ANTIOXIDANTS & REDOX SIGNALING
Volume 11, Number 5, 2009

DOI: 10.1089/ars.2008.2322

Comprehensive Invited Review

Redox Control of Platelet Function

David W. Essex

Reviewing Editors: Robert K. Andrews, Jane Arthur, Subrata Chakrabarti, Richard Phipps, and John C. Still

Department of Medicine and the Sol Sherry Thrombosis Research Center, Philadelphia, Pennsylvania.

Abstract

There has recently been a dramatic expansion in research in the area of redox biology with systems that utilize thiols to perform redox chemistry being central to redox control. Thiol-based reactions occur in proteins involved in platelet function, including extracellular platelet proteins. The αIIbβ3 fibrinogen receptor contains free thiols that are required for the activation of this receptor to a fibrinogen-binding conformation. This process is under enzymatic control, with protein disulfide isomerase playing a central role in the activation of αIIbβ3. Other integrins, such as the α2β1 collagen receptor on platelets, are also regulated by protein disulfide isomerase and thiol metabolism. Low molecular weight thiols that are found in blood regulate these processes by converting redox sensitive disulfide bonds to thiols and by providing the appropriate redox potential for these reactions. Additional mechanisms of redox control of platelets involve nitric oxide that inhibits platelet responses, and reactive oxygen species that potentiate platelet thrombus formation. Specific nitrosative or oxidative modifications of thiol groups in platelets may modulate platelet function. Since many biologic processes are regulated by redox reactions that involve surface thiols, the extracellular redox state can have an important influence on health and disease status and may be a target for therapeutic intervention. *Antioxid. Redox Signal.* 11, 1191–1225.

I. Introduction

Reactions involving thiols and disulfide bonds in extracellular proteins have recently become a focus of study in hemostasis and platelet biology. Disulfide bond cleavage or rearrangement initiated by thiols is a dynamic process that occurs in proteins involved in hemostasis and platelet function (44, 84). Certain disulfide bonds in native proteins

serve as sites for regulation of protein function by the reversible conversion to dithiol groups (44). Moreover, thiol–disulfide exchange is implicated in regulating the function of proteins or receptors involved in hemostasis, thrombosis, and wound healing (2, 49, 124, 231, 289). This process involves a nucleophilic attack by a thiolate anion on a disulfide bond (36, 67, 124).

While redox processes regulate numerous cytoplasmic events, they also regulate biological reactions in the more oxidizing cell surface environment (84). Proteins on the plasma membrane are at the interface between an oxidizing and a reducing environment and many biologic processes are regulated by the redox state of surface thiols. Relatively recent research emphasizes a role for reactions involving thiols and disulfide bonds as part of a dynamic process that regulates cell and protein function (47, 81, 82, 87, 123, 124, 152–154, 156, 231, 236, 237, 289). Extracellular protein disulfide isomerase (PDI) mediates some of these reactions (47, 82, 123, 124, 152–154, 231, 237). Moreover, platelet activation generates thiols from disulfide bonds in surface proteins (31, 83) and platelets contain a transmembrane electron transport system that reduces disulfide bonds (85). Additionally, physiologic concentrations of low molecular weight thiols potentiate platelet activation by generating vicinal thiols from redox sensitive disulfide bonds (83). Thus, thiol-containing proteins on the platelet plasma membrane can be influenced by both cytoplasmic and extracellular reducing equivalents. While protein–protein and cell–protein interactions have been well characterized in hemostasis and wound healing, disulfide bond rearrangement as a specific event in these processes has until relatively recently received little attention.

Nitric oxide (NO) and reactive oxygen species (ROS) also regulate platelet function. NO released from platelets or in the form of *S*-nitrosothiols found in plasma generally inhibit platelet responses, with an increase in platelet aggregation found in states of NO deficiency (169). Evidence is accumulating that ROS have a role in the regulation of platelet function (149), although this role needs to be further defined. Specific nitrosative or oxidative modifications of reactive thiol groups are expected to modulate the function of thiols on the platelet surface or in cytoplasmic platelet proteins.

II. Platelet Function

Platelets are small subcellular fragments found in blood that are involved in the processes of hemostasis, atherosclerosis, and wound healing. In hemostasis, platelet plug formation represents the primary response to vascular injury, with the coagulation cascade and fibrin formation comprising the secondary response. Normal primary hemostasis requires three critical events: platelet adhesion, granule content release, and platelet aggregation (Fig. 1). Upon vascular injury, platelets adhere promptly to collagen fibrils in the vascular subendothelium (234). This initial interaction is mediated by the platelet receptor, glycoprotein Ib (GPIb), which binds to exposed subendothelial collagen fibrils through the adhesive protein von Willebrand factor (vWF) and through the collagen receptor, $\alpha 2\beta 1$, a member of the integrin family of receptors (235, 277). Both of these adhesion receptors have a role in platelet adhesion under low shear rates, but at high shear rates (500 to 800 sec^{-1}) only the interaction of GPIb with immobilized vWF is capable of supporting adhesion (235).

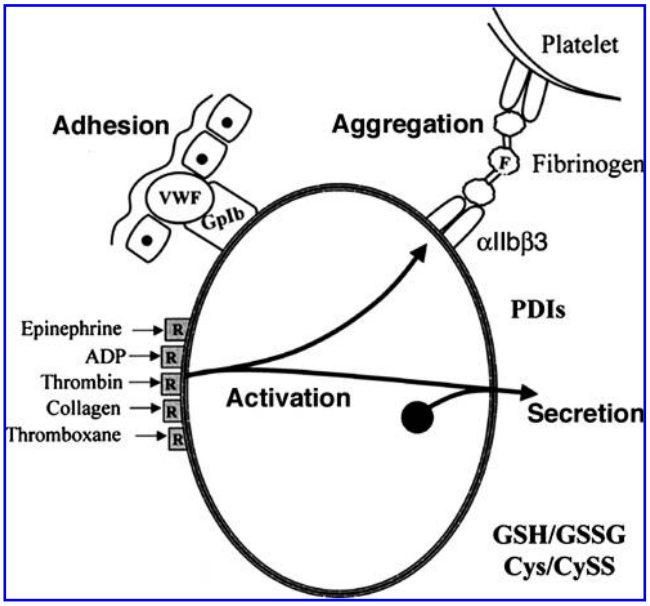

FIG. 1. Normal platelet responses. The basic platelet responses are platelet adhesion, activation, aggregation, and secretion. The primary adhesion receptor under conditions of high shear is GPIb which binds to von Willebrand Factor (vWF). A number of physiologically relevant platelet agonists such as thrombin and collagen activate platelets through primary agonist receptors. Platelet activation leads to inside-out activation of the αIIbβ3 integrin. This allows αIIbβ3 to bind fibrinogen supporting the adhesion of adjacent platelets (aggregation). The secretion reaction involves secretion of the contents of dense granules, α-granules, and lysosomal granules. Protein disulfide isomerases (PDIs) are required for activation of the αIIbβ3 integrin. The extracellular GSH/GSSG and Cys/CySS redox pools regulate the extracellular reactions involving thiols and disulfides.

The glycoprotein VI (GPVI) receptor, a member of the immunoglobulin superfamily, is another collagen receptor that mediates collagen-dependent platelet activation (12, 277). Adherent platelets then release granule contents, including ADP, and generate other mediators of platelet activation/aggregation, such as thromboxane A_2 (235, 277). Collagen, thrombin, ADP, and thromboxane are all important physiologic agonists in the activation of platelets. Similar to other cells, platelet activation is controlled by changes in the level of cyclic nucleotides, calcium influx, hydrolysis of membrane phospholipids, and phosphorylation of intracellular proteins (146).

A. The roles of ADP and thromboxane

The release of ADP from platelet dense granules and the generation of thromboxane from membrane phospholipids as secondary mediators potentiates the activation of platelets as well as the recruitment of additional platelets to the growing thrombus (135). The importance of these pathways in platelet function is evidenced by the fact that therapy with drugs that interrupt the ADP or thromboxane pathways are beneficial to patients with disease syndromes that involve platelet activation (such as coronary artery disease) (72). The drug clopidogrel inhibits ADP activation of platelets by inhibition of the $P2Y_{12}$ ADP purinergic receptor on the platelet surface and is

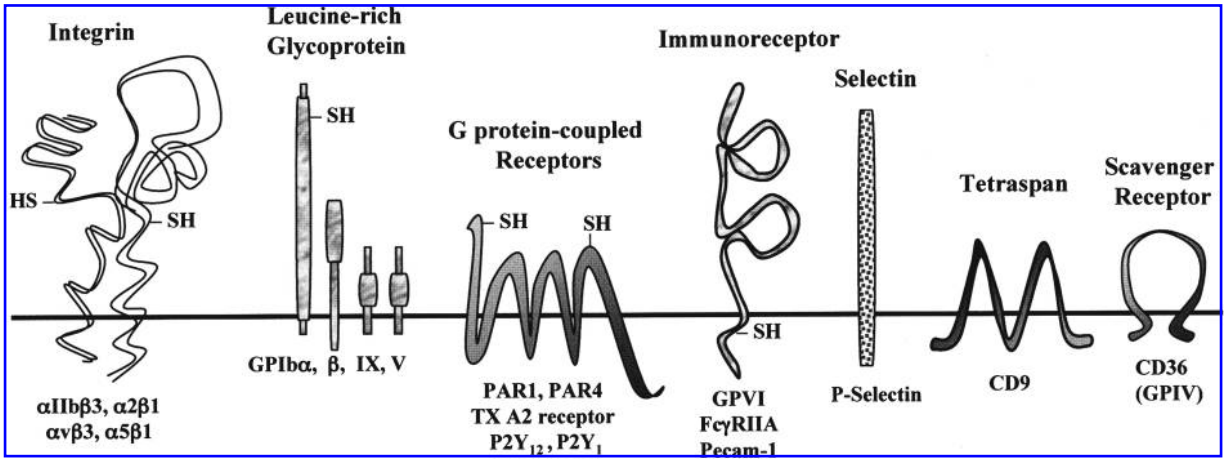

FIG. 2. Schematic illustration of platelet membrane glycoprotein receptor families. The glycoprotein family name is indicated above each structure. Platelet membrane glycoproteins belonging to individual families are listed below each structure. The platelet membrane is represented by the *horizontal line*. Thiols found in some receptors are potential sites of redox regulation (these SHs are not necessarily found in all members of the family).

in widespread use. Aspirin inhibits the formation of thromboxane A2 in platelets by inhibition of cyclooxygenase-1 (1).

ADP is stored in the dense granules of platelets and released upon activation of platelets by a variety of agonists. The principle receptors on the platelet surface for ADP are the $P2Y_1$ and $P2Y_{12}$ G-protein-coupled receptors that are important in platelet shape change, aggregation, and thromboxane A2 generation (198). Co-stimulation of both receptors is required for ADP-induced platelet aggregation (135). Additionally, the $P2Y_{12}$ receptor has an important role in ADP-mediated potentiation of platelet activation mediated by other physiological agonists including collagen, thromboxane A2, and lower doses of thrombin. Thromboxane A2 is an eicosanoid produced in platelets that is another secondary mediator of platelet activation. Thromboxane A2 is generated when arachidonic acid is released from platelet membrane lipids by phospholipase A2. The action of cyclooxygenase-1 is needed for the conversion of arachidonic acid to thromboxane A2. ADP and thromboxane A2 provide positive feedback mechanisms to the initial physiologic hemostatic response, amplifying this response and activating other resting platelets.

B. Platelet receptors and surface proteins

Receptors that are important in platelet adhesion reactions include glycoprotein Ib, a leucine rich glycoprotein, and the $\alpha 2\beta 1$ integrin (Fig. 2). GPIb is a complex of four polypeptide chains; GPIbα is covalently link to GPIbβ, while GPIX and GPV are noncovalently linked in the complex (220). GPIbα is the largest subunit and is the subunit that binds vWF. The platelet integrin that supports platelet aggregation, αIIbβ3, also has a role in platelet adhesion to von Willebrand factor. The principle receptors involved in the initial activation of platelets include the protease-activated receptors, PAR1 and PAR4, and the GPVI collagen receptor. Subsequently the ADP and thromboxane receptors play roles in the potentiation of platelet activation. The PAR1 and PAR4 thrombin receptors as well as the thromboxane and the $P2Y_{12}$ and $P2Y_1$ ADP-receptors are G protein-coupled receptors. The glycoprotein Ib and αIIbβ3 receptors also have roles in platelet activation as ligand binding to these receptors results in signaling into the platelet. Additional receptors with roles in platelet function include P-selectin (a member of the selectin family), platelet endothelial cell adhesion molecule-1 (PECAM-1), and the FcγRIIA receptor (of the immunoglobulin-like superfamily) as well as CD9 and CD36 (GPIV) (219). P-selectin is of interest in that it is a large cysteine-rich membrane protein that contains an epidermal growth factor-like domain (219) and mediates platelet rolling (35). Some receptors such as P-selectin are only expressed on the surface of activated platelets being translocated from the α-granule membrane upon platelet activation. CD40 ligand (CD40L) is another receptor that is translocated to the platelet surface after platelet stimulation (118). Additionally, some proteins such as thrombospondin-1 bind back to the platelet surface after being released from platelets (262). The αIIbβ3, $\alpha 2\beta 1$, GPIb, $P2Y_{12}$, and GPVI (cytoplasmic domain) receptors have free thiol groups (Fig. 2) that are potential sites of redox regulation. It remains to be determined if other receptors are also regulated by reactions involving thiols and disulfides. For a more detailed description of platelet receptors, the reader is referred elsewhere (219).

C. Activation of the αIIbβ3 fibrinogen receptor

Platelet activation leads to activation of the αIIbβ3 integrin (also known as glycoprotein IIbIIIa). Upon activation, αIIbβ3 binds fibrinogen, causing adhesion of adjacent platelets in a process called platelet aggregation, resulting in the formation of the primary hemostatic plug. The αIIbβ3 platelet integrin is part of the integrin family of receptors that are heterodimeric transmembrane receptor complexes, each with an α and β subunit (250). Integrins function in numerous physiological processes as hemostasis, immune responses, and angiogenesis. They function in cell adhesion and signaling by interacting with the extracellular matrix or other cellular receptors. Integrins exist in different activation states that have different affinities for ligands (250). Activation of integrins controls cell adhesion and integrin activation is reported to control

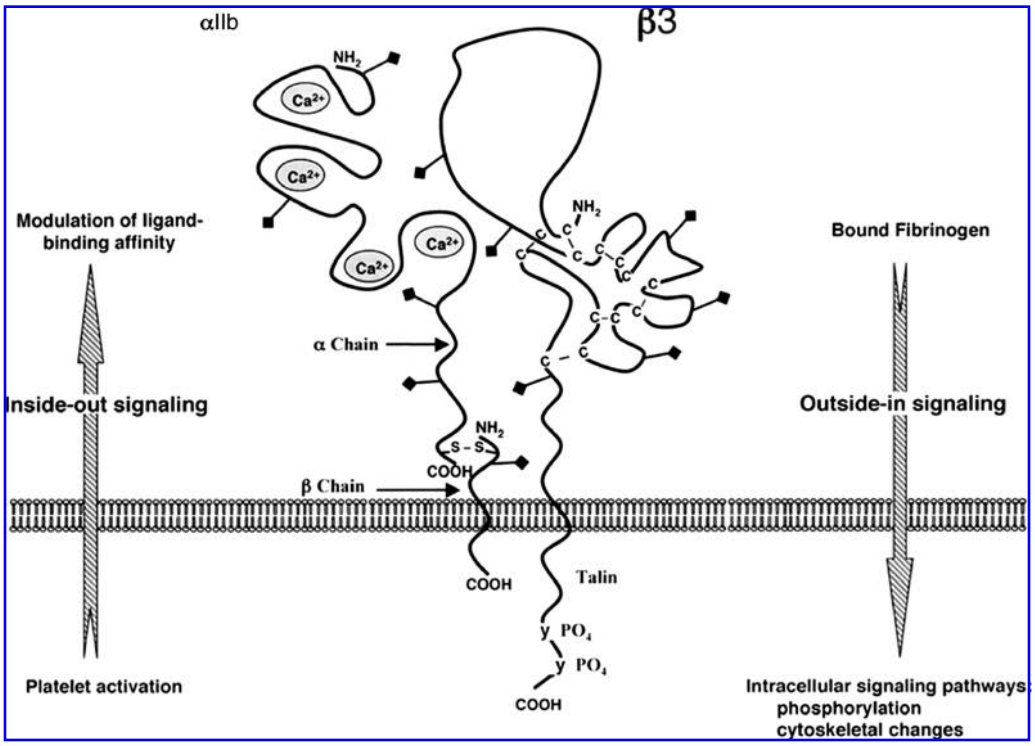

FIG. 3. Model of αIIbβ3. After platelet activation, inside-out signaling converts αIIbβ3 to a ligand-binding conformation. Bound fibrinogen supports platelet aggregation and this induces outside-in signaling that leads to phosphorylation of proteins and cytoskeletal rearrangements. The β3-subunit contains 56 cysteine residues, 32 of which are in a cysteine-rich region. The αIIb subunit contains Ca^{2+}-binding sites and both subunits have carbohydrate linkages (◆). The cytoplasmic tail domain of the β-subunit interacts with talin and contains phosphorylation sites.

metastasis in breast cancer (88). αIIbβ3 is specifically expressed in platelets and has provided the prototype example of integrin modulation as it goes through a transition from a low affinity/avidity state to a state where it effectively binds soluble ligands such as fibrinogen. Engagement of the head domain of talin with the β3-cytoplasmic tail may be a final common effecter in transmitting cytoplasmic signals to the extracellular conformational changes in αIIbβ3 (18).

One general model for activation of the αIIbβ3 integrin is as follows (249, 250): (a) Platelet stimulation leads to changes in αIIbβ3 by inside-out signaling (Fig. 3). These changes in αIIbβ3 are a response to intracellular signaling events and involve the propagation of conformational changes from the cytoplasmic domains of integrins to the extracellular ligand binding site; (b) this results in fibrinogen binding to αIIbβ3 providing; (c) the physical link for aggregation and causing; (d) further conformational changes in αIIbβ3 that lead to outside-in signaling. Outside-in signaling causes additional platelet responses including platelet spreading and clot retraction that involve phosphorylation events and cytoskeletal rearrangements (10).

