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Direct Evidence for the Interaction of Platelet Surface Membrane Proteins GPIIb and III With Cytoskeletal Components: Protein Crosslinking Studies

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When intact platelets are incubated at 37°C with Concanavalin A (ConA), the two major surface membrane proteins GPIIb and III become associated with the Triton-insoluble cytoskeleton [4]. Preincubation of platelets with a variety of metabolic inhibitors, including cytochalasin B, 2-deoxy-D-glucose, and antimycin A or lidocaine, had no effect on the ability of ConA to produce this effect. These results suggested that the ConA-induced anchorage of GPIIb and III to the Triton-insoluble cytoskeleton is a passive process requiring clustering of GPIIb-III molecules but not requiring the metabolic energy of an intact cell. This was supported by experiments that showed that ConA binding to plasma membrane-rich fractions at 37°C could induce association of GPIIb and III with a sedimentable actin-rich, Triton-insoluble membrane matrix. Similar results were obtained when membranes were first isolated from ConA-treated cells. Adding DNAse I, an actin depolymerizing agent, into the Triton extraction buffer inhibited the ConA-induced sedimentation of GPIIb-III and actin by 50% in the presence of Mg^{2+}-ATP. Treatment of ConA-treated membranes with dimethyl-3,3′-dithiobispropiomidate, a bifunctional, reducible protein crosslinking agent, produced Triton-insoluble crosslinked species of discrete molecular weights. When these crosslinked species were analyzed by SDS-PAGE in the presence of β-mercaptoethanol, they were found to be composed of a 180–200 K dalton protein, GPIIb, GPIII, and actin. Crosslinking of these components was equally effective after Triton treatment and indicated as well that the species crosslinked in the intact membrane was stable after Triton extraction.

Addition of crosslinker to membranes *not* treated with ConA produced similar crosslinked species. Analysis of their composition on reduced gels revealed that the amounts of GPIIb and III were reduced greatly (< 10% of the total input GPIIb and III) but that the 180–200 k dalton protein and actin content were similar to that seen with ConA-treated membranes. These results are consistent with the notion that ConA clusters mobile, unanchored molecules of GPIIb-III (~90-95% of the total) around a small fraction of IIb-III that is associated with a submembranous cytoskeleton.

Key words: platelets, receptors, cytoskeleton, actin, membranes

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Numerous studies have provided indirect evidence for interactions between cytoskeletal proteins within the cell cytosol and membrance surface receptors or lectin binding proteins [1–10]. In general, these interactions appear to require clustering of surface proteins or receptors either by crosslinking by multivalent ligands, such as lectins or antibodies [1–4,6,10,11], or by clustering, perhaps induced directly by conformational changes in receptor [7–9].

However, limited information exists concerning the precise molecular interactions involved in attachment of surface receptor to cytoplasmic actin. In the mature erythrocyte, for example, a fraction of the major transmembrane sialylglycoprotein, Band III, is associated with ankyrin [12], which in turn interacts on the cytoplasmic face of the membrane with the spectrin-actin complex [see 13 for a review]. In fact, modulation of the spectrin content or topographical distribution has been shown to perturb the distribution of the surface sialylglycoprotein [14,15].

In other membrane systems, the molecular details of transmembrance cytoskeletal-receptor interactions are relatively sparse. Carraway et al [16] have demonstrated that surface glycoproteins of microvilli derived from tumor lines copurify with actin and probably interact directly with a particular transmembrane glycoprotein. In intestinal microvilli, a 110 k dalton protein has been isolated that interacts with actin and may represent the lateral bridge that connects the membrane with the actin core [17]. However, little is known about the nature of the membrane attachment site.

The present study has shown that ConA could induce interactions between the two major glycoproteins of the platelet plasma membrane (GPIIb and III) and the Triton X-100 (TX-100) insoluble cytoskeleton. Furthermore, it was found that these interactions could be disrupted by the specific actin depolymerizing agent (DNAse I). In this report, the metabolic requirements of this process were examined and found to be insensitive to a variety of metabolic inhibitors. Further, it was shown that ConA can induce similar effects in isolated platelet plasma membranes inducing a DNAse I sensitive co-sedimentation of the membrane cytoskeleton and GPIIb-III. Chemical crosslinking studies suggest that GPIIb-III exist in a molecular complex with actin and several proteins with Mr of 180–200 k daltons.

MATERIALS AND METHODS

Cells

Human platelets were prepared from fresh human blood by the gel filtration method [18] as previously described [4]. Where indicated, cells were surface iodinated with ^{125}I by the lactoperoxidase method as previously described [4].

Plasma Membrane-Rich Fractions

Platelet plasma membranes were prepared from unstimulated or ConA-treated cells by the method of Barber and Jameison [19]. Yields of membrane protein were comparable in both cases.

Reagents

DNAse I (bovine pancreas) was obtained from Sigma (St. Louis, MO) and treated with 2 mM phenylmethylsulfonylfluoride (PMSF) in 20 mM HEPES (pH

7.0)–0.15 M NaCl 1 mM EDTA for 1 hr prior to use. Rabbit skeletal muscle actin was prepared as described by Spudich and Watt [20]. Dimethyl-3-3′-dithiobis propionimidate HCl (DTBP) was obtained from Pierce Chemical. Cytochalasin B and 2-D-deoxyglucose were obtained from Calbiochem (La Jolla, CA). Antimycin A was obtained from Sigma (St. Louis, MO). $Na^{125}I$ and [^{14}C]-serotonin were obtained from Amersham.

