Abnormal Inside-Out Signal Transduction-Dependent Activation of Glycoprotein IIb-IIIa in a Patient With Impaired Pleckstrin Phosphorylation

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Platelet agonist interaction results in activation of glycoprotein (GP) IIb-IIIa complex and fibrinogen binding, a prerequisite for platelet aggregation. Fibrinogen binding exposes new antibody binding sites on GPIIb-IIIa (ligand-induced binding sites; LIBS). Signal transduction events, including pleckstrin phosphorylation by protein kinase C (PKC), are considered to regulate GPIIb-IIIa activation. We studied a 16-year-old white male with lifelong mucocutaneous bleeding manifestations and abnormal platelet aggregation and secretion in response to multiple agonists. Pleckstrin phosphorylation was diminished in response to platelet-activating factor (PAF; 4 and 400 nmol/L) and thrombin (0.05 U/mL). Binding of monoclonal antibodies (MoAbs) 10E5 and A2A9, which bind to both resting and activated GPIIb-IIIa, was normal. Binding of MoAb PAC1, which binds to only activated GPIIb-IIIa, was diminished upon activation with PAF, adenosine diphosphate (ADP), thrombin receptor agonist peptide (SFFLRN), A23187, and 1,2-dioctanoylglycerol (DIOCA). Signal transduction-dependent LIBS expression (studied using MoAb 62) induced by ADP, SFFLRN, and DIOCA and signal transduction-independent LIBS expression induced by RGDPS peptide or disintegrin albolabrin were normal or only minimally decreased, indicating the presence of intact ligand binding sites. We conclude that the patient's platelets have a defect in inside-out signal transduction-dependent GPIIb-IIIa activation due to an upstream defect in the signal transduction mechanisms rather than in the GPIIb-IIIa complex itself. Our findings extend the spectrum of congenital mechanisms leading to impaired aggregation from defects in GPIIb-IIIa per se to aberrations in signaling mechanisms.

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events that modulates GPIIb-IIIa activation through agonist receptors on the platelet surface. In addition, there is evidence for stimulation of outside-in intracellular signaling by ligand-occupied GPIIb-IIIa.25,26

Although the above-cited studies indicate an important role of inside-out signaling in fibrinogen receptor activation, the role of PKC activation in this response is not fully clarified. GPIIIa is phosphorylated on activation, but there is little evidence that it leads to fibrinogen receptor exposure.25 During the course of our studies in patients with congenital defects in platelet function, we identified a patient with markedly impaired, agonist-stimulated phosphorylation of pleckstrin, the protein phosphorylated by PKC. We have used platelets from this patient to examine the relationship between activation of PKC and that of GPIIb-IIIa. Our goals were to determine whether this patient with a defect in signal transduction mechanisms would manifest impaired activation of GPIIb-IIIa and to characterize the mechanisms leading to the markedly abnormal primary wave of aggregation noted in this patient.

MATERIALS AND METHODS

Patient information. Patient K.S. is a 16-year-old white male who was referred at the age of 2 years for the evaluation of a history of easy bruising and epistaxis noted from around 6 to 10 months of age. The bruising was noted in his extremities, abdomen, and face. His father had a history of easy bruising since childhood and thrombocytopenia; he died of acute lymphoblastic leukemia. The patient's paternal grandfather had a history of bleeding symptoms. The patient's two siblings did not have a history of easy bruising. The physical examination did not reveal hemarthropathy. Laboratory evaluation at that time and subsequently have revealed him to have mild thrombocytopenia (platelet counts, 65,000 to 140,000/μL), prolonged bleeding times (9 minutes to greater than 15 minutes), normal prothrombin time and activated partial thromboplastin time, and normal plasma levels of factor VIII coagulation activity, von Willebrand factor antigen, and ristocetin cofactor activity. The bleeding times have been consistently prolonged even with platelet counts of greater than 100,000/μL. The platelet aggregation and secretion studies have been consistently abnormal (Fig 1).

