Protein Disulfide Isomerase and Sulfhydryl-Dependent Pathways in Platelet Activation†

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ABSTRACT: The inhibition of blood platelet aggregation and secretion was studied using covalent thiol reagents, maleimides, or mercuribenzoates, or using inhibitors of protein disulfide isomerase (PDI), bacitracin or antibodies to PDI. As expected, both types of inhibitors were effective against stimulation by normal physiologic stimuli. On the other hand, when stimulation was initiated with the peptide LSARLAF, that specifically activates the integrin αIIbβ3 (the fibrinogen receptor), the PDI inhibitors were without effect. LSARLAF-induced aggregation was, however, inhibited by the sulfhydryl reagents. To further investigate the role of sulfhydryl-containing proteins and αIIbβ3, platelets were labeled with membrane-impermeant sulfhydryl reagents. Nine bands were found labeled on gel electrophoresis. Two of the labeled bands were identified as αIIb and β3. The conclusions are that while PDI is required for platelet aggregation and secretion, an additional sulfhydryl-dependent step or protein is also required. This latter reaction occurs at the level of αIIbβ3. In distinction to most literature reports, at least a subpopulation of αIIbβ3 contains free sulfhydryl groups, consistent with the possibility that it is a substrate for PDI or part of the sulfhydryl-dependent response.

Although we traditionally think of disulfide bonds as structural components in proteins, current evidence also points to the possibility of thiol−disulfide rearrangement as a dynamic process in stimulus−response coupling. A role for protein disulfide isomerase (PDI)[1] in blood platelet physiology was first demonstrated by Detwiler and coworkers (*1*−*5*). Essex and Li showed that inhibition of PDI prevented platelet aggregation and secretion (*6*). This work suggested, as well, that PDI regulated an additional component in the later phases of the platelet response: inhibition of PDI was accompanied by reduced ability of the fibrinogen receptor, the integrin αIIbβ3,[2] to bind PAC-1 (an antibody that recognizes the activated state of αIIbβ3). Recently, Lahav et al. showed that PDI mediated integrin-dependent platelet adhesion to adhesive proteins (*7*). It has been known for some time that platelet aggregation is inhibited by sulfhydryl reagents (*8, 9*) and PDI-catalyzed reactions may be the basis for these early observations. Rearrangement of disulfide bonds may be part of the cascade of events that couples platelet stimulation to the various responses including aggregation and secretion.

Disruption of specific disulfide bonds in αIIbβ3 causes its activation (*10*−*12*). Because it is the receptor for fibrinogen, it is required for the later stages of the stimulus−response sequence, and, in addition, binding of fibrinogen to αIIbβ3 and aggregation can themselves be a stimulus leading to signaling into the platelet (*13*). The reactions of αIIbβ3 may directly involve thiols and disulfides: the integrin may, for example, be a substrate for PDI.

In this report, we used sulfhydryl reagents, as well as PDI inhibitors, to investigate the functional role and possible interactions of PDI and αIIbβ3. We stimulated platelets with collagen or other natural agonists (thrombin or ADP) and with a peptide, LSARLAF (LSA), that stimulates platelet responses by directly activating αIIbβ3 (*14*−*16*). Stimulation by collagen, as in previous work, was inhibited by PDI inhibitors and, not surprisingly, by sulfhydryl reagents. LSARLAF stimulation, in distinction, was not inhibited by PDI inhibitors but was inhibited by the sulfhydryl reagents. Therefore, sulfhydryl-dependent molecules other than PDI are also involved in platelet stimulation. Labeling experiments also showed that about nine proteins including αIIbβ3 on the platelet surface react with thiol-specific reagents.

MATERIALS AND METHODS

Materials. ATP, Chemo-Lumo reagents, ADP, collagen, and epinephrine were purchased from Chronolog Corp. (Havertown, PA). Apyrase, bovine serum albumin, papain, prostaglandin E_1 (PGE_1), protein A, dithiothreitol (DTT), reduced glutathione (GSH), and normal rabbit IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Bacitracin A was a generous gift of Dr. Leo Kesner (Department of Biochemistry, Downstate Medical Center, Brooklyn, NY). Purified α-thrombin and γ-thrombin were gifts of Dr. John Fenton II (Division of Laboratories and Research, New York State Department of Health, Albany,

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSH, reduced glutathione; LSA, peptide LSARLAF; MPB, 3-*N*-maleimidylpropionyl biotin; PCMBS, *p*-chloromercuribenzenesulfonate; PDI, protein disulfide isomerase.

