Effects of Leukocyte-Derived Cathepsin G on Platelet Membrane Glycoprotein Ib-IX and IIb-IIIa Complexes: A Comparison With Thrombin

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Cathepsin G is a serine, chymotrypsin-like protease released by activated polymorphonuclear leukocytes (PMN) that may act as a platelet agonist. The effect of this enzyme on platelet surface glycoproteins (Gp Ib and IIb-IIIa) was evaluated by means of a cytofluorimetric assay, using fluorescein isothiocyanate-labeled monoclonal antibodies (MoAbs) directed at the α chain of Gp Ib (SZ2), at Gp IX or at the complex Gp IIb-IIIa (P2), and the fibrinogen-receptor-specific MoAb PAC-1. In human washed platelets, cathepsin G increased the binding of P2 and PAC-1, decreased the binding of SZ2, but only slightly affected the binding of anti-Gp IX. SZ2 binding decrease was more rapid in cathepsin G- than in thrombin-stimulated platelets, whereas the increase of P2 and PAC-1 binding occurred to a comparable extent with either agonist. In paraformaldehyde (PFA)-fixed and energy-depleted platelets, no effect on either Gp Ib or IIb-IIIa complex was observed with thrombin. At variance, cathepsin G was still able to reduce binding of SZ2, whereas increased binding of P2 or PAC-1 antibodies was not observed. Triton X-100 permeabilization of cathepsin G-treated, PFA-fixed platelets did not restore SZ2 binding at variance with thrombin.

Moreover, platelet incubation with cathepsin G resulted in the loss of ristocetin-induced agglutination in the presence of the von Willebrand factor and in the appearance of Gp Ib-derived proteolytic products in supernatants. After dissociation by EDTA pretreatment of surface Gp IIb-IIIa complexes, cathepsin G still induced increased binding of P2. Aspirin and an adenosine diphosphate scavenger system had only a slight but not significant effect on changes in antibody binding induced by cathepsin G. All these data would indicate that cathepsin G, like thrombin, interacts with platelet-surface Gp, inducing the exposure of the intracellular pool of the Gp IIb-IIIa complex with concomitant expression of a functional fibrinogen receptor. Moreover, it induces a loss of antigenic sites on Gp Ib, but the mechanism involved, a proteolytic cleavage of Gp Ib, is substantially different from that of thrombin. These changes, induced by a product of activated PMN, might reduce the reactivity of platelets to the subendothelium, while increasing their ability to undergo aggregation and release reaction.

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Gp Ib and Gp IIIa are two different glycoproteins that together form a calcium-dependent complex on the resting platelet surface. Gp Ib consists of two disulfide-linked subunits, Gp Ib α (132 Kd), and Gp Ib β (23 Kd). Gp IIIa is a single polypeptide (105 Kd) with extensive intrachain disulfide bonding.

Changes on surface Gp occur after stimulation with different agonists. Gp IIb-IIIa undergoes a conformational change, thus enabling it to bind fibrinogen and other adhesive proteins, such as fibronectin, vitronectin, and thrombospondin.

Proteolytic enzymes, among them several serine proteases, act on the platelet surface, modifying platelet Gp. Gp Ib has been reported to be cleaved by calcium-activated proteases with the release of a large, hydrophilic, carbohydrate-rich fragment termed glyocalcin or by trypsin, pancreatic α-chymotrypsin, and leukocyte-derived elastase with the release of proteolytic products of various lengths. Moreover, exposure of fibrinogen binding sites by elastase, probably through proteolytic cleavage of Gp IIIa, results in spontaneous platelet aggregation by fibrinogen.

Cathepsin G, a serine chymotrypsin-like protease released from the azurophilic granules of activated polymorphonuclear leukocytes (PMN) may act as a platelet agonist. Bykowska et al first mentioned cathepsin G-induced degradation of Gp Ib and IIIa, whereas Pidard et al did not detect any proteolytic effect of cathepsin G on the Gp IIb-IIIa complex. Therefore, it is of interest to clearly define the effects of cathepsin G on the platelet surface glycoproteins and to study some of the mechanism(s) involved.

