

An Altered Platelet Granule Glycoprotein in Patients with Essential Thrombocythemia

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Abstract. The protein profiles of washed platelets from nine patients with essential thrombocythemia were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In four patients, an additional protein band (reduced M_r of 170,000) was clearly identified in both unstimulated platelet preparations and thrombin-released supernatant fractions. This band was also evident, though to a lesser extent, in three more patients, but it could not be located in the two remaining patients nor in any of ten controls. Subsequent characterization of the 170,000 reduced protein in one patient indicated that (a) it was glycosylated, as judged by periodic acid-Schiff staining, and (b) that native protein was a disulfide-linked multimer (possibly trimeric), which (c) partially bound to the activated platelet plasma membrane in the presence of calcium, and (d) was immune precipitated by anti-glycoprotein G antisera. The combined evidence is consistent with the 170,000 reduced protein being a modified form of the normal subunit of the platelet α -granule constituent, glycoprotein G (also termed thrombospondin and thrombin-sensitive protein).

Introduction

Essential thrombocythemia is a myeloproliferative disorder characterized primarily by excessive production of megakaryocyte-platelet elements. The disorder is commonly associated with bleeding and thrombotic tendencies arising to some extent from disturbed platelet function (1-3). Abnormal platelet ag-

gregation (4-6), possibly due to defective stimulus-receptor coupling (7), is often found while decreased platelet adhesiveness to glass bead columns occurs in almost all cases (4). Deficient lipid peroxidation (8), serotonin uptake (3, 5, 9), lipoxigenase activity (10), and prostaglandin D_2 receptor function (11) have also been reported. At the molecular level, alterations in the amount of platelet membrane glycoproteins, particularly in the glycoprotein I and glycoprotein IV molecular weight regions (12, 13), have been demonstrated by carbohydrate staining, although with no apparent correlation to the aggregation response (12) or density distribution (13).

Glycoprotein G (14) (also termed thrombospondin [15] and thrombin-sensitive protein [16]) is a high molecular weight glycoprotein (17) contained in the platelet α -granules (18-20) and is rapidly released on thrombin stimulation (16, 21), along with the other α -granule constituents. It comprises $\approx 2\%$ of total platelet protein (22) and 20-30% of releasable protein (16). The protein is a trimer consisting of equivalent disulfide-linked subunits with M_r of 185,000 as determined by the discontinuous electrophoretic system of Laemmli (23, 24) and M_r of 145,000 by the electrophoretic system of Weber and Osborn (15, 24). Glycoprotein G contains $\approx 5\%$ carbohydrate (16). Binding of glycoprotein G to the activated platelet membrane surface is reported to be calcium-mediated (21), a functional property also attributed to fibrin/fibrinogen (21). Recent reports indicate that glycoprotein G is the platelet endogenous lectin (25) and that fibrinogen is the lectin receptor (26). Binding of glycoprotein G to fibrinogen in a cell-free assay (27) is reported to be inhibited by the same aminosugars and net positively charged amino acids that specifically inhibit the enhanced lectin activity of thrombin-stimulated platelets (27, 28). These same aminosugars and amino acids also inhibit thrombin-induced platelet aggregation (28), suggesting that platelet membrane cohesiveness may involve the formation of lectin-lectin receptor bridging complexes (25, 28). While the precise role of glycoprotein G remains to be defined, the available data are nevertheless consistent with glycoprotein G playing an important role in platelet aggregation. In this study of nine patients with essential thrombocythemia, the molecular basis of the disorder was further investigated by electrophoretic analysis of whole platelet and thrombin-released proteins. An abnormal form of glycoprotein G was identified in most patients (7:9), while in one patient, glycoprotein G occurred in decreased amount.

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Methods

Materials. Hirudin and catalase were obtained from Sigma Chemical Co., St. Louis, MO; benzamidine from Aldrich Chemical Co., Inc., Milwaukee, WI; Na¹²⁵I from New England Nuclear, Boston, MA; lactoperoxidase from Boehringer-Mannheim, Sydney, Australia; hydrogen peroxide from BDH Chemicals Ltd., Poole, England; PANSORBIN (*Staphylococcus aureus* cells) from La Jolla, CA; and molecular weight markers from Bio-Rad Laboratories, Richmond, CA. α -Thrombin was the generous gift of Dr. J. W. Fenton II, Albany, NY.