Drug Inhibition Studies

Gel filtered platelets, surface labeled with ^{125}I, (4×10^8/ml) were preincubated for 15 min at 37°C with metabolic inhibitors at the concentrations indicated in the text in Tyrode's BSS (pH 7.4). ConA was added (100 μg/ml) in 1/20 volume of Tyrode's BSS incubated an additional 15 min without shaking. The cells were lysed by adding 1/0 volume of 10% Triton X-100-40 mM EDTA dissolved in 30 mM Tris-HCl-120 mM NaCl (pH 7.4). After 10 min the Triton lysates were centrifuged for 30 min at 20,000 rpm in SS-34 rotor at 4°C in a Sorvall RC-5 centrifuge (Dupont, Co., Wilmington, DE) at 4°C. The pellet and supernatant fractions were analyzed for protein and GPIIb, III content by SDS-PAGE as previously described [4].

Protocol for Preparing Platelet Plasma Membrane "Cytoskeletons"

Platelet membranes derived from ^{125}I-surface labeled platelets were resuspended in 0.15M NaCl-1mM EDTA-20mM HEPES (pH 7.4) at a protein concentration of 30–50 μg/ml. An 150 μl aliquot was treated with 10 μl of ConA (150 μg/2×10^9 platelet equivalents/ml) or buffer at 37°C or 4°C for 15 min as indicated in the text. Then 1/10 volume of 20% Triton X-100 (Sigma)-0.15 M NaCl-1 mM EDTA-20 mM HEPES (pH 7.4) was added, and the lysates were incubated for 30 min at 37°C and centrifuged in the Sorvall SS-34 rotor at 4°C at 20,000 rpm for 30 min. When present, DNAse I was added to a final concentration of 2 mg/ml just prior to the addition of Triton X-100.

The Triton-insoluble pellets were dissolved in 100 μl of SDS-PAGE sample buffer [4] in the presence and absence of β-mercaptoethanol as indicated.

Crosslinking of Platelet Membranes and Triton Lysates With DTBP

Platelet membranes pretreated with or without ConA for 15 min as described above were treated with freshly prepared 40–800 μg/ml DTBP (final) for 30 min. The protocol was identical to that used in the absence of DTBP except that the pH was adjusted to 8 to enhance the crosslinking reaction. Triton-insoluble pellets were isolated as described above and analyzed either by one dimensional SDS-PAGE on 5% acrylamide mini-slab gels [21] or by two dimensional mini-gel modification of the nonreduced-reduced gels described by Wang and Richards [22] (see below).

SDS-PAGE Analysis

One dimensional. Eight percent acrylamide slab gels were performed by the method of Laemmli [23] in a mini-gel system [21]. Samples were prepared as previously described [4].

Two dimensional. SDS was performed using a mini-gel modification of the procedure of Wang and Richards [22]. Briefly, crosslinked platelet membrane lysate pellets were suspended in SDS-PAGE sample buffer and electrophoresed under nonreducing conditions on either 5% acrylamide tube gels or 1.75% acrylamide/

0.5% agarose tube gels according to Kiehm and Ji [24]. First dimension tube gels were prepared by casting 100 μl gels in Clay Adams 100 μl Micro-Selectapette pipette tips. Dialysis tubing was secured over the bottom of each tube gel to prevent the gels from sliding out of the tubes during electrophoresis. Tube gels then were incubated in reducing buffer containing, 8% SDS/20% βME/4mM EDTA/20% Glycerol/0.1% bromophenol blue, pH 6.8. Tube gels were then laid on 8% SDS-PAGE slab gels with 3% stacking gel and overlaid with 2% agarose and run according to Tobias et al [21].

RESULTS AND DISCUSSION

The Effect of Metabolic Inhibitors on ConA-Induced Association of GPIIb-III With the Platelet Cytoskeleton

Table I shows the effect of a number of metabolic inhibitors on ConA-induced co-sedimentation of GPIIb and III with the Triton X-100-insoluble cytoskeleton. For these experiments, platelets were preincubated with the indicated concentration of inhibitor(s) for 15 min at 37°C. ConA (100 μg/ml) was added and the cells incubated an additional 20 min. A 1/10 volume of 10% Triton X-100 50 mM EDTA-0.1 M Tris HCl-1.5 M NaCl (pH 7.4) (10 × Triton-EDTA buffer) was added. In cells treated with ConA only, nearly all of the lactoperoxidase labeled cell surface glycoprotein IIb and III cosedimented with the actin-rich pellet fraction (Table I). In the absence of ConA, only about 5% of the total IIb and III label was sedimentable confirming our previously reported results [4]. Parallel experiments confirmed that the inhibitors were active inasmuch as 2-deoxyglucose and antimycin A and lidocaine treatment under identical conditions blocked serotonin release (data not shown). Thus, the

TABLE I. Effect of Metabolic Inhibitors on the ConA-induced Association Between GPIIb - III and the Triton-insoluble Cytoskeleton

	Percent of total surface ^{125}I labeled component recovered in Triton pellet[a]	
Inhibitor	GPIIb	GPIII
None	88.2	92.0
Cyto B (25 μg/ml)	87.6	89.4
2-DOG + AA (5mM) (1μg/ml)	89.2	90.2
Lidocaine (1%)	80.3	82.5

[a]Determined by quantitative densitometry of autoradiograms of SDS-PAGE gels. Platelets were incubated at 37°C with the drugs at the indicated concentrations for 15 min. ConA was added (100 μg/ml) and incubation continued for 15 min. 1/10 volume of 10% Triton-EDTA in Tris buffer saline (TBS) was added and after 10 min centrifuged as described in Materials and Methods. Stoichiometric amounts of the resulting supernatant and pellet fractions then were analyzed by SDS-PAGE. In the absence of ConA, 8.2 and 6.4% of GPIIb and III were recovered in the pellet fraction, respectively.

cosedimentation of GPIIb and III induced by ConA treatment of platelets does not appear to require metabolic energy, nor is it sensitive to cytochalasin B.

