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# Physical Proximity and Functional Association of Glycoprotein 1bα and Protein-disulfide Isomerase on the Platelet Plasma Membrane*

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**Platelet function is influenced by the platelet thiol-disulfide balance. Platelet activation resulted in 440% increase in surface protein thiol groups. Two proteins that presented free thiol(s) on the activated platelet surface were protein-disulfide isomerase (PDI) and glycoprotein 1bα (GP1bα). PDI contains two active site dithiols/disulfides. The active sites of 26% of the PDI on resting platelets was in the dithiol form, compared with 81% in the dithiol form on activated platelets. Similarly, GP1bα presented one or more free thiols on the activated platelet surface but not on resting platelets. Anti-PDI antibodies increased the dissociation constant for binding of vWF to platelets by ~50% and PDI and GP1bα were sufficiently close on the platelet surface to allow fluorescence resonance energy transfer between chromophores attached to PDI and GP1bα. Incubation of resting platelets with anti-PDI antibodies followed by activation with thrombin enhanced labeling and binding of monoclonal antibodies to the N-terminal region of GP1bα on the activated platelet surface. These observations indicated that platelet activation triggered reduction of the active site disulfides of PDI and a conformational change in GP1bα that resulted in exposure of a free thiol(s).**

The platelet thiol-disulfide balance is important for platelet function. Perturbation of platelet thiol status effects platelet aggregation and release. The low $M_r$ thiol compounds, reduced glutathione (GSH), cysteine, and 6-mercaptopurine, inhibit platelet aggregation induced by several agonists, while the disulfide-bond reducing agents dithiothreitol and β-mercaptoethanol promote aggregation (1). In addition, reaction of platelet sulfhydryl groups with the thiol specific compounds, diamide and *N*-ethylmaleimide, inhibits *in vitro* aggregation and clot retraction (2–5). These results imply that certain platelet thiol groups are critical for platelet aggregation. Furthermore, the observation that specific depletion of platelet GSH by 1-chloro-2,4-dinitrobenzene only marginally effects platelet aggregability implies that the critical thiol groups are associated with protein (6). In support of this notion, Yamada *et al.* (7) have shown that the anti-platelet aggregation actions of 2,2′-dithiobis(*N*-2-hydroxypropylbenzamide) are mediated through interaction of the compound with platelet protein thiol groups.

Protein-disulfide isomerase (PDI)[1] is a noncovalent homodimer with a subunit molecular mass of 57 kDa that catalyzes thiol-disulfide interchanges that can result in formation, reduction, or rearrangement of protein disulfide bonds. It is generally considered that PDI is important for proper folding and disulfide bonding of nascent proteins in the endoplasmic reticulum (8–10). PDI also functions as the β subunits of prolyl-4-hyroxylase (11, 12) and the β subunit of triglyceride transfer protein complex (13, 14). Bovine aortic endothelial cells (15), rat hepatocytes (16), rat pancreatic cells (17), and human B cells (18, 19) secrete PDI which associates with the cell surface, and murine fibroblasts secrete PDI in response to treatment with calcium ionophore (20). Cell surface PDI has been implicated in reduction of the disulfide-linked diptheria toxin heterodimer (21, 22), cell surface events which trigger entry of the human immunodeficiency virus into lymphoid cells (23), shedding of the human thyrotropin receptor ectodomain (24), and as a cell surface recognition/adhesion molecule during neuronal differentiation of the retina (25). PDI is also on the external surface of the platelet plasma membrane and can catalyze rearrangement of disulfide bonds in scrambled ribonuclease (26, 27).

Increase or decrease in PDI on the surface of HT1080 human fibrosarcoma cells is associated with increase or decrease in cell surface protein thiols (28) and cell surface PDI has been implicated in the increase in surface protein thiol content of human lymphocytes following mitogen activation (19, 29). These observations indicated that secreted PDI can control the redox state of existing exofacial protein thiols or reactive disulfide bonds. We have examined the redox properties of PDI on the platelet plasma membrane.

Platelet activation and aggregation resulted in 440% increase in surface protein thiol groups. Both PDI and the von Willebrand factor receptor, glycoprotein 1bα (GP1bα), expressed free thiols on the activated platelet surface. Moreover, PDI and GP1bα were in close proximity on the activated plate-

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[1] The abbreviations used are: PDI, protein-disulfide isomerase; BSA, bovine serum albumin; CysGly, cysteinylglycine; GP, glycoprotein; FRET, fluorescence resonance energy transfer; GSH, reduced glutathione; HRP, horseradish peroxidase; MPB, 3-(*N*-maleimidylpropionyl)biocytin; PBS, phosphate-buffered saline; PVDF, polyvinyldiethylene fluoride; PAGE, polyacrylamide gel electrophoresis; SSB, sulfosuccinimidobiotin; vWF, von Willebrand factor; PE, phycoerythrin; HPLC, high performance liquid chromatography.

let surface. These findings demonstrated that platelet activation/aggregation triggered reduction of the active site disulfides of PDI and a conformational change in GP1bα that resulted in exposure of a free thiol(s). The close proximity of PDI and GP1bα on the activated platelet surface suggested that PDI may have participated in the conformational change in GP1bα.

## EXPERIMENTAL PROCEDURES

*Reagents and Chemicals*—HEPES, apyrase (grade VII), leupeptin, phenylmethylsulfonyl fluoride, streptavidin-agarose, iodoacetamide, GSH, and dithiothreitol were purchased from Sigma, and Trasylol (aprotinin) from Bayer Australia, Sydney, New South Wales, Australia. D-Phe-Pro-Arg-chloromethyl ketone was obtained from Calbiochem, San Diego, CA. 3-(*N*-Maleimidylpropionyl)biocytin (MPB) was purchased from Molecular Probes, Eugene, OR, and sulfosuccinimidobiotin (SSB) from Pierce. PolyScreen polyvinyldiethylene fluoride (PVDF) transfer membrane, Western blot chemiluminescence reagent, and reflection autoradiography film were purchased from DuPont, Boston, MA. All other chemicals were of reagent grade.

*Proteins*—PDI was purified from human placenta according to the method of Lambert and Freedman (30) with modifications described by Hotchkiss *et al.* (31). Human α-thrombin was prepared as described previously (32), and the active enzyme concentration determined by active site titration (33). Rabbit polyclonal antibodies were developed against purified human placenta PDI in New Zealand White rabbits and affinity purified on a PDI-Affi-Gel 15 matrix (Bio-Rad). Streptavidin-horseradish peroxidase (HRP) was from Amersham Australia, Sydney, NSW, and swine anti-rabbit IgG HRP conjugated antibodies were from Dako Corporation, Carpinteria, CA. All proteins were aliquoted and stored at −80 °C until use.

*Platelet Preparation*—Platelets were isolated from whole blood as described previously (34), with the following modifications. The platelets were washed and incubated at 37 °C for 15 min in $Ca^{2+}$- and bovine serum albumin (BSA)-free Tyrode buffer containing 2 units/ml apyrase to minimize platelet activation. Following two further washes in apyrase-free Tyrode buffer the platelets were resuspended in 20 mM HEPES, 137 mM NaCl, 4 mM KCl, 0.5 mM $Na_2HPO_4$, 0.1 mM $CaCl_2$, pH 7.4, buffer. Activation of platelets was with 30 nM human α-thrombin for 2 min at 37 °C on a rotating wheel. Thrombin and platelet enzymes were quenched with 5 μM D-Phe-Pro-Arg-chloromethyl ketone and 10 μM leupeptin. Platelet releasate was separated from activated platelets by centrifugation at 2,000 × *g* for 20 min at 4 °C. The total protein in releasate from $1 \times 10^9$ activated platelets per ml was 0.14 mg/ml using the BCA protein assay (Pierce). Platelet lysates were prepared by washing platelets twice with phosphate-buffered saline (PBS) by centrifugation at 2,000 × *g* for 20 min at 4 °C, resuspension in 50 mM Tris-HCl, 0.5 M NaCl, pH 8.0, buffer containing 1% Triton X-100, 10 μM leupeptin, 10 μM aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 5 mM EDTA, and sonication at 4 °C. The total protein in $1 \times 10^9$ sonicated platelets per ml was 1.0 mg/ml using the BCA protein assay (Pierce).

