater, MN) or FITC-conjugated nonspecific goat IgG (Atlantic Antibodies), and diluted 16-fold in modified Tyrode's buffer, pH 7.4. The samples were then incubated (22°C for 15 minutes) with a subsaturating concentration of PE-conjugated MoAb 7E3. After identification of platelets by gating on both PE positivity and their characteristic light scatter, binding of FITC-conjugated anti-vWF antibody was determined by flow cytometry. The fluorescence of the sample incu-

bated with the nonspecific goat IgG was subtracted from the fluorescence of the sample incubated with the anti-vWF antibody.

Measurement of Platelet F-Actin Content by Flow Cytometry

FITC-conjugated phalloidin (Molecular Probes), which binds directly and specifically to polymerized (F) actin,[55] was used in a flow cytometric assay to detect platelet F-actin content. Platelets were washed, incubated with cathepsin G, and fixed, as described above. The platelets were diluted to a final concentration of 3,750/μL, incubated at 22°C for 20 minutes with 330 nmol/L FITC-phalloidin and (to permeabilize the platelets) 0.1% Triton X-100, and then analyzed by flow cytometry.

Glycocalicin Assay

Washed platelets were incubated at 22°C for 20 minutes with 0 to 5 μg/mL cathepsin G. After addition of the proteolytic inhibitor aprotinin (final concentration, 100 μg/mL; Sigma), the samples were centrifuged (2,000*g* for 10 minutes) and the supernatants were stored at −80°C. Supernatant glycocalicin fragment was determined by a competitive inhibition assay using MoAb 6D1, as previously described.[53]

Ristocetin-Induced Platelet Agglutination

Platelets (250,000/μL) suspended in autologous plasma were stirred with 1.2 mg/mL ristocetin (BioData) in a Lumi-Aggregation Module Series 10008 (Payton, Buffalo, NY). Platelet agglutination was detected by change in light transmission, as previously described.[30]

Patient With Bernard-Soulier Syndrome

The patient (N.S.) is a previously unreported 5-year-old boy with Bernard-Soulier syndrome, as determined by a bleeding diathesis characterized by petechiae; platelet counts of 60 to 70 × 10^9/L; giant platelets on blood smear; bleeding time greater than 20 minutes; normal platelet aggregation in response to epinephrine, adenosine diphosphate, and collagen, but complete lack of ristocetin-induced platelet agglutination; and markedly reduced total platelet GPIb as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Platelet surface glycoproteins of N.S. as a percentage of normal control platelets (determined by flow cytometry with the MoAbs indicated in parentheses) were as follows: GPIb 0% (6D1), 0% (WM23), 0.1% (AK2), 0.3% (AK3), and 0.6% (TM60); GPIX 6% (FMC25); GPIb-IX complex 8% (AK1); GPIa-IIa 229% (6F1); GPIIb-IIIa 212% (Y2/51) and 183% (7E3); GPIV 302% (OKM5) and 322% (F13).

Statistical Analysis

Experimental results were expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed by analysis of variance and Student's *t*-test using Systat (Systat Inc, Evanston, IL) version 5.02 or Epistat (Tracy L. Gustafson, Round Rock, TX).

RESULTS

Cathepsin G Decreases the Platelet Surface Expression of the vWF Binding Site on GPIbα

Cathepsin G (10 μg/mL) resulted in a rapid time-dependent decrease in the platelet surface expression of GPIb, as determined by flow cytometry with an MoAb (6D1) directed against the vWF binding site on GPIbα (Fig 1). Cathepsin G (10 μg/mL) also resulted in a rapid time-dependent decrease in ristocetin-induced binding of vWF to platelets, which reflects binding of vWF to GPIbα[56] (Fig 1). Three minutes after the addition of cathepsin G to a suspension of washed platelets, the platelet surface expression of GPIb was 20.6% ± 1.6% (mean ± SEM, n = 3) of baseline and the ristocetin-induced binding of vWF to platelets was 0.0% ± 0.0% of baseline. Ten minutes after the addition of cathepsin G, the platelet surface expression of GPIb was 1.7% ± 0.2% of baseline and the ristocetin-induced binding of vWF to platelets remained at 0.0% ± 0.0% of baseline (Fig 1). The cathepsin-induced decrease in the platelet surface expression of the vWF binding site on GPIbα was also demonstrated by the inhibition of ristocetin-induced platelet agglutination (data not shown).