ConA-Induced Association of GPIIb and III With the Triton-Insoluble Fraction of Isolated Platelet Membranes

The inhibitor studies suggested that the effect of ConA is a passive one. If so, it was reasoned that ConA might produce similar effects when bound to isolated membrane vesicles. For this purpose, platelet plasma membranes were prepared from ^{125}I-surface-labeled platelets by the Barber and Jameison method [19]. These membrane fractions were treated with Triton X-100 in EDTA-HEPES buffer and the Triton soluble and insoluble fractions isolated by centrifugation. SDS-PAGE analysis was performed under *nonreducing* conditions in order to enhance the separation of GPIIb and III. Such analysis of fractions prepared from cells pretreated with ConA at 37°C (lane 3) or at 4°C (lane 4) are shown in Figure 1. For comparison, similar Triton pellets (panel A) and supernatant fractions (panel B) are shown which were obtained from plasma membranes obtained from resting platelets (lane 1) or resting plasma membranes treated with ConA at 37°C *after* their preparation (lane 2). In all cases, the major polypeptide species visualized by silver staining in the Triton insoluble fraction were proteins that coelectrophoresed with filamin, myosin heavy chain, GPIIb, GPIII, and actin (Ac), as well as numerous other trace components. When compared with Triton pellets derived from untreated platelet membranes (lane 1), membranes derived from cells pretreated at 37°C with ConA (lane 3) showed significantly larger amounts of myosin, actin binding protein, GPIIb, III, and slightly greater quantities of actin. Triton pellets of membranes obtained in parallel from platelets treated with ConA at 4°C (lane 4) were more comparable to resting membranes although they did contain significantly more actin binding protein. Treatment of plasma membranes from resting cells with ConA (100 μg/ml) at 37°C *after* isolation (lane 2) resulted in Triton pellets that were enriched in GPIIb and III and other unidentified proteins but not enriched in myosin actin or actin binding protein when compared with untreated membranes.

An autoradiogram of the gels shown in Figure 1 is shown in Figure 2. As can be seen, little surface GPIIb or III sedimented with the Triton X-100-insoluble pellet fraction in membranes isolated from unstimulated platelets (Fig. 2A, lane 1). About 90% of the input GPIIb and III was recovered in the Triton supernatant fraction as judged by densiometric analysis (Fig. 2B, lane 1). ConA treatment of such membranes at 37°C increased the amount of Triton insoluble GPIIb-III to levels approaching 80–85% of the total (Fig. 2A, lane 2). Similar results were obtained using membranes obtained from cells pretreated with ConA at 37°C, where more than 90% of total surface GPIIb and III was recovered in the Triton pellet (lane 3). In contrast, incubation of cells with ConA at 4°C decreased the Triton-insoluble surface component of isolated membranes to levels that were about 40–50% of the total surface GPIIb and III.

In summary, the data shown in Figures 1 and 2 indicate that the Triton-insoluble membrane fraction can be obtained in platelets that are enriched in contractile and cytoskeletal proteins. Addition of ConA either prior to membrane preparation at 37°C or afterwards to isolated membranes resulted in insolubilization of the two major ConA binding surface proteins GPIIb and III. Finally, incubation at low temperatures prior to preparation of membranes partially inhibited the ConA effect.

