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 affects platelet aggregation and release. The low Mₗ 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) 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-hydroxylase (11, 12) and the 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 regulation of the disulfide-linked diphtheria toxin heterodimer (21, 22), cell surface events which trigger entry of the human immunodeficiency virus into lymphoid cells (23), shedding of the human thyrotrpin 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 ribonucleoside (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 thios (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 thios 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 platelet surface. Preincubation of the platelet surface with anti-PDI antibodies did not prevent platelet aggregation, indicating that PDI is not an essential factor for this process.

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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-(4-Maleimidophenyl)propionyl-cysteine; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; PAGE, polyacrylamide gel electrophoresis; SSB, sulfosuccinimidobiotin; vWF, von Willebrand factor; PE, phycoerythrin; HPLC, high performance liquid chromatography.
to quench unreacted GSH. All incubations were performed at room temperature in 20 mM HEPES, 0.14 mM NaCl, pH 7.4, buffer.

**Immunoprecipitation of Platelet Surface Glycoproteins—**Resting or activated platelets were labeled with either SSB or MPB, as described above. 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 GPIbα (AK3) or αIIbβ3 (AP2). The platelets were washed three times with PBS, lysed in 10 mM Tris-HCl, 0.15 mM NaCl, pH 8.0, buffer containing 0.5% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 100 μM aprotinin, and 2 mM phenylmethysulfonyl 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 Mr Thiol Compounds—**Platelet releasate was separated from 1.5 × 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, by the BCA protein assay (Pierce, Rockford, IL). The platelet low Mr 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 (Beckton Dickinson, San Jose, CA) with argon ion laser excitation 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 500 events/s with the PE channel being the forward light scatter parameter.

**Binding of vWF to Platelets—**Purified human plasma vWF was a gift from Dr. M. Berndt (39). 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 hydroxyamine 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 Ruggieri and Zimmerman (40). There was no apparent difference in the multimter distribution of unlabeled versus Alexa-488-labeled vWF.

**Fluorescence Resonance Energy Transfer (FRET)—**To assess the proximity of PDI and GPIbα on the platelet surface, the efficiency of FRET between PE-labeled PDI and Cy5-labeled GPIbα was measured by flow cytometry as described previously (41–43). Platelets (2 × 10^9 in

**PDI and GPIbα on the Platelet Plasma Membrane**
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 GPIbα.—**Resting platelets (1 ml of 7 × 10⁹ per ml for MPB and −0.4–2 × 10⁹ per ml for SSB) 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 GPIb-IX-V immunoprecipitated with 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 × 10⁹ in 100 µl of BSA-free Tyrodes buffer) were incubated with 200 µg/ml of either preimmune rabbit IgG Fab fragments or rabbit anti-PDI Fab fragments for 30 min at room temperature. The Fab fragments were made using the Pierce Fab (Fab) Preparation Kit (Pierce). The platelets were activated with thrombin as described above and incubated with 10 µg/ml of the following fluorogenic thiols-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. 1A). 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. Denitometric 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 and EDTA (Fig. 1A). Platelets were labeled with MPB, sonicated, resolved on 12% SDS-PAGE, transfected to PVDF membrane, and blotted with streptavidin peroxidase.

The results represent labeling of 1 × 10⁸ platelets (10% each) with SSB and 3 × 10⁷ platelets (30%) 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 Mr values of ~60,000 and ~120,000 which may be PDI and GPIbα, respectively. The positions of Mr markers are shown at the right and Mr markers are shown on 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 5 × 10⁷ platelets (5 µg). The positions of Mr markers are shown at the right and Mr 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 of the curves. Platelet activation resulted in a 130% increase in labeling with SSB and 440% increase in labeling with MPB.

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.

**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. 1B. 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.

**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. 1A). 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. Denitometric 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 and EDTA (Fig. 1A). Platelets were labeled with MPB, sonicated, resolved on 12% SDS-PAGE, transferred to PVDF membrane, and blotted with streptavidin peroxidase.

The results represent labeling of 1 × 10⁸ platelets (10% each) with SSB and 3 × 10⁷ platelets (30%) 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 Mr values of ~60,000 and ~120,000 which may be PDI and GPIbα, respectively (see below). The positions of Mr markers are shown at the right and Mr 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 of 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. 1A). 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 thiols 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. 2A. 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. 2B). 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 labeling 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. 2B), 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 M₉ thiol compounds secreted by activated platelets. To test this theory, whole platelets and secreted low M₉ thiol compounds were measured.