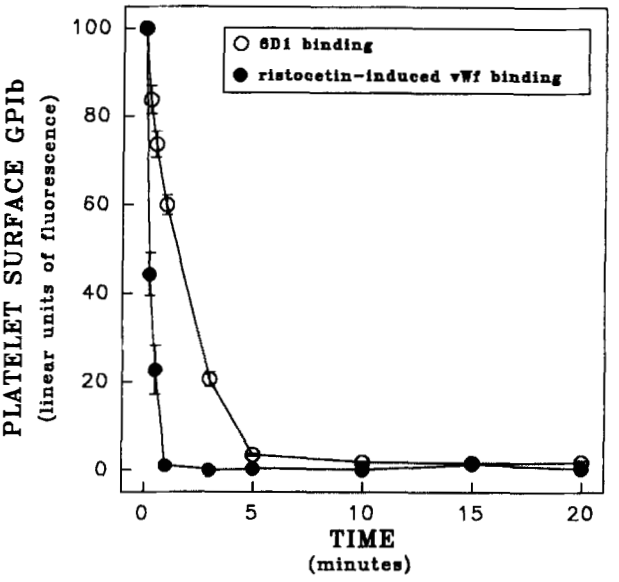

Fig 1. Effect of cathepsin G on the platelet surface expression of GPIb. Cathepsin G (10 μg/mL) was added at the 0 time point to washed platelets incubated at 22°C. Aliquots were fixed with 1% formaldehyde at the indicated time points. Platelet surface binding of the GPIb-specific MoAb 6D1 was analyzed by flow cytometry. In addition, after pooled platelet-poor plasma was added as a source of vWF, the ristocetin-induced binding of vWF to platelets was determined by flow cytometry with a polyclonal anti-vWF antibody, as described in Materials and Methods. The binding of 6D1 and the ristocetin-induced binding of vWF before the addition of cathepsin G were each assigned 100 U of fluorescence. Data are mean ± SEM, n = 3 separate experiments.

Cathepsin G-Induced Decreases in the Platelet Surface Expression of GPIX and the GPIb-IX Complex Were Similar But Less Than the Cathepsin G-Induced Decrease in the Platelet Surface Expression of GPIb

The effect of cathepsin G on the platelet surface expression of different components of the GPIb-IX complex was assessed. Twenty minutes after the addition of 10 μg/mL cathepsin G to a suspension of washed platelets, platelet

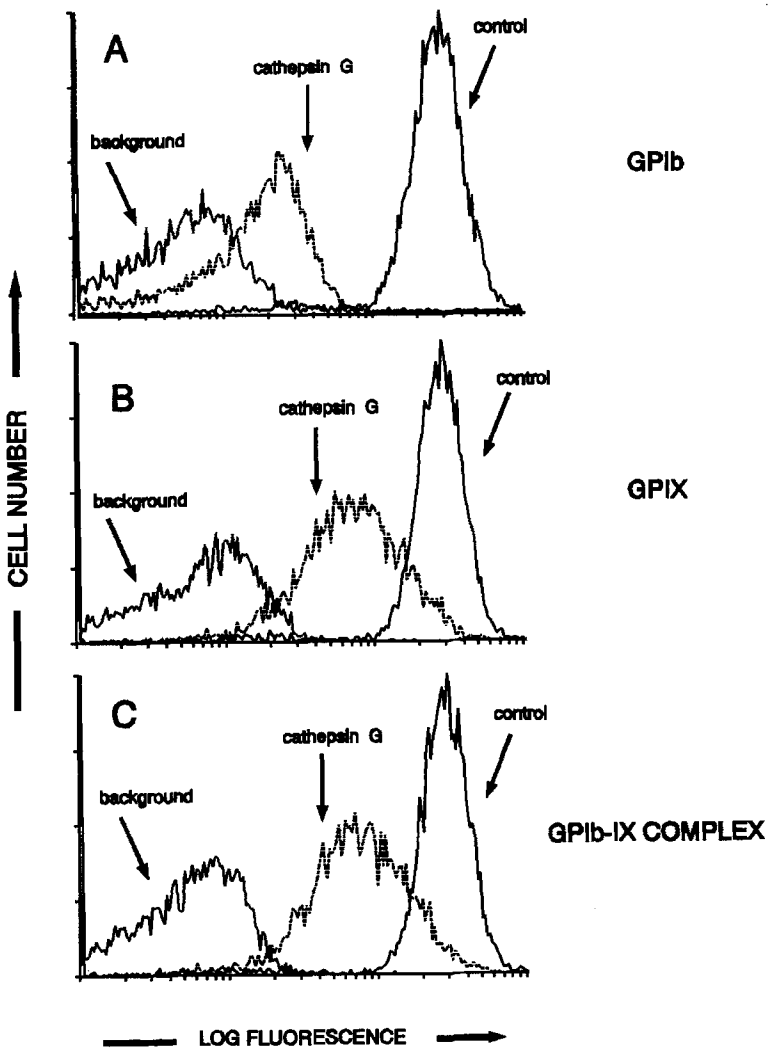

Fig 2. Effect of cathepsin G on the platelet surface expression of the GPIb-IX complex. Washed platelets were incubated at 22°C for 20 minutes with 10 μg/mL cathepsin G and then fixed. Samples indicated as "control" were incubated without cathepsin G. The horizontal axis (log scale) represents platelet surface binding, as determined by flow cytometry, of MoAbs 6D1 (GPIb-specific [A]), FMC25 (GPIX-specific [B]), and AK1 (GPIb-IX complex-specific [C]). "Background" refers to samples incubated with normal mouse IgG rather than with MoAb. The experiment is representative of five so performed.

surface antigen expression compared with baseline was 3.6% ± 0.1% (n = 5) for GPIb (as determined by MoAb 6D1), 24.0% ± 2.3% for GPIX (as determined by MoAb FMC25), and 23.2% ± 1.5% for the GPIb-IX complex (as determined by MoAb AK1). This quantitative difference between the cathepsin G-induced decrease in different components of the GPIb-IX complex is illustrated in Fig 2. In contrast, when thrombin was substituted for cathepsin G, there was a parallel decrease in the platelet surface expression of the different components of the GPIb-IX complex. Thus, 20 minutes after the addition of 1 U/mL thrombin to a suspension of washed platelets, platelet surface antigen expression compared with baseline was 23.7% ± 1.5% (n = 5) for GPIb (as determined by 6D1), 24.3% ± 1.6% for GPIX (as determined by FMC25), and 23.8% ± 1.2% for the GPIb-IX complex (as determined by AK1).