*Labeling of Platelets with SSB or MPB*—Labeling with SSB was a modification of the method of Ingalls *et al.* (35), while labeling with MPB was a modification of the method of Roffman *et al.* (36). Resting or activated platelets (1 ml of ~$7 \times 10^8$ per ml for SSB and ~$0.4–2 \times 10^9$ per ml for MPB) were incubated with SSB (1 mM) or MPB (100 μM) for 30 min at room temperature on a rotating wheel. On some occasions, resting platelets (1 ml of $1.3 \times 10^9$ per ml) were incubated with 50 μM dithiothreitol for 30 min at room temperature prior to labeling with MPB. On another occasion, resting platelets (1 ml of $0.4 \times 10^9$ per ml) were incubated with 20 μg/ml ReoPro (Centocor B.V., Leiden, The Netherlands) and 5 mM EDTA for 30 min at room temperature, prior to activation with thrombin and labeling with MPB. Unreacted SSB was quenched with glycine (2 mM), and unreacted MPB with GSH (200 μM), for 30 min at room temperature. The unreacted GSH was quenched with iodoacetamide (400 μM) for 10 min at room temperature. The labeled platelets were washed twice with PBS and sonicated as described above. An aliquot of labeled platelet sonicate was set aside for Western blotting with streptavidin-HRP. Streptavidin-agarose beads (100 μl of packed beads for SSB or 25 μl of packed beads for MPB) were incubated with the platelet sonicates for 1 h at 4 °C on a rotating wheel to isolate the biotin-labeled proteins. The streptavidin-agarose beads were washed 5 times with 50 mM Tris-HCl, 0.15 M NaCl, 0.05% Triton X-100, pH 8.0, buffer. The beads were suspended in 200 μl (SSB) or 50 μl (MPB) of SDS-Laemmli buffer and boiled for 2 min.

MPB was also used to label purified human placenta PDI. PDI (0.5 μM), or PDI preincubated with dithiothreitol (5 μM) for 30 min, was labeled with MPB (100 μM) for 30 min. Unreacted MPB was quenched with GSH (200 μM) for 10 min and iodoacetamide (400 μM) added for 10 min to quench unreacted GSH. All incubations were performed at room temperature in 20 mM HEPES, 0.14 M NaCl, pH 7.4, buffer.

*Immunoprecipitation of Platelet Surface Glycoproteins*—Resting or activated platelets were labeled with either SSB or MPB as described above and resuspended in PBS containing 1% BSA. The labeled platelets were incubated for 30 min at 4 °C on a rotating wheel with 10 μg/ml of either control murine monoclonal antibody (MOPC21) or murine monoclonal antibodies that recognize either GP1bα (AK3) or αIIbβ3 (AP2). The platelets were washed three times with PBS, lysed in 10 mM Tris-HCl, 0.15 M NaCl, pH 8.0, buffer containing 0.5% Triton X-100, 0.05% Tween 20, 10 μM leupeptin, 10 μM aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 5 mM EDTA, sonicated as described above, clarified by centrifugation at 12,000 × *g* for 30 min at 4 °C, and incubated with sheep anti-mouse coated Dynabeads (Dynal, Victoria, Australia) for 2 h at 4 °C on a rotating wheel. The Dynabeads were washed five times with PBS, resuspended in SDS-Laemmli buffer, and boiled for 2 min.

*SDS-PAGE and Western Blotting*—Samples were resolved on 10, 12, or 5–15% SDS-PAGE under nonreducing conditions according to Laemmli (37), transferred to PVDF membrane, developed according to the manufacturers instructions (DuPont), and visualized using chemiluminescence. Affinity-purified rabbit anti-PDI polyclonal antibodies were used at a final dilution of 1:5000, swine anti-rabbit HRP-conjugated antibodies at 1:1000 dilution, and streptavidin-HRP at 1:2000 dilution.

*Quantitation of SSB- and MPB-labeled Platelet Surface Proteins*—SSB- or MPB-labeled platelet surface proteins were quantitated by densitometry using a Model GS-300 Hoeffer Scientific Instruments scanning densitometer. SSB- or MPB-labeled PDI was quantitated by relating band intensity to a standard curve constructed using purified placenta PDI. Total protein estimations (BCA Protein Assay, Pierce, Rockford, IL) were performed on all platelet samples prior to SSB or MPB labeling and after platelet sonication to correct for platelet loss during the labeling and washing procedures. This loss was always <20%.

*Quantitation of Platelet Low $M_r$ Thiol Compounds*—Platelet releasate was separated from $1.5 \times 10^9$ thrombin-activated platelets in 1 ml by centrifugation at 2,000 × *g* for 20 min at 4 °C. The total protein in releasate and platelet sonicate was 0.21 and 1.5 mg/ml, respectively, using the BCA protein assay (Pierce, Rockford, IL). The platelet low $M_r$ thiol compounds were derivatized with the fluorescent compound, 7-benzo-2-oxa-1,3-diazole-4-sulfonic acid, and resolved by reverse-phase HPLC as described previously (38). The starting sample was diluted 9.45-fold during derivatization and 20 μl was injected onto HPLC.

*Flow Cytometry*—Flow cytometry was performed using a FACStar Plus cytometer (Becton Dickinson, San Jose, CA) with argon ion laser exitation at 488 nm. Emission spectra were collected using a 530 ± 30 nm band pass filter for fluorescein isothiocyanate and Alexa-488, or a 585 ± 45 nm band pass filter for phycoerythrin (PE). Ten thousand platelets were acquired at a flow rate of 500–1000 particles per second.

*Binding of vWF to Platelets*—Purified human plasma vWF was a gift from Dr. M. Berndt (39). The vWF was labeled with the fluorochrome, Alexa-488, according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Briefly, vWF was mixed with Alexa-488 at pH 8.3 with constant stirring at room temperature for 60 min. The reaction was quenched with hydroxylamine and the unconjugated dye removed by dialysis against PBS. The concentration of the vWf-Alexa conjugate was determined by protein assay (BCA Protein Assay, Pierce). vWF and Alexa-488-labeled vWF were resolved on 1% agarose gel electrophoresis according to Ruggeri and Zimmerman (40). There was no apparent difference in the multimer distribution of unlabeled *versus* Alexa-488-labeled vWF.

Washed platelets were prepared as described above and resuspended at $2 \times 10^6$ platelets/ml in PBS containing 1% BSA and 10 mM EDTA. Platelets were incubated with 20 μg/ml of the anti-platelet FcγRIIA receptor monoclonal antibody, IV.3, for 10 min at room temperature and then with 0 to 200 μg/ml of either preimmune rabbit IgG or anti-PDI IgG for a further 30 min at room temperature. The blocked platelets were incubated with 0.1 to 10 μg/ml vWF-Alexa and 1 mg/ml ristocetin (Sigma) for 10 min at room temperature. Unbound vWF-Alexa was removed by washing the platelets three times with PBS containing 1% BSA and the bound vWF-Alexa measured by flow cytometry.

*Fluorescence Resonance Energy Transfer (FRET)*—To assess the proximity of PDI and GP1bα on the platelet surface, the efficiency of FRET between PE-labeled PDI and Cy5-labeled GP1bα was measured by flow cytometry as described previously (41–43). Platelets ($2 \times 10^7$ in

100 μl of PBS containing 1% BSA) were incubated with rabbit anti-PDI polyclonal antibodies (20 μg/ml) and/or the anti-GPIbα monoclonal antibody AK2 (20 μg/ml) for 20 min and washed once with PBS containing 1% BSA. The primary antibodies were labeled by incubating for 20 min in the dark with PE-conjugated donkey anti-rabbit IgG (Becton Dickinson, San Jose, CA) or Cy5-conjugated donkey anti-mouse IgG (Jackson Immunoresearch, West Grove, PA). The samples were diluted 4-fold with BSA-free Tyrodes and kept in the dark until analysis. The FRET efficiency was expressed as the percentage of the emission energy from donor (PE) taken up by acceptor (Cy5). This method can detect proximity in the 2–10 nm range (41–43).