Monoclonal antibodies and disintegrins. Monoclonal antibody (MoAb) PAC1 binds only to the activated form of the GPIIb-IIIa complex and was provided by Dr Sanford Shattil (University of Pennsylvania, Philadelphia, PA) and Joel Bennett (University of Pennsylvania, Philadelphia, PA), respectively. Monoclonal anti-LIBS antibody Ab62 was donated by Dr Mark Ginsberg (Scripps Research Institute, La Jolla, CA).29 These antibodies were labeled with fluorescein isothiocyanate (FITC; Cal Biochem, San Diego, CA) as described elsewhere.29 Mouse IgG and FITC-labeled anti-mouse IgG were purchased from Jackson Immuno Research Laboratory, Inc, Philadelphia, PA. Albolabrin, a snake venom peptide containing RGD sequence (disintegrin), was provided by Dr Barry Caller (Mount Sinai School of Medicine, New York, NY) and Joel Bennett (University of Pennsylvania, Philadelphia, PA), respectively. Albolabrin, a snake venom peptide containing RGD sequence (disintegrin), was provided by Dr Barry Caller (Mount Sinai School of Medicine, New York, NY) and Joel Bennett (University of Pennsylvania, Philadelphia, PA), respectively.

Other reagents. Iloprost (ZK 36,374), a stable prostacyclin analog, was provided by Verdes Laboratories, Cedar Knolls, NJ. Thrombin receptor agonist peptide (TRAP; SFLLRN) was purchased from Bachem Bioscience Inc, King of Prussia, PA. [14C]-Serotonin and [32P]-Pi were purchased from Amersham Corporation, Arlington Heights, IL, and DuPont NEN Research Products, Boston, MA, respectively. Thrombin was purchased from Armour Pharmaceutical Co, Kankakee, IL. Platelet-activating factor (PAF) was from Avanti Polar-lipids, Inc, Alabaster, AL. 1,2-Dioctanoyl-glycerol (DioC8) was purchased from Biomol Research Laboratories, Plymouth Meeting, PA. Fura 2/AM pentacetaeoxymethylster and human fibrinogen were purchased from Cal Biochem, San Diego, CA. For labeling of fibrinogen with FITC, 300 μg of FITC in 5% sodium carbonate, pH 9.5, was reacted with 3 mg of fibrinogen for 45 minutes at room temperature. T1IC-fibrinogen was separated from free FITC using Sephadex G25 column PD-10 (Pharmacia LKB Biotechnology, Piscataway, NJ) and stored at 4°C until used. The α-chymotrypsin type I-S (from bovine pancreas), soybean trypsin-chymotrypsin inhibitor, and all other chemicals used in this study were purchased from Sigma Chemical Co, St Louis, MO.

Aggregation and secretion. Blood was collected in 1/10 volume of 3.8% sodium citrate and centrifuged at 180g for 15 minutes at room temperature to obtain platelet-rich plasma (PRP). Platelet aggregation responses were monitored using a Chrono-Log (Havertown, PA) dual channel aggregometer as described previously.33 The extent of aggregation was expressed as a percentage taking the light transmission through PRP and platelet-poor plasma to be 0% and 100%, respectively. For assaying 3H-serotonin secretion, platelets were incubated for 30 minutes at room temperature with 0.25 μmol/L of 5-hydroxy(side chain-2-14C) tryptamine creatine sulfate (50 μCi/mmol/L). Secretion was expressed as a percent of total 14C-serotonin incorporated that is released after platelet stimulation. The agonists used included ADP (8 μmol/L), PAF (200 nmol/L and 20 μmol/L), TRAP (25 μmol/L), epinephrine (8 μmol/L), arachidonic acid (1 mmol/L), collagen (10 μg/mL), and human fibrinogen (1 mg/mL). ADP and PAF were measured by the luciferase-luciferin assay.34 Thromboxane A2 production was measured in supernatants of PRP after incuba-
tion of PRP for 5 minutes with the agonist, using a radioimmunoassay for thromboxane B2.36