As shown by the single peaks in Fig 2, the cathepsin G-induced decrease in the platelet surface expression of the GPIb-IX complex was not restricted to a distinct subpopulation of platelets.

Effect of Cathepsin G on the Total Platelet Content of GPIb-IX

We next compared the effects of cathepsin G and thrombin on the total platelet content of GPIb-IX and platelet surface GPIb-IX, as determined by flow cytometric analysis of permeabilized and nonpermeabilized platelets (Fig 3). Both cathepsin G and thrombin resulted in marked decreases in the platelet surface expression of GPIb, GPIX, and the GPIb-IX complex (Fig 3A, C, and E). As noted above, cathepsin G, but not thrombin, resulted in a greater decrease in the platelet surface expression of GPIb than GPIX and the GPIb-IX complex (compare Fig 3A with Fig 3C and E). Furthermore, 10 μg/mL cathepsin G resulted in a greater decrease in the platelet surface expression of GPIb than did a maximal concentration of thrombin (1 U/mL; Fig 3A).

In parallel with the cathepsin G-induced decrease in the platelet surface expression of GPIb (Fig 3A), there was a cathepsin G-induced marked decrease in the total platelet content of GPIb (Fig 3B). In contrast, despite the cathepsin G-induced decrease in the platelet surface expression of