*Effect of Anti-PDI Polyclonal Antibodies on MPB Labeling of GP1bα*—Resting platelets (1 ml of ~7 × $10^8$ per ml for SSB and ~0.4–2 × $10^9$ per ml for MPB) were incubated with 200 μg/ml of either preimmune rabbit IgG or rabbit anti-PDI IgG for 30 min at room temperature. The platelets were activated with thrombin, labeled with either SSB or MPB, and the GP1b-IX-V immunoprecipitated using AK3 monoclonal antibodies as described above. The labeled glycoproteins were resolved on 10% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin peroxidase to detect the SSB or MPB label.

*Binding of Anti-GP1b-IX Monoclonal Antibodies to Platelets*—Resting platelets (2 × $10^6$ in 100 μl of BSA-free Tyrodes buffer) were incubated with 200 μg/ml of either preimmune rabbit IgG $F(ab)_2$ fragments or rabbit anti-PDI $F(ab)_2$ fragments for 30 min at room temperature. The $F(ab)_2$ fragments were made using the Pierce $F(ab)_2$ Preparation Kit (Pierce). The platelets were activated with thrombin as described above and incubated with 10 μg/ml of the following fluorescein isothiocyanate-conjugated monoclonal antibodies: MOPC21 (irrelevant control, Becton Dickinson, San Jose, CA), SZ1 (anti-GPIX, Immunotech, Marseille, France), AN51 (anti-GPIbα, Dako Pharmaceuticals, Carpinteria, CA), AK2 (anti-GPIbα, Serotec, Oxford, United Kingdom), HIP1 (anti-GPIbα, Becton Dickinson, San Jose, CA), and SZ2 (anti-GPIbα, Immunotech, Marseille, France). The samples were diluted 4-fold with BSA-free Tyrodes and kept in the dark until analysis.

## RESULTS

*Platelet Aggregation Triggered an Increase in Platelet Surface Protein Thiol Groups*—The membrane impermeable biotinylated thiol-specific reagent, MPB, was used to label sulfhydryl groups on resting or thrombin-activated platelets allowed to aggregate or prevented from aggregating with ReoPro and EDTA (Fig. 1*A*). Platelets were labeled with MPB, sonicated, resolved on 12% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin peroxidase.

The amount of platelet surface proteins labeled with MPB increased markedly upon platelet activation/aggregation. Densitometric analysis of the profiles indicated that 460% more protein was labeled with MPB on activated/aggregated platelets compared with resting platelets. In contrast, far less protein was labeled with MPB on activated platelets prevented from aggregating with ReoPro/EDTA, 60% increased labeling compared with resting platelets. The same result was observed if EDTA alone was used to minimize platelet aggregation (not shown). This result implied that platelet activation and aggregation, not activation alone, triggered reduction of platelet surface protein disulfides or presentation of previously cryptic protein thiols. The intensity of labeling of individual proteins with MPB increased with platelet activation/aggregation. In addition, activation/aggregation-specific MPB-labeled proteins were also observed. This feature of activated platelets was examined in more detail by measuring the changes in total protein on the resting *versus* activated platelet surface and that fraction of protein that presented free sulfhydryl group(s).

*Quantitation of Thiol- versus Amine-containing Platelet Surface Proteins*—Platelet surfaces were labeled with either MPB or SSB. SSB reacts with primary amines and, therefore, is a measure of total surface protein. The ratio of thiol- to amine-labeled surface protein was used to estimate the relative proportion of total platelet surface protein that presented free sulfhydryl group(s). MPB or SSB was incubated with resting or thrombin-activated/aggregated platelets. The labeled platelets were sonicated, resolved on 10% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin-peroxidase to detect the SSB or MPB label. The profiles of the labeled proteins are shown in Fig. 1*B*. The greater prevalence of primary amines compared with sulfhydryl groups on the platelet surface necessitated labeling of 22 times more platelets with MPB than with SSB.

The amount of platelet surface proteins labeled with SSB increased significantly upon platelet activation/aggregation.

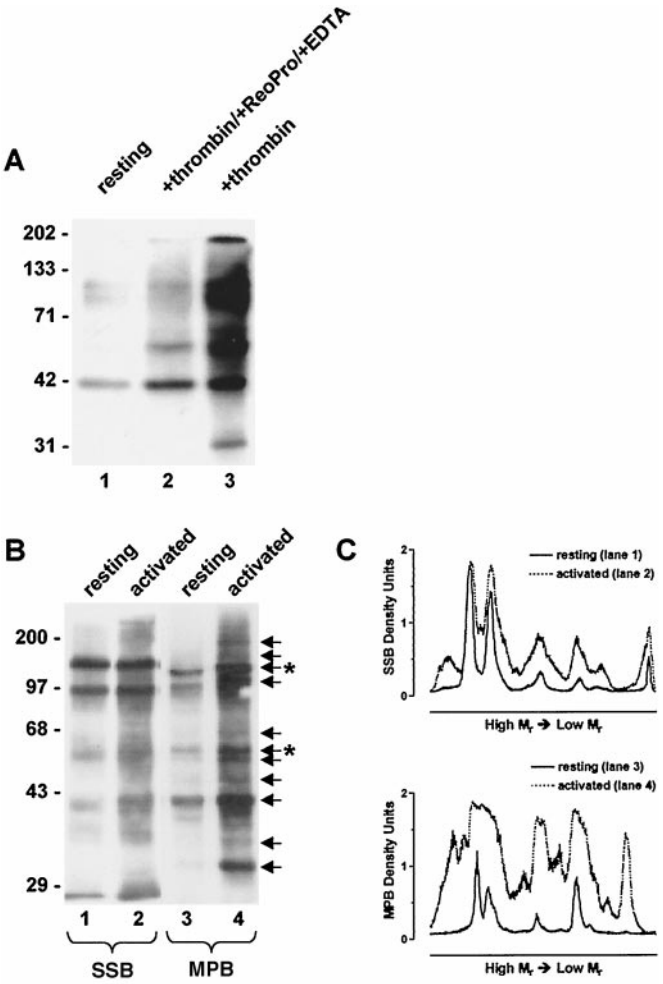


FIG. 1. **Platelet aggregation triggered an increase in platelet surface protein thiol groups.** *A,* the membrane impermeable thiol-specific reagent, MPB, was used to label sulfhydryl groups on resting or thrombin-activated platelets allowed to aggregate or prevented from aggregating with ReoPro (20 μg/ml) and EDTA (5 mM). Platelets were labeled with MPB, sonicated, resolved on 12% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin peroxidase to detect the MPB label. The results represent labeling of 5 × $10^6$ platelets (5 μg). The positions of $M_r$ markers are shown at the *left*. *B,* comparison of primary amines and thiols on the surface of resting *versus* thrombin-activated platelets. Resting and thrombin-activated/aggregated platelet surfaces were labeled with either SSB or MPB as indicated, sonicated, resolved on 10% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin peroxidase to detect the SSB or MPB label. The results represent labeling of 1 × $10^6$ platelets (1 μg) with SSB and 3 × $10^7$ platelets (30 μg) with MPB. The *arrows* at *right* indicate proteins that were labeled with MPB on the activated/aggregated platelet surface but were poorly or not labeled on the resting platelet surface. The *asterisks* indicate proteins with apparent $M_r$ values of ~60,000 and ~120,000 which may be PDI and GP1bα, respectively (see below). The positions of $M_r$ markers are shown on the *left*. *C,* densitometric analysis of the SSB-labeled (*top*) and MPB-labeled (*bottom*) platelet surface proteins shown in *B*. The extent of labeling with either SSB or MPB was calculated from the areas under the curves. Platelet activation resulted in a 130% increase in labeling with SSB and 440% increase in labeling with MPB.