[2] The integrin αIIbβ3 is also called glycoprotein IIb/IIIa.

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NY). Highly purified native RNase from bovine pancreas was obtained from Fluka Chemical Corp. (Milwaukee, WI). PDI was purified from freshly outdated platelets as previously described (*3*) or by using a monoclonal antibody raised against human PDI and coupled to Sepharose using a cyanogen bromide-activated Sepharose column (Pharmacia, Piscataway, NJ). The PDI was eluted at pH 3.0.

The LSARLAF (LSA) peptide and a control peptide, FRALASL (FRA), were synthesized and purified to >95% purity by Research Genetics (Huntville, AL). As previously described, the peptide LSA (0.5−1 mM), but not the control peptide, causes platelet aggregation and secretion by directly activating the integrin αIIbβ3 (*14*−*16*). αIIbβ3 binds specifically to LSARLAF in cell-free assays, and the purified integrin is directly activated by LSA. These authors used the "molecular-recognition hypothesis" to design the LSA peptide to bind to the target residue sequence, 315−321, of αIIb (*14*). Antibodies raised to the 315−321 residue sequence of αIIb (but not control antibodies) inhibited LSA-induced aggregation; a peptide corresponding to this sequence of αIIb removed this inhibition. Calcium chelation was used to dissociate the receptor subunits, rendering the receptor inactive. While LSA normally induced secretion in the absence of stirring or fibrinogen, with dissociation of αIIbβ3 by calcium chelation, the LSA-induced secretion was lost. Additional evidence of the specificity of this peptide for αIIbβ3 is that LSA does not induce release of platelet factor 4 (PF4) from thrombasthenic platelets and the scrambled control peptide does not induce PF4 release from normal platelets *(16*).

Antibodies. Rabbit anti-PDI antiserum was raised against human platelet PDI and was passed through a human albumin−Sepharose affinity column and subsequently through a protein A column as described (*3*). For some experiments, the antibody was affinity-purified on a PDI−Sepharose column (made from high-purity platelet PDI coupled to a cyanogen bromide-activated Sepharose column). The rabbit anti-PDI antibody was characterized, and Fab fragments were prepared using papain as previously described (*3*, *6*). A monoclonal antibody which Western-blots to αIIb was obtained from Immunotec (Clone SZ22, Westbrook, ME) and to the β3 subunit from Zymed Labs Inc. (Clone Y2/51, San Francisco, CA).

Platelet Preparation. Blood was drawn into one-seventh volume of acid−citrate−dextrose solution (ACD), and platelet-rich plasma (PRP) was prepared by centrifugation for 20 min at 300*g* as previously described (*3*, *4*). For aggregation studies, gel-filtered platelets were prepared from the PRP as described elsewhere (*17*) by concentration of the platelets by centrifugation (10 min, 500*g*) followed by gel filtration on a Sepharose 2B column equilibrated in Tyrode's−albumin solution containing 0.137 M NaCl, 2.7 mM KC1, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.5 mM glucose, and 0.2% albumin at pH 7.35. The platelets eluting in the void volume were collected and counted using a hemocytometer. Calcium (1 mM) was added prior to aggregation studies.

For sulfhydryl labeling studies, washed platelets were prepared as previously described (*6*). Briefly, after preparation of the PRP in ACD anticoagulant as described above, the platelets were washed 3 times in the Tyrode's−albumin solution containing calcium (2 mM). The first wash contained heparin (2 units/mL), apyrase (1 unit/mL), and PGE_1 (1 μM); the second wash contained apyrase and PGE_1; the third wash contained only PGE_1. The platelets were resuspended in albumin-free Tyrode's buffer without inhibitors.

PDI Assay. Scrambled RNase was prepared from native RNase, and the PDI assay was performed as previously described (*4*).