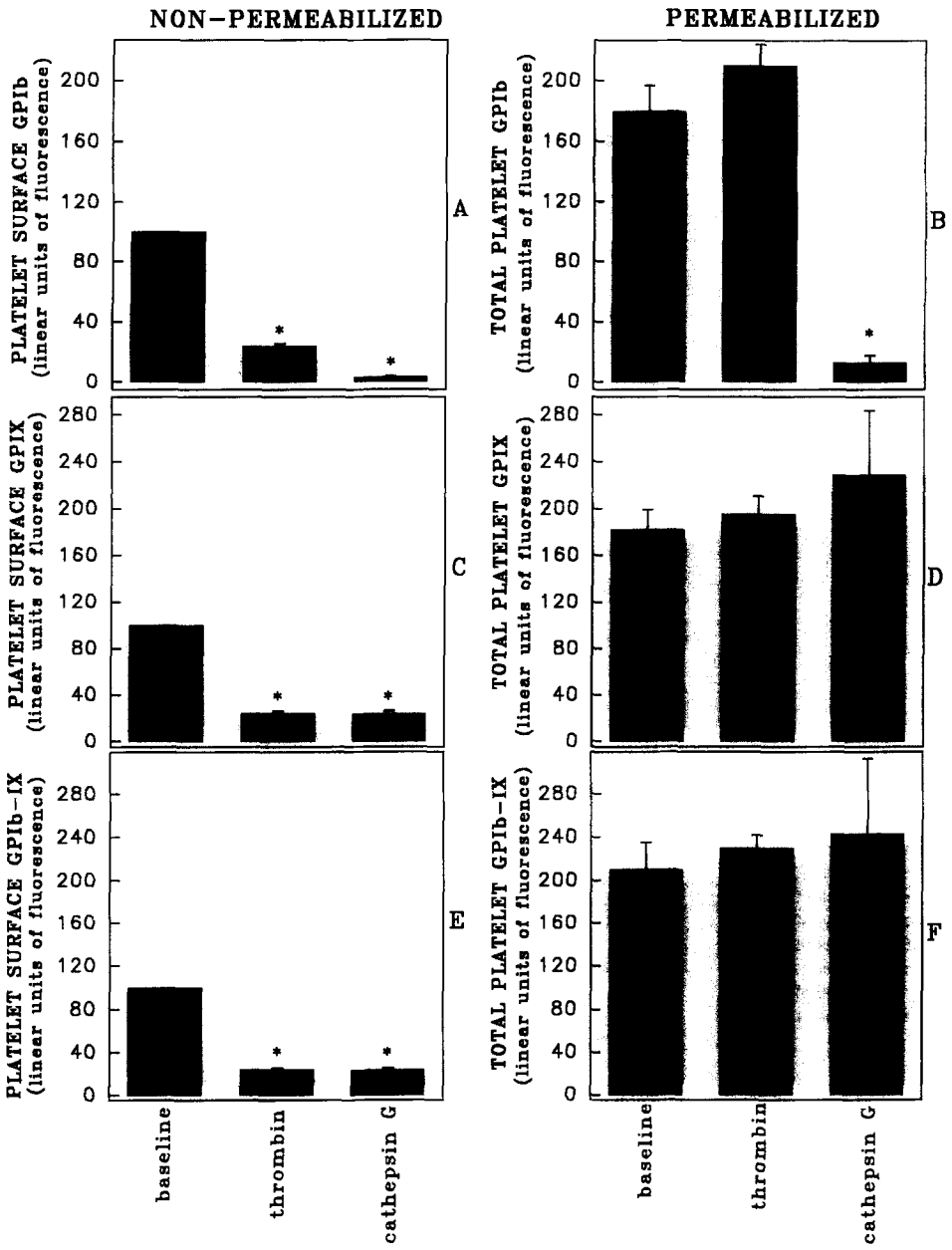

Fig 3. Effect of cathepsin G and thrombin on total platelet GPIb-IX and platelet surface GPIb-IX, as determined by flow cytometric analysis of permeabilized and nonpermeabilized platelets. Washed platelets were incubated (22°C for 20 minutes) with either 10 μg/mL cathepsin G, 1 U/mL thrombin, or buffer only ("baseline"). After fixation and dilution, the platelets were incubated (22°C for 20 minutes) with an FITC-conjugated MoAb (6D1 in A and B, FMC25 in C and D, and AK1 in E and F) in the presence (B, D, and F) or absence (A, C, and E) of 0.1% Triton X-100. Samples were then analyzed by flow cytometry. Antibody binding to baseline, nonpermeabilized samples was assigned 100 U of fluorescence. Data are mean ± SEM, n = 5 separate experiments. *$P < .05$ compared with baseline samples. In addition, for each antibody (6D1, FMC25, and AK1), binding to baseline, permeabilized platelets was significantly increased compared with the binding to baseline, nonpermeabilized platelets.

GPIX (Fig 3C) and the GPIb-IX complex (Fig 3E), cathepsin G did not result in any significant change in the total platelet content of GPIX (Fig 3D) or the GPIb-IX complex (Fig 3F). Despite the thrombin-induced decrease in the platelet surface expression of GPIb, GPIX, and the GPIb-IX complex (Fig 3A, C, and E), thrombin did not result in any change in the total platelet content of GPIb, GPIX, or the GPIb-IX complex (Fig 3B, D, and F). These experiments suggest that, unlike thrombin, cathepsin G resulted in proteolysis of GPIb.

These experiments also demonstrate that, in addition to the previously described non-surface-accessible pool of GPIb (see Michelson and Barnard[31] and Michelson et al[57], and compare the baseline of Fig 3B with the baseline of Fig 3A), platelets have a non-surface-accessible pool of GPIX, as determined by MoAb FMC25 (compare the baseline of Fig 3D with the baseline of Fig 3C). The GPIb and GPIX in this pool are fully complexed, as determined by MoAb AK1 (compare the baseline of Fig 3F with the baseline of Fig 3E).

Cathepsin G Results in Release of a Glycocalicin Fragment From Platelets

To confirm that cathepsin G resulted in proteolysis of GPIb from platelets, the supernatant concentration of glycocalicin, a proteolytic product of the GPIbα,[56] was determined. Incubation of washed platelets at 22°C for 20 minutes

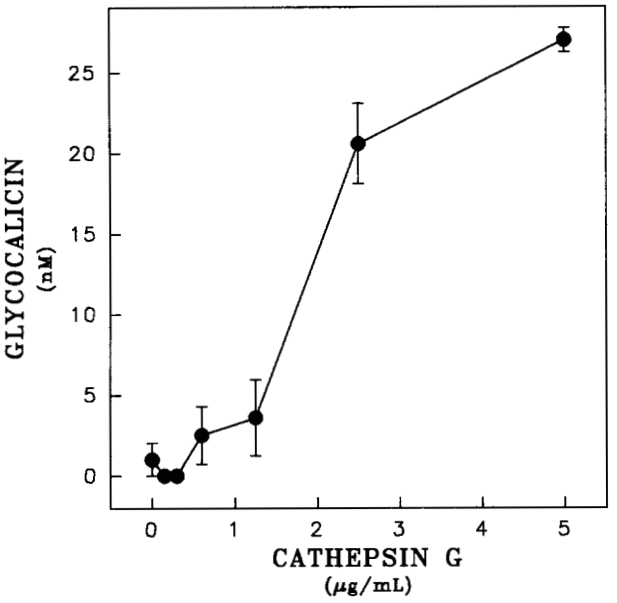

Fig 4. Effect of cathepsin G on the release from platelets of a glycocalicin fragment (proteolytic product of GPIb). Washed platelets were incubated (22°C for 20 minutes) with the indicated concentrations of cathepsin G. After addition of 100 μg/mL aprotinin (a proteolytic inhibitor), the samples were centrifuged (2,000*g* for 10 minutes) and the concentration of glycocalicin in the supernatants determined, as described in Materials and Methods. Data are mean ± SEM, n = 3.

with cathepsin G resulted in a concentration-dependent increase in the glycocalicin content of the supernatant (Fig 4). The maximal observed increase in supernatant glycocalicin of 27 nmol/L more than accounted for the calculated total available platelet surface GPIb pool of 3.7 nmol/L (based on a final concentration of 75,000 platelet/μL and assuming 30,000 copies of GPIb on the platelet surface[56]). These data are consistent with the presence of the previously reported non–surface-accessible pool of GPIb[31,57] and with the cathepsin G-induced marked decrease in this pool (Fig 3B).