Patient history. The patient R.D., a male of 69 yr, has a long history of obstructive airways disease and chronic bronchitis. He initially complained of easy bruising in 1979 and developed a large hematoma after an intramuscular injection. Physical examination showed bruising and the vital signs were within normal limits. There was splenomegaly 4 cm below the left costal margin. The results of investigations at that time included a hemoglobin of 16.6 g/dl, a white cell count of 11.2×10^9 /liter with a normal differential count, and a platelet count of 1278×10^9 /liter. The bone marrow aspirate contained hypercellular fragments and the smears showed hyperplastic erythropoiesis and myelopoiesis with normal maturation and increased numbers of megakaryocytes. The sectioned biopsy confirmed these findings and increased reticulin was demonstrated. The aggregation threshold to ADP and epinephrine was markedly increased, and there was no aggregation response to collagen nor collagen-induced thromboxane B₂ production. These results have been reported elsewhere (7) in a functional study of 20 patients with various myeloproliferative disorders. The patient was treated with busulfan, initially 4 mg and later 2 mg daily, and his platelet count returned to normal levels. At this time, his aggregation defect and collagen-induced thromboxane B₂ production were partially corrected. He has since been free of any bleeding tendency and has remained in good health. Detailed biochemical analysis was carried out on one patient (R.D.) whose history is given above. Eight other patients have also been studied. All of these had clinical and hematological features which are typical of essential thrombocythemia with splenomegaly, such as a variable degree of bruising, platelet counts in excess of 800×10^9 /liter without leukocytosis or erythrocytosis, and hypercellular bone marrow.

Preparation of glycoprotein G and other materials. Glycoprotein G was purified by a modification of the method of Lawler et al. (15). Platelets from 3 U of platelet-rich plasma (supplied by the Red Cross Blood Transfusion Service, Sydney, Australia) were washed as previously described (23) except that the buffer used for the final wash and resuspension contained 0.01 M Tris, 0.15 M NaCl, 10% (vol/vol) acid-citrate-dextrose solution, pH 7.4 (15). The release reaction was induced with 3 U/ml of α -thrombin with stirring for 3 min, and then was stopped with 10 mM benzamidine and 10 U/ml of hirudin. After removal of polymerized fibrin, the cell-free supernatant was loaded onto a 40×2.5 -cm column of Sepharose 4B (Pharmacia, Inc., Uppsala, Sweden) and eluted at 20 ml/h. Column buffer contained 0.01 M Tris, 0.15 M NaCl, 0.001 M benzamidine, 0.001 M CaCl₂, 0.02% (wt/vol) in NaN₃, pH 7.4. Glycoprotein G was further purified by chromatography of glycoprotein G-rich fractions on a 15×1 -cm column of heparin-coupled affigel 15 (Bio-Rad Laboratories). To remove traces of fibrin, the purified glycoprotein G was passed through a 15×1 -cm column of affinity-purified rabbit antifibrinogen antibody coupled to affigel 10. An anti-glycoprotein G antibody was raised in New Zealand white rabbits by subcutaneous injection of 100 μ g of protein mixed 1:1 with Freund's complete adjuvant. At 3-wk intervals, the animals were boosted consecutively with 150 μ g and then, 240 μ g of glycoprotein G mixed 1:1 with Freund's incomplete adjuvant. The animals were bled 10 d after

final injection. The antibody which gave a single precipitin line against glycoprotein G by Ouchterlony analysis was affinity-purified on glycoprotein G coupled to affigel 10 (the antibody was eluted with 0.1 M glycine, pH 2.4). Nonimmune rabbit IgG was purified from serum by three consecutive 0–40% ammonium sulfate fractionations followed by chromatography on DEAE-affigel Blue (Bio-Rad Laboratories).

Platelet preparation and thrombin treatment. Platelets were prepared from freshly drawn blood, washed as previously described (23), and finally resuspended in ETS buffer to 10^9 /ml. ETS buffer contained 0.01 M Tris, pH 7.4 (0.15 M in NaCl and 0.001 M in EDTA). The washed platelets were treated with buffer or 1 U/ml of α -thrombin. Platelets were separated from supernatant by centrifugation at 800 g. The supernatants were removed and the pellets resuspended to their former volume. Pellet and supernatant fractions were retained for electrophoresis.