The effects of cathepsin G were compared with those of thrombin, another physiologic serine protease, whose effects on platelet surface glycoproteins have been widely described. When human washed platelets are activated by
After washing and adjusting platelet count to 5 × 10^7/mL, 50 µL of 1 × 10^8/mL platelet suspension were stimulated with cathepsin G (50 to 400 nmol/L) for 1 minute and fixed with 500 µL 2% paraformaldehyde (PFA) after addition of trypsin-chymotrypsin inhibitor (1 mg/mL). The latter, a second incubation with an FITC-labeled antimouse MoAb was performed. Samples were then analyzed by flow cytometry.

Mean values of fluorescence peaks were compared with those of unstimulated samples, taken as 100%. For further details, see Materials and Methods. Mean and SEM of three to four different experiments are shown. Statistical analysis (ANOVA and Dunnett test) indicated significant differences between cathepsin G (200 to 400 nmol/L cathepsin G).

Thrombin, it is possible to observe a marked decrease in surface binding of antibodies directed at Gp Ib and a marked increase in the binding of antibodies at Gp IIb-IIIa complex with both radioligand binding and with the cyto metric method. Moreover, thrombin treatment decreases vWF binding to the platelet surface. The thrombin-induced reduction of Gp Ib expressed at the platelet surface has been ascribed to a rapid redistribution of Gp Ib-IX complex within the surface-connected membrane system of activated platelets, although several mechanisms of thrombin-induced exposure of a fibrinogen receptor have been hypothesized.

The results reported here indicate that cathepsin G induced changes in Gp Ib and Gp IIb-IIIa similar to those induced by thrombin, but through different mechanisms of thrombotic disease.

**Materials and Methods**

**Antibodies and reagents.** Fluorescein isothiocyanate (FITC)-labeled anti-Gp Ib monoclonal antibody (MoAb), clone SZ2 (able to inhibit ristocetin-induced platelet aggregation in the presence of a vWF binding factor), and FITC-labeled anti-Gp Ib-IIIa MoAb, clone P2 (directed against Gp IIb-IIIa complex), were obtained from Immunotech SA (Marseille, France). FITC-labeled anti-Gp IX was from Becton Dickinson (Milan, Italy). The fibrinogen receptor-specific MoAb PAC-1 was kindly provided by Dr Sanford J. Shattil (University of Pennsylvania School of Medicine, Philadelphia, PA). The peroxidase-linked antimouse IgG used in the Western blot technique and enhanced chemiluminescence (ECL) detection system were from Amersham (Amity, Milan, Italy). Prostaglandin E2 (PGE2), creatine phosphate (CP), creatine phosphokinase (CPK), crystal violet, FITC-labeled antimouse antibody, NaN3, luciferine-luciferase, and human thrombin (3,000 NIH U/mg protein) were obtained from Sigma (St Louis, MO). Cathepsin G (2 U/mg protein, >98% pure), purified from human neutrophils, was from Calbiochem (San Diego, CA). Acetylsalicylic acid (ASA) was provided as Flectadol by Maggioni Winthrop (Milan, Italy). Other chemicals were of reagent grade or higher quality.

**Platelet isolation.** Human venous blood was drawn by venipuncture from adult volunteers, who had not taken any medication for at least 10 days, and with trisodium citrate 3.8% in the ratio of 9 volumes of blood to 1 volume of anticoagulant. Platelet-rich plasma (PRP) was obtained by centrifuging whole blood at 250g for 15 minutes. PRP was twice washed by centrifuging at 1,100g for 15 minutes in the presence of 1 µmol/L PGE2, and the platelets were finally resuspended in HEPES-Tyrode buffer (pH 7.4) containing 1 mmol/L CaCl2. Cell count was adjusted to 1 × 10^8/mL, and the platelet suspension was incubated at 37°C for 1 minute. The agonist was added and mixed by stirring in the aggregometer for 2 seconds. The sample was then incubated at 37°C undisturbed to prevent platelet aggregation. The reaction was stopped at various times by adding hirudin (2 U/mL) or soybean trypsin inhibitor (1 mg/mL) to thrombin- or cathepsin G-stimulated samples, respectively. Platelet suspensions were then incubated with an equal volume of 2% paraformaldehyde (PFA) overnight. CP, CPK, and I-ASA were added to platelet suspensions as specified.