Measurement of pleckstrin and myosin light chain phosphorylation. Blood was collected by venipuncture into 1/10 volume of acid citrate dextrose buffer (85 mmol/L trisodium citrate, 78 mmol/L citric acid, 111 mmol/L dextrose) and centrifuged at 180g for 20 minutes to obtain PRP. PRP was centrifuged at 1,000g for 15 minutes in the presence of hirudin (0.05 U/mL) and apyrase (10 μg/mL) and the platelet pellet was resuspended in 1/3 volume of autologous plasma. Platelet suspension was incubated at 37°C for 60 minutes with [32P]-orthophosphate (0.4 μCi/mL). The platelets were then filtered through a Sepharose 2B column equilibrated with Ca2+-free Tyrode's buffer and analyzed on an Epics Elite flow cytometer (E.I. DuPont CO, Wilmington, DE). The bands on the gel corresponding to the 47-kD band after platelet activation has generally been considered to reflect pleckstrin; however, the change in phosphorylation of the 47-kD band may comigrate, the change in phosphorylation of the 47-kD band after platelet activation has generally been considered to reflect pleckstrin phosphorylation.

Measurement of cytoplasmic Ca2+ concentration. Intracellular ionized calcium concentrations (Ca2+ [pp]) were measured using fura-2 as the indicator, as described previously.46 Fluorescence was recorded (excitation, 340 nm; emission, 470 nm) using a spectrofluorimeter (model LS-5, Perkin Elmer, Oakbrook, IL). The [Ca2+ +]i concentrations were calculated as described previously.46 Flow cytometric analysis. Blood was collected in 1/10 volume of 3.8% sodium citrate from the patient and normal donors who had not taken medication for at least 10 days. PRP was collected by centrifuging the citrated blood at 180g for 15 minutes at room temperature. PRP was added to 12 × 75-mm polypropylene tubes and an appropriate volume of Tyrode's buffer, pH 7.4 (50 mmol/L Hepes, 2 mmol/L MgCl2, 0.3 mmol/L Na3P04, 3 mmol/L KCl, 134 mmol/L NaCl, 12 mmol/L NaHCO3, 0.35% BSA, and 0.1% glucose), was added to make the platelet final concentration 1 × 108/mL. Platelets (50 μL) were incubated with FITC-conjugated MoAbs PAC1 (50 μg/mL), 10E5 (50 μg/mL), A2A5 (50 μg/mL), or Ab62 (50 μg/mL) in the presence of an specific agonist at room temperature for 15 minutes without stirring. Samples were then diluted to 0.5 mL with Tyrode’s buffer and analyzed on an Epics Elite flow cytometer (Coulter Co, Miami, FL). Platelets (10,000 in each sample) were analyzed at 100 mW laser beam (coherent Innova 300) for FITC fluorescence to quantitate the amount of platelet-bound antibody. Results were expressed as mean platelet fluorescence units or as histograms of log platelet fluorescence intensity in arbitrary units on the abscissa and platelet number on the ordinate. In some experiments, the PRP was subjected to gel filtration using sepharose CL 2B column, and platelets were eluted with Tyrode’s buffer (pH 7.4) without added BSA. These platelet suspensions (1 × 108/mL) were used to examine the binding of nonspecific mouse IgG and 10E5 using FITC-labeled anti-mouse IgG as the second antibody. In these experiments, platelet suspensions (50 μL) were incubated with the murine IgG or 10E5 (50 μg/mL) for 15 minutes at room temperature and then with FITC-labeled anti-mouse IgG for 15 minutes. The samples were diluted with Tyrode’s buffer and analyzed by flow cytometry as described above.

Treatment of platelets with α-chymotrypsin. Results in the exposure of fibrinogen binding sites,27-29 which is signal transduction-independent and unaffected by PGI2 and stauorosporine.30-32 We examined the binding of FITC-labeled fibrinogen and PAC1 in platelets treated with α-chymotrypsin and in the presence of Iloprost. Gel-filtered platelets (2 × 108/mL) in Tyrode’s buffer without BSA were incubated at 37°C for 5 minutes with α-chymotrypsin (500 μg/mL) in the presence of 28 nmol/L Iloprost. The α-chymotrypsin was neutralized with a soybean trypsin/chymotrypsin inhibitor (1 mg/mL), and platelets were pelleted by centrifugation. The platelet pellets were resuspended in Tyrode’s buffer containing Iloprost (28 nmol/L). Chymotrypsin-treated platelet suspension (1 × 108/mL; 50 μL) was incubated with FITC-labeled fibrinogen (200 μg/mL) or PAC1 (50 μg/mL) for 15 minutes at room temperature without stirring and was examined for binding of fibrinogen or PAC1 using flow cytometry as described earlier. Control platelets were incubated with Tyrode’s buffer instead of α-chymotrypsin.