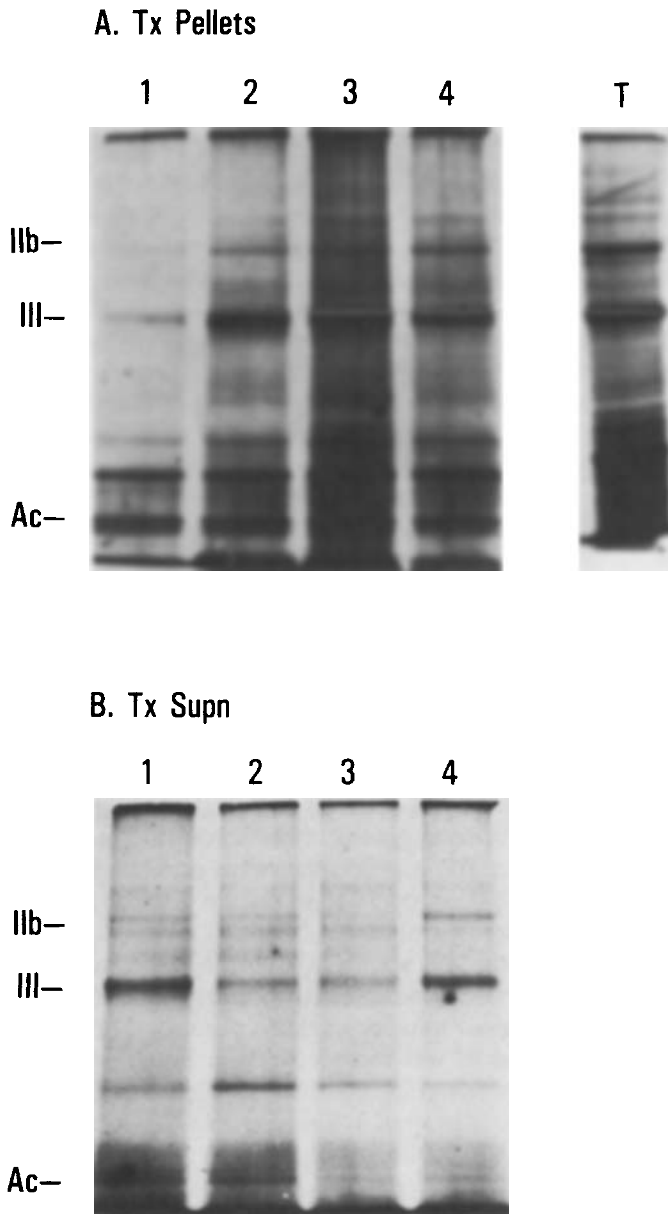

Fig. 1. Silver-stained 8% acrylamide SDS-gel of Stoichiometric amounts of Triton X-100 pellets (P) and supernatants (S) obtained from isolated platelet plasma membranes after centrifugation at 20,000 rpm in a Sorvall SS-34 rotor at 4°C for 30 min. The far right lane (T) in panel A shows the starting membrane preparation at a protein load equivalent to that used in the Triton extraction step. The pellet and supernatant fractions obtained after each treatment are shown in Panel A and B, respectively. Samples were not reduced prior to electrophoresis.

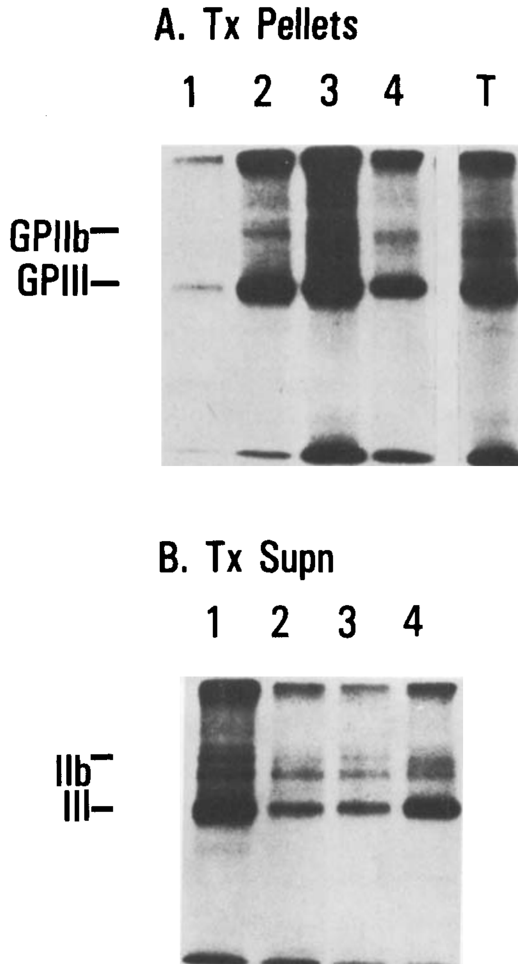

Fig. 2. Autoradiogram of the gel shown in Figure 1 showing the relative distribution of GPIIb and GPIII into Triton insoluble (A) and soluble fractions (B) before and after ConA treatment. Lane 1 is the corresponding Triton pellet and supernatants obtained from membranes derived from unstimulated cells. Lane 2 as lane 1 except the membrane exposed to ConA prior to Triton addition. Lanes 3 and 4 represent samples derived from membranes obtained from intact cells incubated at 37°C and 4°C, respectively, prior to membrane preparation. See Figure 1 legend for details. Samples were not reduced to enhance the separation of GPIII and IIb. Note incubation with either ConA before or after membrane preparation results in increased Triton insolubility.

The effect of actin depolymerization on the association of GPIIb and III with the Triton-insoluble fraction of ConA-treated plasma membrane. The present authors have shown previously that DNase I, which depolymerizes f-actin in solution [26], dissociates GPIIb and III from whole cytoskeleton of ConA-treated platelets [4]. Figure 3 shows the results of an experiment to determine if DNAse I had a similar effect on the Triton insolubility of GPIIb and III induced by ConA treatment of resting plasma membranes. When platelet membranes derived from ^{125}I-surface-labeled platelets were treated at 37°C with ConA in normal Triton-EDTA