Role of Actin Polymerization in the Cathepsin G-Induced Decrease in the Platelet Surface Expression of the GPIb-IX Complex

To examine the effect of cathepsin G (0.1 to 5 μg/mL) on platelet actin polymerization, experiments were performed with FITC-conjugated phalloidin, which specifically stains polymerized (F) actin.[55] These experiments showed that cathepsin G resulted in a concentration-dependent increase in the F-actin content of washed platelets (data not shown).

To examine the role of actin polymerization in the cathepsin G-induced decrease in the platelet surface expression of the GPIb-IX complex, and in the previously reported cathepsin G-induced increase in the platelet surface expression of P-selectin,[6] experiments were performed with 6 μmol/L cytochalasin B, an inhibitor of actin polymerization.[54] Cytochalasin B had no effect on the cathepsin G-induced increase in the platelet surface expression of P-selectin (as determined by MoAb S12; Fig 5A). As expected from our findings of a cathepsin G-induced proteolysis of GPIbα (see above), the decrease in the platelet surface expression of GPIbα induced by 10 μg/mL cathepsin G was not inhibited by cytochalasin B (as determined by MoAb 6D1; Fig 5B). However, the decrease in the platelet surface expression of GPIb induced by 2 to 6 μg/mL cathepsin G was partially inhibited by cytochalasin B (Fig 5B), suggesting that at submaximal concentrations of cathepsin G the decreased platelet surface expression of GPIbα was via both proteolytic and cytoskeletal-mediated mechanisms.

In contrast, the decrease in the platelet surface expression of GPIX (as determined by MoAb FMC25) and of the remnant of the GPIb-IX complex (as determined by MoAb AK1) induced by all tested concentrations of cathepsin G (2 to 10 μg/mL) was completely inhibited by cytochalasin B (Fig 5C and D), suggesting a mechanism involving actin polymerization rather than proteolysis. In the presence of cathepsin G, cytochalasin B actually resulted in an increase in the platelet surface expression of the GPIb-IX complex (Fig 5D), presumably on the basis of increased steric access of AK1 to its binding site as a result of GPIbα proteolysis.

In contrast to these experiments with cathepsin G, cytochalasin B completely inhibited the thrombin-induced decrease in the platelet surface expression of GPIb, GPIX, and the GPIb-IX complex (data not shown), as previously reported.[19]

Effect of PGI_2 on the Cathepsin G-Induced Decrease in the Platelet Surface Expression of the GPIb-IX Complex

To further examine the role of platelet activation in the cathepsin G-induced decrease in the platelet surface expression of the GPIb-IX complex, experiments were performed with PGI_2, an inhibitor of platelet activation. PGI_2 had no effect on the cathepsin G-induced decrease in the platelet surface expression of GPIb, as determined by MoAb 6D1 (Fig 6). In contrast, PGI_2 completely inhibited the cathepsin G-induced decrease in the platelet surface expression of the GPIb-IX complex, as determined by MoAb AK1 (Fig 6).

Effect of Cathepsin G on the Surface Expression of the GPIb-IX Complex on Fixed Platelets

Metabolic inactivation of platelets by fixation with 1% formaldehyde did not inhibit the cathepsin G-induced decrease in the platelet surface expression of GPIb, as determined by MoAb 6D1 (Fig 7). However, cathepsin G resulted in minimal decreases in the binding to fixed platelets of MoAbs TM60 (directed against the thrombin binding site on GPIbα) and WM23 (directed against the macroglycopeptide portion of GPIbα). Fixation abolished the cathepsin G-induced decrease in platelet surface GPIX and the GPIb-IX complex, as determined by MoAbs FMC25 and AK1, respectively (Fig 7). These data show that a metabolically active platelet is required for the cathepsin G-induced decrease in platelet surface GPIX and the GPIb-IX complex, but not for the cathepsin G-induced decrease (proteolysis)