Platelet Aggregation and Release Studies. The experiments were carried out in a Chronolog Lumi-Aggregometer (Chronolog Corp., Havertown, PA) as described elsewhere (*6*). Since luciferase, used to measure ATP release from platelets, is a sulfhydryl enzyme which may be inhibited by sulfhydryl reagents, in some assays released platelet factor 4 (PF4, a platelet α-granule protein) was quantitated. The PF4 was determined with a sandwich ELISA (Asserachrom kit, Diagnostica Stago, Inc., Parsippany, NJ) using a modification of the manufacturer's instructions as described elsewhere (*14*).

Sulfhydryl Reagents and Labeling of Intact Platelet. Sulfhydryl reagents of several chemical classes were employed in these studies. Nonlabeled membrane-impermeant reagents used were *p*-chloromercuribenzenesulfonate (PCMBS) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). PCMBS has been shown to be impermeant to platelets at a 100 μM concentration when incubated with platelets at room temperature for 30 min (*8*). Labeled impermeant reagents employed were the fluorescent Oregon Green maleimide (*18*) and Oregon Green iodoacetamide (Molecular Probes, Eugene, OR) as well as the biotinylated reagent 3-*N*-maleimidylpropionyl biotin (MPB) (Molecular Probes) (*19*). Other sulfhydryl reagents used were *N*-ethylmaleimide (NEM) and 4,4′-dithiodipyridine.

Labeling with the Oregon Green fluorescent reagents was carried out as follows: 40 μM freshly prepared Oregon Green maleimide (*18*) or Oregon Green iodoacetamide was added to washed intact platelets (2×10^8/mL) in Tyrode's buffer (albumin-free), pH 7.4. After a 20 min incubation at 24 °C, the reaction was stopped by the addition of GSH (200 μM) for 10 min and iodoacetamide for 10 min. This was followed by 3 cycles of centrifugation in washing buffer [Tris-buffered saline, EDTA (1 mM), pH 7.4]. In some samples, PCMBS (3 mM) or NEM (5 mM) was preincubated with the platelets (30 min at 37 °C), and in other samples, GSH (1 mM) was preincubated (10 min at 24 °C) with the fluorescent probe. Labeled platelets were resuspended in TBS, EDTA (1 mM), pH 7.4, and 2% SDS-nonreducing sample buffer was added. A total of 5×10^6 platelets were added per well, and after electrophoresis using SDS−(10%) PAGE, the gels were developed using the phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Labeling using the biotinylated reagent MPB was carried out as follows: MPB (20−100 μM) was added to washed platelets in Tyrode's (albumin-free) buffer, pH 7.4, and incubated for 10 min at 4 or 24 °C. The labeling reaction was stopped by addition of GSH and iodoacetamide as above. In some samples, GSH (200 μM) was preincubated for 10 min with the MPB prior to addition of the MPB to the platelets. In other samples, 4,4′-dithiodipyridine (4 mM) (30 min at 37 °C) or PCMBS (0.1−3 mM) was added to the platelets prior to the addition of the MPB. Nonreducing SDS sample buffer was added to the platelet sample and the sample electrophoresed using 2% SDS−(10%) PAGE. The

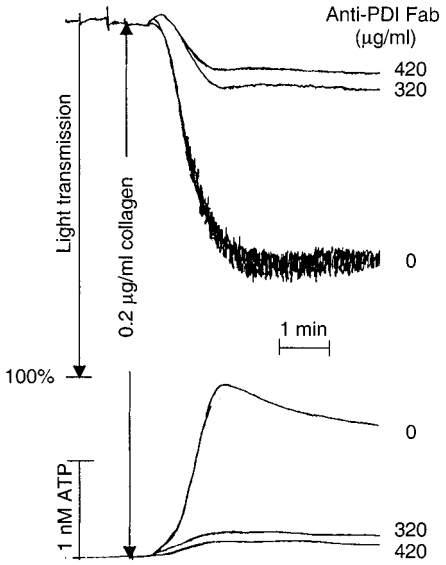

FIGURE 1: Inhibition of collagen-induced aggregation and secretion by anti-PDI Fab fragments. Gel-filtered platelets were treated for 1 min with the indicated concentrations of anti-PDI Fab fragments (purified on a PDI−Sepharose column) and stimulated with collagen (0.2 μg/mL). Responses were recorded in the lumi-aggregometer. Aggregation is shown relative to 100% light transmission which was set with Tyrode's buffer. Fab fragments from nonimmune rabbit IgG (420 μg/mL) gave no inhibition of aggregation or secretion.