Surface labeling of thrombin-treated platelets by pulse-iodination. Iodination of thrombin-treated platelets was performed essentially as previously described (21). Washed platelets were suspended to 10^9 /ml in Tyrode's solution or EDTA-Tyrode's solution and made 1 mCi/ml in Na¹²⁵I and 13.4 μ M in lactoperoxidase prior to addition of either buffer or α -thrombin (1 U/ml final concentration). The platelet suspensions were made 35 μ M in H₂O₂ immediately after thrombin addition and stirred in an aggregometer for 5 s. They were then left unstirred for 55 s before addition of catalase (3.5 μ M final concentration). The platelets were separated from supernatant by centrifugation at 8,730 g in a Beckman microfuge (Beckman Instruments, Inc., Palo Alto, CA) through 15% sucrose prepared in the appropriate Tyrode's solution. Platelet pellets were resuspended to former volume and then added to an equal volume of nonreducing buffer before electrophoresis. Tyrode's solution contained 0.138 M NaCl, 0.0039 M KCl, 0.012 M NaHCO₃, 0.00036 M NaH₂PO₄, 0.005 M glucose, 0.00049 M MgCl₂, 0.0018 M CaCl₂, pH 7.4. EDTA-Tyrode's solution incorporated 0.001 M EDTA in place of MgCl₂ and CaCl₂.

Immune precipitation. Thrombin supernatants from control and patient platelets (10^9 /ml in ETS, 1 U/ml of α -thrombin) were labeled by lactoperoxidase-catalysed iodination (23). Thrombin was inhibited 1 min after platelet stimulation by addition of 2 U/ml of hirudin. Labeled platelet supernatant (200 μ l) was equilibrated with 20 μ l of a 1 mg/ml concentration of either nonimmune IgG or affinity-purified antiglycoprotein G for 2 h at 4°C. Immune precipitation with fixed *S. aureus* cells (PANSORBIN) was then performed essentially as previously described (29).

Electrophoresis and autoradiography. Platelet samples were prepared for electrophoresis by solubilization in 2% (wt/vol) sodium dodecyl sulfate (SDS) in the absence (nonreducing) or presence (reducing) of 5% (vol/vol) 2-mercaptoethanol. Reduced samples were heated at 100°C for 10 min and nonreduced samples at 100°C for 3 min to afford complete solubilization (30). One-dimensional electrophoresis was performed according to the method of Laemmli (31) by using either a 5% linear or a 5–15% or 5–20% exponential gradient of acrylamide in the resolving gel and 3% acrylamide in the stacking gel. Two-dimensional (nonreduced-reduced) electrophoresis was performed as previously described (23). Sample loads were 100 μ l and contained the protein equivalent of 5×10^7 platelets: ≈ 100 μ g in the case of whole platelets, and ≈ 10 μ g for supernatants. Gels were stained for protein with Coomassie brilliant blue (32) and for glycoprotein with periodic acid-Schiff reagent (33). Molecular weight markers were obtained from Bio-Rad Laboratories: myosin (200,000), β -galactosidase (130,000), phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). For autoradiography, stained gels were dried under vacuum, stored at

room temperature next to Cronex 4 medical x-ray film (DuPont Instruments, Wilmington, DE) in cassettes containing intensifying screens, and processed according to manufacturer's instructions.

Results

SDS-polyacrylamide gel electrophoresis of patient R.D.'s platelets under reducing conditions showed a prominent protein band (M_r of 170,000) that is not present in control platelets (Fig. 1, lane 2 vs. lane 1). On stimulation with α -thrombin, the intensity of the 170,000 band decreased in the platelet pellet (lane 4), concomitant with its appearance in the supernatant (lane 8) along with the other α -granule constituents, suggesting that the 170,000 reduced protein was also an α -granule constituent. Furthermore, release of this protein was an active process since the band was not present in the supernatant of unstimulated platelets (lane 6).

Two major possibilities exist for the derivation of this protein band. Firstly, it could represent incorporation of a new protein into the platelet α -granule. A second and more likely explanation is that it represents a modified form of a normal α -granule constituent. With respect to the intensity and molecular weight of the 170,000 reduced protein, the most likely α -granule constituent that is consistent with the latter possibility is glycoprotein G. The following experiments on control and R.D.'s platelets were performed to test this hypothesis.