**Energy-depleted platelet preparation.** PRP was washed twice and, 10 minutes before stimulation, was resuspended in a modified glucose-free HEPES-Tyrode, containing 50 mmol/L 2-deoxyglucose, 0.05% NaN3, and 10 mmol/L gluconic-δ-lactone, to inhibit glycolysis, oxidative phosphorylation, and glycogen phosphorylase, respectively. Total adenosine triphosphate (ATP) levels after energy-depletion treatment measured by luciferine-luciferase in a lumiluminometer (PICA; Chrono-Log, Mascia Brunelli, Milan, Italy) were 27% of controls, similar to the nonmetabolic ATP pool. Because gluconic-δ-lactone is rapidly hydrolyzed at neutral pH, the dry powder was dissolved immediately before addition to the platelet suspension. It induced a small pH decrease from 7.4 to 7.0, which did not affect functional parameters such as aggregation and release reaction by both agonists.

**Platelet staining with MoAbs.** Fixed platelets were washed twice, resuspended at 5 × 10^7/mL in HEPES-Tyrode containing 1 mmol/L CaCl2, and incubated for 30 minutes at 4°C with saturating concentrations of antibodies. The FITC-labeled anti-Gp Ib MoAb used in these experiments (SZ2) recognizes an epitope on the glycopcalcin moiety of Gp Ib and blocks ristocetin-induced binding of vWF to platelet. The FITC-labeled anti-Gp Ib-IIIa (P2) MoAb reacts against the complex both in resting and in stimulated platelets. The FITC-labeled MoAb anti-Gp IX recognized the CD 42a, and the MoAb PAC-1 was directed at the fibrinogen receptor on activated platelet surface. PAC-1 binding was detected by a second FITC-labeled antimouse antibody. Samples were then washed.
Intrinsic platelet fluorescence was tested by quenching with crystal violet, which was prepared by dissolving 2 mg/mL in saline by heating. The solution was then filtered before use on the same day. Platelets (5 × 10^7/mL) suspended in 200 μL of HEPES-Tyrode containing 1 mmol/L CaCl₂ were mixed with 100 μL of crystal violet solution, incubated for 5 minutes in ice, and then washed twice with HEPES-Tyrode. The cells were labeled with MoAbs after the quenching procedure.

Binding of SZ2, P2, and PAC-1 was not significantly different (P > .05) in crystal violet-treated platelets with respect to untreated ones, both in basal and stimulated (400 nmol/L cathepsin G) platelets; only binding of anti-GpIX was reduced by 39.8% and 41.7% in basal and stimulated platelets, respectively (P < .05 by paired student's t-test, n = 4). However, the percent of the mean fluorescence peak for this antibody was not modified by correction for intrinsic fluorescence. In EDTA-treated platelets, the fluorescence peak due to P2 binding was not significantly quenched by crystal violet in either basal or stimulated platelets.

Flow cytometric assessment of platelet surface glycoproteins. Samples were analyzed in a fluorescence-activated cell sorter (FACS)star flow cytometer (Becton Dickinson) equipped with an argon-ion laser. Laser emission was adjusted to deliver 400 mW at 488 nm for excitation. Fluorescence was detected through a 530/30-nm band pass filter. The instrument was calibrated daily for fluorescence and light scatter using 2-μm Calibrite beads (Becton Dickinson). Samples were passed through the laser beam through a 70-μm nozzle at a flow rate between 300 and 500 platelets per second. Logarithmic amplification was used for both fluorescence signal and for light scatter signal. Data were collected and analyzed on a Hewlett Packard computer equipped with a Consort 30 Program (Becton Dickinson).