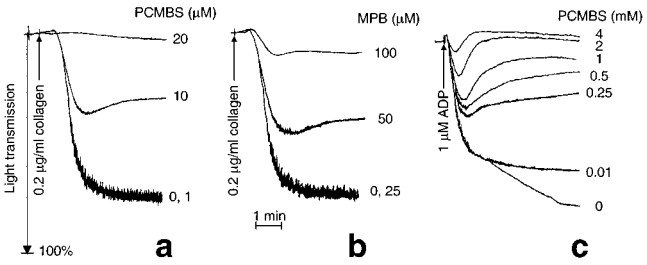

FIGURE 2: Effect of sulfhydryl reagents on aggregation. The sulfhydryl reagents PCMBS (a) or MPB (b) at the indicated concentrations were added to gel-filtered platelets for 1 min at 37 °C and stimulated with collagen. In (c), PCMBS was added to citrated platelet-rich plasma, and ADP was used as the agonist.

sample was transferred to a nitrocellulose membrane, and the biotinylated proteins were detected using streptavidin−horseradish peroxidase (*19*, *20*) with a chemiluminescent substrate (*4*).

RESULTS AND DISCUSSION

Effect of PDI Inhibitors and Sulfhydryl Reagents on Stimulation by Collagen and Other Agonists. Gel-filtered human platelets were treated with antibodies against PDI or the membrane-impermeant sulfhydryl reagents, PCMBS, MPB, or DTNB. Figure 1 shows that anti-PDI Fab fragments inhibit both aggregation and secretion. As reported previously (*6*), we found inhibition of aggregation was correlated with the degree of inhibition of purified PDI. The figure also shows that inhibition of secretion is approximately parallel to inhibition of aggregation, in agreement with previous studies using, as inhibitors, bacitracin or a competing substrate (scrambled RNase).

Figure 2a shows the dose-dependent inhibition of collagen-induced aggregation by PCMBS. This effect is seen with other sulfhydryl inhibitors: Figure 2b shows that the

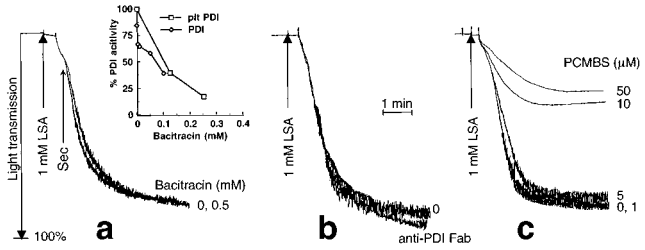

FIGURE 3: Differential effect of bacitracin A, anti-PDI Fab's, and PCMBS on LSARLAF-induced aggregation. (a) shows the absence of an effect of bacitracin on LSARLAF (LSA)-induced stimulation. The inset shows the effect of bacitracin A on the activity of purified PDI and PDI on intact platelets (plt PDI) determined using the sRNase assay. ATP secretion (Sec) occurs at the inflection point of the second wave of aggregation. (b) shows the absence of an effect of anti-PDI Fab's (500 μg/mL) on LSARLAF-induced stimulation [compared to a tracing with no (0) Fab's]; (c) shows the inhibition of aggregation and secretion by the sulfhydryl reagent PCMBS at micromolar (μM) concentrations.

maleimide reagent MPB inhibits aggregation. As with PDI inhibitors, secretion, assayed by ELISA for platelet factor 4 (PF4), was also inhibited. The degrees of inhibition of secretion and aggregation were parallel (not shown). When a higher concentration of collagen was used (0.5 μg/mL), 5-fold more PCMBS (100 μM) or MPB (500 μM) was needed for complete inhibition of aggregation. A third reagent, DTNB, shows similar effects although higher concentrations are needed, consistent with the original literature (*9*, *21*). Inhibition is also not stimulus-specific: α-thrombin stimulation is also repressed by sulfhydryl inhibitors. When higher concentrations of thrombin were used, higher concentrations of sulfhydryl reagent were needed for inhibition.