Phosphorylation of tyrosine residues in platelet proteins regulates platelet function in both early platelet activation events and later activation events caused by outside-in signaling (146). These phosphorylation events are regulated by tyrosine kinases and tyrosine phosphatases.

It has long been known that the poorly-membrane permeant sulfhydryl blocking reagent pCMBS inhibits platelet aggregation (5) and that reduction of disulfide bonds in the fibrinogen receptor by reducing agents induces platelet aggregation (174). Nonetheless, the role of thiols and disulfides in platelet function long remained an underdeveloped area of platelet research. The report of protein disulfide isomerase (PDI) activity being secreted by activated platelets (41) and the localization of a functionally active PDI to the platelet surface suggested functions for PDI not previously thought of (41, 81). PDI was found to mediate platelet aggregation and secretion, and activation of the αIIbβ3 integrin (82). Thiol-based reactions with rearrangement of disulfide bonds are now implicated as part of a process that couples platelet stimulation to various platelet responses, including aggregation and secretion (31, 83, 134, 152–154, 179, 180, 210, 236, 289).

III. Redox Mechanisms

A. Reactive thiols

Free thiols or sulfhydryls can be involved in a variety of reactions. The reactive form of the thiol is the deproteinated S^- form, referred to as a thiolate anion. The propensity for a sulfhydryl group to lose an H^+ depends on the pKa of that thiol group. The thiol groups of most cysteine residues have pKa values around 8.5 ± 0.5 (111) and are thus generally not reactive. However, positively charged neighboring groups from lysine, arginine, and histidine increase the tendency for a thiol to be deproteinated by stabilizing the ionized S^- form.

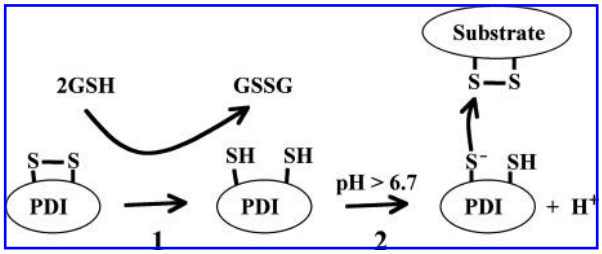

FIG. 4. Mechanisms of PDI activation. The redox state of the GSH/GSSG pool will determine the amount of the PDI active site in the dithiol form (reaction 1). The pH will determine the amount the dithiol form that has the first active site thiol in the thiolate anion form (reaction 2).

Examples of proteins with reactive thiols include proteins involved in disulfide reductase activity, such as thioredoxin, glutaredoxin, and PDI. Thiol proteases such as papain may have a pKa as low as 4.0 (111) and protein tyrosine phosphatases such as phosphatase 1B protein tyrosine have a catalytic site cysteine (pKa ~5.4) that forms a thiolate anion at physiologic pH (162). An exception to reactive thiols having a low pKa is the fast reacting thiol of Cys374 of actin that has a pKa of ~8.4 (279).

B. Extracellular reactions

An example of a reactive thiol group in an extracellular protein is the cysteine 34 residue of albumin that has a pKa of 5.0 (245). The low pKa facilitates oxidative reactions with low molecular thiols (245), reactions with nitric oxide (256), as well as thiol–disulfide exchange reactions (79). Reactions involving the cysteine 34 thiol in albumin typify the reactions that occur with thiols in the extracellular environment.

The first thiol in the active site of PDI has a low pKa (4.5–6.7 range) (36) and thus at physiologic pH is in the reactive thiolate anion form (Fig. 4, reaction 2). This thiolate anion acts as a nucleophile that can attack a sulfur atom that is part of a disulfide bond (36), or release NO from *S*-nitrosothiols (233, 291). In the endoplasmic reticulum where PDI catalyzes disulfide bond rearrangement and disulfide bond formation, the pH is ~7.2 (142). This confers the thiolate anion form on the first thiol in the active site of PDI. Similarly, at an extracellular pH of 7.4 the thiolate anion would predominate for the fraction of PDI that has its active sites in the dithiol form.

C. Oxidized and reduced PDI

The redox environment of PDI will influence the balance between the dithiol and disulfide fractions of the active sites of PDI (Fig. 4, reaction 1). The redox potential of both the cytoplasm and endoplasmic reticulum (ER) is largely determined by glutathione (GSH and GSSG). In the cytoplasm the ratio of GSH to GSSG is ~100:1 maintaining a reduced state (215). In the ER the ratio of GSH to GSSG is 2:1 (107). This oxidizing environment facilitates disulfide bond formation and PDI in this environment can act as an oxidant (208). However, PDI in the ER can also act as a disulfide isomerase, an activity that requires some of the PDI to have the thiol form of the active site (208).

D. Extracellular glutathione

Glutathione is an important modulator of the cellular redox environment but is also found in blood where it could modulate platelet function and integrin activation. The redox potential of the extracellular environment is determined primarily by the GSH/GSSG and the cysteine/cystine (Cys/CySS) redox pools (133), although other extracellular thiols and disulfides will also influence the redox balance. Thiol groups in extracellular platelet membrane proteins change with variation of the GSH/GSSG redox balance over a range that simulates plasma levels and this affects platelet aggregation (83, 85). The extracellular GSH/GSSG and Cys/CySS redox pools are thiol systems that are important in the control of oxidative stress (113, 132). These redox pools become more oxidizing with aging (133).

E. Vicinal thiols

Closely spaced protein thiols—herein called vicinal thiols—are redox-sensitive sites in native proteins that undergo reversible conversions with disulfide bonds under physiologic conditions. The active sites of PDI and similar redox enzymes like thioredoxin contain cysteines that undergo these reversible conversions. Even under mild oxidizing conditions, these cysteine residues are converted to disulfides (24, 70, 110). Vicinal thiols do not need to be in close proximity in the primary amino acid sequence but can be brought into close opposition by protein folding (129). In addition to enzymes whose active sites contain redox-active dithiols, these redox-sensitive sites are found in a variety of proteins involved in the regulation of cellular processes. They include proteins that regulate neutrophil function (23, 280) including NADPH neutrophil oxidase activity (73, 151, 161), platelet function (257), protein tyrosine phosphatase (45), and a variety of other proteins (110, 212, 216). Vicinal thiols are found on cell surfaces (71, 80, 244), with at least 10 to 12 vicinal thiol-containing proteins found on the surface of endothelial or fibrosarcoma cells (71).

F. Allosteric disulfide bonds

Allosteric disulfide bonds have been defined as disulfide bonds that control protein function by mediating conformational changes upon reduction (44). Allosteric disulfide bonds are characterized by the –RHStaple group found mostly in disulfide bonds that link adjacent strands in the same antiparallel β sheet (242). A defining feature of these bonds is the short distance between the α-carbon atoms of the Cys residues so that the –RHStaple bonds have a mean Cα–Cα distance significantly shorter than most disulfides. The torsional energy of the linkage causes a strain on these bonds. An example of this type of disulfide bond based on structural studies is the extracellular Cys186–Cys209 disulfide bond of tissue factor, the integral membrane protein that initiates blood coagulation (44). Studies using cell lines have led to the proposal that this disulfide bond is reduced in the cryptic or inactive form of tissue factor, whereas when the disulfide bond is formed the procoagulant function of tissue factor is activated (3, 43). Thus, the reversible oxidation of these two thiol groups may allow for control of coagulation (3, 43), (an alternative mechanism for tissue factor activation of oxidation-induced exposure of phospholipids instead of disulfide bond formation has

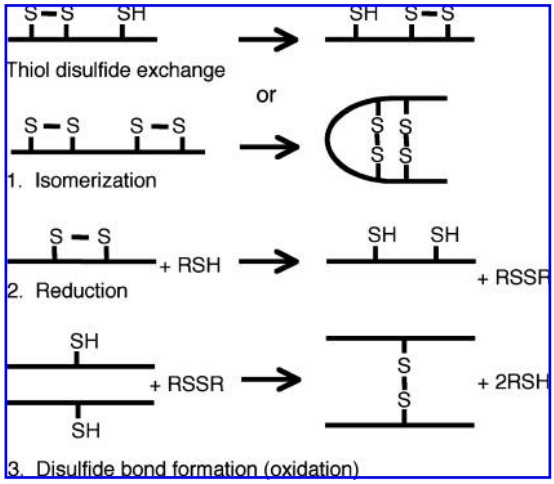

FIG. 5. Reactions catalyzed by PDI. The reaction in (1) illustrates the isomerization of disulfide bond by either an intramolecular thiol–disulfide exchange reaction, resulting in rearrangement of a disulfide bond, or the intramolecular switching of two disulfides bonds. With intramolecular thiol–disulfide exchange reactions, the catalytic thiol (thiolate anion) is in the protein itself; with isomerization of two disulfide bonds the nucleophilic thiolate anion that initiates the reaction can be external to the protein; (2) illustrates the reduction of a disulfide bond, while (3) illustrates disulfide bond formation.

also been proposed (221)). Another example of an allosteric disulfide bond is Cys663–Cys687 in the β-Tail domain (βTD) of the β3 subunit of αIIbβ3 (44). Disruption of this disulfide bridge results in activation of β3 integrins (32).

IV. Protein Disulfide Isomerase and ERP5 in Platelet Function

A. Platelet protein disulfide isomerase

PDI has traditionally been known as an endoplasmic reticulum (ER) protein, but it is now identified on a variety of cell surfaces including lymphocytes and platelets and shown to be functionally active (40, 81, 130, 178, 237). PDI on the surface of T-lymphocytes reduces disulfide bonds in the HIV glycoprotein 120, allowing cell entry (19, 99, 237). PDI was found to be secreted by platelets in response to strong agonists (40, 41) and a pool of PDI was localized to the platelet surface (40, 81). Platelet PDI was found to mediate platelet aggregation and secretion and activation of the αIIbβ3 integrin to a full fibrinogen binding conformation (87). A role for PDI in adhesion of platelets by β_1 and β_3 integrins has also been documented, including adhesion by the α2β1 collagen receptor (152, 154). A physical and functional relationship of PDI to the adhesion receptor glycoprotein Ib on the platelet surface has also been reported (31).

PDI is the prototypic enzyme of what is now known to be a PDI-family of enzymes (77, 274). PDI can catalyze three different reactions, isomerization of disulfide bonds, including thiol–disulfide exchange, reduction, and oxidation (107, 173, 247) (Fig. 5). The oxidation of sulfhydryls to disulfides occurs primarily in the endoplasmic reticulum, while PDI on the cell surface has been thought to primarily catalyze reduction or isomerization of disulfide bonds (208). Human PDI has a

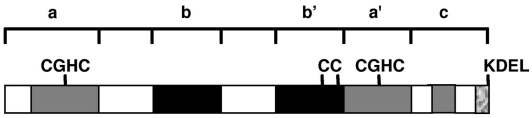

FIG. 6. Model of human PDI. Symbols: a, a′ internally homologous regions with sequence homology to thioredoxin; b, b′ internally homologous regions; c, highly acidic C-terminal region of the polypeptide. The active site sequences (CGHC), additional cysteine residues (C), and the C-terminal endoplasmic reticulum retention signal (KDEL) are indicated.

single subunit with 491 amino acids in the mature protein and has two regions of internal homology, each of which contains an active site with the sequence of Cys-Gly-His-Cys (Fig. 6). The active site Cys residues are a disulfide in equilibrium with a dithiol, with the dithiol form catalyzing isomerization or reduction. The disulfide form of PDI catalyzes the oxidation of thiols to disulfides. In addition to the four cysteines at the active sites, there are two other cysteine residues. PDI contains a substrate-binding site in the b′ domain that has been mapped to a small hydrophilic binding pocket located where the active site is found (77, 224). An interaction of PDI at this site with its substrate appears to be required for catalytic activity (62, 98, 281). PDI also has a C-terminal tetrapeptide sequence, Lys-Asp-Glu-Leu (KDEL), which functions as an endoplasmic reticulum retention signal in cells.

The finding that intramolecular and intermolecular thiol–disulfide exchange reactions occurred in the adhesive protein thrombospondin-1 in the supernatant solution of activated platelets led to the hypothesis that platelets secreted PDI (41, 254). The amino acid sequence for the first 33 amino acids of purified platelet PDI was identical to that predicted by the chromosome DNA (40). PDI was localized to the external surface of platelets by immunologic and functional studies (40, 81). PDI was found to be released from platelets activated with calcium ionophore or high dose α-thrombin in a microvesicle form. An additional pool of PDI in platelets becomes exposed on or near the platelet surface during the process of activation/aggregation (31). It was estimated that there were $\sim$2,400 molecules of PDI on the resting platelet and this increased to $\sim$6,500 on activated/aggregated platelets. Platelet PDI contains the C-terminal KDEL ER retention signal (87).

B. The role of PDI in platelet responses

A role for platelet surface PDI in aggregation and secretion and platelet adhesion is now well documented with the role of extracellular PDI being recently extended to *in vivo* platelet thrombus formation (47). Using Fab fragments from a rabbit antibody specific for PDI (40, 81), or a competing substrate of PDI (scrambled RNase), we found that inhibition of PDI on the platelet surface resulted in inhibition of platelet aggregation (Fig. 7). Neither the control normal rabbit Fab fragments, nor the control native RNase that does not interact with PDI inhibited platelet aggregation (81). A monoclonal antibody, RL90, that inhibits PDI activity, also inhibited platelet aggregation (153). Using flow cytometry and an antibody specific for activated αIIbβ3 (called PAC-1), rabbit anti-PDI Fab fragments (82) or the monoclonal anti-PDI (153) were found to inhibit the activation of αIIbβ3.

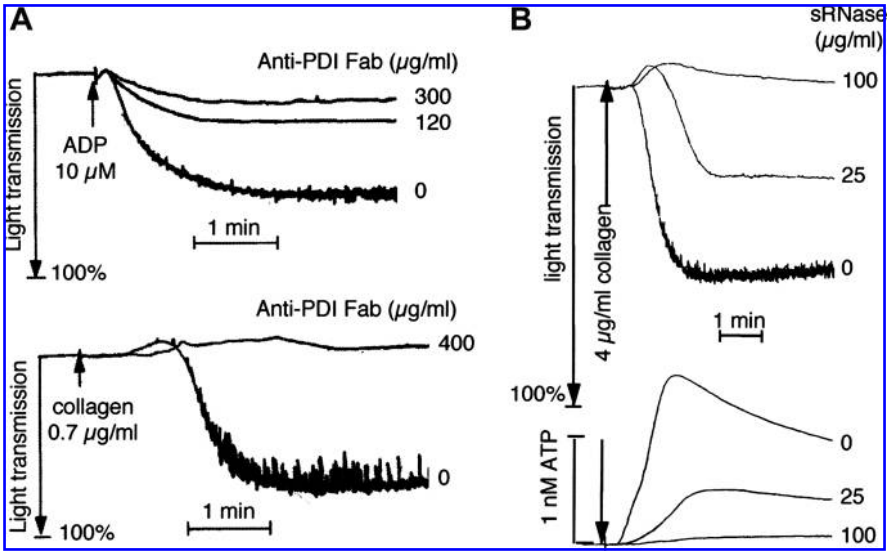

FIG. 7. PDI is required for platelet aggregation. The experiments in (**A**) show that anti-PDI Fab fragments made from a polyclonal rabbit antibody inhibit platelet aggregation induced by ADP and collagen. (**B**) shows that the competing substrate scrambled RNase (sRNase) also inhibits platelet aggregation. In these studies fibrinogen was added to washed platelets being stirred. After the activating agent (collagen or ADP) was added, platelet aggregation was recorded as an increase in light transmission. The anti-PDI antibody was specific for PDI in platelets by Ouchterlony double immunodiffusion and by Western blot analysis (40, 82). (Reprinted with permission from Essex DW, Li M (82).

The involvement of PDI in activation of a second platelet integrin, $\alpha 2\beta 1$, a collagen receptor, has been studied in detail (154). This study used a collagen-based synthetic peptide that contains the $\alpha 2$ I-domain recognition motif, GFOGER, in type I collagen (144) shown to be specific for binding to integrin $\alpha 2\beta 1$ (78, 145). This allowed examination of platelet adhesion via $\alpha 2\beta 1$ separately from other adhesive receptors for collagen such as GPVI. The non-integrin GPVI-specific synthetic collagen related peptide (CRP) allowed GPVI to be tested as an integrin-independent substrate. Both the Fab fragments of the polyclonal anti-PDI, (previously shown to inhibit PDI activity and agonist induced platelet aggregation (81, 82)) and the monoclonal anti-PDI antibody inhibited the integrin dependent adhesion to GFOGER. They did not inhibit adhesion to the non-integrin dependent CRP. Adhesion to type I collagen or to GFOGER was also inhibited by sulfhydryl blocking agents while adhesion to CRP was not (154). These data with the reports on the effect of PDI on $\alpha IIb\beta 3$ suggest that PDI catalyzed rearrangement of disulfide bonds may be general mechanism for members of the integrin family.

PDI probably targets a thiol-containing platelet surface protein in platelet aggregation. The thiol-containing $P2Y_{12}$ ADP receptor is involved in aggregation induced by most agonists. By performing platelet aggregation in the absence of contributions from the $P2Y_{12}$ receptor, a PDI-catalyzed event in a surface protein distinct from the $P2Y_{12}$ receptor was implicated (180). A monoclonal antibody to PDI inhibited γ-thrombin and calcium ionophore-induced aggregation. As calcium ionophore activates platelets by calcium flux independently of primary agonist receptors, it is likely that a PDI-catalyzed event occurs near the final $\alpha IIb\beta 3$-dependent events of aggregation. To study signaling-independent activation of $\alpha IIb\beta 3$, Mn^{2+} was used as an activator of $\alpha IIb\beta 3$ in platelets. Mn^{2+} directly shifts purified integrins from their inactive to active form (165). The anti-PDI antibody inhibited Mn^{2+}-induced activation of $\alpha IIb\beta 3$. The results with Mn^{2+}—a direct activator of $\alpha IIb\beta 3$—suggest that a PDI-catalyzed reaction in $\alpha IIb\beta 3$ is necessary for activation of this integrin on platelets. Others have documented a greater role for PDI in the activation of $\alpha IIb\beta 3$ as opposed to the initial agonist-induced stimulatory events (153). The monoclonal anti-PDI antibody had an inhibitory effect on activation of $\alpha IIb\beta 3$ with the expression of a non-integrin dependent marker of platelet activation (P-selectin) being inhibited to a lesser degree.