Ristocetin-induced platelet agglutination. Human washed platelets were PFA-fixed, washed, and resuspended at 1 × 10⁹/mL in HEPES-Tyrode buffer containing 1 mmol/L CaCl₂. Five hundred microliters of platelet suspension was incubated at 37°C under constant stirring (1,000 rpm) with 3.7 μg/mL of human purified vWF (a kind gift from Dr Augusto Federici, A. Bianchi Bonomi Hemophilia and Thrombosis Center, Milan, Italy) for 5 minutes. Ristocetin (1 mg/mL) was then added, and platelet agglutination was followed by recording the increase in light transmission in an aggregometer (PICA; Chrono-Log). Cathepsin G (200 or 400 nmol/L) was added to the platelet suspension for 10 minutes before vWF.

Electrophoretic blotting. Washed platelets were resuspended at 5 × 10⁹/mL in HEPES-Tyrode buffer in the presence of 1 mmol/L CaCl₂ and then treated with saline or 200 to 400 nmol/L cathepsin G or 0.25 U/mL thrombin. The reaction was stopped at 15, 30, 60, and 300 seconds by adding trypsin-chymotrypsin inhibitor (1 mg/mL) or hirudin (2 U/mL) and by cooling samples. After sample centrifugation (7,000g for 2 minutes), both pellet and supernatant (100 μL/lane) were processed on 10% acrylamide gel slabs. Proteins were then transferred from gels to nitrocellulose sheets and incubated with anti-Gp Ib SZ2 (5 μg/mL), followed by detection with a second, sheep horseradish peroxidase-conjugated antimouse antibody (1:500 dilution) using the ECL system.
RESULTS

Effects of cathepsin G and thrombin on platelet binding of MoAbs directed against Gp Ib and IIb-IIIa. Activation of human washed platelets with 50 to 400 nmol/L cathepsin G for 1 minute yielded a statistically significant, concentration-dependent reduction of platelet surface binding of SZ2, an FITC-labeled MoAb against the α chain of Gp Ib (Fig 1). However, it also resulted in a clear increase in the platelet surface binding of the MoAbs directed at the Gp IIb-IIIa complex, at FITC-labeled P2, or at the functional fibrinogen receptor, PAC-1 (Fig 1). In contrast, the binding of FITC-labeled against Gp IX was only slightly affected by cathepsin G. Taking advantage of the cytofluorimetric method that analyzes single cells, a gradual shift of a single peak was observed by increasing concentrations of the agonist (Fig 2), indicating that cathepsin G-induced changes in Gp Ib and IIb-IIIa were not limited to a restricted platelet number but to the whole platelet population.

Preliminary experiments with thrombin-activated platelets showed a concentration-dependent increase of fluorescence peak of PAC-1 and P2 (data not shown) with a plateau being reached at 0.25 to 0.5 U/mL thrombin (2.2 to 4.5 nmol/L). Therefore, on a molar basis, cathepsin G appeared to be a less potent agonist than thrombin.

Comparison between the time course (5 to 420 seconds) of the modification induced by 200 nmol/L cathepsin G and that induced by 0.25 U/mL thrombin showed a reduction of platelet surface SZ2 binding at 5 minutes (6.9% ± 4.6% and 38.4% ± 3.9% of basal values, respectively; mean ± SEM of 3 to 6 different experiments), which was more rapid in cathepsin G- than in thrombin-activated samples (Fig 3). On the other hand, both agonists induced an increase of binding of P2 at 5 minutes (222.0% ± 23.7% and 194.8% ± 4.6% of basal values, for cathepsin G and thrombin, respectively; mean ± SEM; n = 3 to 6) and PAC-1 at 5 minutes (399.8% ± 59.6% and 280.9% ± 96.0% of basal values, for cathepsin G and thrombin, respectively; mean ± SEM; n = 3 to 5) with similar time-courses, reaching a plateau within 5 to 7 minutes after stimulation (Fig 3).

Effect of cathepsin G and thrombin on PFA-fixed or ATP-depleted platelets. To study the effect of agonist treatment in metabolically inactive platelets, cathepsin G- and thrombin-induced changes in Gp Ib and IIb-IIIa were also studied in PFA-fixed or ATP-depleted platelets. Thrombin treatment (0.25 U/mL) did not modify the binding of either SZ2, P2, or PAC-1 antibodies to PFA-fixed or ATP-depleted platelets. Cathepsin G failed to increase the binding of P2 and PAC-1 to PFA-fixed or ATP-depleted platelets, but it still reduced the binding of SZ2 in the same way as it did in metabolically active control cells. These results may indicate that GpII-IIIa complex exposure and fibrinogen receptor expression by both agonists strictly require functionally and metabolically active cells, whereas cathepsin G, unlike thrombin, can alter the expression of SZ2 epitope in metabolically inactive platelets (Fig 4).