Densitometric analysis of the profiles indicated that 130% more protein was labeled with SSB on activated/aggregated platelets compared with resting platelets. In contrast, 440% more protein was labeled with MPB on activated/aggregated platelets compared with resting platelets (see also Fig. 1*A*). The ratio of thiol- to amine-labeled protein on resting *versus* activated/aggregated platelets was a measure of the relative increase in surface protein thiols with respect to total surface protein. Platelet activation/aggregation resulted in a ~190% increase in surface protein thiol groups. At least 11 proteins presented free thiol(s) that were labeled with MPB on the activated/aggregated platelet surface but were poorly or not labeled on the resting platelet surface.

The finding that platelet activation/aggregation resulted in significantly more labeling of certain platelet surface proteins with MPB, implied that activation influenced the redox state of the thiol/disulfide groups of these proteins. PDI is a platelet surface protein (26, 27) whose activity is regulated by the redox state of its active site dithiols/disulfides (31). Moreover, PDI has been shown to regulate the redox state of proteins on the surface of cultured human fibroblasts (28) and lymphocytes (29). These findings suggested that PDI might similarly regulate the redox state of platelet surface proteins. We investigated the consequence of platelet activation/aggregation for the redox state of platelet surface PDI.

*Platelet Activation Resulted in Reduction of Surface-bound PDI*—The specificity of labeling of oxidized *versus* reduced PDI with MPB is shown in Fig. 2*A*. The active site dithiols of PDI purified from liver (44) or placenta (31) are oxidized. MPB did not label purified placenta PDI, as expected, but did label PDI activated by a low concentration of dithiothreitol (5 μM).

The ratio of thiol- to amine-labeled PDI was used to estimate the relative proportion of total platelet surface PDI that contained active site dithiols (Fig. 2*B*). MPB or SSB was incubated with resting or thrombin-activated/aggregated platelets and the biotin-labeled proteins purified by affinity chromatography on streptavidin-agarose beads. The labeled proteins were resolved on 10% SDS-PAGE, blotted with anti-PDI polyclonal antibodies, and quantitated using densitometry. Control reactions omitting the label were performed to ensure that no unlabeled PDI was being nonspecifically carried through the procedure (not shown).

Platelet PDI had the same apparent mass of ~57 kDa as placenta PDI on SDS-PAGE (Fig. 2*B*), and they share the same amino-terminal sequence (26, 45). Amine-labeled platelet surface PDI increased 170% upon platelet activation/aggregation (2,430 ± 120 to 6,480 ± 320 molecules of PDI per platelet). In contrast, thiol-labeled PDI increased 750% upon platelet activation/aggregation (620 ± 50 to 5,250 ± 240 molecules of PDI per platelet). Therefore, 26% of the total PDI on resting platelets was in a reduced conformation compared with 81% on activated/aggregated platelets (Table I). It should be noted that PDI contains two active site dithiols/disulfides and that this technique does not distinguish between reduction of one or both of these disulfides. Therefore, the reduced PDI measured on the platelet surface is the sum of PDI molecules containing either one or two active site dithiols.

We hypothesized that the active site disulfides of platelet surface PDI might be reduced by low $M_r$ thiol compounds secreted by activated platelets. To test this theory, whole platelet and secreted low $M_r$ thiol compounds were measured.

*Characterization of Platelet Low $M_r$ Thiol Compounds*—Platelets contain low $M_r$ thiol compounds, in particular GSH and cysteinylglycine (CysGly) (for example, see Ref. 46). We compared the low $M_r$ thiol content of thrombin-activated/aggregated platelets and activated platelet releasate from two healthy individuals. The releasate from $1.5 \times 10^9$ thrombin-activated platelets/ml contained <0.2 μM low $M_r$ thiol compounds (Fig. 3*A*). The average concentrations of cysteine, cysteinylglycine, and GSH in normal plasma are 9, 3, and 5 μM, respectively (47). This result indicates that platelets are not a significant source of plasma low $M_r$ thiols and implied that platelet surface PDI was not reduced by secreted low $M_r$ thiol compounds. These observations suggested that activation/aggregation-dependent reduction of platelet surface PDI was me-

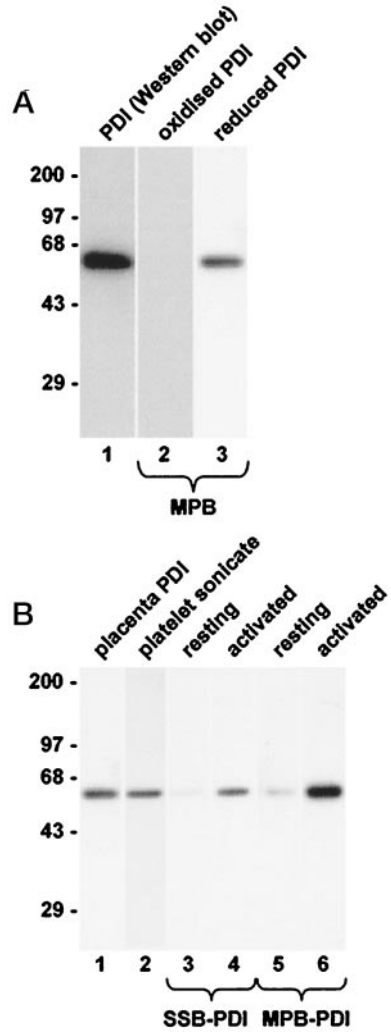


FIG. 2. **Platelet activation/aggregation resulted in an increase in both total and reduced platelet surface PDI.** *A,* labeling of PDI with MPB. Purified placenta PDI (oxidized PDI) or placenta PDI incubated with 5 μM dithiothreitol (reduced PDI) was labeled with MPB, 100 ng of the PDI resolved on 10% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin peroxidase to detect the MPB label. Placenta PDI (500 ng) blotted with anti-PDI polyclonal antibodies is shown in *lane 1* and migrated at the expected $M_r$ of 57,000. Oxidized PDI was not labeled with MPB (*lane 2*), whereas PDI activated with dithiothreitol incorporated MPB (*lane 3*). The positions of $M_r$ markers are shown at the *left*. *B,* resting platelets and thrombin-activated/aggregated platelets were labeled with either SSB or MPB as indicated, sonicated, and incubated with streptavidin-agarose beads to collect the biotin-labeled proteins. The labeled proteins were resolved on 10% SDS-PAGE, transferred to PVDF membrane, and blotted with anti-PDI polyclonal antibodies. The results represent labeling of $1.6 \times 10^8$ platelets (160 μg) with SSB (*lanes 3* and *4*) and $6.8 \times 10^8$ platelets (680 μg) with MPB (*lanes 5* and *6*). Control reactions omitting the SSB and MPB labeling were performed to confirm that no unlabeled platelet PDI was being nonspecifically carried through the procedure (data not shown). Controls are purified placenta PDI (100 ng, *lane 1*) and sonicated whole platelets ($8.3 \times 10^6$ platelets, 8 μg, *lane 2*). The positions of $M_r$ markers are shown at *left*.

TABLE I
*Quantitation of amine- and thiol-labeled platelet surface PDI on resting and thrombin-activated/aggregated platelets*

| | Molecules of surface PDI per platelet | | % PDI reduced |
|---|---|---|---|
| | Amine-labeled PDI[a] | Thiol-labeled PDI[b] | |
| Resting platelets | 2,430 ± 120[c] | 620 ± 50 | 26 |
| Activated/aggregated platelets | 6,480 ± 320 | 5,250 ± 240 | 81 |
| % Increase upon activation | 170 | 750 | |

[a] Total platelet surface PDI was estimated by labeling platelets with the membrane-impermeable amine-reactive reagent, SSB.
[b] Reduced platelet surface PDI was estimated by labeling platelets with the membrane-impermeable thiol-reactive reagent, MPB.
[c] The data is presented as the mean ± range from platelets from two healthy subjects.

diated by protein-catalyzed events.