RESULTS

Platelet aggregation and secretion. Platelet aggregation and secretion were studied in the patient on multiple occasions over several years with consistently abnormal results. Platelet aggregation and secretion in PRP in response to ADP (8 μmol/L), PAF (250 μmol/L), and U46619 (2 μmol/L) were impaired (Fig 1), as were responses to epinephrine (8 μmol/L), arachidonic acid (1 mmol/L), and collagen (1.25 μg/mL; not shown). The most striking abnormalities were observed in response to PAF (200 μmol/L and 20 μmol/L) and U46619 (2 μmol/L; Fig 1); the primary wave or initial aggregation was also markedly blunted. Diminished responses were also observed in response to cell-permeable activators, divalent ionophore A23187 (10 μmol/L), and PKC activator DiC4 (200 μmol/L; Fig 1). The slopes of initial aggregation were blunted in the patient compared with those of normal subjects. The mean values (two to three studies) of the slopes in the patient and the range of values in five normal subjects are as follows: ADP (8 μmol/L): patient, 34 arbitrary units (U); normals, 45 to 73 U; PAF (20 μmol/L): patient, 25 U; normals, 58 to 100 U; A23187 (10 μmol/L): patient, 27 U; normals, 55 to 100 U; and DiC4 (200 μmol/L): patient, 7 U; normals, 17 to 25 U. Exposure of platelets to a combination of DiC4 with PAF or ADP enhanced the aggregation and secretion responses but not to the level noted in normal PRP. However, both responses to a combination of 200 μmol/L of DiC4 and 10 μmol/L A23187 were entirely normal.

Platelet dense granule content and thromboxane A2 production. Content of ATP and ADP and the ATP:ADP ratio in patient’s platelets were normal, thereby excluding dense granule storage pool deficiency. Thromboxane A2 production in PRP was normal in response to 1 μM thrombin (35 pmol/108 platelets; normal range, 21 to 146 pmol/108 platelets; n = 11) and 1 nmol/L arachidonic acid (patient, 167 pmol/108 platelets; normal range, 128 to 709 pmol/108 platelets; n = 8).
Phosphorylation of pleckstrin and myosin light chain and calcium mobilization. In the patient, platelet pleckstrin phosphorylation was markedly diminished after activation with PAF (4 and 400 nmol/L) and thrombin (0.05 U/mL) (Fig 2). At higher thrombin concentrations (5 U/mL), it was also lower than in normal controls; at 30 seconds, it was 2.15-fold of basal in the patient compared with 3.62- to 6.59-fold in 14 normal subjects. At 1 minute, pleckstrin phosphorylation was 3.03- and 2.77-fold of basal in two studies in the patient compared with 3.64- to 7.32-fold in 15 normal subjects. Pleckstrin phosphorylation was diminished in response to 1 μmol/L A23187 (patient, 1.72- and 1.21-fold of basal; v 2.26- to 3.61-fold in six normal controls). After activation with 200 μmol/L DiCa, pleckstrin phosphorylation was normal in the patient. In response to ADP, we observed very minimal or inconsistent pleckstrin phosphorylation even in normal platelets. Myosin light chain phosphorylation was impaired in response to PAF (400 nmol/L) and thrombin (0.05 U/mL). With thrombin (0.5 U/mL), myosin phosphorylation at 1 minute showed a 1.8-fold increase over basal in the patient compared with 2.36- to 3.94-fold over basal in nine normal subjects. In response to 200 μmol/L DiCa and 1 μmol/L A23187, myosin phosphorylation at 1 minute also was blunted in the patient. Studies on Ca\(^{2+}\) mobilization using fura-2-loaded platelets showed that basal and peak [Ca\(^{2+}\)] were normal in the patient after platelet activation with thrombin (0.05 and 0.5 U/mL) and ADP (25 μmol/L) in the presence of 1 mmol/L external Ca\(^{2+}\).