More subtle platelet responses can be seen when platelet aggregation is performed in citrated plasma, and biphasic aggregation becomes apparent (*22*). Using ADP as the agonist, the second phase, or irreversible aggregation, is inhibited by PCMBS (Figure 2c). First-wave aggregation, on the other hand, persists even at very high concentrations (2−4 mM). This is consistent with previous results with inhibitors of PDI (*6*) and suggests that first-wave responses are independent of both PDI and other potential sulfhydryl proteins. [It should be noted that the effective concentration of sulfhydryl reagent in plasma is lower (about 20-fold) than the concentration added (*8*), a finding confirmed in the present aggregation studies.]

Effect of PDI Inhibitors and Sulfhydryl Reagents on Stimulation by the Peptide LSARLAF. The αIIbβ3-activating peptide LSA has been shown to be a stimulus of platelet aggregation and secretion (*14*−*16*). In preliminary studies, this peptide was found to produce a biphasic response with a short primary phase followed by a secondary response with ATP secretion occurring at the inflection point for the secondary response. The scrambled control peptide FRALASL (FRA) did not induce aggregation. We next tested the effect of two kinds of agents, PDI inhibitors and sulfhydryl reagents, on LSA-induced activation.

Figure 3a shows that LSA-induced aggregation was not inhibited by bacitracin A at a concentration of 0.5 mM, almost an order of magnitude greater than the concentration required for half-maximal inhibition of PDI (inset to Figure 3a). Controls showed complete inhibition of irreversible

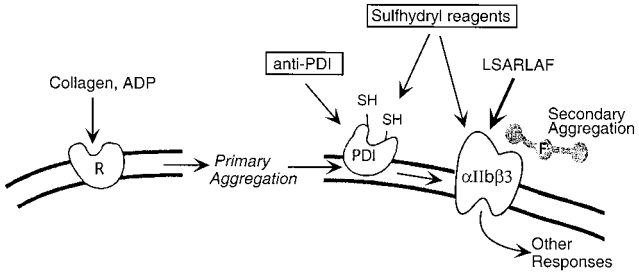

FIGURE 4: Model for the role of PDI and sulfhydryls in platelet aggregation and secretion.

aggregation induced by standard agonists at 0.25 mM bacitracin A. These findings suggest that LSA-induced aggregation is independent of PDI. This was confirmed by experiments using concentrations of Fab fragments higher than required for strong inhibition of purified PDI (400 μg/mL inhibited PDI activity by 95%). Similar concentrations inhibited collagen-induced aggregation. These Fab fragments were, again, without effect on aggregation stimulated by LSA (Figure 3b). The lack of involvement of PDI, which is a sulfhydryl enzyme, fortuitously allowed us to assess the role of sulfhydryl-dependent pathways in at least one kind of stimulation.

In distinction to this lack of inhibition by PDI inhibitors, the sulfhydryl inhibitor PCMBS caused strong inhibition of LSA-induced aggregation and secretion (Figure 3c). The target for this sulfhydryl reagent here is not PDI since the PDI inhibitors used above were not inhibitory. It is important to note that primary phase aggregation induced by LSA, like primary aggregation with normal stimuli, is not inhibited by PCMBS up to the highest concentration tested, 0.5 mM.

Proposed Model. A model for platelet activation (Figure 4) summarizes results and deductions from the work presented here and from other studies in the literature. A particular focus of the model is the interrelations of PDI and the integrin αIIbβ3. Although not directly depicted, important cytoplasmic events leading to conformational changes in αIIbβ3 are likely involved (*13*). The major points are as follows:

1. Primary or reversible aggregation is the first step in platelet stimulation by weak agonists or low concentrations of strong agonists. It is presumed to also take place during strong stimulation, where it is hidden by secondary aggregation. This step is known to involve interactions of αIIbβ3 and fibrinogen (*13*). The key observation is that this step is not inhibitable by either PDI inhibitors or sulfhydryl reagents.

2. PDI is required for irreversible aggregation and secretion by normal stimuli. The major support for this idea, shown in this work (Figure 1) and previous studies (*6*), is that these responses are inhibited with antagonists to PDI.