(a) Glycoprotein G has a subunit M_r of 185,000 (23) and exists as a disulfide-linked trimer (17, 23). On SDS-polyacrylamide gel electrophoresis of α -granule proteins under nonreducing conditions, the highest molecular weight protein band is glycoprotein G (18). When the thrombin supernatant from the patient's platelets was examined under nonreducing conditions

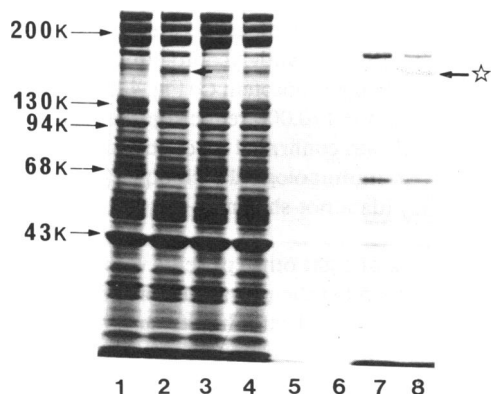


Figure 1. An exponential 5–15% SDS-polyacrylamide gel of control and patient's (R.D.) platelet pellets and supernatants run under reduced conditions and stained for protein with Coomassie brilliant blue. Lanes 1–4, pellets; lanes 5–8, supernatants; lanes 1 and 5, control, unstimulated; lanes 2 and 6, patient, unstimulated; lanes 3 and 7, control, thrombin-stimulated; lanes 4 and 8, patient, thrombin-stimulated. Star indicates 170,000 band.

(Fig. 2), the 170,000 protein band no longer electrophoresed at this molecular weight (lane 2). Instead, the patient's preparation showed extra protein bands immediately below that of glycoprotein G, indicating that the 170,000 reduced protein was part of, or existed as, a disulfide-linked multimer similar to glycoprotein G. This was confirmed by two-dimensional nonreduced-reduced SDS-polyacrylamide gel electrophoresis (compared with Fig. 3 *d*).

(b) Both the glycoprotein G subunit and the 170,000 reduced protein stained for carbohydrate with periodic acid-Schiff reagent (patient's thrombin supernatant, reduced SDS-polyacrylamide gel electrophoresis, data not shown).

(c) In the presence of the chelating agent, EDTA, all the α -granule proteins are released into the supernatant. However, in the presence of exogenous calcium ions, the released glycoprotein G and fibrinogen (or fibrin in the case of α -thrombin) are partially bound and become major constituents of the activated platelet membrane surface (21). To determine if the 170,000 reduced protein shared this functional property, control and patient's platelets were thrombin-stimulated and pulse-radioiodinated, and then, the platelet pellets were examined by using two-dimensional nonreduced-reduced SDS-polyacrylamide gel electrophoresis, a procedure which clearly separates glycoprotein G (and fibrinogen) from the intrinsic membrane glycoproteins (18, 20). The Coomassie blue-stained gels of unstimulated control and patient's platelets are shown in Fig. 3 *a* and *d*, respectively. The 170,000 reduced protein occurred as a distinct spot below that of the glycoprotein G subunit, confirming that both derive from disulfide-linked species of similar molecular weight. In control and patient's unstimulated platelets, only the intrinsic membrane glycoproteins were iodinated (Fig. 3 *b* and *e*, respectively). The 170,000 reduced protein was not detectable on the surface of the patient's unstimulated platelets (Fig. 3 *e*) but was intensely labeled in the thrombin supernatant (not shown),

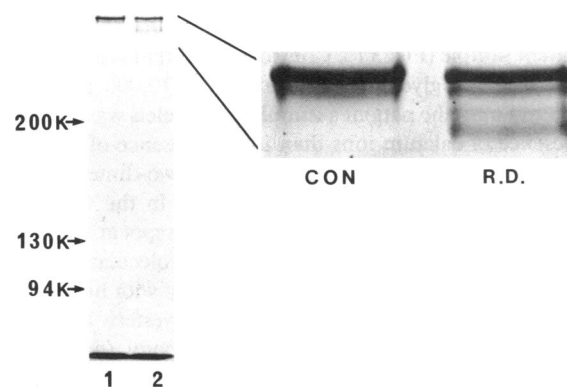


Figure 2. A linear 5% SDS-polyacrylamide gel of control and patient's (R.D.) thrombin supernatants run under nonreduced conditions and stained for protein with Coomassie brilliant blue. Lane 1, control (CON); Lane 2, patient (R.D.). Insert represents a fivefold magnification of high molecular weight region.