More subtle aspects of platelet activation are seen in citrated plasma when the primary aggregation response is distinguished from a stronger secondary aggregation response. When this biphasic aggregation was examined using epinephrine or ADP, the principle effect of the PDI inhibitor bacitracin on platelets was inhibition of the second or irreversible phase of aggregation and the accompanying secretion (82). There was little inhibition of the primary response, even with concentrations of bacitracin that strongly inhibit platelet surface PDI activity. These results suggest that primary aggregation is largely independent of PDI and that transmission to second wave aggregation requires a conformational change that is facilitated by PDI.

C. In vivo *studies of PDI*

Cho *et al*. (47) documented a critical role for extracellular PDI in thrombus formation in mice. This study used a laser-induced vessel injury in the cremaster muscle. While no PDI was noted on the normal vessel wall, upon laser induced injury vessel wall PDI was rapidly expressed (15 s) within the developing thrombus. This expression of PDI was prior to significant platelet accumulation which began at ~30 s. The source of the PDI expressed prior to platelet accumulation may be endothelial cells or other cells; plasma may also be a source (87). Platelets may be a source of PDI that accumulates later in thrombus formation. PDI played a role in both platelet accumulation and fibrin deposition, because both processes were blocked when PDI was inhibited.

To determine whether PDI has a role in platelet accumulation that is independent of coagulation or whether PDI has a role in fibrin formation that is independent of its role in platelet function fibrin deposition and thrombus formation was studied in PAR4−/− mice (47). The PAR4 receptor is a G

protein-coupled platelet receptor for thrombin and, while mice lacking this receptor have some initial platelet accumulation upon laser-induced arteriolar injury, this remains minimal. Platelets form a juxtamural thrombus in these PAR4−/− mice but platelet activation occurs only after a long delay, suggesting that platelets do not play a role in supporting fibrin generation. Since the fibrin generation that occurs in these mice is similar to that found in wild-type mice, other cell membranes are likely supporting coagulation. Inhibition of PDI in these mice by the inhibitory anti-PDI antibody completely blocks fibrin accumulation, implying that PDI is required for thrombin generation. Interestingly, inhibition of PDI also blocks formation of the small juxtamural thrombi formed in these mice. Since these small thrombi are independent of platelet activation by thrombin, a role for PDI in platelet function that is independent of its role in thrombin generation is suggested. Using tail bleeding times as another measure of platelet function the anti-PDI antibody RL90 in appropriate doses completely inhibited thrombus formation.

It is likely that PDI has a role in activation of the αIIbβ3 fibrinogen receptor *in vivo* that is similar to its role *in vitro* (82, 179). Free thiols in αIIbβ3 are required for platelet aggregation (179) and potential sites of PDI regulation of αIIbβ3 are the free thiols localized to the disulfide knot of the β3 subunit between residues 400 and 650 (289). The Cys 663–687 disulfide bond in the β3-subunit is another potential site of PDI regulation. This bond is predicted to be an allosteric disulfide bond (with a high potential energy) that can be easily cleaved (44), and disruption of this disulfide bond results in constitutive activation of the integrin to a fibrinogen binding conformation (32).

A parallel study using ligation-induced endothelial denudation of the carotid artery of mice also found that PDI had a central role in thrombus formation (231). This study focused on the role of PDI in fibrin generation via tissue factor activation. In this model, platelets were a major source for the PDI in the vessel lesion with disrupted cells in the damaged vessel wall also contributing PDI at the lesion site. PDI initiated coagulation through formation of a disulfide bond at Cys186/Cys209 in the extracellular portion of tissue factor. The proposed mechanism was an isomerization reaction that proceeded by cleavage of a mixed disulfide of gluathathionylated Cys209 with subsequent disulfide bond formation between Cys186 and Cys209. The reaction was catalyzed by the reduced form of PDI.

D. PDI on platelet-derived microparticles

In *ex vivo* studies in humans, PDI was found on the surface of platelet-derived microparticles, the most abundant type of microparticle in blood (229). Platelet microparticle PDI was catalytically active and capable of promoting platelet aggregation. Interestingly, this study also found that patients with type II diabetes had elevated levels of these microparticles and PDI on the surface of these microparticles was capable of degrading insulin by reduction of the disulfide bond linking the A and B chains of insulin.

E. Role of ERP5 in platelet function

Another member of the PDI family, ERP5, was found in platelets (134). ERP5 is a 48 kD protein that contains two PDI-like active sites (CGHC motifs). ERP5 lacks the substrate binding b′ domain of PDI and may be an efficient oxidase (77). ERP5 was on the surface of nonstimulated platelets and, like PDI, an additional pool was recruited to the surface with platelet activation. Using polyclonal antibodies to ERP5 that inhibited activity of purified ERP5, Jordan *et al.* (134) showed inhibition of collagen and convulxin-induced aggregation. A role for ERP5 in activation of αIIbβ3 and exposure of P-selectin, a marker of α-granule secretion, was found. Calcium mobilization from intracellular sites was not affected by incubating the platelets with anti-ERP5, showing that inhibition of ERP5 affects responses that occur after the initial agonist receptor interaction. A physical association of ERP5 with αIIbβ3 was found by immunoprecipitation. Thus, platelets contain other members of the PDI family that seem to have a similar role as the traditional PDI in regulating activation of αIIbβ3, although non-overlapping roles are also possible.

F. The generation of thiols by a redox mechanism

In regard to the role of PDI one question is, how is PDI activity controlled? The thiols of the active site of PDI are in equilibrium with a disulfide bond. A shift to the thiol form increases PDI activity. Since the mechanism of thiol–disulfide exchange requires the nucleophilic attack of a free thiol as the first step, a redox mechanism may be required to generate or maintain free thiols in the active site of PDI. The demonstration that activated/aggregated platelets show an increase in surface sulfhydryls and, in particular, PDI sulfhydryls in the absence of secreted GSH (31), supports this concept. These findings, together with the increase in thiols in the activated form of αIIbβ3 (289), suggest the possibility of a redox system in the platelet membrane similar to those found on the plasma membrane of other cells (141, 284). When using purified proteins in assays that examine PDI catalyzed thiol disulfide exchange or cleavage of disulfide bonds, added GSH is generally required to shift the active site of PDI to the thiol form (41, 81, 87, 105, 124, 191). However, added GSH is not required for PDI activity in platelet aggregation (82), or for surface PDI activity in other cells (130, 178, 237, 291). This suggests that in studies that use purified proteins, GSH plays a role in activating PDI that some cellular mechanism normally provides. The glutathione reductase activity found on platelets that generates thiols from disulfide bonds (85) may be a marker for a more general mechanism that generates thiols in PDI and αIIbβ3 or other surface proteins.

In addition to possible cellular mechanisms for activating PDI, extracellular low molecular weight thiols are also likely to affect PDI activity. Plasma contains a concentration of GSH that begins to generate thiols in purified PDI (105). Therefore, in plasma GSH may work together with cellular redox mechanisms to control activity of platelet PDI.

Whereas platelets contain large amounts of PDI (81), and platelet PDI contributes to fibrin generation *in vivo* (231), the amount on the platelet surface appears to be a fraction of the total platelet pool (Lee D and Essex DW, unpublished observations) and may be substantially less than αIIbβ3. In this regard, it is important to note that an interaction of surface exposed PDI with a substrate does not necessarily need to be 1:1 to be functionally significant. One protein disulfide isomerase molecule could in principle be involved in activation of more than one molecule of substrate. This is especially true for PDI catalyzed thiol–disulfide exchange because the active site of PDI retains its thiol status. Also, the thiol status of

PDI may be regenerated by a platelet transplasma membrane oxidoreductase or reducing equivalents from other sources (31, 85). Additionally, not all αIIbβ3 receptors on platelets necessarily need to be activated by a PDI-catalyzed event for the initial steps required for platelet aggregation. Consistent with this possibility, it is known that people with the heterogeneous deficiency state of αIIbβ3 have essentially normal platelet functions (201). Moreover, there are examples of functional interplay between receptors and proteins on the platelet surface where there is a low amount of co-localization of the lower abundance protein/receptor with the higher abundance receptor (258, 268). One example is the interaction of the relatively low abundance FcγRIIa receptor with the GPIb complex (258). It is therefore possible that relatively few PDI molecules have a functional association with a highly abundant receptor such as αIIbβ3.

A role of platelet surface PDI *in vitro* has been confirmed *in vivo* where other sources of PDI may also contribute to thrombus formation (231). Additionally, it is possible that PDI on platelet-derived microparticles may contribute as a source of PDI for platelet activation *in vivo* (229).

G. A role for platelet PDI in NO release

In addition to a role for PDI in activation of αIIbβ3 and platelet aggregation, both cell and platelet surface PDI have been shown to mediate NO release from *S*-nitrosothiols (233, 291). S-nitrosothiols form a reservoir of NO and NO inhibits platelet adhesion and aggregation. In the study by Root *et al*. (233) exposure of washed human platelets to *S*-nitrosoglutathione (GSNO) resulted in saturable denitrosation. PDI inhibitors, including the monoclonal anti-PDI antibody, inhibited the denitrosation activity. GSNO was found to have a dual inhibitory effect on platelets mediated through PDI. First, the NO released from GSNO inhibited platelet activation by activating guanylate cyclase resulting in a decrease of calcium flux. Second, GSNO competes with the same PDI active site that catalyzes disulfide bond rearrangement required in αIIbβ3 for irreversible aggregation.

Bell *et al.* (22) found a central role for PDI in mediating delivery of nitric oxide-redox derivatives into platelets. In this study the role of several enzymes and transporters implicated in NO delivery was investigated in platelets. Platelet PDI activity was required for the rapid delivery of NO into platelets, while the glutathione peroxidase and L-amino acid NO transporter systems did not have a significant role in platelet NO delivery.

H. Summary

In summary, the discovery of an extracellular PDI on platelets pointed to functions for PDI in cells and platelets not previously recognized (41, 81). It is now recognized that extracellular PDI has an important role in platelet function. Specifically, PDI mediates activation of the αIIbβ3 integrin to a fibrinogen binding conformation and mediates platelet adhesion through the α2β1 integrin. In contrast, PDI or thiols do not have a role in adhesion through the non-integrin, GPVI (154). PDI has a role in signaling independent activation of αIIbβ3 by Mn^{2+} suggesting that the effect of PDI on integrins may be direct (180). A direct interaction of PDI with the αvβ3 integrin on endothelial cells has similarly been suggested (261). The homologous ERP5 may also exert a direct effect on αIIbβ3 (134). Platelet surface PDI also catalyzes cleavage of the *S*-NO bond releasing NO from *S*-nitrosothiols (233). *S*-nitrosothiols may both provide a source of NO as well as be a competitive inhibitor of PDI in limiting platelet thrombus formation.

V. Thiol-Containing Platelet Proteins

A. Platelet surface proteins

1. Cell surface thiols as sites of redox regulation. Thiol-containing proteins on the plasma membrane are at the interface between an oxidizing and a reducing environment. Exofacial protein thiols (surface thiols) are kept in a reduced state by cytoplasmic or extracellular reducing equivalents and surface PDI (130, 157, 238, 291). Biological processes may be regulated by the redox state of surface thiols, including entry of HIV (188), platelet aggregation (83), integrin-mediated adhesion (152, 157), and receptor shedding (23, 293). Surface receptor function is modulated by thiol groups in the IL-8 (240), VIP (92), retinoic acid (61), and glucortocoid (192) receptors. Because of their accessibility, exofacial thiols offer a preferential target not only for oxidants but also for mild reducing agents such as GSH and *N*-acetylcysteine. Treatment with glutathione or *N*-acetylcysteine affects cytoplasmic signaling pathways and increases the expression of surface thiols and integrin-mediated cell adhesion (157) as well as integrin-mediated platelet aggregation (83). Consequently, the extracellular redox balance can have an important influence on health and disease status and, as such, redox sensitive surface receptors may be a target for therapeutic intervention (65, 74, 157, 244).

2. Platelet surface thiols. Early reports demonstrate a role for platelet surface sulfhydryl groups in platelet responses and identified several classes of sulfhydryls on the platelet surface. The membrane impermeant sulfhydryl reactive reagent p-chloromercuribenzene sulfonate (pCMBS) inhibited platelet aggregation induced by ADP (5, 176), collagen, and thrombin (117). By measuring the kinetics of binding of p-chloro[^{203}Hg]mercuribenzoate (PCMB), at least two classes of thiols were found on purified platelet membranes (9). Spin-labeled probes detected four major classes of sulfhydryl groups on intact platelets based on mobility (232). Using the membrane impermeant 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) 3.1×10^{-18} moles of SH were found per platelet (117), while pCMBS reacted with 10×10^{-18} moles of SH per platelet (5). Using radioactive sulfhydryl agents, several groups identified four to nine labeled proteins on the platelet surface (8, 200, 227). However, the proteins and their reactive sulfhydryls that are involved in platelet responses were until relatively recently largely unknown.

3. Actin as a surface exposed thiol–protein. Interestingly, earlier studies identified actin as a surface exposed thiol-containing protein of platelets. Using two separate approaches, Nachman and Ferris documented the presence of thiol labeling of surface exposed actin (200). Actin was identified as one of six labeled proteins when platelet membrane extracts were labeled with ^{14}C-iodoacetamide. Additionally, a polyclonal antibody raised to thrombasthenin (a complex of actin and myosin) was found to inhibit the labeling of actin on intact platelets by the impermeant reagent [^{203}Hg] pCMBS.

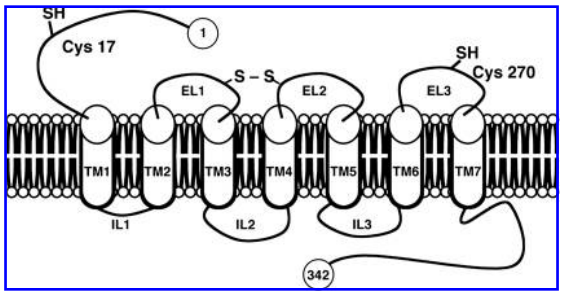

FIG. 8. Model of the P2Y12 ADP receptor. The P2Y12 receptor is a G protein-coupled receptor that contains seven transmembrane (TM) domains with extracellular (EL) and intracellular (IL) loops. The receptor contains four extracellular cysteine residues. Two of these (Cys97 and Cys175) form a disulfide bond between the first and second extracellular loops. The other two, Cys17 and Cys270, contain thiols that are accessible to thiol reagents and are targets for the anti-platelet drug, clopridogrel.

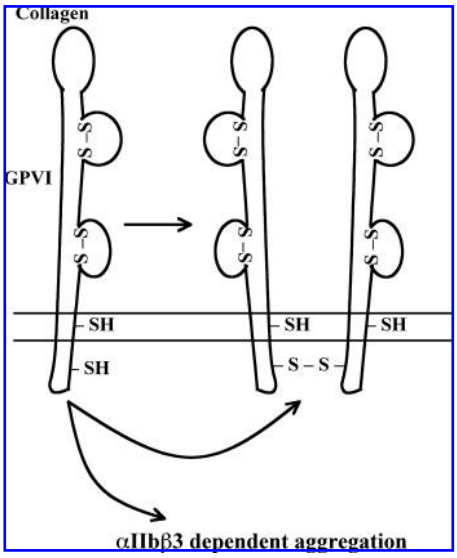

FIG. 9 Disulfide dimerization of GPVI. Ligand binding to GPVI induces rapid disulfide bond formation between Cys338 residues in the cytoplasmic domain of GPVI resulting in a homodimer. GPVI-dependent signaling events result in activation of αIIbβ3 and platelet aggregation.

Another group also localized actin to the exterior surface of the platelet membrane (27) using a polyclonal antibody to actin. While actin is primarily a cytoplasmic protein, it is highly abundant (~25% of the total platelet protein), and platelets have a pool of actin under (93), or embedded in (227), the platelet plasma membrane. Actin was also recently identified as a surface exposed thiol-containing protein in peripheral blood mononuclear cells (157). Based on these reports, it is likely that some of this thiol-containing cytoskeletal protein is surface exposed in platelets, although its function in this location remains unknown.

4. A role for sulfhydryls in the $P2Y_{12}$ platelet ADP receptor. A platelet receptor of major importance to platelet function that contains extracellular thiols is the $P2Y_{12}$ ADP receptor (Fig. 8) (68). Thiols at both Cys17 and Cys270 of the $P2Y_{12}$ receptor are the targets of thiol reagents like pCMBS, although, Cys 270 appears to be the most important. The active metabolites of a commonly used platelet inhibitor, clopidogrel, have a reactive sulfhydryl group that forms disulfide bridges with Cys17 and/or Cys 270. This results in inactivation of the receptor, apparently by blocking the binding of ADP to the $P2Y_{12}$ receptor (interference of redox exchange between Cys17 and Cys270 was also a postulated mechanism). In contrast to the $P2Y_{12}$ ADP receptor, the corresponding extracellular cysteines of a homologous platelet ADP receptor, P2Y1, are disulfide-linked and therefore not inhibited by thiol reagents such as pCMBS or clopidogrel. ADP secreted from platelets plays a role in platelet activation by other agonists such as collagen or low dose thrombin through the $P2Y_{12}$ receptor (128, 143, 150). Therefore, this receptor provides a potential site for regulation of platelet function by redox reactions.

5. Role of thiols and PDI in the function of the glycoprotein Ib adhesion receptor. Both platelet surface PDI and the glycoprotein Ibα (GPIbα) platelet adhesion receptor contain exposed thiols (31). Upon platelet activation/aggregation thiol labeling in glycoprotein Ibα increased (31). This labeling is thought to be in Cys65 of the second leucine rich-repeat of the *N*-terminal region of GPIbα because this residue is predicted to be unpaired (270). The increase in labeling was thought to be due to a conformational change in GPIbα, resulting in the exposure of existing thiols. Inhibition of PDI by anti-PDI antibodies, led to changes in binding of anti-GPIbα monoclonal antibodies to the activated platelet surface. This suggests a role for PDI in conformational changes in GPIbα. In studies employing primary antibodies to PDI and GPIbα and secondary antibodies labeled with PE and Cy5, the possibility of a physical proximity of the two proteins was suggested by fluorescent resonance energy transfer (FRET). The estimated stoichiometry of the PDI: GPIbα molar ratio was 1:4. Since platelet adhesion to vWF activates αIIbβ3 by signaling through the GPIb-IX-V complex (139), it is possible that under some conditions a PDI-related change in glycoprotein Ib may affect activation of the αIIbβ3 integrin.