To further test this hypothesis, the susceptibility of the active site disulfides of platelet surface PDI to reduction by dithiothreitol was examined. Incubation of resting platelets with 50 μM dithiothreitol for 30 min did not result in any further reduction of platelet surface PDI (Fig. 3*B*). In contrast, incubation of purified PDI with 5 μM dithiothreitol for 30 min was sufficient to reduce the active site disulfides (Fig. 2*A*). This result implied that the redox state of platelet surface PDI was refractory to extracellular reducing agents.

The results shown in Figs. 1–3 demonstrated that the active site dithiols/disulfides of PDI were in the dithiol state on the activated/aggregated platelet surface. At least 11 proteins contained free thiol(s) and were labeled with MPB on the activated/aggregated platelet surface but were poorly or not labeled on the resting platelet surface (see Fig. 1*B*). These proteins were potential substrates for PDI. One of these proteins had a $M_r$ of ~120, which is the approximate $M_r$ of the platelet receptor for von Willebrand factor (vWF), GP1bα.

*Labeling of Thiol(s) in GP1bα on the Activated Platelet Surface*—Resting platelets and thrombin-activated/aggregated platelets were labeled with either SSB or MPB and the GP1bα or αIIbβ3 immunoprecipitated using either AK3 or AP2 monoclonal antibodies, respectively. The labeled glycoproteins were resolved on 10% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin peroxidase to detect the SSB or MPB label (Fig. 4). Immunoprecipitation of GP1bα resulted in co-immunoprecipitation of the GP1bβ and GPIX components of the GP1b-IX·GP-V complex.

GP1bα, GP1bβ, and GP-IX were labeled with SSB on resting and activated platelets. GP1bα was labeled with MPB on activated platelets, but was not labeled on resting platelets (Fig. 4*A*). Neither GP1bβ nor GP-IX were labeled by MPB on resting or activated platelets. αIIbβ3 is not known to contain unpaired cysteines, therefore it was an appropriate control for MPB labeling. Accordingly, αIIbβ3 was labeled with SSB but not with MPB on resting and activated platelets (Fig. 4*B*).

The results shown in Figs. 1–4 suggested that PDI and GP1bα may have been physically and/or functionally associated on the platelet surface. This hypothesis was tested by measuring the effect of anti-PDI antibodies on binding of vWF to platelet GP1bα.

*Inhibition of Binding of vWF to GP1bα on the Platelet Surface by Anti-PDI Polyclonal Antibodies*—In initial experiments we observed that incubation of platelets with anti-PDI antibodies at concentrations >200 μg/ml resulted in platelet activation measured by presentation of P-selectin (48). Platelet activation by 300 μg/ml anti-PDI antibody was blocked completely by the murine anti-FcγRIIA antibody, IV.3, at concentrations of either 50 or 20 μg/ml. This result indicated that a small fraction of aggregated IgG in the anti-PDI antibody preparation was binding to the platelet FcγRIIA receptor and triggering activation.

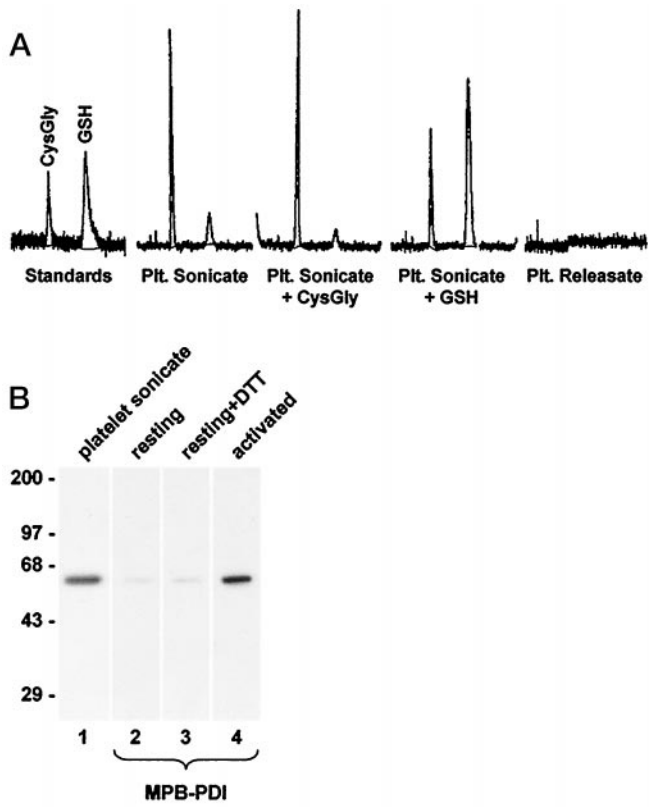


FIG. 3. **Platelet surface PDI is refractive to reduction by low $M_r$ thiols.** *A,* HPLC profiles of platelet low $M_r$ thiol compounds. Platelet low $M_r$ thiol compounds were derivatized with 7-benzo-2-oxa-1,3-diazole-4-sulfonic acid and separated by reverse-phase HPLC (38). The *left hand* trace show the standards, CysGly and GSH, followed by thrombin-activated/aggregated platelet sonicate, the same platelet sonicate spiked with either 2 μM CysGly or GSH to confirm the identity of the platelet thiols, and finally the releasate from the activated/aggregated platelets. The chromatograms represent thiols from $3 \times 10^6$ thrombin-activated/aggregated platelets (3 μg), or the releasate from the same platelets (0.42 μg). The starting sample contained $1.5 \times 10^9$ thrombin-activated/aggregated platelets/ml (1, 500 μg), or the releasate from the same platelets (210 μg). The HPLC procedure will detect a lower limit of ~0.2 μM GSH or CysGly in the starting sample. This pattern was repeated on three separate occasions. *B,* platelet surface PDI was not reduced by dithiothreitol. Resting platelets (*lane 2*), resting platelets incubated with 50 μM dithiothreitol for 30 min at room temperature (*lane 3*), and thrombin-activated/aggregated platelets (*lane 4*) were labeled with MPB, sonicated, and incubated with streptavidin-agarose beads to collect the biotin-labeled proteins. The labeled proteins were resolved on 10% SDS-PAGE, transferred to PVDF membrane, and blotted with anti-PDI polyclonal antibodies. The results represent labeling of $5.2 \times 10^8$ platelets (520 μg). The control for the Western blot is sonicated whole platelets ($8.3 \times 10^6$ platelets, 8 μg, *lane 1*). The positions of $M_r$ markers are shown at *left*.

Washed platelets were incubated with 20 μg/ml IV.3 and 0 to 200 μg/ml control or anti-PDI IgG and the binding of Alexa-labeled vWF was measured by flow cytometry (Fig. 5). The anti-PDI IgG reduced binding of vWF to platelets. The effects were on the apparent dissociation constant for vWF binding with no discernible effect on the apparent stoichiometry. The apparent dissociation constant and maximal binding of vWF was 1.0 ± 0.2 μg/ml and 98 ± 6% in the presence of control IgG, and 1.5 ± 0.2 μg/ml and 97 ± 5% in the presence of 50 μg/ml anti-PDI IgG. This corresponded to molar dissociation constants of 0.5 ± 0.1 and 0.8 ± 0.1 nM, respectively, assuming a weight average $M_r$ for vWF of $2 \times 10^6$. This affinity is in good agreement with other estimates of vWF binding (Ref. 49 and references therein).