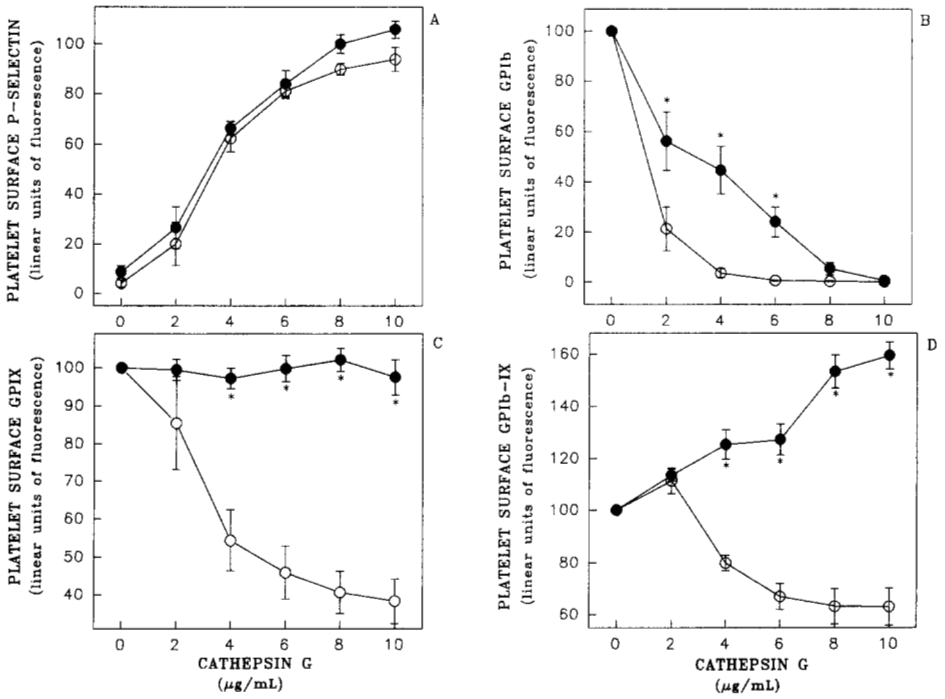

Fig 5. Effect of inhibition of actin polymerization on cathepsin G-induced modulation of the platelet surface expression of P-selectin and the GPIb-IX complex. Washed platelets were (●) or were not (○) incubated (22°C for 15 minutes) with 6 μmol/L cytochalasin B (an inhibitor of actin polymerization). The platelets were then incubated (22°C for 20 minutes) with the indicated concentration of cathepsin G and fixed. The vertical axis represents platelet surface binding, as determined by flow cytometry, of MoAbs S12 (A), 6D1 (B), FMC25 (C), and AK1 (D). In (A), S12 binding after incubation (22°C for 20 minutes) with 1 U/mL thrombin was assigned 100 U of fluorescence. In (B), (C), and (D), the binding of 6D1, FMC25, and AK1 in the absence of cathepsin G or thrombin was assigned 100 U of fluorescence. Data are mean ± SEM, n = 5 separate experiments. *$P < .05$ for samples incubated with cytochalasin B compared with control samples not incubated with cytochalasin B.

in platelet surface GPIb. Furthermore, these data suggest that the cathepsin G-induced proteolysis of GPIbα includes the 6D1 epitope but not the TM60 or WM23 epitopes.

Platelet Surface GPIb Is Not Required for the Cathepsin G-Induced Modulation of the Platelet Surface Expression of Either P-selectin or the Remainder of the GPIb-IX Complex

To address the question as to whether cathepsin G-induced proteolysis of GPIb is required for the cathepsin G-induced cytoskeletal-mediated redistribution of the remainder of the GPIb-IX complex or cathepsin G-induced degranulation, two sets of experiments were performed. First, washed platelets were incubated (22°C for 30 minutes) with *Serratia marcescens* metalloprotease at 2.5 μg/mL. As determined by flow cytometry with MoAb 6D1, the platelet surface GPIb content of *Serratia* protease-treated platelets was 0.1% ± 0.1% (mean ± SEM, n = 6) of control (non-*Serratia* protease-treated) platelets. *Serratia* protease treatment of platelets had no effect on cathepsin G-induced degranulation, as determined by the platelet surface expression of P-selectin (Fig 8A). *Serratia* protease treatment of platelets resulted in increased platelet binding of the GPIb-IX complex-dependent MoAb AK1 (Fig 8B), presumably because of increased steric access of AK1 to its binding site as a result of GPIbα proteolysis. However, *Serratia* protease treatment of platelets had no effect on the cathepsin G-induced decrease in the platelet surface expression of the remnant of the GPIb-IX complex (Fig 8B).

Second, experiments were performed with the platelets of a patient with Bernard-Soulier syndrome, an inherited deficiency of the GPIb-IX complex.[56] The cathepsin G-induced increase in the platelet surface expression of P-selectin was the same for Bernard-Soulier platelets as for normal control platelets (data not shown).

DISCUSSION

In this study, we have shown that neutrophil cathepsin G decreases the platelet surface expression of the GPIb-IX complex, as determined by 3 independent methods: immunologic (flow cytometry with GPIb-IX-specific MoAbs), ligand binding (exogenous vWF in the presence of ristocetin), and functional (ristocetin-induced platelet agglutination). Unlike thrombin,[16,18-20,25] cathepsin G resulted in proteolysis of the vWF binding site on the α chain of platelet GPIb (defined by MoAb 6D1[34,35]), as determined by increased supernatant glycocalicin fragment (a proteolytic product of GPIbα[56]); decreased total platelet content of GPIb; and lack