3. The original observation of inhibition of PDI (*6*) also indicated a decreased ability of αIIbβ3 to bind PAC-1 (an antibody recognizing the activated state of αIIbβ3), suggesting a causal link between these two proteins. A similar relationship is suggested in the recent report of Lahav et al. that integrin-mediated adhesion is dependent on PDI (*7*).

4. The peptide LSARLAF has been shown to bind to αIIbβ3 and to directly stimulate aggregation and secretion. The model in Figure 4 assumes that this peptide mimics a step in the normal stimulus response pathway (*14*−*16*).

The model thus proposes a sequence of events: receptor activation leads to primary aggregation, which involves ligand binding to αIIbβ3. This, in turn, activates PDI (or results in the presentation of a target disulfide to PDI). PDI presumably catalyzes a disulfide exchange reaction that either involves αIIbβ3 or leads indirectly to an increase in its affinity for fibrinogen. This step is by-passed by LSA stimulation, which is not inhibited by antagonists to PDI. LSA-mediated stimulation, however, is inhibited by sulfhydryl reagents, indicating that αIIbβ3 activation is dependent on some sulfhydryl(s) other than those in PDI. The final phase of the model shows that the activated αIIbβ3−fibrinogen complex represents not only the nucleation site for aggregation but also a point of outside−inside signaling leading to other responses (*13*).

This model raises two major questions: How is PDI activity controlled? What is the target for PDI? In regard to control of PDI activity, the recent demonstration by Burgess et al. that activated/aggregated platelets show a dramatic increase in surface sulfhydryls and, in particular, PDI sulfhydryls (*23*) supports the idea that PDI is activated by the reduction of PDI active site disulfides. One candidate for the reducing agent is an NADH/oxidoreductase system analogous to that found in other plasma membrane systems (*24*).

The substrate of PDI is not known, but it must have a thiol or disulfide to participate in reactions catalyzed by PDI. The fact that stimulation by the αIIbβ3-specific peptide LSA is inhibitable by sulfhydryl reagents suggests that αIIbβ3 and/or proteins with which it is associated may be such substrates. To pursue this question, we labeled thiols on the platelet surface.

Labeling of Platelet Surface Thiols. The results described above show that platelet function is inhibited by reagents that react with thiols. This is true of LSA stimulation (which is not blocked by anti-PDI), indicating that if PDI is a target for the sulfhydryl reagents, it is not the only one. Sulfhydryl labeling experiments in platelets have been reported in the past, usually using purified membranes, and typically 4−6 sulfhydryl-reactive bands have been found (*25*, *26*). These have generally not been identified. We labeled intact platelets with membrane-impermeant sulfhydryl reagents: fluorescent Oregon Green maleimide (Figure 5a, lane 1) (*18*) and Oregon Green iodoacetamide (Figure 5b, lane 1) and the biotinylated 3-*N*-maleimidylpropionyl biocytin (MPB) (Figure 5c, lane 1) (*19*, *20*). The three sulfhydryl reagents showed similar labeling patterns of about nine proteins. Specificity was shown by inhibiting labeling with preincubation of the reagent with GSH (to block the reactive group; Figure 5a−c) or preincubation of the platelets with a different type of sulfhydryl reagent, the mercuribenzoate PCMBS (to block platelet sulfhydryls, Figure 5a,b). *N*-Ethylmaleimide also blocked labeling with the iodoacetamide reagent (Figure 5b), and 4,4′-dithiodipyridine blocked labeling by MPB (Figure 5c). The size of the proteins labeled ranged from about 30 kDa to ≥200 kDa and included several bands in the 60−150 kDa size range. When the cells were permeabilized with Triton X-100 before labeling, a major increase in labeling was seen.

At this point, we turned our attention to αIIbβ3. Both αIIb and β3 contain disulfide bonds. αIIb contains 18 cysteine residues (*27*), and β3 contains 56, most of which are in a