Binding of MoAbs 10E5, A2A9, and PAC1 by flow cytometry. Binding of MoAbs 10E5 and A2A9 (which bind to both activated and resting forms of GPIIb-IIIa) by patient platelets was comparable with that in normal platelets (Fig 3), suggesting that normal levels of GPIIb-IIIa were present on patient platelets. In control experiments, we examined the binding of a nonspecific mouse IgG and antibody 10E5 to patient and normal platelets using FITC-labeled anti-mouse IgG as the second antibody. The binding of nonspecific mouse IgG and antibody 10E5 was comparable in the patient and normal platelets (not shown), indicating that the patient platelets did not have a nonspecific increase in binding of IgG and that the observed comparable binding of antibodies 10E5 and A2A9 was not due to enhanced nonspecific binding of IgG by patient platelets.

The binding of PAC1 (which binds to only the activated form of GPIIb-IIIa) was markedly decreased in patient plate-
lets after stimulation with PAF (200 nmol/L and 20 μmol/L; Fig 4). A decrease in PAC1 binding was also observed in response to 5 μmol/L ADP, 25 μmol/L TRAP, 100 μmol/L DiCa, and 5 μmol/L A23187 (Fig 4). Interestingly, after exposure to a high concentration of ADP (60 μmol/L) or a combination of DiCa (100 μmol/L) with A23187 (5 μmol/L), PAC1 binding was comparable with normal control. These studies on PAC1 binding were performed on three separate occasions with similar results. The mean fluorescence values obtained in the patient were expressed as a percentage of the corresponding values in concurrently studied normal subjects. Taking all three studies, the following mean (±SD) values were obtained for the patient: PAF (200 nmol/L), 11% ± 5%; PAF (20 μmol/L), 11% ± 9%; ADP (5 μmol/L), 50% ± 18%; ADP (60 μmol/L), 86% ± 22%; TRAP (25 μmol/L), 34% ± 21%; DiCa (100 μmol/L), 41% ± 3%; and A23187 (5 μmol/L), 33% ± 4%. In both normal and patient platelets, presence of iloprost completely inhibited ADP-induced PAC1 binding (not shown), indicating that it is dependent on signal transduction mechanisms. These studies reveal that although the platelets have normal amounts of GPIIb-IIIa (as recognized by MoAbs 10E5 and A2Aα), there is a defect in its activation as shown by impaired PAC1 binding.

Signal transduction-independent LIBS expression. MoAb 62 (anti-LIBS antibody) binding was monitored in normal platelets by inducing the LIBS expression with RGDS peptide (1 nmol/L) and the disintegrin albolabrin (5 μg/mL), in the presence and absence of Iloprost, which blocks signal transduction processes. Both these peptides were found to be potent inducers of LIBS expression, and Iloprost had only a slight inhibitory effect (Fig 5), indicating that LIBS expression induced by these peptides is signal transduction-independent. In patient platelets, MoAb 62 binding by albolabrin and RGDS peptide were only slightly decreased compared with that in normal platelets (Fig 5), indicating that the patient platelets have an essentially intact ligand-binding ability, as well as the capacity to express LIBS by signal transduction-independent mechanisms.

Signal transduction-dependent LIBS expression. Signal transduction-dependent LIBS expression through cell activation was induced by ADP (5 μmol/L), TRAP (25 μmol/L), PAF (200 nmol/L and 20 μmol/L), DiCa (100 μmol/L) and A23187 (5 μmol/L) (Fig 6). In normal platelets, Iloprost completely blocked LIBS expression induced by these agonists (Fig 6), indicating its dependence on signal transduction mechanisms. In patient platelets, MoAb 62 binding induced by ADP, TRAP, and DiCa in the absence of Iloprost was comparable with that in normal platelets. It was minimally decreased with A23187. In contrast, a striking decrease was noted with PAF 200 nmol/L and 20 μmol/L (Fig 6). Thus, the most striking abnormality in LIBS expression occurred in response to the agonist (PAF) with the most impaired PAC1 expression.

