

Evidence for the Selective Association of a Subpopulation of GPIIb-IIIa with the Actin Cytoskeletons of Thrombin-activated Platelets

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Abstract. Activation of blood platelets triggers a series of responses leading to the formation and retraction of blood clots. Among these responses is the establishment of integrin-mediated transmembrane connections between extracellular matrix components and the actin cytoskeleton of the platelet. Here we report that a specific subpopulation of the major platelet integrin, glycoprotein IIb-IIIa (GPIIb-IIIa) (also referred to as $\alpha_{\text{IIb}}\beta_3$ integrin), becomes incorporated into the detergent-insoluble actin cytoskeleton of platelets during the platelet activation response. The cytoskeletal association of GPIIb-IIIa is independent of platelet aggregation and fibrin sedimentation and is sensitive to cytochalasin D treatment. As determined by Western immunoblot analysis, ~22% of the total cellular GPIIb-IIIa becomes associated with the actin cytoskeleton upon thrombin activation in a manner that is independent of the detection of talin, α -actinin,

or vinculin in the complex. We found that the cytoskeleton-associated GPIIb-IIIa is derived from an intracellular source since it is not available for lactoperoxidase-catalyzed radioiodination before platelet activation. Two intracellular sources of GPIIb-IIIa are present in resting platelets: GPIIb-IIIa associated with the α -granule secretory compartment as well as surface-inaccessible domains of the surface-connected canalicular system. Interestingly, α -granule secretion, which occurs in thrombin-activated platelets and results in the translocation of intracellular GPIIb-IIIa to the plasma membrane, appears to be required for the cytoskeleton incorporation of GPIIb-IIIa that we observe. Collectively, our data provide evidence that a subpopulation of GPIIb-IIIa derived from an intracellular source is selectively linked to the actin cytoskeleton of platelets upon thrombin activation in the absence of platelet aggregation.

PLATELETS are anucleate cells that participate in the processes of hemostasis and thrombosis. Platelets exhibit highly regulated adhesion. They circulate in the bloodstream in a resting, nonadhesive state. Upon exposure to stimuli at the site of injury to a blood vessel, platelets become activated, undergo a dramatic shape change, and develop the capacity to adhere to each other and to the blood vessel wall (for review see Zucker and Nachmias, 1985). Subsequent platelet aggregation, which occurs when activated platelets interact with each other, as well as the platelet-dependent retraction of the fibrin clot, prevent further bleeding from the site of injury.

When platelets become activated and adhesive they establish a transmembrane connection between their contractile machinery and the extracellular matrix, a process that is essential for platelet-mediated clot retraction. This highly regulated process is thought to proceed via an activation-dependent reorganization of proteins to generate a transmembrane link between extracellular fibrin and the actin cytoskeleton of the platelet. One group of proteins capable of mediating interactions between cytoplasmic proteins and

the extracellular environment is the integrin family. Integrins are transmembrane, heterodimeric ($\alpha\beta$) glycoproteins that serve as receptors for extracellular matrix components (for review see Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Albelda and Buck, 1990). It has recently been shown that the cytoplasmic domain of the receptor is also essential for certain aspects of integrin function. In vitro binding studies as well as analysis of cells transfected with β -integrin deletion constructs have revealed that the cytoplasmic domain of integrin is required both for receptor localization at focal contacts (Solowska et al., 1989; Marcantonio et al., 1990) and for association with the cytoskeleton (Horwitz et al., 1986; Tapley et al., 1989; Otey et al., 1990). The duality of integrin function makes proteins in this family likely candidates for mediating transmembrane interactions between extracellular matrix components and the cytoplasmic actin cytoskeleton.

The major extracellular matrix receptor on the platelet surface, the glycoprotein complex GPIIb-IIIa = $\alpha_{\text{IIb}}\beta_3$ (GPIIb-IIIa)¹, is a member of the integrin family of cell adhesion molecules consisting of the α_{IIb} and β_3 subunits.

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1. *Abbreviations used in this paper:* GPIIb-IIIa, glycoprotein IIb-IIIa = $\alpha_{\text{IIb}}\beta_3$ integrin; PVDF, polyvinylidene difluoride; SCCS, surface-connected canalicular system.

GPIIb-IIIa has been identified as a platelet fibrinogen receptor (Bennett and Vilaire, 1979; Marguerie et al., 1979; Bennett et al., 1983; Parise and Phillips, 1985) as well as a receptor for fibronectin (Ginsberg et al., 1983; Parise and Phillips, 1986), von Willebrand factor (Ruggeri et al., 1982), and vitronectin (Pytela et al., 1986). GPIIb-IIIa may also interact with other extracellular matrix components, such as collagen (Kotite et al., 1984; Shadle et al., 1984). Ligand binding by GPIIb-IIIa is dependent on platelet activation (for review see Phillips et al., 1988; Plow and Ginsberg, 1989). Recent studies suggest that activation of platelets by thrombin and other agonists leads to a conformational change in the GPIIb-IIIa complex which enables GPIIb-IIIa to function as an active receptor (Shattil et al., 1985b; Frelinger et al., 1988; O'Toole et al., 1990; Du et al., 1991). The highly regulated process of platelet aggregation is known to be mediated by GPIIb-IIIa binding to fibrinogen (Bennett and Vilaire, 1979; Marguerie et al., 1979; Peerschke et al., 1980). Additionally, GPIIb-IIIa has been shown to be associated with the actin cytoskeleton in aggregated platelets (Phillips et al., 1980; Painter and Ginsberg, 1982). GPIIb-IIIa is therefore believed to link the actin cytoskeleton of platelets to extracellular matrix components.