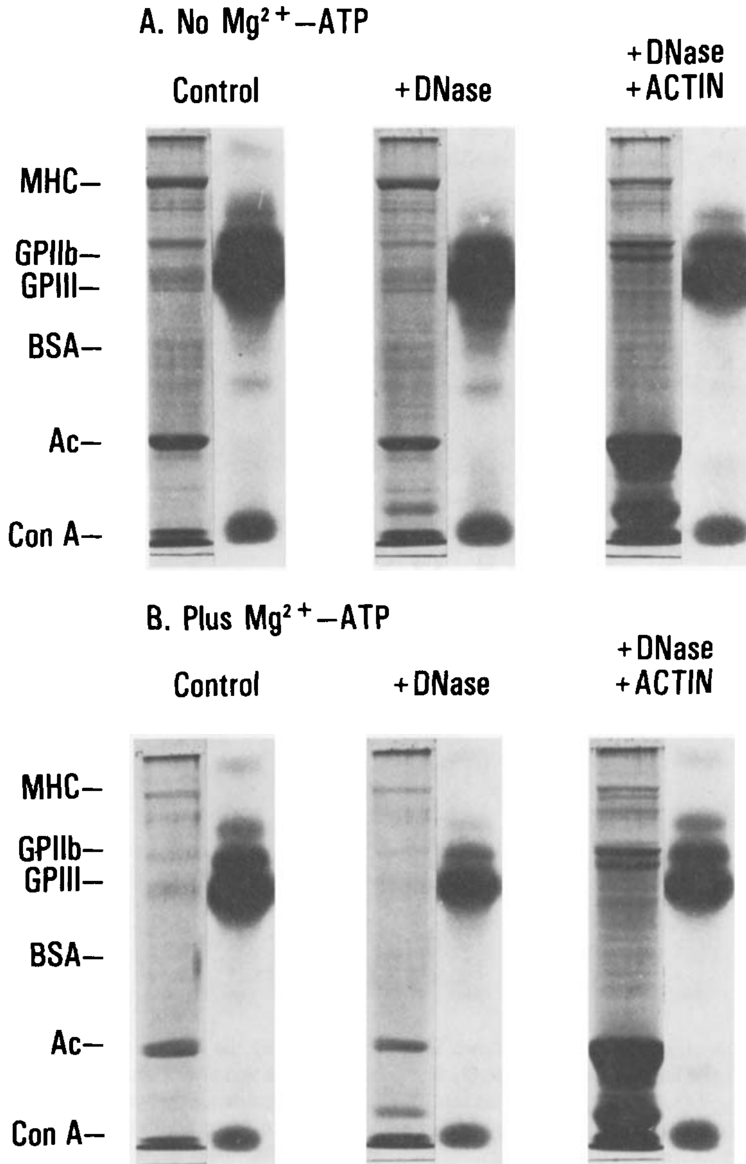

Fig. 3. Treatment of Triton lysates of membranes with DNAse I (2mg/ml) decreases the amount of Triton-insoluble GPIIb-III and actin (Ac). A) Plasma membranes were exposed to ConA (150 μg/2 $\times$ 10^9 equivalents) in 20 mM HEPES-0.1 M NaCl-1 mM EDTA (pH 7.4) buffer for 15 min at 37°C. 1/10 volume of 20% Triton X-100 which contained nothing (control) or 4 mg/ml PMSF-treated DNAse I (+DNAse) or DNAse I (4mg/ml) and rabbit actin (5mg/ml) to inhibit DNAse-mediated depolymerization of actin. After incubation at 37°C for 30 min, Triton-insoluble pellets were harvested by centrifugation and analyzed by SDS-PAGE (8%) under reduced conditions. Left lane of each pair is Coomassie Blue stained, and the right lane is the corresponding autoradiogram. B) Identical to A above except $MgCl_2$ and ATP were added to the Triton-EDTA lysis buffer to yield a final free Mg^{2+} concentration of 2mM and an ATP concentration of 5mM. Note that in the presence of Mg^{2+}-ATP, a protein comigrating with myosin heavy chain was lost from the Triton pellet, whereas the actin and GPIIb-III content in the control was relatively unchanged. Under these conditions, DNAse I inhibited the ConA-induced insolubilization of GPIIb and III about 50%.

buffer, then the bulk of GPIIb and III sedimented with the Triton insoluble membrane fraction (Fig. 3A, control lanes). The left lane of each pair shows the Coomassie Blue staining pattern paired with the autoradiographic image on the immediate right. Addition of DNAse (+DNAse) to the Triton EDTA buffer 2 mg/ml decreased the amount of sedimentable actin and GPIIb and III only slightly (~ 10–15% of control). This small decrease appeared to be specific, as addition of a slight molar excess of rabbit f-actin (Fig. 3A; +DNAse, + actin) to the DNAse I solution blocked the decrease in GPIIb and III seen with DNAse I alone.

Because of the large amount of endogeneous myosin associated with the platelet membranes, it was suspected that the small effects seen within DNAse I might be due to a protective effect of myosin bound to membrane actin. To test this hypothesis, $MgCl_2$ in excess over the EDTA concentration and ATP (5 mM) was included in the Triton buffer in order to dissociate the myosin from the detergent-insoluble actin complex. Figure 3B shows that Mg^{2+}-ATP effectively elutes the 200 k dalton species that comigrated with myosin heavy chain while only slightly decreasing the amount of sedimentable actin recovered (Fig. 3B, control). Addition of DNAse I under these conditions resulted in a 50–55% reduction in the amount of GPIIb and III associated with the Triton insoluble pellet fraction (Fig. 3B + DNAse). As the corresponding Coomassie Blue stained lane shows, the amount of sedimentable actin was reduced by a comparable amount. Addition of rabbit f-actin completely prevented this effect (Fig. 3B + DNAse, + actin). In all cases, proteins lost from the pellet fractions were quantitatively recovered intact in the corresponding supernatant fraction (not shown).

Thus, the cosedimentation of f-actin and platelet surface glycoproteins in ConA-treated plasma membranes is dependent on the polymerization state of actin. In addition, as previously shown, DNAse I has no effect on ConA binding to GPIIb and III *per se* [4]; these results indicate that the cosedimentation of GPIIb and III with the Triton-insoluble fraction is not the result of ConA-induced precipitation of GPIIb or III. Finally, the Mg^{+2}-ATP results confirm that the major 200 k dalton protein is myosin and show that it is probably bound to the membrane via f-actin and does not play a role as far as the binding of GPIIb and III to the Triton insoluble fraction is concerned.