Using the label 3-(N-maleimidylpropionyl)biocytin (MPB) and conditions that maximize labeling of αIIbβ3 (Section VI) we found thiols in four more intensely labeled bands as well as other less intense bands (179). The major bands were identified as GPIbα, αIib, and β3, as well as a band that contained actin. Labeled PDI can also be detected, with platelet activation or immunoprecipitation enhancing the detection (31, 180).

6. Disulfide dimerization of the glycoprotein VI collagen receptor. A subpopulation of the GPVI collagen receptor undergoes rapid disulfide dimerization (within 10 s) upon treatment with collagen (13) (Fig. 9). In contrast to the extracellular thiols on platelet surface proteins this disulfide crosslink involves the penultimate residue of GPVI cytoplasmic tail, Cys-388. The disulfide crosslinking preceded signaling through GPVI. Since the reactive thiols here were in the cytoplasmic tail of the GPVI receptor, it is possible that an oxidative submembranous environment may exist in platelets that is conducive to GPVI–GPVI disulfide bond formation. GPVI also has four extracellular Cys residues that form two

structural disulfide bonds (277) that do not appear to be redox regulated (153).

7. The effect of platelet activation on labeling of thiols. Using the impermeant biotinylated maleimide reagent, MPB, at least 11 platelet surface proteins were labeled (31). When platelets were stimulated and allowed to aggregate in the presence of the biotinylated maleimide, MPB, the sulfhydryl labeling increases in ~11 platelet surface proteins, including PDI (31). The increase in sulfhydryl labeling is not accounted for by an increase in surface exposure of the same proteins. This implies that sulfhydryls are generated in these proteins during platelet activation/aggregation. Importantly, only a small amount of thiols on PDI were in the dithiol form on resting platelets, compared with 81% in the dithiol form on activated platelets. Similarly, we found a several-fold increase in labeling of platelet surface PDI with platelet activation (180). Our studies were performed in the absence of aggregation of the platelets, suggesting that the generation of thiols in PDI may be an early event in platelet activation pathways. The increase in labeling could not be accounted for by translocation of proteins from internal stores or by secretion of GSH (31), raising the possibility that an NAD(P)H-oxidoreductase system similar to that found on cell membranes of other cells generates thiols in PDI (141, 284).

8. Effect of thiosulfinates on platelet surface thiols. The thiosulfinates are compounds from allium found in vegetables such as garlic and onions that contain a reactive S-S = O group. When added to platelets, an increase in free thiol groups on the platelet surface is found with inhibition of activation of αIIbβ3 (16). Garlic contains similar sulfur containing compounds such as allicin that may have a role in inhibiting platelet activation (37).

9. Role of surface sulfhydryls in platelet responses. Dose-dependent inhibition of collagen-induced aggregation was demonstrated using either the membrane impermeant sulfhydryl reagent pCMBS or MPB, an impermeant sulfhydryl reagent of the maleimide class (86). When ADP was used as the agonist the second phase, or irreversible aggregation, was inhibited by pCMBS while first wave aggregation persisted. This suggests that first wave responses are independent of both PDI and other potential sulfhydryl proteins. Activation of αIIbβ3 was almost completely inhibited by pCMBS, however, expression of a marker of platelet activation, P-selectin, was inhibited by pCMBS to a substantially smaller degree (153). Thus, activation of αIIbβ3 appears to be more dependent on a sulfhydryl containing protein compared to the initial stimulatory events.

B. Secreted proteins

1. Thiol-containing adhesive proteins. A variety of platelet proteins secreted from α-granules contain thiol groups including the adhesive glycoproteins thrombospondin-1 (TSP1), vitronectin, fibronectin, and von Willebrand factor (vWF). These thiol groups have been implicated in the function of these proteins. PDI catalyzes thiol–disulfide exchange in TSP1 (124) and vitronectin (87) and these proteins have a role in platelet responses such as aggregation (14, 69, 163). Fibronectin has a role in platelet adhesion and possibly in platelet aggregation (48). vWF is the major adhesive protein for platelets under conditions of high shear rates. Thiol groups in vWF are involved in thiol–disulfide exchange reactions and this appears to have an important role in regulating vWF size and binding to platelets (49).

2. Thrombospondin 1. TSP1 is a large multifunctional adhesive protein that makes up 25% of the α-granule contents and when secreted has a reactive thiol at Cys974 (124). It functions as an extracellular matrix protein implicated in growth and proliferative processes as well as hemostasis and fibrinolysis. Upon secretion some of the TSP1 binds back to the platelet surface (124, 262). TSP1 also binds extracellular matrix proteins and proteins involved in hemostasis and fibrinolysis, such as fibrinogen, fibronectin, plasminogen, collagen, cathepsin G, and platelet-derived growth factor. For some proteins, binding is highly dependent on the conformational state of TSP1 that is determined by the calcium concentration or disulfide pairings (122).

Upon secretion in the presence of calcium, Cys974 is the only cysteine with a free thiol in TSP1, but upon calcium depletion inter or intramolecular thiol–disulfide exchange reactions occur. Protein–protein interactions appear to have a similar effect as the depletion of calcium, and probably facilitate randomization of the free thiol at Cys974 to other cysteines residues by thiol–disulfide exchange (67). Indeed, an antibody specific for the conformation that TSP1 takes in an EDTA-containing buffer recognizes TSP1 bound to the surface of platelets, even in a calcium-containing buffer (124). This implies that protein–protein interactions of TSP1 with its platelet surface receptor mimic the effect of calcium chelation.

TSP1 in the supernatant solution of activated platelets undergoes both intramolecular and intermolecular thiol–disulfide exchange reactions (Fig. 10). Detwiler and colleagues initially felt that it was unlikely for thiol–disulfide exchange reactions to occur in TSP1 released from activated platelets in the absence of a catalyst (254). Subsequently, PDI was found to be secreted in the platelet supernatant and inhibitors of PDI inhibited PDI-catalyzed thiol–disulfide exchange reactions (41). When PDI was purified from platelets, it was found to catalyze both inter and intramolecular thiol–disulfide exchange reactions in TSP1 (124, 191). Treatment of TSP1 with *N*-ethylmaleimide (NEM) prior to the addition of PDI blocks the ability of PDI to convert TSP1 to the EDTA-dependent conformation. This distinguishes the catalytic activity of the active sites of PDI from its noncatalytic chaperone functions. TSP1 that is bound to the platelet surface has the conformation that results from a PDI-catalyzed intramolecular thiol–disulfide exchange reaction (124).

PDI-catalyzed thiol–disulfide exchange reactions in TSP1 may also be of a functional significance for the role of TSP1 in cell adhesion. The intramolecular thiol–disulfide exchange reaction occurs in the region of the RGD sequence in TSP1 that mediates specific cell adhesive abilities of TSP1 (124).

PDI also catalyzes the formation of an intermolecular complex with thrombin–antithrombin (thrombin-AT) in which TSP1 becomes disulfide linked to thrombin (Fig. 10) (67, 191). Since thrombin contains three pairs of disulfide bonds with no unpaired cysteines (76), the formation of the ternary complex must occur via thiol–disulfide exchange, with TSP1 providing the nucleophilic thiolate anion that attacks a disulfide bond in thrombin. While the catalytic site of

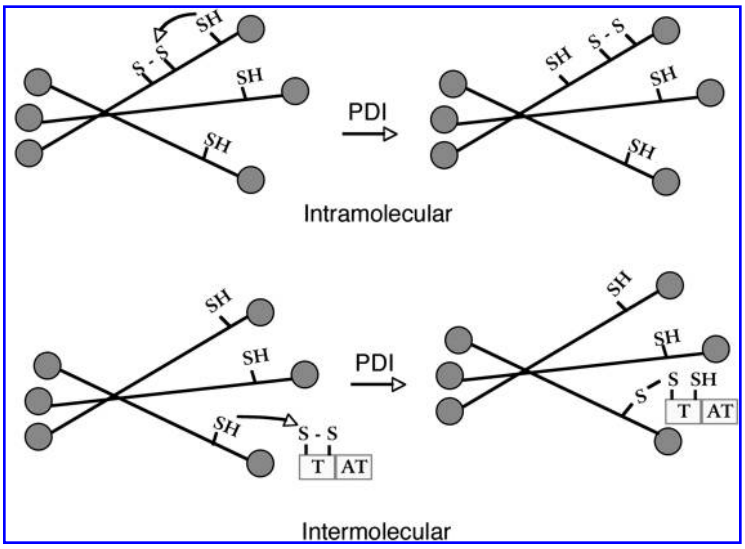

FIG. 10. Thiol–disulfide exchange reactions in thrombospondin-1. The *top figure* illustrates an intramolecular thiol-disulfide exchange reaction in TSP1. The *bottom* illustrates an intermolecular thiol–disulfide exchange reaction that results in the linking of TSP1 to thrombin in the thrombin–antithrombin complex (TAT). These reactions were catalyzed by PDI in the supernatant solution of activated platelets. TSP1 is made up of three identical polypeptide chains.

thrombin is inhibited by antithrombin, thrombin retains biologic activities against cells that are independent of its catalytic site. Thus, this reaction would provide a mechanism to localize biologically active molecules to the extracellular matrix or platelet thrombus.

3. Vitronectin. Vitronectin is a thiol-containing multifunctional 75 kD glycoprotein present in plasma and the extracellular matrix. It is also present in platelet α-granules in a form that is functionally distinct from the plasma form (243). Binding sites for vitronectin are exposed on platelets following stimulation (269) and upon platelet activation by thrombin, some of the vitronectin secreted from α-granules binds back to the platelet (217). Platelet-bound vitronectin appears to have a role in platelet aggregation (14).

Vitronectin has at least one free reactive thiol group (58, 226) and becomes disulfide-linked (271, 272) to the thrombin molecule of the thrombin–AT complex (63). This reaction is analogous to the reaction in which TSP1 forms a disulfide-linked complex with thrombin-AT. It is catalyzed by PDI and vitronectin supplies the reactive thiol group (87). The formation of a ternary vitronectin–thrombin–AT complex may facilitate the final clearance and translocation of thrombin-containing complexes in the vascular system (225). In this process, a conformational change in the vitronectin part of the ternary complex endows it with heparin binding properties so that the ternary complex binds to cell surface sites on endothelial and other cells.

4. Fibronectin. Fibronectin is an important component of cell matrices and is abundant in plasma (48). Fibronectin is also secreted from platelet α-granules and can be found on the surface of thrombin-activated platelets but not inactivated platelets. Fibronectin has a role in platelet adhesion and possibly in aggregation. Fibronectin has a CXXC motif and intrinsic PDI-like activity (156) that may play a role in the formation of high molecular weight disulfide-linked multimers in the extracellular matrix.

5. von Willebrand factor. vWF supports platelet adhesion at the site of vascular injury and has a unique and very important role in initiating platelet adhesion under conditions of high shear stress (235). vWF is a large disulfide-linked multimeric structure of varying sizes with the larger size multimers being the most adhesive. The platelet GPIb receptor binds vWF which in turn binds to type VI collagen in the subendothelium. vWF can be secreted into plasma by endothelial cells. Platelets also secrete vWF from their α-granule contents at sites of vascular injury. Some vWF multimers contain exposed free thiol groups, and the size and function of vWF is regulated by physiologic levels of shear that induces thiol–disulfide exchange reactions (49). vWF binding to platelets increases with the shear-induced thiol–disulfide exchange in vWF. These reactions involve up to nine Cys residues of the D3 and C domains (at positions 889, 898, 2448, 2451, 2490, 2491, 2453, 2528, and 2533).

6. Other thiol-containing proteins. The secreted platelet proteome includes nonadhesive thiol-containing proteins. Proteins known to contain thiols that are found in the secreted platelet proteome include albumin, actin, and tubulin (55) as well as thioredoxin and PDI (64).

Platelets are a rich source of the thiol-containing transforming growth factor β1 (TGF–β1) at sites of vascular injury and the pathologic effects of TGF-β1 include stimulation of collagen production from fibroblasts leading to tissue fibrosis (2). TGF-β1 is secreted from platelet α-granules in a latent biologically inactive form that contains a latency-associated peptide that is disulfide bonded to latent TGF-β binding protein. A recent report showed that shear-induced activation of latent TGF-β1 was dependent on thiol–disulfide exchange (2). This potentially provides a novel mechanism for *in vivo* activation of TGF-β1.

C. Cytoplasmic proteins

There are many thiol-containing cytoplasmic proteins in platelets with the high concentration of cytoplasmic GSH maintaining most cysteine residues in these proteins as thiol groups (215). The role of thiol groups in a number of these proteins has been studied and some of these thiol groups are likely to be relevant to platelet function. Actin contains six

thiol groups with the C-terminal free thiol at Cys374 being accessible on the surface of the protein and therefore the most reactive (59). Glutathionylation of Cys374 is essential for spreading and cytoskeletal organization (89). Additionally, the β-tubulins contain thiol groups (39) with β1 tubulin being an important structural protein involved in microtubule assembly in platelets (218). Thiols in β-tubulins are involved in the regulation of β-tubulin function (131).

Protein tyrosine phosphatases are a large family of soluble and membrane bound enzymes that contain an essential Cys residue at their active site motif. The active site thiol has a low pKa (5.4) maintaining the sulfur group in the thiolate anion form. This form is essential for the catalytic activity of protein tyrosine phosphatases, which requires a phosphocysteine intermediate (45). This reactivity also leaves the phosphatase susceptible to reversible oxidative modifications that regulate activity of these phosphatases (Section X) (45). Protein–tyrosine kinases and phosphatases in platelets amplify signals from cell surface receptors (127). Protein–tyrosine phosphatase 1B is required for αIIbβ3 dependent platelet spreading on fibrinogen and clot retraction (10) and an 18 kD low molecular weight phosphotyrosine phosphatase regulates FcγRIIA mediated platelet activation (177).

N-ethylmaleimide-sensitive factor (NSF) is an ATPase with alkylation-sensitive cysteine residues that is a critical component in vesicular membrane trafficking. NSF interacts with soluble *N*-ethylmaleimide-sensitive factor attachment receptors (SNARES) to mediate secretion of platelet granule contents (197). NSF disassembles complexes of soluble NSF attachment protein receptors allowing recycling of SNARES and subsequent rounds of fusion of vesicular membranes.

In addition to kinases (104), phosphatases, and NSF, platelets contain a variety of other thiol-containing proteins with roles in platelet function. Calpain is a cytoplasmic thiol protease in platelets that has a role in platelet aggregation, secretion, and spreading (57). Platelets also contain caspases 3 and 9 (54, 167) that have redox-sensitive cysteine residues in their active site (199, 283). Platelet caspases have a role in thrombin-induced apoptotic events in platelets (167) and in platelet activation (54). Finally, K^+ and Ca^{2+} channels contain thiol groups that are subject to redox regulation (283). These channels have a role in procoagulant responses in platelets (282).

VI. Thiols and Disulfide Bonds in Platelet Integrins

Both subunits of the platelet fibrinogen receptor, αIIb and β3, contain disulfide bonds. αIIb contains 18 cysteine residues and β3 contain 56 cysteine residues. Thirty-two of the cysteines in β3 are in a cysteine-rich region in the extracellular portion of the molecule now referred as the integrin-epidermal growth factor (I-EGF) domain (265, 287). This region contains four I-EGF repeats and begins one cysteine earlier than previously proposed (265). In addition to the cysteine residues in the I-EGF repeats, structurally important cysteine residues exist elsewhere in αIIbβ3 (267). The cysteine residues of integrins are highly conserved, underscoring their importance.

All of the cysteines in αIIbβ3 have generally been assumed to be disulfide-bonded but relatively recent results show that some exist as free thiols. The idea that the cysteines in αIIbβ3 are entirely in the disulfide form is based partly on work noting an "apparent lack of free thiol group" in the purified β3 (75) and αIIb (33) subunits using the reagent DTNB. Several groups have now reported labeling of sulfhydryls in both αIIb and β3 (83, 86, 179, 227, 289). There are several possible explanations for the differences in results, including the use of labeling reagents inherently more sensitive than DTNB, and the fact that SH groups in some sulfhydryl-containing proteins are inaccessible to DTNB for steric reasons. Additionally, sulfhydryl groups are subject to oxidation during purification procedures (273) and conditions that disrupt or dissociate the αIIbβ3 subunits are required to easily detect labeling of αIIbβ3 on intact platelets with certain reagents (179).

A. Functional role for disulfides in platelets

Cleavage of disulfide bonds appears to be involved in conversion of αIIbβ3 to a fibrinogen-binding conformation. Concentrations of the reducing agent dithiothreitol (DTT) above 1 m*M* caused slow progressive platelet aggregation after a lag period of 1–4 min, provided that fibrinogen was present (174, 176). DTT activates purified αIIbβ3 to a fibrinogen-binding state by reduction of disulfide bonds within the integrin's cysteine-rich repeats (288). This leads to global conformational changes in both αIIb and β3 and exposure of fibrinogen binding sites.

Activation of αIIbβ3 can also be effected by disruption of certain disulfide bonds in the β3 subunit including, the "long-range" disulfide bond Cys5-435 (259) (now redefined as Cys13-435) (285), the disulfide bond involving Cys 560 in the third cysteine rich EGF repeat (236), or Cys 598 of the fourth repeat of the cysteine-rich region (42). Activation can also be effected by a noncysteine point mutation in the extracellular cysteine-rich repeat region of β3 (137, 138) and substitutions in amino acids neighboring certain cysteines in the cysteine-rich region of the β subunits of integrins (292). This indicates that the cysteine-rich regions cooperate to restrain the integrin in the inactive conformation. Using [^{14}C] iodoacetamide labeling of the purified αIIbβ3 receptor, labeling was reported in 2.6 and 4.4 free cysteines of the nonactivated and activated forms of the receptor, respectively, suggesting that reduction of a disulfide bond is associated with αIIbβ3 activation (289). Together these results point to the involvement of disulfide bond cleavage in activation of αIIbβ3.