Binding of fibrinogen and PAC1 to α-chymotrypsin-treated platelets. To obtain additional information on the ligand-binding ability of patient platelets, we studied the binding of FITC-labeled fibrinogen and PAC1 in platelets treated with α-chymotrypsin, which is known to induce fibrinogen binding that is not affected by elevation in cAMP by agents such as prostaglandin E1.15,37,38 The binding of FITC-labeled fibrinogen in patient platelets was comparable.
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Fig 5. Signal transduction-independent LIBS expression (Ab62 binding). Normal (N) or patient (P) PRP (1 x 10^9 platelets per milliliter) was incubated with FITC-labeled antibody Ab62 (50 μg/mL) and either albolabrin (5 μg/mL) or RGDS (1 mmol/L) in the absence (top panel) and presence (bottom panel) of Iloprost (ZK 36,374) at room temperature for 15 minutes without stirring. The histogram R (broken line) represents Ab62 binding in the absence of albolabrin or RGDS to both normal and patient platelets. Antibody binding to platelets was measured as indicated in legend to Fig 4.

with that in normal platelets in the presence of Iloprost (Fig 7). The binding of PAC1 was also comparable in similarly treated patient and normal platelets (Fig 7).

DISCUSSION

The major finding in this report is that the patient’s platelets have a defect in receptor-mediated signal transduction-dependent activation of GPIIb-IIIa that manifests as impaired aggregation in response to multiple agonists and impaired secretion in response to weak agonists (ADP, PAF, and epinephrine). Elegant studies exploiting several specific antibodies have permitted delineation of a series of discrete events that are related to platelet aggregation. These include (1) agonist-induced activation of the GPIIb-IIIa complex leading to the exposure of ligand (fibrinogen)-binding site, (2) ligand binding, and (3) post-occupancy events that follow ligand binding, including expression of the LIBS. In our patient, GPIIb-IIIa complexes are present in normal amounts on the platelet surface, as evidenced by normal binding of MoAbs 10E5 (Fig 3) and A2A9 and of FITC-labeled fibrinogen to chymotrypsin-treated platelets (Fig 7). However, GPIIb-IIIa activation is impaired, as revealed by diminished
Given the lifelong history in our patient, we suggest that the abnormality is congenital, supported also by a lifelong history of easy bruising and thrombocytopenia in the patient’s father (deceased).

The findings in our patient extend the spectrum of potential congenital platelet mechanisms leading to impaired aggregation from abnormalities in GPIIb-IIIa per se to aberrations in upstream events of platelet signal transduction that govern its activation. Thus, defects in primary aggregation may be viewed as related to defects in (1) the GPIIb-IIIa complex per se (which includes Glanzmann thrombasthenia and variants) and (2) GPIIb-IIIa function, resulting from abnormalities in the intracellular signaling events that regulate its activation. Our patient exemplifies the second mechanism and, to our knowledge, such a congenital defect has hitherto not been described. It is tempting to speculate that defects in aggregation (primary), often observed in patients with bleeding manifestations, may be more likely due to abnormalities in intracellular signaling events than in GPIIb-IIIa molecule. Thus, it is conceivable that some of the patients described by Lages and Weiss 43 with impaired initial aggregation responses to weak agonists, such as ADP, epinephrine, and U46619, also have abnormal integrin activation related to defective signal transduction events. In one of these patients, abnormalities in phosphoinositide hydrolysis 44 and pleckstrin phosphorylation were subsequently described. 45 Kornecki et al46 have described a patient with markedly abnormal platelet aggregation, normal levels of radiolabeled platelet surface glycoproteins, and markedly impaired 125I-fibrinogen binding sites. However, pleckstrin phosphorylation was normal. 46

Our studies in normal platelets extend previous reports 16-19 that LIBS expression may be induced by signal transduction-dependent and independent mechanisms. LIBS expression after exposure to smaller peptides (RGDS and albolabrin) occurred largely independent of intracellular events, in contrast with that mediated by surface receptor agonists such as ADP, PAF, and thrombin, which was strongly dependent on signal transduction. In our patient, ADP- and thrombin-induced LIBS expression was normal (Fig 6), even though PACI binding was abnormal (Fig 4). RGDS 19 and albolabrin 45 increase binding of MoAb 62 to platelets by changing conformation of αIIbβ3, and expressing the LIBS epitope. Although anti-LIBS epitope and the ligand-binding site are distinct structures, there is a cooperativeness between these sites: ligand binding promotes LIBS expression and binding of anti-LIBS antibody enhances ligand binding. 19 Moreover, fibrinogen binding to its receptor enhances cellular signaling leading to the increased fibrinogen-binding affinity to GPIIb-IIIa. 25 For these reasons, it is difficult to completely separate signal transduction-dependent and signal transduction-independent LIBS expression, and LIBS expression may not be totally independent. It is conceivable that slightly decreased binding of a MoAb 62 antibody to patient platelets stimulated by RGDS and albolabrin (Fig 5) may also reflect the defect in intracellular activation mechanisms.