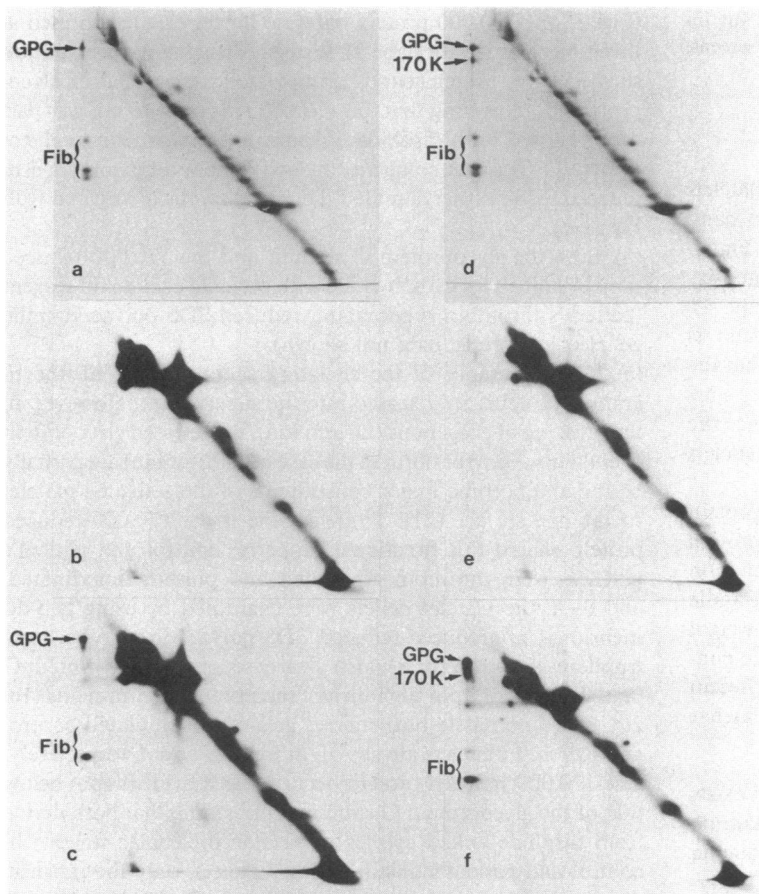


Figure 3. Two-dimensional, linear 7.5% SDS-polyacrylamide gels of control (*a-c*) and patient's (R.D.) (*d-f*) platelets, surface radioiodinated with lactoperoxidase, and run nonreduced in the horizontal direction and reduced in the vertical direction. *a* and *d*, Coomassie brilliant blue-stained gels of unstimulated platelets; *b* and *e*, corresponding autoradiographs of the labeled unstimulated platelets; *c* and *f*, autoradiographs of labeled platelets stimulated with thrombin in the presence of calcium. *GPG*, glycoprotein G; *Fib*, fibrin(ogen).

which is consistent with its putative internal localization before stimulation. In control and patient's platelets aggregated with α -thrombin in the presence of exogenous calcium ions, both glycoprotein G and fibrin were present on the activated platelet surface (Fig. 3 *c* and *f*, respectively) as was the 170,000 protein in the patient sample (Fig. 3 *f*). Control experiments confirmed that the amount of glycoprotein G and the 170,000 reduced protein bound onto the patient's stimulated platelets was greater in the presence of calcium ions than in the presence of EDTA (data not shown). The control samples in the two-dimensional gels showed a lightly labeled or stained smear in the 180,000 region below glycoprotein G, but lacked a discrete spot at 170,000 (Fig. 3 *a* and *c*). A broad band with reduced molecular weight of 180,000 was also observed electrophoretically with high protein loads of purified glycoprotein G and on western blots of intact platelets with antiglycoprotein G antibody (data not shown). Thus, while normal controls may contain small amounts of the 170,000 band, the levels are below the detection limit of the electrophoretic methods employed in this study. (*d*) The immunological relatedness of the 170,000 reduced protein to glycoprotein G was tested by reacting thrombin supernatant proteins radioiodinated by lactoperoxidase with either