Effect of permeabilization on SZ2 binding to cathepsin G-treated platelets. The thrombin-like mechanism of Gp Ib internalization was tested in cathepsin G- or thrombin-stimulated platelets by measuring SZ2 binding after PFA treatment and Triton X-100 permeabilization. This treatment resulted in a small but not significant increase in SZ2 binding to unstimulated platelets, confirming that permeabilization made internal Gp Ib accessible to the antibody. However, this treatment failed to restore SZ2 binding in cathepsin G-stimulated platelets (Fig 5), as it did in throm-
bin-stimulated platelets (data not shown and Hourdillé et al29).

Western blot analysis of proteolytic products of Gp Ib in cathepsin G- and thrombin-stimulated platelets. A possible proteolytic effect of cathepsin G on Gp Ib was tested by the Western blot technique, using the same unlabeled MoAb SZ2. As shown in Fig 6 (left panel), treatment of human washed platelets with 400 nmol/L cathepsin G resulted in a time-dependent appearance of detectable amounts of 3 bands of molecular weights between 60 and 45 Kd in platelet supernatant. Similar results were obtained with 200 nmol/L cathepsin G (data not shown). In parallel experiments, no proteolytic products were detectable in supernatants of thrombin (0.25 U/mL)-treated platelets (Fig 6, right panel).

Effect of cathepsin G on ristocetin-induced platelet agglutination. PFA-fixed platelets, when treated with 400 nmol/L cathepsin G, failed to agglutinate in the presence of human purified vWF and ristocetin, whereas in control samples an agglutination wave was recorded and macroscopic clumps were observed (Fig 7). A similar effect was obtained by using 200 nmol/L cathepsin G (data not shown).

Exposure of intraplatelet Gp IIb-IIIa pool by cathepsin G. To investigate whether cathepsin G-induced Gp IIb-IIIa exposure involves only external or also intracellular Gp IIb-IIIa pools, surface Gp IIb-IIIa complexes were irreversibly dissociated by incubating platelets with 4 mmol/L EDTA (37°C for 30 minutes). Samples were then washed, resuspended in calcium-containing buffer, and finally stimulated with cathepsin G. Under these conditions, the fluorescence peak of unstimulated platelets was very close to the autofluorescence peak, showing no or very limited binding of P2 to platelet surface, indicating the efficacy of EDTA treatment. Cathepsin G (200 nmol/L) was able to induce a shift of fluorescence peak in EDTA-treated samples, sug-
suggesting that the increase of P2 binding is due to the exposure of additional Gp Ib/IIa complex, deriving from an internal pool (Fig 8).

Contribution of adenosine diphosphate (ADP) and thromboxane A2 (TXA2) to platelet surface Gp changes induced by cathepsin G. Cathepsin G-induced platelet activation is partially due to amplification by TXA2 and ADP release.21,35 To verify the role of these amplifiers in cathepsin G-induced platelet-surface Gp changes, I-ASA-treated (60 minutes at room temperature) platelets were incubated with the ADP scavenger system CP/CPK. This treatment, which completely prevented TXB2 formation and ADP-induced platelet aggregation, affected only slightly but not significantly the Gp Ib/IIa and fibrinogen receptor exposure brought about by cathepsin G at 5 minutes (Table 1). Binding of SZ2 in cathepsin G-stimulated samples was unaffected by I-ASA and CP/CPK treatment. Similar results were obtained when 1 mmol/L ATP was used, instead of the CP/CPK system (data not shown).