A number of studies have linked PKC activation to GPIIb-IIIa activation 12,14,15. In our patient, we provide evidence of a defect in pleckstrin phosphorylation in response to throm-
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bin and PAF (Fig 2). Our findings strongly support previous findings in normal platelets,23-27 that activation of GPIIb-IIIa is, indeed, regulated by intracellular events. We propose that the observed defective integrin activation in our patient is related to the upstream abnormality in intracellular events including pleckstrin phosphorylation. These findings may provide additional support for a role of PKC in GPIIb-IIIa activation, at least in response to some agonists. The precise mechanisms by which PKC regulates GPIIb-IIIa activation await clarification. Interestingly, in our patient, Dic-2-induced pleckstrin phosphorylation was normal, implying that the yet-undefined defect lies further upstream and not directly in either PKC or in its substrate, pleckstrin.

ADP and PAF are both weak platelet agonists,47 and ADP is a particularly weak inducer of phospholipase C activation,48 with some studies not detecting hydrolysis of 32P-labeled phosphoinositides.49 In our studies in normal platelets, pleckstrin phosphorylation in response to ADP was very low compared with that with other agonists (not shown). Yet, our studies show GPIIb-IIIa activation with ADP was more intense than with PAF. In the patient, PAC1 binding, GPIIb-IIIa activation, and initial slopes of aggregation are more strikingly abnormal with PAF, and, in fact, high ADP concentrations lead to near normal PAC1 binding, while a similar effect is not noted with PAF. This may suggest that ADP and PAF induce GPIIb-IIIa activation by different mechanisms, and PAF may have a greater dependence on products of phospholipase C activation than ADP. There is evidence of major differences in another platelet responses (Ca2+ mobilization) induced by these agonists.50 Sage and Rink51 have demonstrated that an increase in [Ca2+]i after activation with PAF, thrombin, and U46619 occurs after a delay of 200 to 400 milliseconds, while with ADP, it is markedly shorter, occurring without measurable delay. These studies have suggested that ADP evokes Ca2+ influx using a different transduction system, more closely coupled to Ca2+ entry system, than by other agonists, where the delay is sufficient for one or more biochemical steps to occur between activation and Ca2+ flux. Activation of GPIIb-IIIa by different agonists may be differentially regulated by intracellular activation events. ADP may be relatively more effective in inducing GPIIb-IIIa expression also by direct effects on membrane. The apparent lack of correlation between pleckstrin phosphorylation and GPIIb-IIIa activation observed after activation with ADP leads us to conclude that ADP-induced fibrinogen receptor exposure may be only minimally dependent on PKC activation, and more importantly, that such a relationship is clearly agonist-dependent. Our observations are compatible with the conclusions that the impaired aggregation in our patient arises (1) due to an abnormality in GPIIb-IIIa activation despite the presence of adequate amounts of this integrin on platelet surface with intact ligand-binding ability, and (2) that it is related to a defect in the signal transduction mechanisms that couple agonist receptors to GPIIb-IIIa activation rather than in the GPIIb-IIIa complex itself. The finding that pleckstrin phosphorylation is impaired upon platelet activation lends support to the paradigm that, at least in response to some agonists (eg, PAF, thrombin), PKC activation constitutes one of the mechanisms that modulate GPIIb-IIIa activation and fibrinogen binding. However, such a relationship may not be equally operative for other agonists (eg, ADP). Our patient provides strong evidence that abnormal aggregation may result not only from defects in GPIIb-IIIa per se but also from aberrations in upstream events of platelet signal transduction.

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