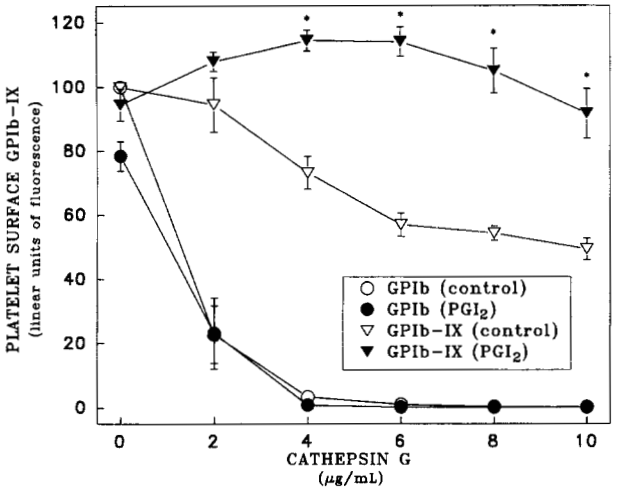

Fig 6. Effect of PGI_2 on the cathepsin G-induced decrease in the platelet surface expression of GPIb-IX. Washed platelets were (solid symbols) or were not (open symbols) incubated (22°C for 30 minutes) with 10 μmol/L PGI_2. The platelets were then incubated (22°C for 20 minutes) with the indicated concentration of cathepsin G and fixed. The vertical axis represents platelet surface binding, as determined by flow cytometry, of MoAbs 6D1 (GPIb-specific) (○, ●) and AK1 (GPIb-IX complex-specific) (▽, ▼). The binding of 6D1 and AK1 in the absence of PGI_2 and cathepsin G was assigned 100 U of fluorescence. Data are mean ± SEM, n = 6 separate experiments. *$P < .05$ for samples incubated with PGI_2 compared with samples not incubated with PGI_2.

of effect of either cytochalasin B (an inhibitor of actin polymerization[54]), PGI_2 (an inhibitor of platelet activation), or prior fixation of the platelets. Molino et al[21] in a manuscript published after the original submission of the present study,

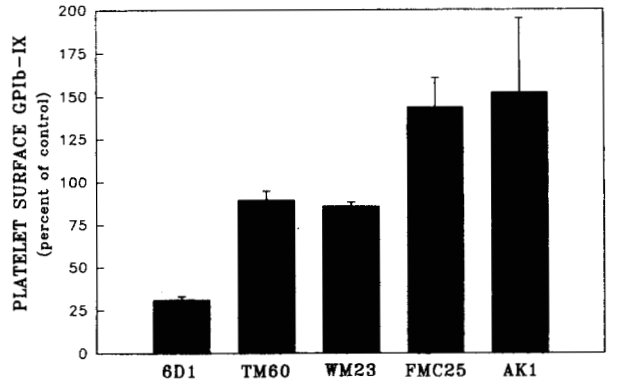

Fig 7. Effect of cathepsin G on the surface expression of GPIb-IX on fixed platelets. Washed platelets were fixed with 1% formaldehyde before incubation (22°C for 20 minutes) with 10 μg/mL cathepsin G. The samples were then incubated with a saturating concentration of a GPIb-IX-specific MoAb (6D1, TM60, WM23, FMC25, or AK1). 6D1, TM60, and WM23 are GPIb-specific; FMC25 is GPIX-specific; and AK1 is GPIb-IX complex-specific. Antibody binding was analyzed by flow cytometry. Antibody binding in the absence of cathepsin G was assigned 100 U of fluorescence. Data are mean ± SEM, n = 3 separate experiments.

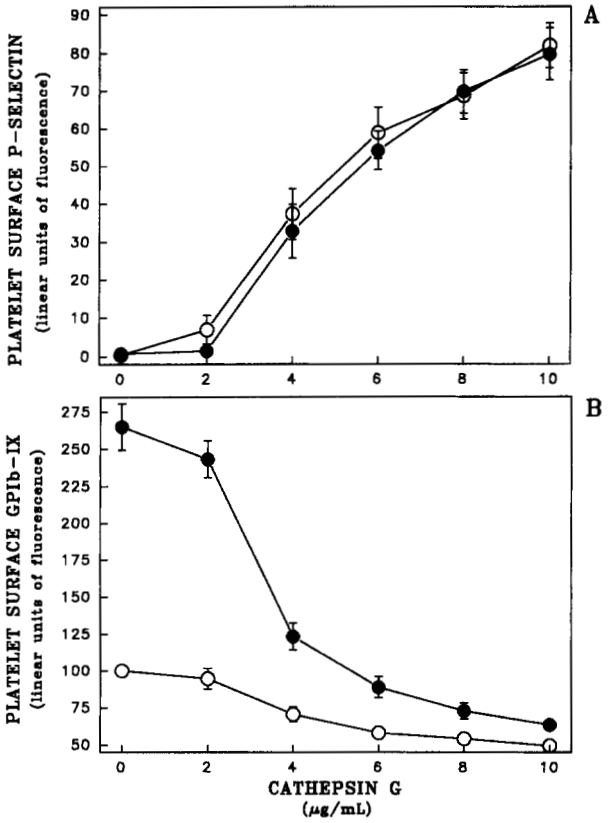

Fig 8. Effect of proteolysis of GPIb on cathepsin G-induced modulation of the platelet surface expression of P-selectin and the remainder of the GPIb-IX complex. Washed platelets were (●) or were not (○) incubated (22°C for 30 minutes) with 2.5 μg/mL *Serratia marcescens* protease. The platelets were then incubated (22°C for 20 minutes) with the indicated concentration of cathepsin G and fixed. The vertical axis represents platelet surface binding, as determined by flow cytometry, of MoAbs S12 (A) and AK1 (B). In (A), S12 binding to non-*Serratia* protease-treated platelets after incubation (22°C for 20 minutes) with 1 U/mL thrombin was assigned 100 U of fluorescence. In (B), AK1 binding to non-*Serratia* protease-treated platelets in the absence of cathepsin G or thrombin was assigned 100 U of fluorescence. Data are mean ± SEM, n = 6 separate experiments.