DISCUSSION

Thrombin is known to induce changes in the Gp complex Ib-IX affecting the ability of platelets to bind vWF28 and in the Gp Ib/IIa complex, resulting in the exposure of a functional fibrinogen receptor.30,31

Our results show that another naturally occurring serine protease, the PMN-derived cathepsin G, like thrombin, modifies the platelet membrane Gp in a concentration-dependent manner, reducing the number of Gp Ib recognized by the antibody used and increasing the exposure of the Gp Ib/IIa complex and enabling it to bind fibrinogen. These cathepsin G-induced changes, as clearly shown by cytofluorimetry, are not restricted to subpopulations of platelets.

Whereas a constant 1:1 ratio of Gp Ib:IX was kept by thrombin-induced platelet activation,27 cathepsin G induced a reduction of anti-Gp IX binding less marked than that of Gp Ib. Therefore, different mechanisms may be involved in cathepsin G- or thrombin-induced Gp Ib modifications. This was confirmed by experiments with both fixed and energy-depleted platelets.

It is known that thrombin-induced fibrinogen-receptor exposure depends on intraplatelet second messengers.30,31 In agreement with this finding, no increase in P2 or PAC-1 binding was observed in either functionally or metabolically inactive platelets treated with thrombin. Similar negative results were obtained with cathepsin G in both PFA-treated and ATP-depleted cells, suggesting the requirement of metabolically active cells for Gp Ib/IIa exposure and fibrinogen receptor expression. This would imply that a possible proteolytic cleavage of Gp Ib/IIa complex by cathepsin G23,24 would not result in acquisition of competence to bind fibrinogen. On the contrary, reduction of binding of SZ2, triggered by cathepsin G, occurred on PFA-fixed and energy-depleted platelets in the same way as on control samples.

In contrast, SZ2 binding decrease was not observed after thrombin treatment of PFA-fixed or ATP-depleted platelets. Thrombin-induced downregulation of Gp Ib has been reported to require the active site of the enzyme,26,28,29 but not to be associated with the release of proteolytic products.26-28 This would imply that proteolytic activity of thrombin is required to activate the cell and to switch on mechanisms able to reduce the expression of Gp Ib, rather than to directly modify the conformational aspects of the complex. Therefore, receptor cleavage,40 followed by Gp Ib conformational changes or more probably Gp Ib movement within the surface-connected membrane system,29 with consequent loss of antibody binding sites, may be involved in the above-mentioned regulatory effect of thrombin. This is not the case of cathepsin G, because binding of SZ2 was not restored after Triton X-100 permeabilization of cathepsin G-stimulated samples. Therefore, under the conditions in which the internal pool of Gp Ib was made available to the antibody (as shown by the increased binding of SZ2 in unstimulated Triton X-100 permeabilized samples), restoration of anti-Gp Ib binding to thrombin- (data not shown and Hourdilé et al29) but not to cathepsin G-stimulated samples was observed (Fig 5). This indicates that the disappearance of SZ2 binding sites in cathepsin G-stimulated samples is not due to a redistribution in the open canalicular membrane system, as occurs with thrombin, but to a direct effect on Gp Ib.

Although occupation of the antibody binding site by cathepsin G might be hypothesized, Western blot analysis shows a time-dependent appearance of bands corresponding to a molecular weight of 60 to 45 Kd in supernatants of cathepsin G- but not of thrombin-stimulated samples, a
clear indication of proteolytic cleavage of Gp Ib by the former agonist. In conclusion, cathepsin G, unlike thrombin, alters the expression of Gp Ib by proteolysis rather than by internalization.

A functional consequence of the action of cathepsin G on Gp Ib is the loss of its vWF-receptor function. In fact, cathepsin G treatment of platelets caused a loss of agglutination by vWF in the presence of ristocetin. This would imply that, in the presence of activated PMN, vWF-mediated platelet adhesion to the subendothelium could be prevented.

Thrombin not only activates the Gp IIb-IIIa present on the outer leaflet of the platelet membrane but also exposes the complexes that are present in the alpha granules and mainly in the open canalicular system. To verify the possible involvement of the internal pool of Gp IIb-IIIa during platelet activation by cathepsin G, external Gp IIb-IIIa complexes were dissociated by EDTA treatment of platelets at 37°C. Calcium deprivation of platelets at 37°C also induces sequestration of the more internal segments of the canalicular system from the extracellular milieu. Internal Gp IIb-IIIa complexes are consequently protected from dissociation and exposed to the external surface after agonist stimulation. In our experiments, EDTA-treated platelets showed basal P2 binding lower than that of untreated platelets.