Relatively recent structural studies of integrins have advanced our knowledge of the conformational changes associated with integrin activation. Xiong *et al*. (286) found that in the presence of Ca^{2+}, the crystal structure of the extracellular segment of the αvβ3 molecules show a compact v-shaped structure. One leg of the v-shaped form ends in the large globule corresponding to the integrin headpiece while the other leg corresponds to the tailpiece. Takagi *et al*. (267) showed that upon activation the bent conformation extends in a "switchblade"-like fashion to a very different, completely extended conformation. This conformation has a globule head and two long tails, similar to electron microscopy (EM) images previously reported.

Activation of the αIIbβ3 integrin involves a transition from the bent over inactive conformation to an extended intermediate affinity (closed) conformation to a high affinity (open) conformation (285) (Fig. 11). There are multiple proposed intermediates between these three conformations. The domains of the α and β-subunits form a "headpiece" and two "tails". The headpiece of αIIbβ3 is formed by an interaction

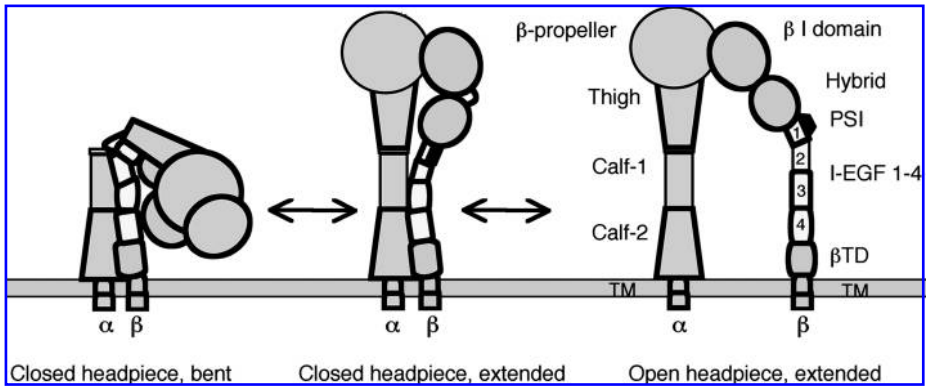

FIG. 11. Structure of the αIIbβ3 integrin and conformational changes associated with regulation of its affinity for fibrinogen. There are three main activation states of αIIbβ3 (285). The bent-over conformation represents a low affinity state. The extended conformations have a "closed" headpiece that represents an intermediate affinity state and an "open" headpiece that represents the high affinity state. The α-sub--subunit has four major domains: the β-propeller, thigh, calf 1, and calf 2 domain. The β-subunit contains the βI-domain followed by the hybrid, PSI, I-EGF, and βTD domains.

between the β-propeller domain of the α-subunit and the βI-domain of the β-subunit. The βI-domain of the β-subunit is the major ligand recognition site of αIIbβ3 (264). This domain is followed by the hybrid and PSI domains (with PSI standing for plexins, semaphorins, and integrins). There is no change in the hybrid/PSI interface during rearrangement of the headpiece between closed and open conformations (172). The rigidity of this interface is thought to be partially reinforced by nearby disulfide bonds (172). There are four cysteine rich I-EGF domains. The second and third I-EGF domains serve as a fulcrum for the switchblade-like opening motion believed to be associated with integrins going from the bent over conformation to the active conformation (20). The I-EGF domains are followed by a membrane proximal βTD. After a transmembrane domain both αIIb and β3 have short cytoplasmic domains.

Structural studies have recently resulted in a reassignment of disulfide bonds in the PSI domain of the β3 subunit αIIbβ3 (285) compared to earlier assignments (34). The long range Cys5 to Cys435 is now believed to link Cys13 to Cys435. The interface between the hybrid and PSI domain of β3 appears to be rigid. This rigidity is supported by the nearby Cys13–Cys435, Cys16–Cys38, and Cys26–Cys49 disulfides in the PSI domain, and the Cys406–Cys433 disufide bond in the hybrid domain (285).

Results from EM and NMR structural studies are consistent with the concept that disulfide isomerization plays a role in integrin activation. First, there is weak electron density on EM of the cysteine-rich EGF domains, EGF 1, EGF 2, and the first ten residues of the EGF-3 domain, of the β subunit of αvβ3. This suggests that these domains are disordered (20). Second, in the extended or active form of αvβ3, the lower half of the β-leg containing the cysteine-rich EGF domains was very difficult to visualize by EM (266). This indicates that the lower β-leg exists in many alternative conformations. Third, there are discrepancies in the disulfide connectivity in the EGF 3 domain of the crystal structure of intact αvβ3 compared to the NMR disulfide pairings of this EGF 3 module (20, 286). These apparent discrepancies may reflect actual differences due to instability of disulfide bonds in this region and different methodologies used to prepare and study the samples.

It has been suggested that inside-out activation of integrins is primarily triggered through protein movements in the β subunit (11). This is based on the findings that three of the cysteine-rich EGF β-domains in αvβ3 that are linked to activation are disordered and presumably flexible, and that the vast majority of activating antibodies for integrins map to the β subunit (125). Taken together, these findings imply instability of disulfide bonds in these regions and suggest the involvement of disulfide rearrangement in activation of the integrin. A recent report identified a population of strained disulfide bonds in the native resting structure of αIIbβ3 (278). These strained disulfides disappeared with Mn^{2+}-induced activation, but partially reappeared following reversal of the activated conformation of αIIbβ3 by *S*-nitrosylation.

B. Mutational studies of Cys residues in the EGF domains

Kamata *et al.* (136) systematically mutated the cysteine residues in the β3-subunit of αIIbβ3 to serine. They found that the disulfide bonds required to maintain αIIbβ3 in an inactive state were clustered in the cysteine rich EGF domains. Disruption of only a single disulfide bond in these domains could fully activate αIIbβ3. In contrast, when disulfide bonds outside the EGF domains were disrupted, only 2 of 13 tested resulted in activation. This study shows that intact disulfide bonds in the EGF domains serve to maintain the integrin in the inactive state.

The consequences of single or double cysteine substitutions of nine disulfide bonds in EGF-3, EGF-4, and βTD of the β-subunit of αIIbβ3 were recently studied (193). Disruption of disulfide bonds in the EGF-3 domain resulted in a constitutively active αIIbβ3. This implies that these bonds stabilize the inactive αIIbβ3 conformation. Mutants of the Cys-567–Cys-581 bond in EGF-4 were inactive even in the presence of αIIbβ3-activating antibodies, implying that this bond is required for activating αIIbβ3. Disruption of Cys-560–Cys-583 bond located at the interface between the EGF-3 and EGF-4 domains, or of the Cys-608–Cys-655 in the βTD, caused activation of αIIbβ3 only when Cys-560 or Cys-655 of either pair was mutated but not when the partner cysteine (Cys-583, Cys-608) or both cysteines were mutated. This suggests that the free sulfhydryls of Cys-583 and Cys-608 are involved in a thiol–disulfide exchange reaction that activates αIIbβ3. While it is not yet clear how these studies in an expression system relate to the activation of αIIbβ3 in platelets, they provide important insights into the disulfide bonds of the domains

studied and indicate that these bonds play variable structural and regulatory roles in αIIbβ3.

C. Functional role for thiols in αIIbβ3

While *p*-chloromercuribenzene sulfonate (pCMBS) has been used in most studies on platelet surface thiols, the specific thiol–proteins that pCMBS reacts with to inhibit aggregation have not been well defined. Since the thiol-containing $P2Y_{12}$ ADP receptor is involved in most types of platelet aggregation, we used the ADP scavenger apyrase and the $P2Y_{12}$ receptor antagonist 2-MeSAMP to examine thiol-dependent reactions in the absence of contributions from this receptor. In the absence of the $P2Y_{12}$ pathway, pCMBS inhibited aggregation induced by either convulxin or γ-thrombin. Convulxin activates platelets through the GPVI collagen receptor of the immunoglobulin superfamily; γ-thrombin activates platelets through the G-protein-coupled PAR4 thrombin receptor, independently of the thiol-containing GPIb (252). Because the GPVI and PAR4 receptors are markedly different in structure, it is unlikely that the main site of inhibition by pCMBS is a primary agonist receptor. Furthermore, the extracellular portion of the GPVI receptor does not contain thiols (12) and adhesion through this receptor is independent of thiols (154).

When we used a peptide (LSARLAF) that activates platelets without involvement of PDI (83), pCMBS inhibited aggregation even after removal of the $P2Y_{12}$/ADP pathway. This indicates the involvement of a thiol–protein other than PDI or $P2Y_{12}$ in platelet aggregation. We previously found that addition of this peptide to platelets induced a brief, primary phase of aggregation followed by a release reaction (86). This is consistent with the reported direct activation of αIIbβ3 by LSARLAF (66) (peptide–protein interactions may result in thiol–disulphide exchange (194)) and suggests that pCMBS blocks a thiol-dependent reaction in or near αIIbβ3. pCMBS was also found to inhibit aggregation induced by calcium ionophore—an agent that activates independently of primary agonist receptors. Similar results were found for convulxin, γ-thrombin, the LSARLAF peptide, and calcium ionophore when the platelets were incubated with indomethacin to inhibit thromboxane production. Thus, these studies excluded the involvement of thiol proteins with known roles in platelet aggregation, $P2Y_{12}$ and PDI, and suggest a role for thiols at or near the final αIIbβ3-dependent events of aggregation.

D. Reactive thiols in the purified αIIbβ3 integrin

The inhibition of platelet aggregation by impermeant thiol blocking reagents raises the possibility that a thiol–disulfide exchange reaction occurs in platelet surface proteins during aggregation. There is evidence that a thiol–disulfide exchange reaction occurs in purified αIIbβ3 when it is activated by the reducing agent DTT (289). Treatment of the nonactive form of αIIbβ3 with DTT was shown to induce binding to an integrin-specific RGD ligand. However, incubation of the nonactive form of αIIbβ3 with a maleimide sulfhydryl reagent blocked the ability of DTT to activate the integrin. This implies that the mechanism of DTT-induced activation of αIIbβ3 is not only due to disulfide cleavage but also to thiol–disulfide exchange. Additionally, using a reduced RNase assay, intrinsic thiol-isomerase activity was found in purified αIIbβ3 in the presence of EDTA (210). This provides evidence for a reactive disulfide in αIIbβ3 and suggests at least a catalytic amount of free sulfhydryl. These authors noted that the motif of the PDI active site CGXC is found once in each of the four cysteine-rich EGF repeats of the β3 subunit and postulated that the PDI-like activity originated from these sites.

E. Labeling and function of thiols in αIIbβ3

Both the αIIb and β3 subunits can be labeled with sulfhydryl reagents (83, 86, 289). Using a maleimide reagent, both subunits of purified αIIbβ3 were labeled but only the β3-subunit was labeled on intact platelets (289). Optimal labeling of αIIbβ3 on intact platelets with the reagent 3-*N*-maleimidylpropionyl biotin (MPB) was found to occur on intact platelets under conditions known to disrupt and inactivate the receptor (5 m*M* EDTA, 60 min, 37°C) (83). Since EDTA does not by itself generate new sulfhydryls, the increased labeling in these studies suggests cryptic sulfhydryls that are sterically hindered from reacting with the labeling reagent we used. Labeled sulfhydryls in the β3 subunit have been localized to a 30 kD fragment (amino acids residues 400–650) containing the cysteine rich EGF domains of the integrin (289).

Using intact platelets we studied thiols in αIIbβ3 with pCMBS and MPB (179). Disruption of the receptor was required to obtain labeling of thiols in αIIb and β3 with MPB but not with pCMBS. Specificity of labeling for thiols in the αIIb and β3 subunits was confirmed by identification of the purified proteins by mass spectrometry and by inhibition of labeling with DTNB. In contrast to MPB, pCMBS preferentially reacted with thiols in αIIbβ3 and preferentially blocked aggregation under physiologic conditions. Similarly, pCMBS preferentially inhibited signaling-independent activation of αIIbβ3 by Mn^{2+}. These results suggest that the thiols in αIIbβ3 that are blocked by pCMBS are important in activation of this integrin.

Low-dose glutathione (2–10 μmol/L) generates free thiols in αIIb and β3 and potentiates platelet aggregation (83, 85). The experiments just mentioned indicate that blocking thiols in αIIbβ3 has the opposite effect. The contrasting findings between generation and inhibition of thiols in αIIbβ3 with the findings that pCMBS blocks signaling-independent activation of αIIbβ3 with Mn^{2+}, strongly suggest that a thiol-dependent reaction in the αIIbβ3 integrin is required for its activation. Consistent with this conclusion, pCMBS has been found to inhibit activation of αIIbβ3 by an anti-LIBS antibody that converts αIIbβ3 to the high affinity state conformation without platelet activation (153). As mentioned, modification of free thiols in purified αIIbβ3 with *N*-ethylmaleimide was shown to block activation of αIIbβ3 induced by the reducing agent DTT (289), implying that a thiol–disulfide exchange reaction in αIIbβ3 is required for its activation. Moreover, in a cell expression system free thiols in Cys-583 or Cys-608 of the β3 subunit of αIIbβ3 appear to be involved in a thiol–disulfide exchange reaction in activation of αIIbβ3 (193).

F. Thiol-labeling studies on activated αIIbβ3

Sulfhydryl labeling studies performed on the purified αIIbβ3 integrin found an increase of 2-sulfhydryls in the activated form of the integrin (289). We found a two- to three-fold increase of labeling in the β3 subunit of αIIbβ3 on intact platelets with platelet activation (83). This increase could not be explained by an increase in receptor number and is therefore due to exposure or generation of new sulfhydryls. There

was no detectable increase in labeling of the α subunit. Since the activated form of the αIIbβ3 receptor has two additional thiols (289), and since sulfhydryls appear to be generated in other platelet surface proteins with platelet aggregation (31), the increase we found in β3 with activation may be due to generation of thiols during activation.

G. Role of thiols and PDI in activation of other integrins

PDI and extracellular sulfhydryls groups have also been implicated in integrin-dependent platelet adhesion (152), with pCMBS giving strong inhibition and anti-PDI partial inhibition of platelet adhesion by both β1 and β3 integrins. Removal of pCMBS prior to adhesion resulted in the restoration of adhesion, indicating that new sulfhydryls are exposed in response to adhesion. PDI and thiols are required for activation of the α2β1 platelet integrin (154) and have been implicated in activation of the αvβ3 vitronectin receptor in endothelial cells (261). Platelets have low levels of the αvβ3 integrin and the α5β1 integrin that functions as the receptor for fibronectin (134). Both of these integrins possess endogenous thiol-isomerase activity (210) suggesting that they contain catalytic thiols and reactive disulfide bonds. Thus, a thiol disulfide exchange reaction in αIIbβ3 appears to be required for activation of this receptor (179, 289), and it is likely that similar mechanisms are involved in the activation of other integrins.

VII. The Role of Glutathione and Other Low Molecular Weight Thiols in Platelet Function

A. Intracellular glutathione

Cytoplasmic glutathione modulates cell and platelet function with millimolar concentration found in cells, the vast majority of which is in the reduced form. GSH is thus by far the most abundant low molecular weight thiol in cells, and intracellular glutathione has a role in platelet activation. Platelets contain 11–15 nanomoles of GSH per 10^9 platelets with >90–95% of intraplatelet glutathione being found in the reduced form (112, 119). Conversion of cytoplasmic GSH to GSSG (by diamide) inhibits platelet aggregation and induces disulfide crosslinking of cytoskeletal proteins (28, 186, 253). Thus, cytoplasmic GSH appears to have a fundamental role in the regulation of platelet activation, probably by maintaining the sulfhydryl status of cytoplasmic proteins. Since platelets do not secrete GSH (31), platelet sources of GSH do not appear to have a direct role in extracellular redox reactions.

B. The effect of extracellular glutathione and other low molecular weight thiols on platelet aggregation

While glutathione is an important modulator of the cellular redox environment, it is also found in blood where it could modulate platelet function and integrin activation. Although cells contain mmol/L concentrations of total glutathione, earlier reports found only $\sim$10–25 μmol/L in plasma (6, 158, 160, 181, 184). More recent reports have suggested that this is even lower ($\sim$3 μmol/L) (133). However, a large fraction in plasma is also in the reduced form, with the GSH/GSSG ratio in plasma being in the 4:1 to 13:1 range (6, 133, 158, 160, 181). The major source of plasma glutathione is the liver and about two-thirds of plasma glutathione is metabolized by the kidneys (105). Plasma glutathione levels as well as the ratio of GSH to GSSG are altered in disease states, including fasting alcoholism, cirrhosis, and malignancy (86, 160, 184). The role for plasma glutathione in extracellular redox reactions has not been well studied.

Plasma also contains other low molecular weight thiols (cysteine, cysteine–glycine, and homocysteine), mostly in disulfide forms (106, 181). In plasma, the level of total cysteine ($\sim$200 μmol/L) is higher than that of glutathione, with cysteine being the most abundant low molecular weight thiol in plasma. Most of the cysteine is present in the disulfide form as cystine ($\sim$90 μmol/L) with the cysteine level being lower ($\sim$13 μmol/L) (106). Cysteine is also present as protein-cysteine mixed disulfides (60–70 μmol/L), or cysteine–glutathione mixed disulfides ($\sim$23 μmol/L). Cysteinylglycine is produced in plasma when GSH is cleaved by γ-glutamyl transpeptidase (also known as γ-glutamyl transferase) (189). Plasma cysteinylglycine (total concentration $\sim$30μmol/L) is also distributed between reduced, oxidized, and protein-bound forms (7, 181). The total amount of homocysteine species is $\sim$10 μmol/L, with about two-thirds being protein bound and only low amounts (<1 μmol/L) of reduced homocysteine ($\sim$30% is in a free oxidized fraction) (276).