Crosslinking of GPIIb and III to cytoskeletal proteins with bifunctional protein crosslinking reagents. The DNAse I results suggest a direct or indirect physical linkage between actin and GPII-III. In order to determine if direct protein-protein interactions were involved, ConA-treated plasma membranes were treated with a reducible, bifunctional protein crosslinker, dimethyl-3,3′-dithiobisproprioimidate (DTBP). Figure 4A shows the effect of increasing concentrations of DTBP on ConA-treated plasma membranes derived from resting platelets as judged by SDS-PAGE analysis on nonreduced 5% acrylamide gels. As the concentration of cross-linker increased, a major crosslinked species appeared that just entered the running gel. Concomitantly, myosin heavy chain disappeared, and GPIIb and III decreased slightly as judged by scanning densitometry. Crosslinker at 40 μg/ml appeared to yield a significant amount of crosslinked species that just entered the nonreduced gel; thus, larger aliquots of this sample were subjected to two-dimensional gel electrophoresis (5% nonreduced, 8% reduced) as described by Wang and Richards [21]. As shown in Figure 4B, the major crosslinked species that just entered the nonreduced gel was composed of GPIIb, III, actin and myosin, and a number of other components. More significantly, lower nonreduced molecular weight species were noted (Fig. 4B,

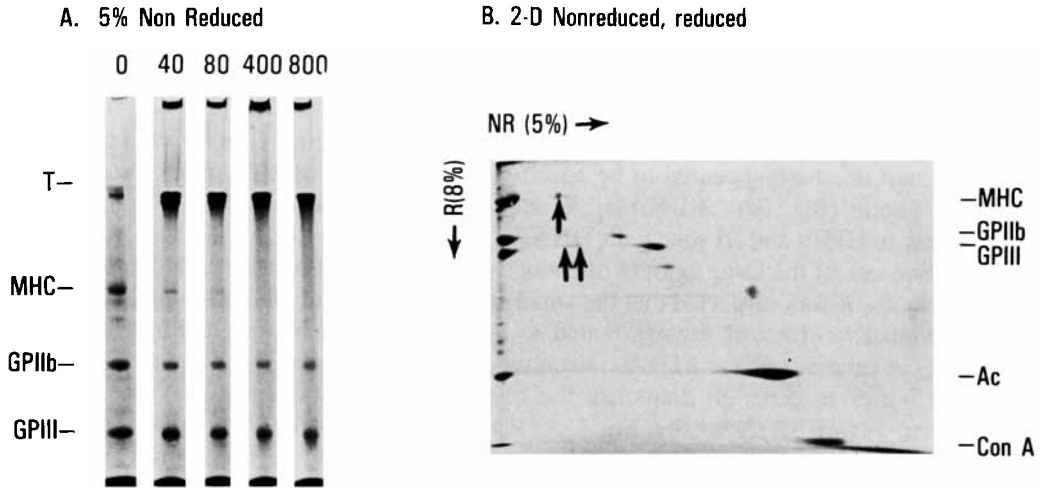

Fig. 4. Effect of DTBP on ConA-treated plasma membranes derived from unstimulated platelets. A) Effect of increasing concentrations of DTBP on the production of high molecular weight protein species. Electrophoresis was performed on 5% acrylamide in the *absence* of reducing agent. Numbers refer to the final concentration of DTBP used in μg/ml. ConA-treated membranes prepared as described in Figure 1 were incubated prior to Triton lysis. After quenching the reaction with the 10 mM ammonium chloride, Triton pellets and supernatants were isolated by centrifugation. Note the production of large molecular weight species with increasing concentration of crosslinker. B) A Wang-Richards 2-D gel of the 40 μg/ml sample shown in panel A. The nonreduced (NR) first dimension, which originates at the upper left hand corner, was 5% acrylamide. Note the presence of spots (arrows), falling well off the diagonal, that are composed of GPIIb, GPIII, and a 190/230 k dalton doublet (upper arrow). Larger molecular weight crosslinked species present near the top of the nonreduced gel are composed of similar components as well as actin (ac) and myosin (MHC). Both gels stained with Coomassie Blue.

arrows) that upon reduction were found to be composed of a doublet around 190–220 k daltons (upper arrow) and GPIIB and III (lower arrow). This component was not assessible for labeling to lactoperoxidase^{125}I surface labeling in intact cells (not shown).

The majority of the crosslinked surface GPIIb and III just entered the running gel in the nonreduced dimension, raising the possibility that differing homoaggregates were not separating in the first dimension. To explore this possibility, TX-100 lysates of membranes treated with or without ConA prior to crosslinking were subjected to electrophoresis in the nonreduced dimension on 1.25% acrylamide-0.5% agarose in order to increase the molecular weight separation range. These gels then were subjected to electrophoresis in the second dimension after reduction. As Figure 5A shows, Triton pellets of DTBP crosslinked membranes from unstimulated cells, reveal multiple crosslinked species which upon reduction were found to be composed of IIb, III, actin and several polypeptides of molecular weight around 200 k daltons. When ConA pretreated membranes were analyzed in an identical manner, similar results were obtained, with the exception that much more GPIIb and III were crosslinked into higher molecular weight species (Fig. 5B). The amounts of actin and the 200 k