![Fig 6. SZ2 immunoblotting of pellets and supernatants of cathepsin G- and thrombin-stimulated platelets. Washed platelets were treated with saline (lanes 1, 6, 11, and 16) or with 400 nmol/L cathepsin G at 15 seconds (lanes 2 and 7), 30 seconds (lanes 3 and 8), 60 seconds (lanes 4 and 9), and 300 seconds (lanes 5 and 10); or with 0.25 U/mL thrombin at 15 seconds (lanes 12 and 17), 30 seconds (lanes 13 and 18), 60 seconds (lanes 14 and 19), and 300 seconds (lanes 15 and 20). Platelet pellets (lanes 1 through 5 and 11 through 15) were separated by centrifugation from platelet supernatants (lanes 6 through 10 and 16 through 20) and processed on a 10% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions. Immunoblotting was performed using unlabeled SZ2, as described under Materials and Methods. This is representative of five to seven experiments.

![Fig 7. Effect of cathepsin G on PFA-fixed platelet agglutination by ristocetin and vWF. Washed, PFA-fixed platelets were incubated at 37°C under continuous stirring with buffer (A) or 400 nmol/L cathepsin G (B) for 10 minutes and then 3.7 μg/mL of human purified vWF was added. After 5 minutes, ristocetin (1 mg/mL) was added and agglutination was recorded as an increase of light transmission. This is representative of five experiments.
lets, with the fluorescence peak close to that of intrinsic cell fluorescence (Fig 8). In contrast, a great increase in binding was observed when EDTA-treated platelets were exposed to cathepsin G. These results indicate that cathepsin G, like thrombin, is able to expose internal pool of Gp IIb-IIIa.

A secondary purpose of this study was to examine a possible contribution of endogenous mediators of platelet function such as ADP and thromboxane A$_2$ on the observed membrane glycoprotein changes. Cathepsin G has been shown to be a strong platelet agonist, but in the range of concentrations used, its effects on platelets, measured in terms of aggregation, serotonin secretion, and intracellular ionized calcium increase, are partly due to released ADP and, to a lesser extent, to TxA$_2$ production.$^{21,33}$ Both agonists are known to induce expression of the fibrinogen receptor.$^{30,43}$ Our experiments show that cathepsin G-induced increase of P2 and PAC-1 antibody binding (after 5 minutes of stimulation) is only slightly inhibited in I-ASA– and CP/CPK–treated platelets. This indicates that the contribution of cyclooxygenase metabolites and ADP to cathepsin G–induced fibrinogen receptor exposure is minor. In agreement with the finding that the effect of cathepsin G on Gp Ib is not dependent on intracellular pathways, aspirin and CP/CPK treatment did not modify the anti-Gp Ib binding. Similar results were obtained when ATP at 1 mmol/L was used instead of the CP/CPK system.

In conclusion, cathepsin G modifies platelet membrane Gp, reducing the expression of Gp Ib but exposing additional complex Gp IIb-IIIa and expressing the functional fibrinogen receptor. The former event, unlike that induced by thrombin, is fast, does not depend on intracellular activation mechanism(s) or platelet metabolism, and is accompanied by a proteolytic cleavage of the Gp. The latter seem to depend on platelet activation and to be unrelated to intracellular amplification mechanisms. In view of the pivotal role played by platelet membrane Gp in the interaction with other cells, subendothelium, coagulation factors, and other proteins, cathepsin G–induced changes in Gp Ib and Gp IIb-IIIa may be relevant for a better understanding of the pathophysiology of hemostasis and ischemic disease. This study suggests that this enzyme is able to induce platelet Gp modifications similar to those induced by thrombin. These changes may be important in shifting the reactivity of platelets from interaction with subendothelium to aggregation and release reaction. Although the mechanism(s) of action of either enzyme are quite different, the possibility should be considered that platelet reactivity may be modified in the absence of thrombin generation, if PMN are adequately activated.

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