nonimmune IgG or affinity-purified antiglycoprotein G antibody. Although some nonspecific binding of glycoprotein G and fibrin to *S. aureus* cells occurred in the presence of non-immune IgG (Fig. 4, odd lanes), antiglycoprotein G IgG specifically immune precipitated glycoprotein G (Fig. 4, even lanes) and in the patient sample, the 170,000 reduced protein (Fig. 4, lanes 4 and 8). This result was confirmed on control and R.D.'s supernatants by using an immunological technique (34) based on western blotting (35) (data not shown).

Preliminary screening of eight other patients with essential thrombocythemia demonstrated the prevalence of the 170,000 reduced protein in this disorder. Electrophoretic analysis of unstimulated whole platelets (not shown) and the corresponding α -thrombin supernatants (Fig. 5) revealed that, in three patients (M.V., G.W., and H.K.), the intensity of the 170,000 reduced band was similar to or greater than that in R.D. In three more patients (C.B., A.D., and H.H., the latter two not shown in Fig. 5), this band was evident though with less intensity than in R.D. The 170,000 reduced band could not be identified in the remaining two patients (E.S. and A.S.), nor in any of ten normal controls. In the case of E.S., there appeared to be a deficiency

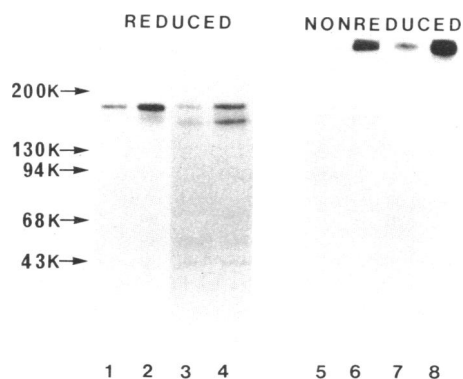


Figure 4. Control and patient's (R.D.) thrombin supernatants were radioiodinated and immune precipitation performed by using either nonimmune IgG or antiglycoprotein G (immune) IgG. This figure is an autoradiograph of the solubilized immune precipitates derived from the same volume of labeled supernatant and run on an exponential 5–15% SDS-polyacrylamide gel under reduced (lanes 1–4) and nonreduced conditions (lanes 5–8). Lanes 1 and 5, control, non-immune; lanes 2 and 6, control, immune; lanes 3 and 7, patient, nonimmune; lanes 4 and 8, patient, immune.

in the amount of glycoprotein G released with respect to the other α -granule components. The electrophoretic profile of E.S.'s unstimulated platelets indicated that glycoprotein G was present in decreased amount in this patient's platelets (not shown).

Discussion

A platelet protein not present in normal platelets, with reduced M_r of 170,000, has been identified in patients with essential thrombocythemia. Characterization of the 170,000 reduced

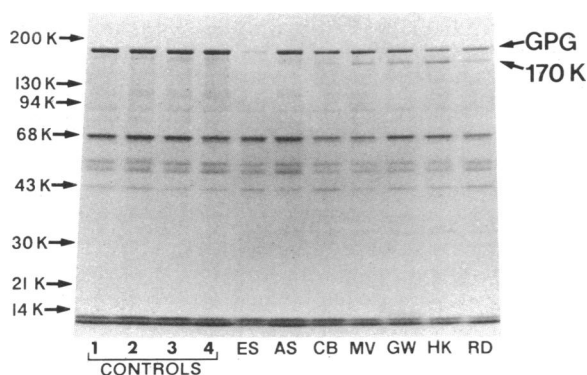


Figure 5. An exponential 5–20% SDS-polyacrylamide gel of control (lanes 1–4) and patient's (lanes 5–11) thrombin-released supernatants run under reducing conditions and stained for protein with Coomassie brilliant blue. Four of ten controls studied are represented. The 170,000 band was identified in two patients (A.D. and H.H.) not included in this figure; the intensity of the bands was similar to that found in C.B. (lane 7). *GPG*, glycoprotein G.