Characterization of Platelet Low M₉ Thiol Compounds—Platelets contain low M₉ thiol compounds, in particular GSH and cysteinylglycine (CysGly) (for example, see Ref. 46). We compared the low M₉ thiol content of thrombin-activated/aggregated platelets and activated platelet releasate from two healthy individuals. The releasate from 1.5 × 10⁹ thrombin-activated platelets/ml contained <0.2 μM low M₉ thiol compounds (Fig. 3A). 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 M₉ thiols and implied that platelet surface PDI was not reduced by secreted low M₉ thiol compounds. These observations suggested that activation/aggregation-dependent reduction of platelet surface PDI was me-
TABLE I  
Quantitation of amine- and thiol-labeled platelet surface PDI on resting and thrombin-activated/aggregated platelets

<table>
<thead>
<tr>
<th>Molecules of surface PDI per platelet</th>
<th>Amine-labeled PDI</th>
<th>Thiol-labeled PDI</th>
<th>% PDI reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting platelets</td>
<td>2,430 ± 120</td>
<td>620 ± 50</td>
<td>26</td>
</tr>
<tr>
<td>Activated/aggregated platelets</td>
<td>6,480 ± 320</td>
<td>5,250 ± 240</td>
<td>81</td>
</tr>
<tr>
<td>% Increase upon activation</td>
<td>170</td>
<td>750</td>
<td></td>
</tr>
</tbody>
</table>

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

The results shown in Figs. 1–3 demonstrated that the active site dithiols/disulfides of PDI were in the dithiol state on the platelet surface. This hypothesis was tested by measuring the effect of anti-PDI antibodies on binding of vWF to platelets.

Table I shows the quantitation of amine- and thiol-labeled platelet surface PDI on resting and thrombin-activated/aggregated platelets.

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Fig. 3. Platelet surface PDI is refractive to reduction by low M₄ thiols. A, HPLC profiles of platelet low M₄ thiol compounds. Platelet low M₄ 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 × 10⁸ thrombin-activated/aggregated platelets (5 μg), or the releasate from the same platelets (0.42 μg). The starting sample contained 1.5 × 10⁸ thrombin-activated/aggregated platelets/ml (1, 500 μg/ml), 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 × 10⁶ platelets (520 μg). The control for the Western blot is sonicated whole platelets (8.3 × 10⁶ platelets, 8 μg, lane 1). The positions of M₄ markers are shown at left.

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.

Ironically, despite 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.

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 M₄ for vWF of 2 × 10⁶. 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
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 (enhancement). In our experiments we monitored the fluorescence intensity on the donor side (enhancement). In our experiments we monitored the fluorescence intensity on the donor side (enhancement). In our experiments we monitored the fluorescence intensity on the donor side (enhancement). In our experiments we monitored the fluorescence intensity on the donor side (enhancement).

**Donor, Corrected & Donor, Corrected**

The efficiency of FRET can be calculated from the relationship: FRET efficiency = 1 − (F<sub>donor,corrected</sub>−F<sub>donor,corrected</sub>) / F<sub>donor,corrected</sub>.

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-
To assess the proximity of plasma membrane measured by FRET. Platelet surface activation was the result of specific events on the activated 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 × 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.

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...
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 WCXCXCK 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 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 when we have reported.

No detectable low 

M₃ 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 Cys₆⁵, 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 Cys₆⁵. It is noteworthy that substitution of Cys₆⁵ 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

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, S21 (part ii) binds to GPIX in complex with GP1bα, while AN51 (part iii), AK2 (part iv), HIP1 (part v), and S22 (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.
thiols in GP1bα or other components of the GP1b-IX-GP-V complex that were inaccessible or refractive to labeling by MBP 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-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 of GP1bα, resulting in exposure of a free thiol(s). The fact that PDI and GP1bα antibodies were perturbing a PDI-catalyzed conformational change in GP1bα and a change in GP1bα led to exposure of a free thiol(s) and disulfides of PDI and a conformational change in GP1bα. Disulfides of PDI and a conformational change in GP1bα were in close proximity on the activated platelet surface. In

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 ligand. Platelet activation triggers a conformational change in αIIbβ3 which enables ligand binding (57). Similarly, platelet activation/aggregation triggered reduction of active the 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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