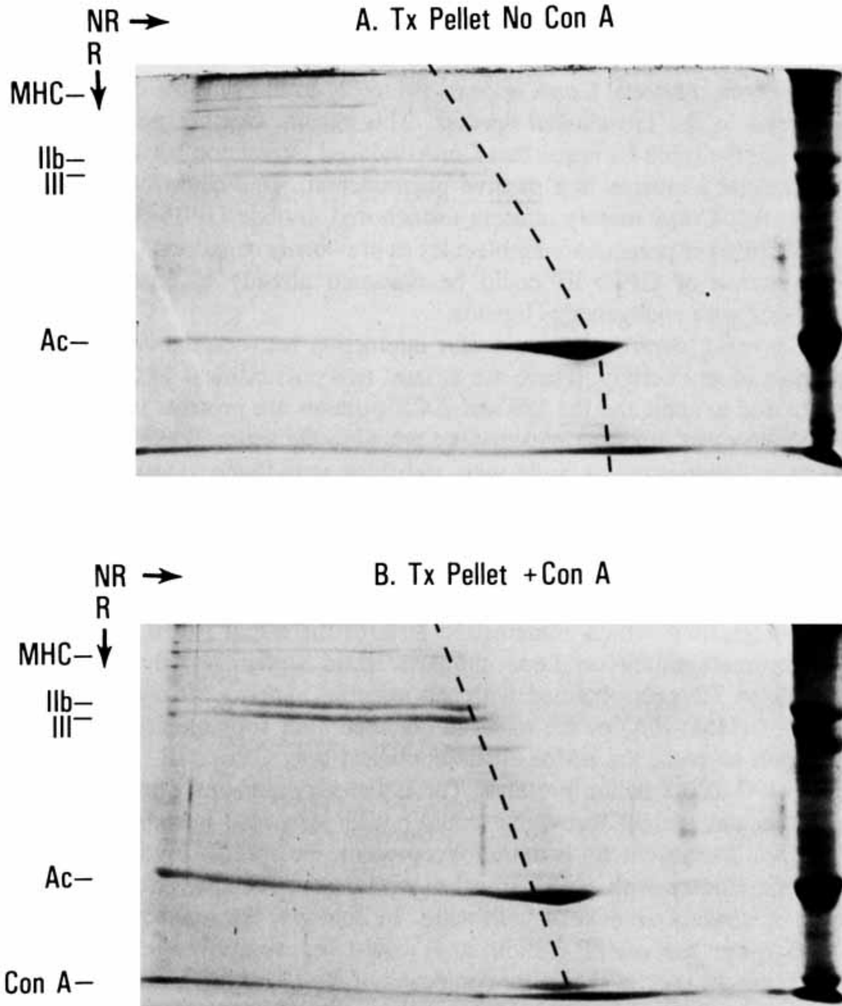

Fig. 5. Two dimensional Wang-Richards gels of Triton pellets of platelet membranes treated with 100 μg/ml DTBP. Membranes were treated with buffer (A) or with ConA (B) for 15 min at 37°C, prior to crosslinking and Triton X-100 extraction and isolation of Triton pellets. The first, nonreduced dimensions were performed using 1.25% acrylamide-0.5% agarose SDS with a Laemmli buffer system. The second, reduced dimension was run on an 8% SDS acrylamide gel, stained with Coomassie Blue. Note the multipile crosslinked species that fell off the diagonal (dashed line) that were composed of GPIIb, III, actin and, 180–230 k dalton proteins. The only apparent effect of ConA is to increase the amount of GPIIb-III crosslinked into the high molecular weight aggregates.

dalton components were similar to those seen in membranes not previously exposed to ConA. Of all the crosslinked components shown in the Coomassie Blue stained gel, only GPIIb and III showed significant ^{125}I surface label, indicating that these components (including actin) are unavailable for lactoperoxidase labeling in intact cells.

These results indicate that crosslinked species are formed that are composed of surface glycoprotein GPIIb and III, actin, and several components of molecular

weights around 180–230 k daltons. Furthermore, as Carraway et al [16] found with isolated cell microvilli, ConA treatment is not necessary for the demonstration of such interactions. Instead, ConA appears primarily to increase the *amount* of GPIIb-III recovered in the crosslinked species. This result, together with the metabolic inhibitor results (Table I), argue that ConA-induced interaction between GPII and III and cytoskeletal elements is a passive phenomenon. This could be rationalized by postulating that ConA merely clusters unanchored, mobile GPIIb-III around a small fraction (~ 10%) of preanchored molecules as previously suggested [4]. Alternatively, a small fraction of GPIIb-III could be clustered already by interaction of these glycoproteins with endogeneous ligands.

The precise hierarchy of molecular interaction between the various proteins in the complex is less certain. There are at least two possibilities: 1) GPIIb and III are directly bound to actin and the 180 and 200K proteins are proteins with actin binding properties (spectrin, for example) that are bound to the actin; 2) alternatively, the 180 and/or 200 k dalton proteins could serve to bridge actin to the transmembrane surface glycoproteins.