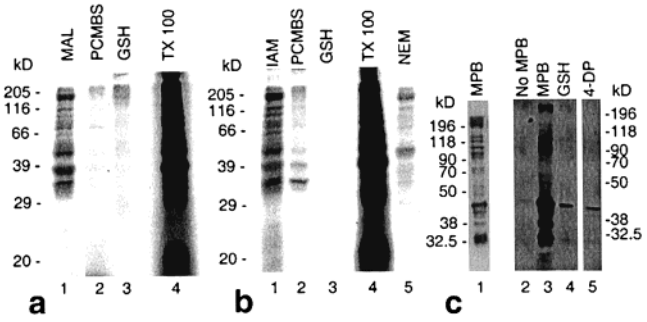

FIGURE 5: Labeling of intact platelets with sulfhydryl reagents. Panels a and b show the labeling pattern for two fluorescent reagents, Oregon Green maleimide (MAL) and Oregon Green iodoacetamide (IAM), and panel c shows the pattern with the biotinylated maleimide reagent (MPB). Labeling was performed as described under Materials and Methods. The labeling pattern for each reagent is shown in lane 1 of each gel. Controls for the fluorescent probes were as follows: To show specificity for sulfhydryls, platelets were preincubated with competing reagents of a different type; preincubation with the mercribenzoate PCMBS is shown in lane 2 of (a) and (b); the iodoacetamide reagent in (b) was also preincubated with NEM, lane 5. As an additional control, the effect of permeabilizing the cells with Triton X-100 (1%) (TX 100), added before the labeling agents, is seen in lane 4 of (a) and (b). Results for the biotinylated reagent MPB are shown in (c). The sample in lane 1 shows results of labeling platelets with 20 μM MPB. In lane 2, MPB was omitted (No MPB). In lanes 3−5, MPB was incubated at a higher concentration (100 μM), and controls show the effect of preincubating with GSH (200 μM, lane 3) or the platelets with the sulfhydryl reagent 4,4′-dithiodipyridine (4-DP) (lane 4). Although not shown in (c), PCMBS also inhibited MPB labeling.

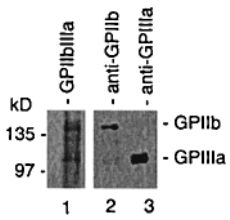

FIGURE 6: Identification of αIIb (GPIIb) and β3 (GPIIIa) from labeled platelets. Labeling of intact platelets was performed using MPB (20 μM) for 10 min at 4 °C, after which the samples were subjected to concanavalin A and heparin affinity chromatography as described elsewhere (*38*). Development was as for the MPB-labeled samples in Figure 5. Western blots of whole platelets using antibodies against αIIb (lane 2) and β3 (lane 3) are shown for comparison.

cysteine-rich region in the extracellular portion of the molecule. It has generally been assumed that these are all disulfide-bonded (*28*−*31*), but recent results suggest that some may exist as free thiols; Qingqi and Stracher labeled both αIIb and β3 with [^{3}H]NEM (*32*), and a recent abstract raised the question of a potential thiol in a subpopulation of the β3 subunit (*33*). Figure 6 shows that subunits of αIIbβ3 purified from our samples of intact platelets are labeled, indicating that at least a subpopulation of these proteins has a free thiol. Label was consistently incorporated into the β3 subunit. We think that our ability to identify the sulfhydryl is because fluorescent and biotinylated reagents are more sensitive than previous spectrophotometric methods (*34*, *35*) and the fact that purified systems are susceptible to oxidation (*36*).

In a recent publication, Yan and Smith, using the purified receptor, found sulfhydryl labeling in both of the subunits of αIIbβ3 (*37*). When the labeling was performed on intact platelets, only the β3 subunit was labeled. These authors found that labeling in the purified receptor was greater in the activated form of the receptor compared to the non-activated form. They also showed that the labeling was localized to the cysteine-rich repeat region of the β3 subunit. In contrast, Burgess et al. did not find labeling in αIIbβ3 (*23*). The reasons for this are not clear but may involve differences in the experimental conditions used.

While we did not quantitate the amount of label incorporated into αIIbβ3, it may be that only a subpopulation of the receptor was labeled in our experiments. This would not be inconsistent with a global response involving αIIbβ3 because new sulfhydryls may be generated in this receptor during the activation process. Newly generated sulfhydryls would also be inhibited by the sulfhydryl reagents. While the role of PDI may involve a direct functional interaction with αIIbβ3, PDI may, alternatively, participate in a more general redox reaction involving several proteins. In conclusion, our findings in conjunction with the findings of Yan and Smith support the concept that a sulfhydryl-dependent reaction at the level of αIIbβ3 is necessary for platelet responses.

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