In the presence of subthreshold concentrations of agonist, concentrations of GSH (10 μmol/L) approximating physiologic concentrations stimulated platelet aggregation and secretion (83). GSH could not by itself stimulate aggregation. Other low molecular weight thiols, cysteine and cysteinylglycine, also potentiated irreversible aggregation, but concentrations higher than GSH were generally required. A stimulatory effect of the sulfhydryl or reduced form of homocysteine was also found on aggregation at 1–3 μmol/L concentrations and was maximal at 4–5 μmol/L concentrations. The effect of homocysteine is of special interest, because homocysteine is a known risk factor for vascular disease. Levels of the reduced form of homocysteine that potentiate aggregation have been reported in end stage renal disease, a disease with unusually high rates of cardiovascular morbidity and death (121).

One possible mechanism for the effect of the low molecular weight sulfhydryl compounds is that they are inducing a partially activated state of the platelet integrin αIIbβ3, which lowers the threshold for other agonists to induce a fully active conformation. Both GSH and cysteine had a stimulatory effect on activation using subthreshold levels of the direct αIIbβ3 agonist peptide LSARLAF (83). GSH (10 μmol/L) by itself induced an over twofold increase in sulfhydryl labeling in the β3-subunit, confirming an effect on αIIbβ3. A 1.6–1.7-fold increase in labeling of αIIb was also found with GSH. Since GSH by itself does not cause platelet aggregation, this sulfhydryl generation does not by itself fully activate the receptor. It rather appears to prime the αIIbβ3 receptor for activation by a second step that is presumably a thiol-disulfide exchange reaction (Fig. 12). This reaction is catalyzed by PDI or ERP5.

The effect of GSH and other low molecular weight thiols on platelet aggregation that we found contrasts with the effect of the reducing agent DTT on platelet aggregation and activation of the αIIbβ3 receptor (175, 176, 294). DTT in the presence of fibrinogen causes a slow progressive platelet aggregation without secretion of alpha or dense granule contents. No agonist is needed. GSH by itself does not cause aggregation, and the aggregation tracings produced by the effect of GSH follow the pattern of the agonist used (*e.g.*, collagen or ADP) (83).

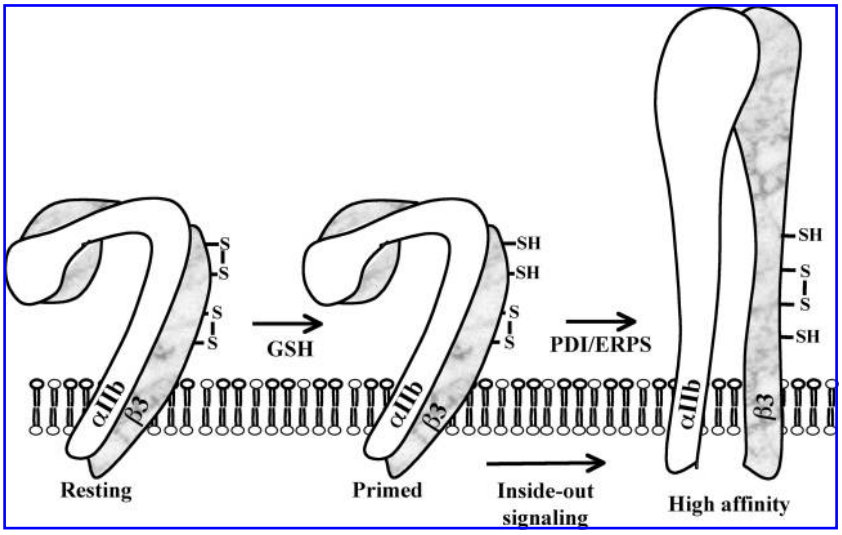

FIG. 12. Model for redox regulation of αIIbβ3 function. This model depicts the transformation of αIIbβ3 from a resting conformation with low affinity for fibrinogen to a conformation that is primed by GSH (or GSH/GSSG) for conversion to the high affinity state. The primed conformation has a redox sensitive disulfide bond that is cleaved by GSH to form additional thiols. This conformation is essentially unchanged from the resting conformation, however, the newly generated thiols participate in a reaction that results in the high affinity state. A PDI/ERP5 catalyzed event is required for the conversion to the high affinity state. The structural changes are initiated by inside-out signaling.

Additionally, the effect of DTT is only found with concentrations over 1 mmol/L, much higher than the concentrations of GSH used in our studies (despite DTT being a better reducing agent (108)). Therefore, mechanistically the understanding of the role of thiol groups in our studies is an advancement on the studies with DTT. Most importantly, the effect of GSH appears to be physiologic. Not only is GSH in plasma, but its effect on platelets is found around physiologic plasma GSH concentrations (83).

To determine if the requirement was for reducing equivalents or for a redox potential (ratio of GSH to GSSG), aggregation was further studied with the addition of low concentrations of GSSG to the GSH. With a ratio of GSH/GSSG of 5/1, similar to that of blood, the addition of GSSG potentiated the stimulatory effect as compared to GSH alone (83). This indicates that the effect of glutathione on aggregation is not simply by GSH reducing disulfide bonds; there is rather a requirement for a certain redox potential.

C. Platelets have a transplasma membrane flavoprotein reductase

When GSSG alone was added to platelets, surprisingly we found that it also potentiated platelet aggregation (80). Most of the GSSG was found to be converted to GSH by a flavoprotein dependent platelet surface mechanism. This provided an appropriate redox potential for platelet activation. The addition of GSSG to platelets generated sulfhydryls in the β-subunit of the αIIbβ3 fibrinogen receptor, suggesting a mechanism for facilitation of agonist-induced platelet activation (85). Importantly, a concentration of GSSG as low as 2 μmol/L potentiated aggregation. Since the majority of GSSG is converted to GSH, the concentrations of GSH and GSSG would be well within the lower concentrations of GSH and GSSG more recently described in plasma (133).

In regard to the conversion of GSSG to GSH by platelets, recent reports have raised the possibility that platelets have a transplasma membrane oxidoreductase that can reduce extracellular disulfide bonds in redox sensitive membrane proteins (31, 83, 289). While there is little information about such systems in cells, a form of a cell membrane NADH oxidase (25, 196) in plant cells has been reported to have disulfide reductase activity (52). Cells and platelets contain flavoprotein–electron transport systems such as NADPH oxidase on neutrophils; however, the electron acceptor is molecular oxygen (not disulfide bonds) and the product is superoxide anion (O_2^-) (15, 148, 159).

Thus, the conversion of GSSG to GSH by platelets suggests a novel surface flavoprotein glutathione reductase activity that may be a marker for the more general disulfide reductase activity already suggested. This activity appears to provide the appropriate redox potential for platelet activation and may modulate the local redox environment at sites of vascular injury where platelets are found in high concentrations. Redox sensitive proteins on platelets whose function may be modulated by the redox environment include αIIbβ3 and PDI (82).

It has been known for some time that platelets can generate O_2^- implicating an NAD(P)H oxidase (182). A recent report found that collagen-induced platelet activation generated superoxide anions that facilitated platelet recruitment (148). Similar to the NAD(P)H oxidase found in neutrophils, this study found the platelet transplasma membrane electron transport system contains $p47^{phox}$ and $p67^{phox}$ and involves a flavoprotein. It is not known if there is any relationship of this transmembrane electron transport system with the one that converts GSSG to GSH.

The conversion of GSSG to GSH by platelets was found to be completely dependent on PDI (230). These findings imply that PDI mediates the transport of cytoplasmic reducing equivalents to the extracellular disulfide bond in GSSG (Fig. 13).

D. A role for vicinal thiols

The susceptibility of disulfide bonds in αIIbβ3 to low concentrations of GSH (10 μmol/L) is unusual and suggests the presence of a disulfide bond that can undergo reversible dithiol/disulfide conversions. To test for a role of vicinal thiols in surface proteins in platelet activation, we used phenylarsine oxide (PAO), a reagent that binds to vicinal sulfhydryls. PAO was found to inhibit platelet aggregation (83). The membrane-impermeable sulfonic acid dithiol 2,3-dimercaptopropane sulfonic acid (DMPS) removes PAO from its target (23). Reversibility of the inhibitory effect of PAO by

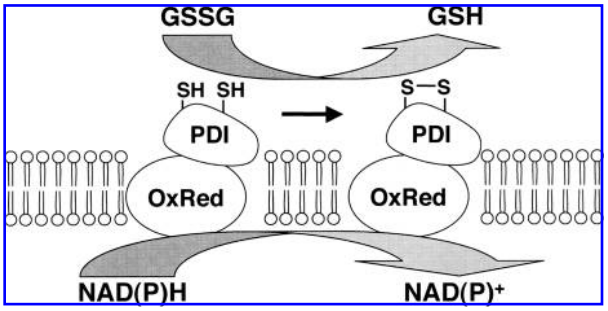

FIG. 13. Platelet membrane oxidoreductase that converts GSSG to GSH is PDI dependent. Platelets contain a transmembrane mechanism that converts extracellular GSSG to GSH. The source of the electrons needed for this is cytoplasmic NAD(P)H. The PDI active site would normally become oxidized to a disulfide bond during the conversion of GSSG to GSH. However, the transmembrane oxidoreductase may maintain the thiol form of PDI, although this is not shown here. This figure summarizes the results of two studies showing that (a) intact platelets convert GSSG to GSH (85) and (b) platelet surface PDI converts GSSG to GSH (230).

DMPS confirmed that PAO was not nonspecifically affecting platelets and that its effect is on vicinal thiols on the platelet surface.

E. Vicinal thiols in αIIbβ3

Possible targets of PAO on platelets include the known vicinal thiols of the PDI active site or potential vicinal thiols in αIIbβ3. The vicinal thiols in the active site of PDI are expected to react with PAO and this has been demonstrated experimentally (71). To test for a possible effect of PAO on αIIbβ3, we incubated platelets with PAO under different conditions and looked for inhibition of the labeling with MPB. PAO inhibited the labeling of the αIIb and β3 subunits in both the nonactivated and activated platelets and platelets treated with a low concentration of GSH (10 μmol/L) (80). This suggests that both αIIb and β3 contain vicinal thiols and that platelet activation results in exposure or generation of vicinal thiols in the β3-subunit. Since vicinal thiols are in equilibrium with disulfide bonds, these findings imply that there are redox sensitive sites in both αIIb and β3 that can be regulated by platelet redox mechanisms or by low molecular thiols found in the external redox environment.

F. Effect of glutathione on αIIbβ3-mediated cell adhesion

A recent study done under flow conditions found that the concentrations of GSH approximating physiologic levels (20 μmol/L) inhibited αIIbβ3-mediated adhesion of CHO cells and platelets (17). These results differed from the previous observation that low concentrations of GSH-enhanced platelet aggregation (83). The inhibitory effect on cell and platelet adhesion was found for cell adhesion to either fibrinogen or vWF, suggesting that this effect was probably not due to an effect on the ligand. This study therefore suggests that GSH has distinct effects on agonist-induced activation of αIIbβ3 and on the strength of the αIIbβ3-ligand bonds when exposed to fluid shear stress.

G. N-acetylcysteine and platelet function

N-acetylcysteine is a reduced thiol used in the treatment of different medical conditions. In pharmacologic doses (~1–5 mmol/L) it potentiates platelet inhibition by nitroglycerin (168) and by nitric oxide (255). The effects of *N*-acetylcysteine were associated with increasing intracellular platelets cyclic GMP levels, likely by inducing the formation by *S*-nitrosothiol adducts that activated guanylyl cyclase. Pretreatment with *N*-acetylcysteine potentiated the inhibition of intravenous nitroglycerin on platelet thrombus formation in a canine model of coronary artery stenosis (90), suggesting a possible therapeutic role for *N*-acetylcysteine. It may be that *N*-acetylcysteine, similar to high concentrations of GSH (83), inhibits platelet aggregation by additional mechanisms such as cleavage of structurally important disulfide bonds.

VIII. Nitric Oxide and Platelet Function

Although nitric oxide (NO) was originally discovered as a vasodilator produced by the endothelium, it also has potent antiplatelet activity (169). By activating guanylate cyclase and inhibiting calcium influx, NO inhibits the processes of platelet adhesion, activation, and aggregation. The inhibitory effect on platelet activation of endothelial-derived NO and *S*-nitrosothiols are similar to that of prostacyclin, however, NO additionally inhibits platelet adhesion. This may be mediated by inhibition of binding of the platelet integrin αIIbβ3 to von Willebrand factor (190).

S-nitroso-albumin is an important *in vivo* reservoir in blood for endothelial NO from which low molecular weight *S*-nitroso-cysteine and *S*-nitroso-glutathione are derived (169). *S*-nitroso-thiols can be transferred to or from *S*-nitroso-albumin *in vivo* (166, 241). Cell surface PDI catalyzes the release of NO from extracellular S-nitrosothiols, providing a possible mechanism for the intracellular transfer of NO (228, 291). Indeed, platelet surface PDI appears to be the primary mechanisms for intracellular transfer of NO from *S*-nitrosothiols (22). Platelet surface PDI denitrosates *S*-nitrosothiols at the same PDI active site that catalyzes disulfide exchange in αIIbβ3 (233). Moreover, GSNO as a substrate of PDI is a competitive inhibitor of PDI-catalyzed disulfide exchange involving the integrin and ligand. Thus, *S*-nitrosothiols inhibit platelets by two mechanisms: NO produced from PDI denitrosation can inhibit by activation of guanylate cyclase and *S*-nitrosothiols directly inhibit PDI-catalyzed reactions in αIIbβ3. *S*-nitrosylation of thiols in the active sites of PDI is another potential mechanism of NO inhibition of PDI (Section X) (197).

Several lines of evidence have implicated nitric oxide deficiency in platelet activation and arterial thrombosis *in vivo* (169). Nitric oxide deficiency is associated with arterial thrombosis in animal models. Inhibition of endothelial NO synthase in human volunteers shorten bleeding times, as a measure of platelet function (251). Additionally, platelets from patients with acute coronary events when studied *ex vivo* produced much less NO than those from patients with stable angina pectoris (97). This raises the possibility that impaired platelet-derived NO production contributes to the genesis of acute coronary events. Evidence for a causal relationship between NO deficiency and platelet activation is from two brothers with childhood strokes (one boy had a stroke at ages 13 and 22 months, the other at age 15 months). The boys were

found to have a complete lack of platelet inhibition by NO provided by an NO donor. The underlying defect was a deficiency of plasma glutathione peroxidase (GPx-3) an enzyme that catalyzes the reduction of lipid peroxides to lipid alcohols (95). In the absence of GPx-3 the lipid peroxides are converted to lipid peroxide radicals instead of lipid alcohols. Because lipid peroxide radicals inactivate NO (by the formation of lipid peroxinitrites), increased platelet activation and thrombosis is seen.

Although platelets produce less NO than endothelial cells (115), they are reported to be a source of NO and platelet NO limits the recruitment of platelets to platelet-rich thrombi (94, 96) However, *in vivo* studies on the role of platelet NO produced by platelet eNOS have provided interesting but seemingly paradoxical results. Mice that are deficient in eNOS whose platelets do not generate NO have shorter bleeding times than wild-type mice and *ex vivo* experiments confirmed the importance of platelet-derived NO in attenuating platelet recruitment (96). However, this was not associated with spontaneous thrombosis in the mice. Additionally, eNOS-deficient mice were not found to die from an increase in thrombotic tendency but from cardiac failure (the female mice in this study had a normal life span) (164). Furthermore, the platelets of eNOS deficient mice do not express more P-selectin or produce more thromboxane B_2, both markers of platelet activation (96, 126). Also, no difference was seen in ADP-induced aggregation between wild type and eNOS-deficient mice (126).

A difficulty in straightforward interpretation of studies on platelets and thrombosis in eNOS-deficient mice is that there may be physiologic compensation for the absence of an individual NOS gene. The compensation may be by another NOS gene (164) but may also be by non-NO dependent mechanisms. For example, using a carotid artery thrombosis model in mice, it was found that eNOS-deficient mice paradoxically had a prolongation of time to occlusion compared to wild-type mice (126). NO normally inhibits release of tissue plasminogen activator (tPA) from the Weibel–Palade storage compartment in endothelial cells, and a lack of NO-dependent inhibition of Weibel–Palade body release of tPA was found in these studies. This was associated with increased fibrinolysis of the thrombus. Such compensatory mechanisms may partially explain the absence of obvious thrombosis and normal life spans found in eNOS-deficient mice. For these reasons it has been questioned whether eNOS null mice are the appropriate model for studying this enzyme (206).

The complexity of the role of platelet NO in platelet function is further highlighted by evidence suggesting that lower concentrations paradoxically activate platelets apparently by a cGMP-mechanism while higher concentrations of NO inhibit platelet function (183). These authors found a role for platelet NO in one type of platelet secretion that is dependent on aggregation. Aggregation-dependent platelet secretion amplifies platelet activation induced by low concentrations of agonists. Low concentrations of NO promoted platelet secretion and aggregation with higher concentrations of NO inhibiting platelet activation. These authors suggest that NO plays a biphasic role in platelet activation and propose a revision of the role that NO plays in platelet signaling.

In summary, NO from both endothelial cells and platelets seems to have important inhibitory effect on platelets. However, much remains to be elucidated about the role of endothelial and platelet-derived NO in platelet function, and in a recent report the researchers were not able to detect NOS in platelets (100). The inhibitory effects of platelet-derived NO that have been described appear to be important in the later platelet recruitment phase of thrombus formation as opposed to the initial platelet activation events (94, 96, 115). The timing of this inhibitory effect of NO is of special interest because the effect of platelet-generated superoxide (O_2^-) also appears to be a delayed effect that potentiates platelet recruitment (148). These opposing effects of NO and O_2^- on platelet recruitment may serve to balance the platelet component of thrombus formation by ensuring adequate hemostasis while at the same time limiting thrombus propagation.

IX. Reactive Oxygen Species in Platelet Function

A variety of cell sources, including platelets, generate reactive oxygen species (ROS) and these ROS have an effect on platelet activation. Cells in the vessel wall (endothelial cells, vascular cells, smooth muscle cells, and fibroblasts) provide a continual, low-level release of ROS. While ROS indirectly affect platelets by reacting with nitric oxide (NO), a potent inhibitor of platelet activation (170), ROS can also directly effect platelet activation.