In order to investigate these two possibilities, membranes derived from unstimulated, surface iodinated platelets were treated with DTBP, and the Triton-insoluble pellet fraction was isolated.The pellet fraction was sonicated, recentrifuged, and the supernatant fraction, which contained >80% of the initial IIb-III, was purified by affinity chromatography on Lens culinaris lectin-Sepharose 4B. Figure 6 shows Wang-Richard 2D gels obtained with the material eluted with α-methyl-D-mannopyranoside (αMM) (6A) or the material obtained after subsequent elution with SDS (6B). As can be seen, the αMM eluate contained only GPIIb, III, and actin, and no detectable 190–220 k dalton proteins. These three components were crosslinked into higher molecular weight forms that could not be separated into discrete molecular-sized species. Because actin is not a glycoprotein, the specific elution of this protein from a lectin column with αMM, together with the crosslinking results, indicates that GPIIb-III is directly associated with actin. In contrast, the material that was eluted with SDS (after the αMM elution step) could be crosslinked into discrete, high molecular weight species that were composed of IIb-III, actin, and the 190 and 220 k dalton proteins seen previously in the crosslinked membranes (6B). Because actin and GPIIb-III can be isolated as a crosslinkable complex in the absence of the 190 and 220 k dalton proteins, these results clearly favor a model whereby IIb-III directly interact with actin with the two higher molecular weight species bound as accessory proteins. The fact that the crosslinked species are much larger when these proteins are present is consistent with the possibility that they are actin crosslinking proteins rather than linkage proteins. Because the attachment of GIIb-III to the actin-containing membrane complex is DNAse I sensitive (Fig. 3), it is also hypothesized that actin is present in the F form.

What might be the physiological role of the observed interaction between membrane actin and GPIIb-III? It is widely believed that GPIIb-III is the platelet receptor for fibrinogen (and fibrin) in activated platelets. The present data indicate that a small subfraction of GPIIb-III may be preanchored to membrane actin filaments. In addition, clustering of unanchored GPIIb-III by multivalent ligands such as ConA, antibodies, or potentially fibrin, would allow for the direct anchorage of the majority of surface GPIIb-III to the contractile apparatus in order to allow for subsequent clot retraction.

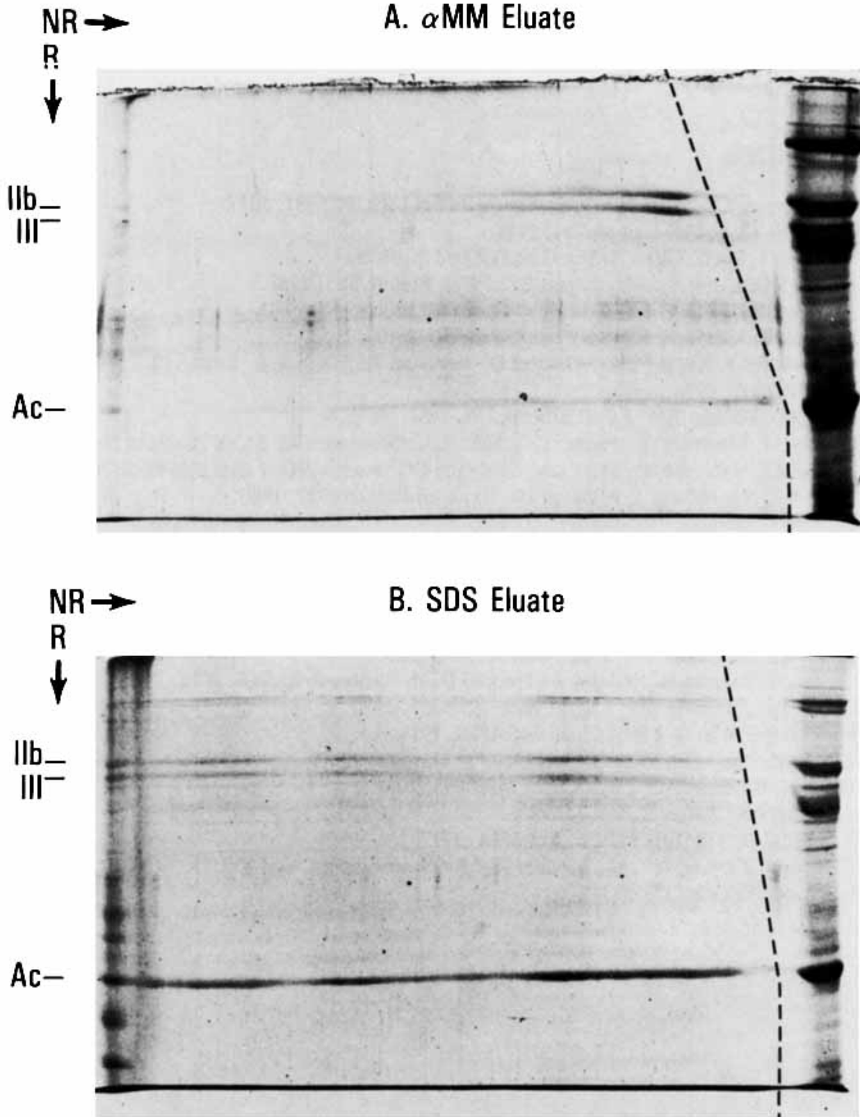

Fig. 6. Two dimensional Wang-Richards gels of Lens lectin-Sepharose 4B eluates after crosslinking with DTBP. The material was prepared by isolating Triton insoluble pellets of resting membranes. These pellets were subjected to sonication in Triton-EDTA, recentrifuged, and the supernatant applied to a Lens-lectin-Sepharose column. Fraction A is the material eluted with 0.5M αmethyl-D-mannopyranoside (αMM) and Fraction B is the remaining column-bound material eluted with 1% SDS.

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