similarly found that cathepsin G resulted in proteolysis of GPIbα. In the present study, in contrast to the findings with MoAb 6D1, cathepsin G resulted in minimal decreases in the binding to fixed platelets of MoAbs TM60 (directed against the thrombin binding site on GPIbα[37-39]) and WM23 (directed against the macroglycopeptide portion of GPIbα[36,40]). These data suggest that the cathepsin G-induced proteolysis of GPIbα includes the 6D1 epitope but not the TM60 or WM23 epitopes.

Furthermore, we now show that, in addition to its proteolytic effect on GPIbα (present study and Molino et al[21]), cathepsin G results in a decrease in the platelet surface expression of GPIX and the remnant of the GPIb-IX complex (defined by MoAbs FMC25 and AK1) via a cytoskeletal-mediated redistribution rather than via proteolysis, as determined by lack of change in the total platelet content of

GPIX and the GPIb-IX complex, and complete inhibition by cytochalasin B, PGI_2, and prior fixation of platelets.

Cathepsin G binds to platelets via an as yet unidentified specific receptor.[58] In this study, we used two models of GPIb-deficient platelets (*Serratia* protease-treated platelets and platelets from a patient with Bernard-Soulier syndrome) to examine whether platelet surface GPIb is required for the cathepsin G-induced modulation of the platelet surface expression of either P-selectin or the remainder of the GPIb-IX complex. *Serratia* protease results in virtually complete, selective proteolysis of the glycocalicin portion of platelet surface GPIbα (this study, Cooper et al[27] and Yamamoto et al[28]), without any effect on platelet surface GPIX, the remainder of the GPIb-IX complex, or any other surface GP,[28] and without causing platelet activation.[28] The platelets of our patient with Bernard-Soulier syndrome, an inherited deficiency of GPIb-IX,[56] have a greater than 99% reduction in platelet surface GPIb and a 94% reduction in platelet surface GPIX. The results of the experiments with these two models of GPIb-deficient platelets showed that neither platelet surface GPIb nor cathepsin G-induced proteolysis of GPIb are required for the cathepsin G-induced cytoskeletal-mediated redistribution of the remainder of the GPIb-IX complex or for cathepsin G-induced degranulation. Thus, cathepsin G activates platelets and redistributes the remnant GPIb-IX complex via a GPIbα-independent pathway. In contrast, the thrombin-induced cytoskeletal-mediated redistribution of the GPIb-IX complex and thrombin-induced degranulation proceed via both GPIbα-dependent and GPIbα-independent pathways.[28,59] The fact that cathepsin G-induced proteolysis of GPIb is not required for cathepsin G-induced platelet activation (this study) is comparable to the fact that thrombin-induced proteolysis of GPV is not required for thrombin-induced platelet activation.[60]

There are many interactions between neutrophils and platelets.[61,62] For example, P-selectin, a component of the platelet α granule membrane that is only expressed on the platelet surface membrane after degranulation,[10,43] mediates adhesion of activated platelets to neutrophils and monocytes.[63-65] The precursor of neutrophil-activating peptide-2, a cytokine that causes neutrophil degranulation and chemotaxis, is released by activated platelets.[66] Another example is platelet activation by cathepsin G, a serine protease released by the azurophilic granules of stimulated neutrophils.[1-6] It has recently been reported that cathepsin G increases the platelet surface expression of P-selectin[6] and increases the exposure of the fibrinogen binding site on the platelet surface GPIIb-IIIa complex.[6,21] Although thrombospondin 1,[22] antiserine proteases,[23] and plasma[6] are all inhibitors of cathepsin G, the effects of cathepsin G on platelets can occur during close contact between neutrophils and platelets in a protective microenvironment (eg, thrombosis and local inflammation).[23,24] The present finding that neutrophil cathepsin G decreases the platelet surface expression of the GPIb-IX complex, together with the previous finding that neutrophil elastase decreases the platelet surface expression of GPIb,[32,33] suggests that, in a protective microenvironment, neutrophils play a role in transforming platelets from a state favoring adhesion to damaged vessel walls (mediated by vWF binding to the GPIbα component of the GPIb-IX complex[56]) to a state favoring platelet-to-platelet aggregation (mediated by the binding of fibrinogen and other ligands to the GPIIb-IIIa complex[67]) and platelet-to-leukocyte adhesion (mediated by P-selectin[63-65]).

In summary, neutrophil cathepsin G modulates the platelet surface expression of the GPIb-IX complex both by proteolysis of the vWF binding site on GPIbα and by a cytoskeletal-mediated redistribution of the remainder of the complex. Neither platelet surface GPIb nor cathepsin G-induced proteolysis of GPIbα is required for the cathepsin G-induced cytoskeletal-mediated redistribution of the remainder of the platelet surface GPIb-IX complex or for cathepsin G-induced platelet degranulation.

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