These findings supported an association between PDI and

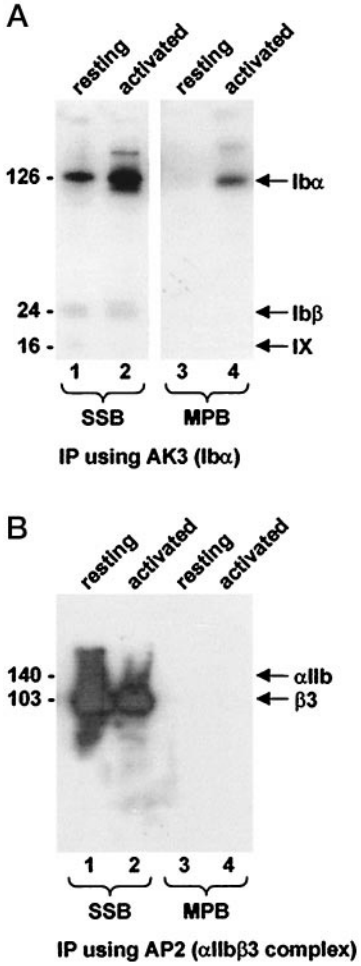


FIG. 4. **Labeling of GP1bα with MPB on the activated platelet surface.** Resting platelets and thrombin-activated/aggregated platelets were labeled with either SSB or MPB as indicated, sonicated, and the GP1bα or αIIbβ3 immunoprecipitated using either AK3 or AP2 monoclonal antibodies, respectively. The labeled glycoproteins were resolved on 10% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin peroxidase to detect the SSB or MPB label. The results of labeling of GP1bα are shown in *A*, while the results of αIIbβ3 labeling are shown in *B*. The figure represents labeling of $4 \times 10^8$ platelets (400 μg). GP1bα, GP1bβ, and GP-IX were labeled with SSB on resting and activated platelets. Platelet activation resulted in a 180% increase in labeling of GP1bα with SSB. GP1bα was labeled with MPB on activated platelets, but was not labeled on resting platelets. Neither GP1bβ nor GP-IX were labeled by MPB on resting or activated platelets. αIIbβ3 was labeled with SSB but not with MPB on resting and activated platelets.

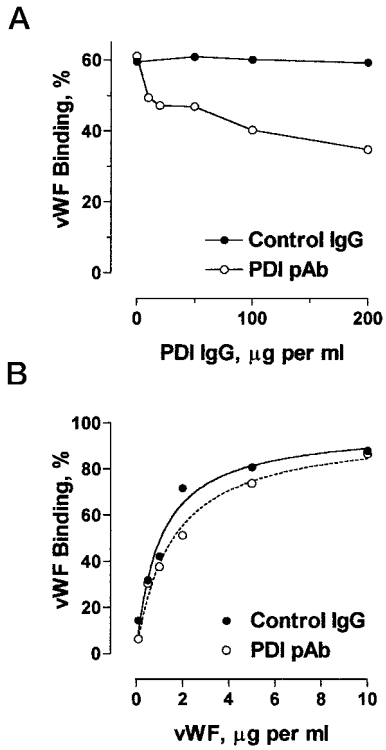


FIG. 5. **Inhibition of binding of vWF to GP1bα on the platelet surface by anti-PDI polyclonal antibodies.** Washed platelets were incubated with 20 μg/ml of the anti-platelet FcγRIIA receptor monoclonal antibody, IV.3, for 10 min at room temperature and then with 0 to 200 μg/ml of either preimmune rabbit IgG or anti-PDI IgG for a further 30 min at room temperature. The blocked platelets were incubated with 0.1–10 μg/ml vWF-Alexa and 1 mg/ml ristocetin for 10 min at room temperature and the bound vWF-Alexa measured by flow cytometry. The effect of increasing control IgG (●) or anti-PDI IgG (○) on binding of 5 μg/ml vWF-Alexa to platelets is shown in *A*. The effect of 50 μg/ml of either control IgG or anti-PDI IgG on the binding of 0.1–10 μg/ml of vWF-Alexa to platelets is shown in *B*. The *lines* in *B* represent the best fit of the data to the rectangular hyperbolic binding equation by nonlinear least squares regression. The apparent dissociation constant and maximal binding of vWF was 1.0 ± 0.2 μg/ml and 98 ± 6% in the presence of control IgG (—) and 1.5 ± 0.2 μg/ml and 97 ± 5% in the presence of 50 μg/ml anti-PDI IgG (···). In another experiment the apparent dissociation constant and maximal binding of vWF was 0.9 ± 0.4 μg/ml and 83 ± 6% in the presence of control IgG and 1.5 ± 0.4 μg/ml and 84 ± 10% in the presence of 50 μg/ml anti-PDI IgG (data not shown).

GP1bα on the platelet surface. To examine directly the proximity of these proteins, FRET between PE-labeled PDI and Cy5-labeled GP1bα on the platelet surface was measured by flow cytometry (41–43).

*Physical Proximity of PDI and GP1bα on the Platelet Plasma Membrane Measured by FRET*—FRET efficiency is the probability that an excited donor molecule will transfer its energy to an acceptor molecule in a non-radiative process and can be determined either on the donor side (quenching) or the acceptor side (enhancement). In our experiments we monitored the fluorescence intensity on the donor side. First, all fluorescence intensities were corrected for autofluorescence (Fig. 6*A, unlabeled*), that is the mean fluorescence intensity of unlabeled cells was subtracted from the mean fluorescence intensity of labeled cells. Second, the mean fluorescence intensity of sample labeled only with the donor secondary antibody was subtracted from the mean fluorescence intensity of sample labeled with donor primary and secondary antibodies and acceptor secondary antibody (Fig. 6, anti-PDI/PE, AK2-PE). This value was $F_{\text{donor,corrected}}$ and is corrected for nonspecific binding of the donor PE-conjugated secondary antibody, which was negligible. Third, the mean fluorescence intensity of sample labeled only with the donor secondary antibody and the sample labeled only with the acceptor primary and secondary antibodies was subtracted from the mean fluorescence intensity of sample labeled with both donor and acceptor primary and secondary antibodies (Fig. 6, anti-PDI/PE, AK2/Cy5-PE-AK2/Cy5). This value was $F_{\text{FRET,corrected}}$ and is corrected for the spectral contribution of acceptor Cy5 in the donor PE detection channel, which was negligible. FRET efficiency was calculated from the relationship; FRET efficiency = $1 - (F_{\text{FRET,corrected}}/F_{\text{donor,corrected}})$.

A FRET efficiency of 14.8 ± 4.9% (1 S.D.) was calculated from three experiments. A 14.8% FRET efficiency means that on average the PE fluorescence intensity of the FRET sample (labeled with both donor and acceptor molecules) was 14.8% less than that of the donor sample (labeled with donor molecule only). A FRET efficiency above 5% is considered significant (41–43), therefore, our value of 14.8 ± 4.9% should be consid-

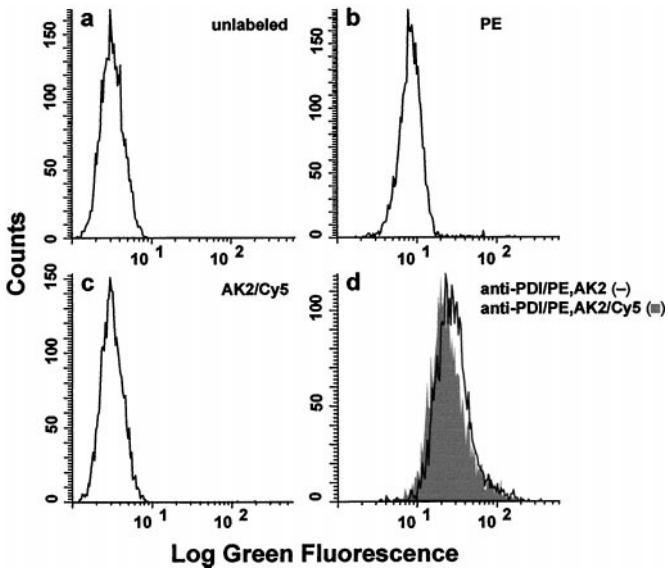


FIG. 6. **Physical proximity of PDI and GP1bα on the platelet plasma membrane measured by FRET.** To assess the proximity of PDI and GP1bα on the platelet surface, the efficiency of FRET between PE-labeled PDI and Cy5-labeled GP1bα was measured by flow cytometry. The contribution of autofluorescence was determined using unlabeled cells (*a*). The binding of the PE-conjugated secondary antibody in the absence of primary donor antibody is shown in *b*, while the overlap of acceptor Cy5 in the donor PE channel is shown in *c*. FRET between PE-labeled PDI and Cy5-labeled GP1bα is shown in *d*. A FRET efficiency of 14.8 ± 4.9% (1 S.D.) was calculated from three experiments.