The term ROS refers to several molecules that may affect cellular or platelet function with O_2^- being central to ROS chemistry. O_2^- may be converted to other biologically active ROS molecules by either enzymatic or nonenzymatic reactions. Superoxide dismutase (SOD) converts O_2^- to hydrogen peroxide (H_2O_2) and O_2^- can also react rapidly with NO to form peroxynitrite ($ONOO^-$). The reaction rate of O_2^- with NO is about three times faster than the rate of conversion of O_2^- to H_2O_2 (283), a finding of possible physiologic relevance. H_2O_2 is broken down to water and oxygen by catalase, and glutathione peroxidase (GPx) also degrades H_2O_2 by oxidizing GSH to GSSG in the reaction: $2GSH + H_2O_2 \rightarrow GS-SG + 2H_2O$. H_2O_2 can also react in the Fenton reaction with ferrous iron (Fe^{2+}) to generate the hydroxyl radical, •OH, and ferric iron (Fe^{3+}).

In studies in which platelets were exposed to O_2^--generating enzymatic systems, O_2^- was found to reduce the threshold for platelet activation to thrombin, collagen, ADP, or arachidonic acid (149). In these studies it was believed that O_2^- itself (and not other ROS species) facilitated platelet activation. In addition to directly facilitating platelet activation, O_2^- can react with NO to form $ONOO^-$. This results in the decreased availability of NO as an inhibitor of platelet activation.

Although its role *in vivo* is unclear, peroxynitrite is itself reactive with aromatic amino acids such as tyrosine (30) or thiols in proteins (213) and administration of $ONOO^-$ to platelets inhibits ADP, thrombin, collagen, or other stimuli-induced platelet aggregation (171, 195, 290). However, the effect of $ONOO^-$ on platelets is complex as it paradoxically activates platelets in normal buffer and inhibits platelets when plasma is added (29, 195). The inhibitory effect of $ONOO^-$ in plasma was likely mediated by the conversion of $ONOO^-$ by thiols to NO and/or an NO donor such as GSNO or *S*-nitrosoalbumin (195).

As pointed out by Krotz *et al.*, it is important to interpret studies cautiously on the effect of adding H_2O_2, O_2^- or $ONOO^-$ to platelets for at least two reasons (149). First, the

concentrations of ROS that have been used may be supraphysiologic and have a toxic effect on cells. Second, the antioxidant capacity of the buffer used in the specific ROS assay can affect the experimental results. Adding a specific ROS to platelets in a buffer without antioxidant capacity may provide information about a direct effect of the ROS on platelets. On the other hand, the use of plasma or blood may more accurately reflect the *in vivo* situation because plasma and blood contain antioxidant capacity.

A. Platelet-derived ROS

The first observation that platelets themselves release O_2^- was by Marcus in 1977 (182) and several months later by Handin (116). Subsequent reports using unstimulated or stimulated platelets documented the release of O_2^-, H_2O_2, and •OH from platelets (147). ROS produced from platelets may, like low levels of ROS produced from other cells, behave as a second messenger. The amount of platelet NAD(P)H-oxidase-derived O_2^- is similar to that produced from endothelial cells, but much less than that produced from neutrophils (147). While high concentrations of ROS are used in host defense for bactericidal activity of phagocytic cells, there is evidence that lower concentrations (1%–2% of granulocyte generation) are used for cell signaling (203).

As is the case with ROS derived from other cells, platelet-derived ROS can potentially be produced from different enzymatic sources. NAD(P)H oxidase is a major producer of ROS in platelets and is similar to the better characterized NAD(P)H oxidase found in neutrophils. The NAD(P)H oxidase of neutrophils is composed of five major components that coalesce on the cell membrane to form an electron transport chain that results in the formation of O_2^- (15). $gp91^{phox}$ and $p22^{phox}$ are membrane-localized components, while $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ are cytoplasmic components that translocate to the cytoplasmic surface of the membrane. The complex binds NAD(P)H and flavin adenine dinucleotide (FAD). Platelets contain the $p22^{phox}$, $p67^{phox}$ and $gp91^{phox}$, and $p47^{phox}$ subunits of NAD(P)H oxidase (148, 223, 246).

NAD(P)H oxidase in platelets is activated by physiologic platelet agonists. Stimulation of platelets with collagen increases platelet NAD(P)H-oxidase activity and the O_2^- produced potentiates the recruitment of platelets to a growing thrombus *in vivo* (148). The kinetics of platelet ROS production are delayed when collagen is used as the agonist (148). There is normally a 1–2 min delay in the aggregation response of platelets stimulated with collagen, and O_2^- production by collagen-stimulated platelets occurred with a delay of 3–5 min (148). This suggests that the role platelet O_2^- has in the initial steps of platelet aggregation is minimal (148). In this study, platelet-released O_2^- increased the availability of ADP resulting in additional platelet recruitment and an increase in late thrombus growth. Similarly platelet NAD(P)H superoxide generation in response to activation by a thrombin receptor activating peptide (TRAP) prevented late thrombus disaggregation normally caused by platelet-derived NO (53). Other studies have also found that platelet-derived O_2^- may act as an NO scavenger that removes the inhibitory effects of NO on platelets (38, 263).

Collagen-induced platelet aggregation was also found to be associated with a burst of H_2O_2 production that appeared to act as a second messenger by stimulating arachidonic acid metabolism and phospholipase C pathway (222). Removal of H_2O_2 by catalase inhibited thromboxane A2 production, release of arachidonic acid from the platelet membrane, and inositol 1,4,5P_3 (IP3) formation.

In addition to platelet NAD(P)H-oxidase, endothelial cell nitric oxide synthase (eNOS), and xanthine oxidase may also contribute to the production of ROS in platelets. Platelets contain several other flavoprotein-dependent systems capable of generating ROS including cytochrome p450 and cyclooxygenase. Mitochondria in platelets may also generate superoxide at two sites in the electron transport chains, NADH dehydrogenase and coenzyme Q (283). The role of these platelet ROS-producing systems in platelet activation remains to be determined.

In addition to a role for collagen in generation of ROS in platelet thrombus formation, the platelet agonist thrombin may also have a role in this process (21, 53), although not all studies have been able to document a role for thrombin (148, 222). NAD(P)H oxidase inhibitors and superoxide scavengers reduced the production of intracellular ROS produced by platelets in response to a variety of agonists (21). The ROS that were produced potentiated the activation of the αIIbβ3 platelet integrin but did not affect alpha and dense granule release or platelet shape change. Thrombin-induced activation of the αIIbβ3 integrin was decreased by the NAD(P)H inhibitors diphenylene iodonium (DPI) and apocynin (although rather high concentrations of these inhibitors were used). These inhibitors as well as scavengers of O_2^- also had an inhibitory effect on platelet aggregation and thrombus formation on collagen in a flow chamber under high shear conditions. The effect of these inhibitors was independent of the nitric oxide/cyclic guanosine monophosphate (NO/cGMP) pathway suggesting that the effect of ROS in this study was not by scavenging NO but rather by another mechanism.

In summary, increasing evidence points to a physiologic role for ROS and in particular O_2^- in the enhancement of platelet aggregation and recruitment. Likely mechanisms that are involved are the regulation of redox sensitive ectonucleotidases on platelets or endothelial cell membranes (resulting in increased availability of ADP) and/or the scavenging of platelet NO by O_2^- (Fig. 14). Since patients with chronic granulomatous disease who have deficiencies in subunits of NAD(P)H oxidase that cause defective bactericidal activity of neutrophils do not have obvious platelet defects, the role of ROS *in vivo* needs to be further defined.

A series of interesting studies by the group of Nardi and Karpatkin (204) have documented a role for platelet NAD(P)H oxidase-generated ROS in the platelet destruction found in HIV-1-related immunologic thrombocytopenia (ITP). In early stage HIV-1 infection, ITP is secondary to increased peripheral destruction of platelets, whereas patients with AIDS have a larger component of decreased platelet production mediating the thrombocytopenia (202). Circulating immune complexes found in these patients contain an antiplatelet GPIIIa antibody that targets amino acid residues 49–66 of the GPIIIa or β3 subunit of αIIbβ3. Binding of the antibody to this site results in the production of hydrogen peroxide by the NAD(P)H oxidase pathway in platelets and the H_2O_2 produced causes platelet fragmentation (204). The mechanism by which anti-GPIIIa 49–46 ligation resulted in

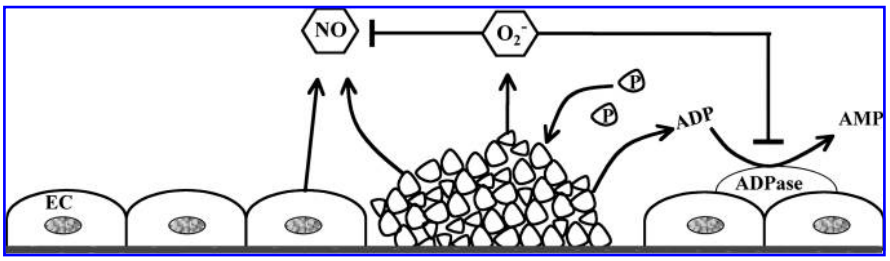

FIG. 14. Superoxide enhances platelet recruitment to a growing thrombus. Platelets adhere to exposed subendothelium and are activated by collagen. Activated platelets release O_2^-, which potentiates thrombus growth by scavenging endothelial, or platelet-derived NO. O_2^- also inhibits removal of ADP released from activated platelets by inhibiting a redox-sensitive ecto-ADPase on the endothelial cell membrane.

activation of NAD(P)H oxidase is by activation of the platelet 12-lipoxygenase (12-LO) with formation of the 12(S)-HETE product.

Two other inducers of platelet particle formation, calcium ionophore (A23187) and phorbol myristate acetate (PMA), were also found to stimulate ROS production in platelets by an identical manner as the anti-GPIIIa 49-66 antibody (205). Dexamethasone successfully treats the thrombocytopenia in HIV-ITP patients and therapeutic concentrations of dexamethasone inhibit ROS/NAD(P)H oxidase-mediated platelet particle formation. Dexamethasone inhibited NAD(P)H oxidase, as well as platelet phospholipase A2 (PLA2) and 12-lipoxygenase (12-LO) (205), by inhibition of translocation of PLA2, 12-LO, and the $p67^{phox}$ component of NAD(P)H oxidase from the platelet cytoplasm to the platelet membrane for induction of ROS. In summary, platelet particle formation can be induced by platelet generation of ROS and platelet PLA2, 12-LO, and NAD(P)H oxidase translocation to the membrane as well as ROS production are all inhibited by dexamethasone.

X. Oxidoreduction of Protein Thiols in Redox Regulation

A. Known modification of thiols

We have discussed intramolecular thiol–disulfide exchange reactions and the conversion of vicinal thiols to disulfides bonds, as reactions involving thiols. However, protein thiols also participate in a number of other physiologically relevant reactions. These include *S*-thiolation (glutathionylation/cysteinylation), *S*-nitrosylation, and oxidation to sulfenic (SOH), sulfinic (SO_2H), and sulfonic (SO_3H) acids. Many of the reactions involving thiols are markers of oxidative stress but may also regulate protein function in a way analogous to phosphorylation of proteins (91, 102, 103, 114, 185). Redox modifications of thiols can be viewed as a continuous processing from milder reversible forms to more severe irreversible forms (Fig. 15). Many of the reversible modifications that occur with milder nitrosative or oxidative stress are being recognized as signaling mechanisms in cells. The more severe oxidative modifications such as sulfonic acid (SO_3^-) formation are associated with toxicity causing pathologic changes in the affected tissues (50, 51). There is not necessarily a strict cut off between modifications that are used in signaling and modifications that cause toxicity. For example, the formation of *S*-nitrosothiols is a reversible modification, but *S*-nitrosylation of PDI in neurons has been related to pathologic abnormalities in patients (275). While these reactions have been primarily studied in cells, the same enzymes or proteins that are known to undergo these modifications are also found in platelets and several studies suggest these reactions have a role in platelet function.

B. Oxidation of thiols to disulfides

As mentioned already, vicinal thiols can be reversibly converted under conditions of mild oxidative stress to disulfide bonds, and this involves changes in protein structure or function. The active sites of PDI and similar enzymes undergo redox cycling between the thiol and disulfide forms, with the thiol form of PDI catalyzing thiol–disulfide exchange or cleavage of a disulfide bond. A shift to the disulfide form would allow PDI to oxidize thiols to disulfides. The platelet integrin, $\alpha IIb\beta 3$, also appears to have vicinal thiols that can reversibly convert to disulfide bonds depending on the redox state of the platelet environment (80). This may function to regulate the activity of these thiols. Other examples of proteins in which the conversion of vicinal thiols to disulfide bonds regulates protein function are low molecular weight protein tyrosine phosphatase (inactivates enzyme) (45) and Src tyrosine kinase (activates enzyme) (104). Both of these enzymes are found in platelets (177, 211).

C. S-thiolation

S-thiolation of proteins involves the formation of a mixed disulfide between a reactive protein thiol and a low molecular weight thiol such as glutathione, cysteine, cysteinylglycine, or

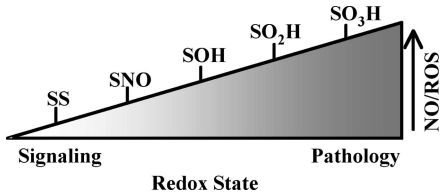

FIG. 15. Oxidative modification of protein thiols. Cysteine thiols can undergo a range of nitric oxide (NO) dependent modifications as well as oxidation by reactive oxygen species (ROS). These modifications can be viewed as a continuum that relates levels of reactive species (NO species or ROS) to the form and consequences of the modification. The progression from SS to *S*-nitrosylation to sulfenic acid (SOH), to sulfinic (SO_2^-) and irreversible sulfonic (SO_3^-) acids represents a graded transition from signaling functions through nitrosative and oxidative stress to toxicity or pathology.

homocysteine. Glutathione is the most abundant intracellular low molecular weight thiol and is the best-studied form of *S*-thiolation (60). However, extracellular proteins are predominantly *S*-cysteinylated due to the higher abundance of cysteine/cystine in plasma. For example, the predominant modification of hemoglobin in red blood cells is *S*-glutathionylated hemoglobin, while plasma proteins such as albumin are mainly *S*-cysteinylated.

D. S-glutathionylation

Protein *S*-glutathionylation is a reversible binding of glutathione to a protein thiol. *S*-glutathionylation of protein thiols generally renders the protein inactive, although glutathionylation of Cys67 of the HIV-1 protease increases activity several fold and also stabilizes the protease (114). *S*-glutathionylation also protects the protein thiols from irreversible oxidation to sulfinic or sulfonic acids during more severe oxidative stress. *S*-glutathionylation can occur by several mechanisms. These include thiol–disulfide exchange between the protein thiol and GSSG or the conversion of a protein sulfenic acid (PSOH) or a protein *S*–nitrosothiol (PSNO) to a *S*-glutathionylated cysteine by reaction with GSH (60).

In the appropriate reducing GSH/GSSG environment, *S*-glutathionylation is reversible in either an enzyme-dependent or independent manner. The principle enzyme that removes glutathione from mixed sulfides is glutaredoxin, although thioredoxin or protein disulfide isomerase can cause deglutathionylation (60, 231). *S*-glutathionylation has been demonstrated in several diseases including aging and cataract formation (in which lens proteins such as the crystallins becomes glutathionylated), as well as in Alzheimer's disease (60).

1. *S*-glutathionylation of actin. *S*-glutathionylation of actin is of particular interest in that it is involved in the regulation of β-actin function during integrin-mediated cellular adhesion (89) and is induced by oxidative stress in platelets (59). During the process of adhesion, fibroblasts release H_2O_2 that causes oxidation of actin by formation of a mixed disulfide between Cys374 of actin and glutathione (89). Cys374 is susceptible to glutathionylation because of its surface exposure on the protein (even with a pKa of $\sim$8.4 (60)). *S*-glutathionylation of actin is essential for cell spreading and the formation of actin stress fibers.

Oxidant stress of platelets with diamide caused an increase in *S*-glutathionylated proteins in platelets and this correlated with inhibition of ADP-induced platelet aggregation (59). The main *S*-glutathionylated cytoskeletal protein was actin. The mechanism of *S*-glutathionylation was thought to be due to initial oxidative activation of actin thiol groups (with loss of an electron) to an S^+, which further reacted with GSH to form the *S*-glutathionylated protein.

E. Regulation of platelet function by formation of protein S-nitrosothiols

NO is generated and released from platelets during platelet activation, providing a negative feedback with inhibition of continuing platelet recruitment (94). While a major mechanism of NO regulation of platelet activation is through activation of guanylate cyclase with an increase in cGMP, NO also regulates platelets by cGMP-independent pathways. An additional mechanism by which NO could modulate platelet function is by reacting with protein thiols to form *S*-nitrosothiols. NO inhibits exocytosis of dense granules, α-granules, and lysosomal granules of platelets by *S*-nitrosylation of *N*-ethylmaleimide-sensitive factor (NSF) (197). Cys264 is the redox sensor for NSF. Interestingly, NO- and H_2O_2-dependent modifications of the thiol at Cys264 appeared to have distinct, slightly differing, effects on NSF function (187).

1. *S*-nitrosylation of PDI. Several studies, including one using human tissue, have documented *S*-nitrosylation of thiols in PDI. NO was shown to react with PDI, presumably by reacting with thiols in the active sites of PDI (291). Treatment of a megakaryocyte cell line (Meg-01 cells) with GSNO caused loss of labeling of surface thiols in most proteins including PDI (although high concentrations of GSNO were used) (248). The GSNO-mediated thiol modification was reversible as addition of GSH restored thiol labeling. *S*-nitrosylation of PDI causes dysregulation of protein folding in the endoplasmic reticulum (275). Moreover, in this study *S*-nitrosylated PDI was found in brain tissue of patients suffering from Alzheimer's disease and Parkinson's disease but not in normal brains. This was the first report documenting *S*-nitrosylation occurring in a protein *in vivo*, suggesting that the *in vitro* findings with platelet PDI may also occur *in vivo* under conditions of nitrosative stress.