protein in one patient, R.D., strongly suggests that it is a clipped form of platelet glycoprotein G (reduced M_r of 185,000), a disulfide-linked trimer (17, 23) which partially binds to the activated platelet surface in the presence of calcium ions (21). Glycoprotein G appears to be an α -granule protein since it is absent in platelets from patients with the Gray Platelet syndrome along with other known α -granule constituents (18–20). The present data suggests that the 170,000 reduced protein is also an α -granule constituent since it could not be labeled on the unstimulated platelet surface (Fig. 3), but appeared in the supernatant on thrombin stimulation (Fig. 1). The 170,000 reduced protein was part of, or existed as a disulfide-linked multimer (Figs. 2 and 3) and was partially bound to the activated platelet surface in the presence of exogenous calcium ions (Fig. 3). Furthermore, the 170,000 reduced protein is immune-precipitated by antiglycoprotein G antibody (Fig. 4). The combined evidence is consistent with this protein being a clipped form of the normal subunit of glycoprotein G.

While the 170,000 reduced protein appears to be prevalent in patients with essential thrombocythemia (Fig. 5), the ratio of glycoprotein G to the clipped form varied widely (almost 1:1 in the case of H.K. to 6.5:1 in the case of C.B., as judged by scanning densitometry of Coomassie blue-stained gels of α -thrombin supernatants). In the case of R.D., staining for protein by Coomassie blue (Fig. 1) and for carbohydrates by periodic acid-Schiff reagent indicated a ratio of \sim 2:1 (by densitometry), whereas the immune precipitation data (Fig. 4) and a comparison of counts associated with [125 I]-labeled thrombin supernatant proteins suggested a ratio closer to 1:1. While the pattern of four bands in the nonreduced gel (Fig. 2, lane 2) could derive from either of these ratios together with a random disulfide-linked assembly of 185,000 and 170,000 subunits, the intensity of staining is more consistent with a 2:1 ratio. The discrepancy between the labeling and staining results presumably reflects an increased exposure of tyrosine residues in the clipped subunit. The 170,000 reduced protein could be demonstrated in R.D.'s platelets on all occasions studied over a 2-yr period, and in G.W.'s platelets over a 6-mo period, while the intensity of this band relative to glycoprotein G remained constant during these periods of time.

Several possibilities exist as to the origin and nature of the 170,000 reduced protein. It could derive from a transcriptional defect, or alternatively, a posttranscriptional modification of the parent molecule involving either a glycosylation defect or proteolytic clipping. Staining of the 170,000 reduced protein with periodic acid-Schiff reagent suggested that a glycosylation defect is unlikely. If proteolysis is the case, then it must occur either in the α -granules or before assembly of α -granule constituents since the clipped form is present in unstimulated platelets (Fig. 1). In support of this, prolonged incubation of glycoprotein G with thrombin (4 U/ml) in the presence of calcium has been reported to hydrolyze the 185,000 subunit to a 160,000 cleavage product (24).

The apparent molecular weight clip of 15,000 (reduced) did not appear to affect calcium-dependent binding of modified

glycoprotein G to activated platelets (Fig. 3). Preliminary experiments to characterize a functional consequence of the observed cleavage have been equivocal. Patients H.K. and G.W. had normal levels of thrombin-enhanced platelet-bound and supernatant lectin activities (25, 36). Neither glycoprotein G nor a thrombin-generated proteolytic mixture of 170,000 and 140,000 (reduced) derivatives of glycoprotein G (24) had any effect on platelet aggregation induced by threshold or higher concentrations of ADP, collagen, or epinephrine. While the role of glycoprotein G in platelet function is not yet clear, the reported binding to fibrinogen (27), possibly via a lectin-binding site, suggests a role in platelet cohesion (25, 28). It remains possible that one or more of these binding sites may be absent or altered in the modified protein, resulting in a form which may impede normal function. The inability in preliminary experiments to observe a functional consequence of cleavage may be due to the concentration disadvantage of exogenous vs. endogenous released glycoprotein. Glycoprotein G has recently been reported to also be synthesized by endothelial cells (37) and fibroblasts (38). Whether these cells from patients with essential thrombocytopenia also produce a modified form of glycoprotein G and whether this altered form in platelets correlates with the reported functional abnormalities in this disorder is under current investigation.

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