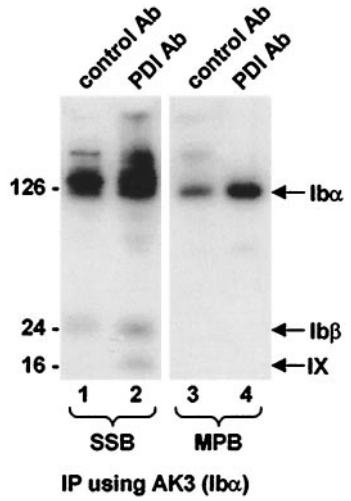


FIG. 7. **Enhancement of labeling of GP1bα on the activated platelet surface by anti-PDI polyclonal antibodies.** Resting platelets were incubated with 200 μg/ml of either preimmune rabbit IgG or rabbit anti-PDI IgG for 30 min, activated with thrombin, labeled with either SSB or MPB, and the GP1b-IX-GP-V immunoprecipitated using AK3 monoclonal antibodies. The labeled glycoproteins were resolved on 10% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin peroxidase to detect the SSB or MPB label. The figure represents labeling of $4 \times 10^8$ platelets (0.4 mg). The presence of anti-PDI polyclonal antibodies resulted in a 80% increase in labeling of GP1bα with SSB and a 130% increase in labeling with MPB. The experiment was performed on two separate occasions with the same qualitative result.

ered as significant. There was no FRET between PE-labeled preimmune IgG and Cy5-labeled GP1bα.

It is unlikely that the FRET between PE-labeled PDI and Cy5-labeled GP1bα occurred by chance. The surface area of a platelet is 22 $\mu m^2$ and there are 25,000 molecules of GP1bα on the platelet surface (49). Therefore, one molecule of GP1bα will be found in an area of 890 $nm^2$. Assuming an even lattice-like arrangement, the receptors will be 30 nm apart. This is well in excess of the upper limit of the Förster energy transfer (8–10 nm).

The results shown in Fig. 4 implied that GP1bα underwent a conformational change upon platelet activation resulting in the exposure of a free thiol(s). To test whether PDI might be involved in this conformational change, resting platelets were incubated with anti-PDI antibodies, activated with thrombin, and labeled with either SSB or MPB or incubated with anti-GP1b-IX monoclonal antibodies.

*Enhancement of Labeling of GP1bα on the Activated Platelet Surface by Anti-PDI Polyclonal Antibodies*—Resting platelets were incubated with 200 μg/ml of either preimmune rabbit IgG or rabbit anti-PDI IgG for 30 min, activated with thrombin, labeled with either SSB or MPB and the GP1b-IX·GP-V immunoprecipitated using AK3 monoclonal antibodies. The labeled glycoproteins were resolved on 10% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin peroxidase to detect the SSB or MPB label (Fig. 7). The presence of anti-PDI polyclonal antibodies resulted in a 80% increase in labeling of GP1bα with SSB and a 130% increase in labeling with MPB.

Incubation of resting platelets ($1 \times 10^9$ platelets/ml) with dithiothreitol-activated PDI (0.5 μM) for 10 min, or 200 μg/ml rabbit anti-PDI IgG for 30 min, at 37 °C did not cause exposure of the free thiol in GP1bα (not shown). These findings suggested that the conformational change in GP1bα upon platelet activation was the result of specific events on the activated platelet surface.

*Enhancement of Binding of Anti-GP1bα Monoclonal Antibodies to the Activated Platelet Surface by Anti-PDI Polyclonal Fab Fragments*—Resting platelets were incubated with 200 μg/ml of either preimmune rabbit IgG $F(ab)_2$ fragments or rabbit anti-PDI $F(ab)_2$ fragments for 30 min, activated with thrombin, incubated with 10 μg/ml fluorescein isothiocyanate-conjugated anti-GP1b-IX monoclonal antibodies and the bound antibody measured by flow cytometry. The GP1b-IX·GP-V complex is in close proximity to the FcγRIIA receptor on the platelet surface (48). To eliminate potential complications of anti-PDI polyclonal antibody binding to the FcγRIIA platelet receptor, $F(ab)_2$ fragments of the anti-PDI antibodies were made and used. The binding of one anti-GP-IX monoclonal antibody and four anti-GP1bα monoclonal antibodies was measured. SZ1 binds to GPIX in complex with GP1bα. AN51 binds to the N-terminal flank of GP1bα (residues 1–35), while AK2 binds to the first leucine-rich repeat, HIP1 to the second leucine-rich repeat, and SZ2 to the anionic sulfated tyrosine sequence (residues 269–282) (50).

The anti-PDI $F(ab)_2$ fragments did not effect binding of the irrelevant control antibody, MOPC21. The binding of SZ1, AN51, AK2, and HIP1 to activated platelets significantly increased upon incubation with anti-PDI $F(ab)_2$ fragments, while the binding of SZ2 decreased (Fig. 8*A*). Quantitation of the effects of anti-PDI polyclonal $F(ab)_2$ fragments on the binding of the anti-GP1b-IX monoclonal antibodies is shown in Fig. 8B. The anti-PDI $F(ab)_2$ fragments did not effect binding of any of the antibodies to resting platelets (not shown).

## DISCUSSION

Platelet activation/aggregation resulted in 440% increase in exofacial protein thiol groups. This increase was dependent on platelet aggregation, as negligible increase in surface protein thiol groups was observed if activated platelets were prevented from aggregating. The increase in surface thiols was due to expression of new thiol-containing proteins on the platelet surface, generation of thiols by reduction of disulfide bonds in existing proteins, or presentation of previously cryptic protein thiols. At least 11 proteins presented free thiol(s) that were