2. *S*-nitrosylation potentially regulates the function of αIIbβ3. It is possible that *S*-nitrosylation of thiols in αIIbβ3 may regulate the function of this integrin. The combination of 3 mmol/L GSH and NO was found to inactivate purified αIIbβ3 (289). Moreover, a recent study using purified αIIbβ3 with GSNO as the NO donor (albeit, again with high concentrations of GSNO), found *S*-nitrosylation of αIIbβ3 by raman spectroscopy (278). In this study, sterically strained disulfide bonds were found in native resting αIIbβ3. These strained disulfides were lost with Mn^{2+}-induced activation of αIIbβ3. Treatment of Mn^{2+}-activated αIIbβ3 with NO resulted in conversion of αIIbβ3 back to the resting state with partial recovery of the strained disulfide bonds. Loss of SH groups in αIIbβ3 was also found with NO treatment, presumably due to modification of SH groups with NO. The formation of *S*-nitrosothiols in the active site of platelet PDI could inhibit PDI function and the formation of *S*-nitrosothiols in αIIbβ3 could inhibit a thiol–disulfide exchange reaction. It is also important to keep in mind that that *S*-nitrosothiol modulation of protein function does not necessarily involve transnitrosation or thiolation. For example, structural changes in fibrinogen were caused by an allosteric effect of GSNO on fibrinogen that did not involve chemical changes in fibrinogen (4).

F. Potential modification of platelet thiols by peroxynitrite

Peroxynitrite ($ONOO^-$), formed when NO reacts with superoxide anion released from platelets, is a sulfhydryl oxidant known to effect platelet function (195). In addition to nitration of cytosolic proteins (171, 213), peroxynitrite can react with both low molecular weight and protein thiols in platelets (29, 209). This could contribute to platelet sulfhydryl oxidization, potentially modulating platelet function. When studying

washed platelets, higher concentrations of peroxynitrite (50–400 μmol/L) stimulated platelet aggregation and decreased extracellular thiols. In contrast, lower, more physiologically relevant concentrations (50–100 μmol/L), inhibited platelet aggregation due to the conversion of $ONOO^-$ to NO or an NO donor (29). In another study, peroxynitrate (100 μmol/L) inhibited platelet aggregation of washed platelets and depleted low molecular weight thiols such as glutathione, probably by two-electron oxidation leading to disulfide formation (209, 213). Peroxynitrite also induces disulfide bonds between the α and β tubulin subunits (β1 tubulin is a structural protein in platelets (218)) a reaction found to be repaired by thioredoxin in cells (155).

G. Formation of sulfenic, sulfinic, and sulfonic acids

The oxidation of thiols to sulfenic, sulfinic, and sulfonic acid represents progressive oxidation states (Fig. 15) of a protein thiol with the formation of some sulfinic acids and sulfonic acid being irreversible (120). These forms of oxidation have been primarily studied *in vitro* and cells, however, they have been documented to occur in tissue from human samples. For example, sulfonic acid modification of thiols in superoxide dismutase and ubiquitin-carboxyl terminal/hydroxylase has been found in brain tissue from patients with neurodegenerative diseases (50, 51). The more severe oxidation state represented by sulfonic acid suggests that progressive oxidation of the sulfur atom has occurred from the milder forms of oxidation (120).

H. Oxidative modification of enzymes and proteins found in cells and platelets

Thiol-containing protein tyrosine phosphatases including low molecular weight protein tyrosine phosphatase and protein tyrosine phosphatase 1B have thiol groups in their active site that are subject to redox regulation. Src and Ret tyrosine kinases and Shc proteins are also susceptible to redox regulation. Phosphatases are generally inactivated by ROS by the formation of sulfenic acid (162) or a disulfide bond (45) involving the active site cysteine. Kinases, on the other hand, are generally activated by ROS by the oxidation of SH groups to a disulfide bond, causing a conformational change necessary for their activation (104). Although redox regulation of these enzymes has generally been studied in nonplatelet cells, most of the phosphatases and kinases studied have roles in platelet signaling events (10, 127, 177, 211). It, thus, seems likely that similar redox regulation of these enzymes would occur in platelets.

I. Regulation of cell adhesion and spreading by integrin-induced ROS

In redox regulation of integrin signaling, ROS that are released during cell adhesion act specifically on thiols in cytoplasmic proteins such as low molecular weight phosphotyrosine phosphatase (46) or the tyrosine kinase Src (104). Oxidation of thiols in these proteins regulates cell spreading through downstream effects on other proteins, suggesting that integrin-induced ROS act as second messengers resulting in cytoskeletal rearrangement. For example, in low molecular weight protein tyrosine phosphatase, the Cys-17 thiol forms an intramolecular S–S bond with Cys-12 of the catalytic site upon exposure to H_2O_2 (45). This inactivates the enzyme and prevents further irreversible oxidation. Treatment with reducing agents rescues the catalytic activity. In human platelets low molecular phosphotyrosine phosphatase functions as a negative regulator of FcγRII-mediated cell activation (177).

Src tyrosine kinases are also central components to adhesive responses that are required for cell spreading on the extracellular matrix. In response to integrin ligation, ROS act as intracellular messengers that potentiate cytoskeletal rearrangement and cell spreading (104). Src tyrosine kinase is oxidized and activated after integrin ligation. Src has an early nonthiol-dependent activation phase caused by Tyr527 dephosphorylation. It also has a late activation phase that depends on integrin-dependent ROS being produced. The ROS produced cause oxidation of thiols at Cys245 and Cys487 (conserved among all Src family members) to a disulfide bond. This results in strong activation of Src. c-Src tyrosine kinase is the most abundant Src family member found in platelets (211). In platelets, c-Src binds directly to the β3-subunit of αIIbβ3 and, fibrinogen binding to αIIbβ3 triggers c-Src activation in outside-inside signaling through αIIbβ3.

J. Redox regulation of protein tyrosine phosphatase 1B

Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitous nonreceptor tyrosine phosphatase. The low pKa of the catalytic cysteine of PTP1B both enhances its function as a nucleophile and renders it susceptible to inactivation by H_2O_2 (162, 239). The phosphatase is rendered inactive when the active site thiolate anion is converted to sulphenic acid. PTP1B is an essential positive regulator of platelet integrin signaling (10). In platelets, PTP1B is required for dephosphorylation of c-Src tyrosine 529, c-Src activation, platelet spreading, and thrombus formation.

K. Other thiol-containing proteins

Several other redox regulated thiol-containing proteins are found in cells and platelets. The Shc proteins are adaptor proteins that function in propagation of intracellular signals from activated tyrosine kinases (109). $p66^{Shc}$ has an *N*-terminal phosphotyrosine-binding domain that is activated through reversible tetramerization by the formation of two disulfide bonds (101). Glutathione and thioredoxin can reduce and inactivate $p66^{Shc}$. $p66^{Shc}$ has been identified as the primary protein binding to the tyrosine phosphorylated β3 subunit of αIIbβ3 in platelets during outside-in integrin platelet signaling (56).

Other thiol-containing proteins are also redox regulated. α-Helix thiols (Cys707 and Cys697) of the contractile protein myosin have been implicated as structural elements facilitating conformational changes in the myosin head through crosslinking of the reactive sulfhydryl groups (207). Myosins are important proteins in the contractile response of platelets (214). Thiol modifications can regulate the function of K^+ and Ca^{2+} ion channels (283) and these channels are found in platelets (282). Although not yet studied in platelets, the Ret tyrosine kinases are receptor-type tyrosine kinases with thiols that form disulfide dimers on intracellular domains (140). This

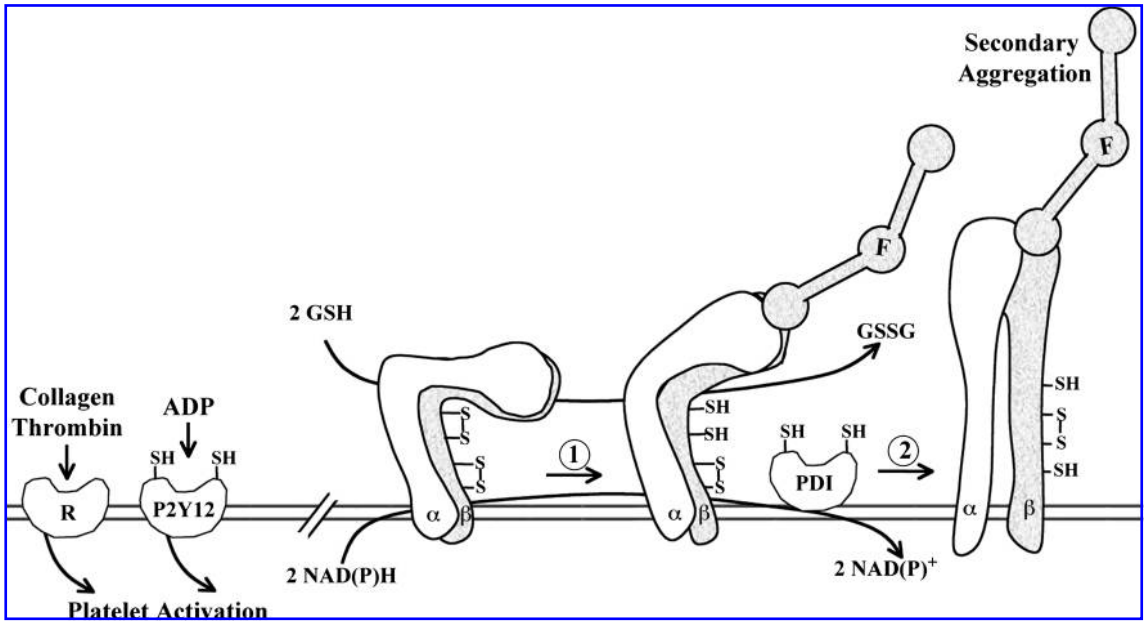

FIG. 16. Working model of the role of sulfhydryls and PDI in platelet function. The platelet fibrinogen receptor αIIbβ3 (αβ) is shown in three different activation states. The nonactivated state is on the *left side*. Redox sensitive disulfide bonds in the EGF domain of β3 are depicted (although not shown the nonactive receptor also contains free thiols). Agonist induced stimulation leads to cytoplasmic events resulting in inside-out signaling and an initial ligand binding interaction of fibrinogen (F) with the receptor (1). A PDI catalyzed event then converts αIIbβ3 to the high affinity conformation (2) represented by secondary platelet aggregation. During platelet activation, sulfhydryls are generated in αIIbβ3 as well as in the active site of PDI from cytoplasmic reducing equivalents supplied by NAD(P)H. GSH or other low molecular weight thiols in the external redox environment also generate sulfhydryls in both αIIbβ3 and PDI facilitating the reactions shown.

causes activation of these protein kinases. Finally, the p53 tumor suppressor protein is susceptible to oxidation of the free thiol of Cys182 that undergoes glutathionylation resulting in structural changes (260).

XI. Summary and Working Model

Although we traditionally think of disulfide bonds as structural components in proteins, current evidence also implicates thiol–disulfide rearrangement as a dynamic process in stimulus–response coupling. Numerous lines of evidence now confirm early findings that sulfhydryl groups in platelet surface proteins are required for platelet responses. PDI or PDI-like enzymes mediate activation of αIIbβ3 (47, 82, 134, 153) as well as other platelet integrins, including the α2β1 collagen receptor (152, 154). GSH at concentrations (and a ratio of GSH to GSSG) normally found in blood potentiates platelet aggregation (85, 86). The effect of GSH was found with several platelet agonists, suggesting that the effect is on the later phases of the platelet stimulation pathway as opposed to primary agonist receptors. The effect of low molecular weight thiols on LSARLAF-induced aggregation raises the possibility of a reaction at the level of αIIbβ3. The finding that GSH increases sulfhydryls in the β3-subunit shows that one effect of GSH is on αIIbβ3.

Several lines of evidence suggest that a transplasma membrane oxidoreductase generates thiols from disulfides on the platelet surface. Such a mechanism could both stimulate PDI activity as well as prime other proteins such as αIIbβ3 for thiol–disulfide exchange reactions. Vicinal thiols in equilibrium with disulfides in αIIbβ3 and PDI provide specific redox sites able to respond to either a transplasma membrane oxidoreductase or to changes in thiols in the external redox environment. Other reactions that thiols are involved in such as the formation of *S*-nitrosothiols, are likely to modulate this system.

Mechanistically, a thiol–disulfide exchange reaction in αIIbβ3 seems to be required for this receptor to attain a full ligand binding conformation. This is because reagents like pCMBS or NEM that prevent thiols from being involved in thiol–disulfide exchange reactions, inhibit both activation of the purified integrin and platelet aggregation induced by direct activation of the integrin (86, 289). pCMBS reacts with thiols in αIIb and blocks signaling-independent activation by this receptor, suggesting that these thiols are required for activation of αIIbβ3 (179). Also, GSH generates sulfhydryls in β3 and lowers the threshold for agonists to induce aggregation but does not by itself induce aggregation. This suggests that a second thiol-dependent reaction in αIIbβ3 is required (83).

A. Working model

The working model from the above data (Fig. 16) focuses on inter-relations of PDI and the integrin αIIbβ3 in platelet aggregation. It is likely that the PDI/sulfhydryl surface events work together with cytoplasmic pathways that lead to conformational changes in αIIbβ3 through inside-out signaling (18, 250). The major points are as follows: Agonist stimulation causes platelet activation in which cytoplasmic events (not

shown here) lead to inside-out signaling that results in a low affinity binding of fibrinogen to αIIbβ3 (1); this is followed by further conformational changes in αIIbβ3 that are facilitated if not caused by a PDI or ERP5 catalyzed event (2). The PDI catalyzed event is probably a thiol–disulfide exchange reaction, although reduction of a disulfide bond could be involved. Thiol disulfide rearrangement, depicted in αIIbβ3, facilitates formation of the high affinity/avidity state. The interaction of PDI with αIIbβ3 may be direct (180), at least for a population of αIIbβ3. Rearrangement of disulfide bonds or sulfhydryl generation may be part of a cascade of events that couples platelet stimulation to the various responses including aggregation and secretion. External GSH or a transplasma membrane NAD(P)H-dependent reductase could generate sulfhydryls in PDI or αIIbβ3 facilitating the reactions.

The sulfhydryl containing $P2Y_{12}$ ADP receptor is also an important site where sulfhydryl reagents could block ADP-dependent platelet responses (68). Sulfhydryl reagents could potentially block responses at $P2Y_{12}$, αIIbβ3 or PDI.

A more general model would take into account the role of PDI in activation of the α2β1 integrin (154). In this model an initial interaction of an extracellular ligand with the α2β1 or αIIbβ3 integrin initiates a PDI catalyzed event which induces a full ligand binding conformation in the integrin: ligand→αβ→PDI→αβ*. While this model again focuses on extracellular events, transmembrane signaling events may also be involved. In summary, stimulus–response coupling in platelets and, in particular, PDI modulation of the integrin αIIbβ3, can be seen as models for other systems in cell biology. PDI regulation of the platelet α2β1 collagen receptor suggests that affinity regulation of integrins by PDI is a general mechanism. Both the generation of thiols, as well as thiol–disulfide exchange, appear to be mechanisms in the activation of integrins.

The findings discussed in this review are likely to relate to platelet function in patients. The effect of low molecular weight thiols on platelet function occurs at levels of these thiols that closely approximate physiologic concentrations, and the redox balance of low molecular weight thiols is altered in a variety of diseases. Moreover, it is likely that at the site of atherosclerotic vascular disease and plaque rupture that ROS and thiol-based redox reactions will interact. ROS have an important role in the events surrounding plaque rupture and thrombus formation (26) and ROS can regulate the function of thiol groups of proteins (104). The characterization of the role of thiols, disulfide exchange reactions, and PDI-like enzymes in platelet responses clearly needs to be further developed. Whether inhibitors of PDI will be selective enough in the inhibition of platelet function for the development of new antiplatelet agents, or whether the use of PDI inhibitors will be limited by inhibition of nonplatelet integrins remains to be explored.

Acknowledgments

This work was supported by funding from the American Heart Association and Veterans Administration.

Abbreviations

βTD, β-tail domain; CD40L, CD40 ligand; CRP, collagen related peptide; Cys, cysteine; CySS, cystine; DMPS, 2,3-dimercaptopropane sulfonic acid; DPI, diphenylene iodonium; DTNB, 5, 5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; EM, electron microscopy; eNOS, endothelial cell nitric oxide synthase; ER, endoplasmic reticulum; FAD, flavin adenine dinucleotide; Fe^{2+}, ferrous iron; Fe^{3+}, ferric iron; FRET, fluorescent resonance energy transfer; GPIb, glycoprotein Ib; GPIbα, glycoprotein Ibα; GPVI, glycoprotein VI; GPx, glutathione peroxidase; GSH, reduced glutathione; GSNO, *S*-nitrosoglutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; I-EGF, integrin-epidermal growth factor; ITP, immunologic thrombocytopenia; 12-LO, 12-lipoxygenase; MPB, 3-(*N*-maleimidylpropionyl)biocytin; NEM, *N*-ethylmaleimide; NO, nitric oxide; NSF, *N*-ethylmaleimide-sensitive factor; O_2^-, superoxide anion; •OH, hydroxyl radical; $ONOO^-$, peroxynitrite; PAO, phenylarsine oxide; PAR, protease-activated receptors; pCMBS, p-chloromercuribenzene sulfonate; PDI, protein disulfide isomerase; PECAM-1, platelet endothelial cell adhesion molecule-1; PLA2, platelet phospholipase A2; PMA, phorbol myristate acetate; PTP1B, protein tyrosine phosphatase 1B; PSNO, protein *S*-nitrosothiol; PSOH, protein sulfenic acid; ROS, reactive oxygen species; SNARES, soluble *N*-ethylmaleimide-sensitive factor attachment receptors; SOD, superoxide dismutase; SOH, sulfenic acid; SO_2H, sulfinic acid; SO_3H, sulfonic acid; TGF–β1, transforming growth factor β1; thrombin-AT, thromin-antithrombin; tPA, tissue-plasminogen activator; TSP1, thrombospondin-1; vWF, von Willebrand factor.

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Address reprint requests to:
David W. Essex M.D.
Temple University Medical School
Room 300C OMS, 3400 North Broad Street
Philadelphia, PA 19140

E-mail: david.essex@temple.edu

Date of first submission to ARS Central, October 3, 2008; date of final revised submission, November 22, 2008; date of acceptance, December 6, 2008.

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