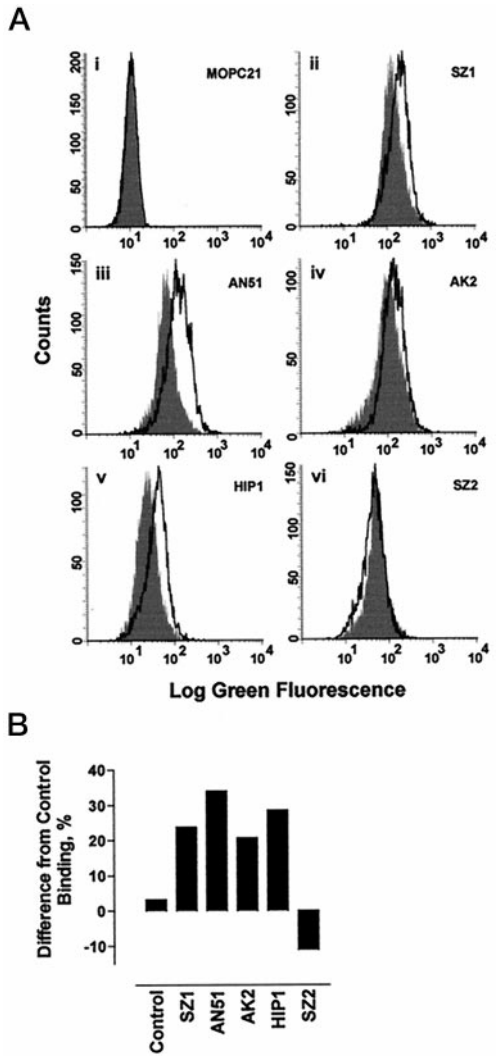


FIG. 8. **Enhancement of binding of anti-GP1bα monoclonal antibodies to the activated platelet surface by anti-PDI polyclonal F(ab)$_2$ fragments.** *A*, resting platelets were incubated with 200 μg/ml of either preimmune rabbit IgG F(ab)$_2$ fragments (filled histograms) or rabbit anti-PDI F(ab)$_2$ fragments (lined histograms) for 30 min, activated with thrombin, incubated with 10 μg/ml fluorescein isothiocyanate-conjugated anti-GP1b-IX monoclonal antibodies and the bound antibodies measured by flow cytometry. MOPC21 (*part i*) is an irrelevant control antibody, SZ1 (*part ii*) binds to GPIX in complex with GP1bα, while AN51 (*part iii*), AK2 (*part iv*), HIP1 (*part v*), and SZ2 (*part vi*) bind to epitopes in GP1bα (see text for antibody epitopes). *B*, quantitation of the effects of anti-PDI polyclonal F(ab)$_2$ fragments on the binding of the anti-GP1b-IX monoclonal antibodies shown in *A*. Monoclonal antibody binding in the presence of control IgG F(ab)$_2$ fragments was gated at 50%, and the binding in the presence of anti-PDI F(ab)$_2$ fragments was measured. The experiment was performed on three separate occasions with the same qualitative result.

labeled on the activated/aggregated platelet surface but were poorly or not labeled on the resting platelet surface. One of these proteins was PDI.

The ability of PDI to catalyze disulfide interchange in proteins resides in two very reactive surface exposed dithiols/disulfides which share the common sequence WCGPCK and have a redox potential of −110 mV (8–10). Secreted PDI controls the redox state of existing exofacial protein thiols or reactive disulfide bonds on the surface of human fibroblasts (28) and lymphocytes (29). The redox status of PDI on resting and thrombin-activated/aggregated platelets was investigated by labeling platelets with a thiol- or amine-reactive membrane impermeable probe.

There were ~2,400 molecules of amine-labeled PDI on the resting platelet surface *versus* ~6,500 molecules on the activated/aggregated platelet surface, an increase of 170%. This result implied that platelet activation/aggregation resulted in recruitment of PDI to the platelet surface and/or that a fraction of the PDI on the resting platelet surface was refractive to labeling with SSB. Chen *et al.* (26) reported no clear difference in the amount of PDI on resting *versus* activated platelets by immunogold labeling and electron microscopy, which suggested that the 170% increase we observed was perhaps due to labeling differences between resting and thrombin-activated/aggregated platelets. There is evidence that resting platelets contain a surface connected compartment, perhaps located in the platelet surface-connected canalicular system, which can be freely entered by some compounds but not others (51). It may be that the structure of SSB confers limited access to a pool of PDI on the resting platelet surface that becomes accessible following thrombin activation, which could account for the observed increase in total PDI on the surface of activated/aggregated platelets.

PDI containing free sulfhydryl groups increased 750% upon platelet activation/aggregation, and by comparison with amine-labeled PDI, 81% of the PDI on activated platelets was in a reduced conformation compared with 26% on resting platelets. This result indicated that platelet activation/aggregation triggered reduction of the active site disulfides of surface PDI. Although every effort was made to minimize activation/aggregation of the resting platelets, activation/aggregation of a fraction of the platelets during the washing procedure was almost unavoidable. The small amount of reduced PDI on the surface of resting platelets may have represented the fraction of the total platelets that is activated/aggregated. Therefore, the difference in amount of reduced PDI on the surface of resting *versus* activated/aggregated platelets may have in fact been greater then we have reported.

No detectable low $M_r$ thiol compounds were secreted upon platelet activation/aggregation and PDI on the surface of resting platelets was refractory to reduction by 50 μM dithiothreitol. These observations suggested that reduction of platelet surface PDI was triggered by a secreted or plasma membrane protein or proteins. It was possible that platelet surface PDI was intrinsically reduced but that the active site dithiols were masked by a protein at the platelet surface that was displaced upon platelet activation/aggregation. However, the activated/aggregated platelet surface contained 440% more protein thiol groups other than those of PDI, which implied a more general reduction event. The report that existing lymphocyte surface thiols are involved in the generation of additional surface thiols (29) supports the notion that the active site disulfides of PDI may have been reduced by an existing platelet surface protein whose activity was controlled by platelet activation/aggregation. One possibility is the plasma membrane NADH-oxidoreductase system (52) which has been implicated in reduction of extracellular protein disulfide bonds.

Another protein labeled with MPB on the activated/aggregated platelet surface but not labeled on the resting platelet surface was GP1bα. GP1bα is part of the GPIb-IX·GP-V complex which mediates adhesion of platelets to vessel wall von Willebrand factor at high wall shear (53). The thiol labeled in GP1bα on the activated platelet surface was perhaps the unpaired cysteine at position 65 in the second leucine-rich repeat near the N terminus (54). This result indicated that $Cys^{65}$, or another cysteine, was not exposed on the resting platelet surface and implied that platelet activation was associated with a conformational change in GP1bα which exposed $Cys^{65}$. It is noteworthy that substitution of $Cys^{65}$ for Arg in the N-terminal region of GP1bα impairs binding of vWF to GP1bα (55). It should be noted, however, that there may have been other free

thiols in GP1bα or other components of the GPIb-IX·GP-V complex that were inaccessible or refractive to labeling by MPB on the platelet surface.

PDI and GP1bα were in close proximity on the activated platelet surface. Anti-PDI antibodies increased the dissociation constant for binding of vWF to platelets by ~50% and PDI and GP1bα were sufficiently close on the platelet surface to allow FRET between chromophores attached to PDI and GP1bα. There are approximately 6,500 molecules of PDI (Table I) and 25,000 molecules of GP1bα (49) on the activated platelet surface, therefore the PDI:GP1bα molar ratio was ~1:4. There are two molecules of GP1bα, GP1bβ, and GP-IX and one molecule of GP-V in a GP1b-IX·GP-V complex on the platelet surface (53). This translates to an average of one molecule of PDI for every two $(GP1b\text{-}IX)_2$·GP-V complexes.

Incubation of resting platelets with anti-PDI antibodies followed by activation with thrombin enhanced labeling and binding of three monoclonal antibodies to the N-terminal region (residues 1–74) of GP1bα on the activated platelet surface. In contrast, binding of a monoclonal antibody to the anionic sulfated tyrosine sequence (residues 269–282) was inhibited by anti-PDI antibodies. The enhanced labeling and antibody binding to GP1bα in the presence of anti-PDI antibodies may have been due to simple displacement of PDI from GP1bα by the anti-PDI antibodies, however, it was possible that the anti-PDI antibodies were perturbing a PDI-catalyzed conformational change in GP1bα. The observation that anti-PDI antibodies caused enhanced binding of three monoclonal antibodies but decreased binding of another to GP1bα supported a PDI-facilitated conformational change. The nature of this putative conformational change is unknown, but it is noteworthy that PDI can catalyze net formation, net rearrangement, or net reduction of protein disulfide bonds depending on the nature of the protein substrate, the redox conditions, and the presence of other thiols and disulfides (10).

These studies point to a control mechanism that is analogous to activation of platelet αIIbβ3 (56). On the resting platelet surface αIIbβ3 is in a conformation that does not bind its ligands. Platelet activation triggers a conformational change in αIIbβ3 which enables ligand binding (57). Similarly, platelet activation/aggregation triggered reduction of the active site disulfides of PDI and a conformational change in GP1bα that resulted in exposure of a free thiol(s). The fact that PDI and GP1bα were in close proximity on the activated platelet surface suggested that PDI may have influenced the conformational change in GP1bα. An important question is what consequence the conformational change in GP1bα has for its interaction with vWF. It is possible that the change causes displacement of vWF from GP1bα. This might facilitate platelet spreading.

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