In addition to mediating a structural connection between extracellular and cytoplasmic compartments, integrins may participate directly in transmembrane signal transduction. In a variety of cells, the binding of integrins to their extracellular ligands leads to the activation of signaling molecules including tyrosine kinases, ion channels, and transcription factors that could regulate adhesion-stimulated changes in cell behavior. In platelets for example, the tyrosine kinase pp125^{FAK} is activated by integrin engagement (Lipfert et al., 1992). Interestingly, the activation of pp125^{FAK} is blocked if the actin cytoskeleton is perturbed by pretreatment of the platelets with cytochalasin D (Lipfert et al., 1992), an observation that points out the importance of the actin cytoskeleton in the signal transduction response. Taken together with the demonstration that tyrosine kinase activity can be stimulated by integrin clustering (Kornberg et al., 1991), an event that is widely postulated to require a cytoskeletal linkage *in vivo*, it appears that integrin signaling potential may depend on both receptor engagement and cytoskeleton attachment.

The molecular mechanism by which actin is linked to integrins in platelets has not been defined. In other cell types, such as fibroblasts, a number of proteins have been identified that are thought to mediate actin-membrane interactions (for review see Burrige et al., 1988; Crawford and Beckerle, 1990; Beckerle and Yeh, 1990; Geiger and Ginsberg, 1991; Luna et al., 1992). Talin has been shown to interact with the cytoplasmic domain of the $\beta 1$ subunit of integrin (Horwitz et al., 1986; Buck and Horwitz, 1987) as well as with vinculin (Otto, 1983; Burrige and Mangeat, 1984). Vinculin, in turn, can associate with the actin cross-linking protein, α -actinin (Belkin and Kotliansky, 1987; Wachsstock et al., 1987). This series of protein-protein interactions could form a bridge between the cytoplasmic actin cytoskeleton and the transmembrane receptors for extracellular matrix components, thereby linking the contractile machinery of the cell to the substratum. Other mechanisms have also been proposed by which actin-membrane interactions could be mediated in cells. For example, α -actinin, which has been shown to bind to $\beta 1$ and $\beta 3$ integrin subunits *in vitro* (Otey et al.,

1990), could serve to link integrin directly to the actin cytoskeleton or actin itself may be directly linked to the membrane (Painter et al., 1985b).

We have used a biochemical approach to examine the regulated development and molecular composition of integrin-cytoskeleton linkages in platelets. Here we report that 22% of the cellular GPIIb-IIIa becomes associated with the actin cytoskeleton of thrombin-activated, nonaggregated platelets. This is a novel finding since previously it was reported that the linkage of GPIIb-IIIa to the actin cytoskeleton was a late response to agonist that depended on platelet aggregation (Phillips et al., 1980; Painter and Ginsberg, 1982). Furthermore, we show here that the incorporation of GPIIb-IIIa into the actin cytoskeleton is a selective process involving a specific subpopulation of GPIIb-IIIa derived from an intracellular pool.

Materials and Methods

Platelet Isolation and Activation

Washed human platelets were prepared from freshly drawn whole blood from aspirin-free, adult donors essentially as described by Fox and Phillips (1982) except that we centrifuged the platelet-rich plasma at 560 g for 20 min, washed the platelets two times in a 37°C citrate-glucose-sodium chloride solution (13 mM sodium citrate dihydrate, 30 mM glucose, 120 mM sodium chloride, pH 7), and isolated the platelets after each wash by centrifugation at 560 g for 15 min. Platelets were resuspended in Tyrode's buffer (138 mM NaCl, 2.9 mM KCl, 12 mM NaHCO₃, 0.36 mM Na₂HPO₄, 5.5 mM glucose, 1.8 mM CaCl₂, and 0.49 mM MgCl₂) at a concentration of 10⁹ platelets/ml (as counted using a hemacytometer) and allowed to incubate at 37°C for 30–60 min to restore a resting, discoid shape (Nachmias, 1980; Fox et al., 1984). Platelets were activated with either thrombin (0.1 U/ml, Calbiochem-Novabiochem Corp., La Jolla, CA) or ADP (100 μ M, Sigma Chem. Co., St. Louis, MO) for 15 min in the absence of stirring to prevent platelet aggregation. One minute before detergent lysis, 5 mM EDTA/5 mM EGTA was added to resting and activated platelet samples to increase the stability of the actin cytoskeletons during their isolation. We obtain essentially identical results if divalent cations are chelated 15 min before platelet activation. To examine the importance of actin filament assembly, resting platelets were treated with cytochalasin D (Sigma Chem. Co.) in ethanol (2.5 $\times 10^{-5}$ M cytochalasin D in 0.5% ethanol) or ethanol alone for 1 h immediately after their isolation.

Platelet activation and aggregation were monitored using a Model 500 Lumi-Aggregometer (Chrono-Log Corp., Havertown, PA). Additionally, small aliquots of resting and activated platelets were taken from the experimental samples and fixed in preparation for light microscopic observation. Fixed samples were viewed by phase contrast microscopy to confirm that the resting platelets exhibited a discoid shape and that thrombin-activated samples had undergone a shape change but were not aggregated. If these conditions were not met, the samples were discarded.

Isolation and Analysis of Platelet Lysates

Platelets were lysed 15 min after activation. Detergent-soluble and insoluble platelet fractions were prepared using a modification of the method described by Phillips et al. (1980). Intact resting and activated platelets in solution were lysed by mixing with one volume of a 2 \times Triton lysis buffer containing 2% Triton X-100, 0.01 M EGTA, 0.01 M EDTA, 0.1 M Tris-HCl, pH 7.4, and the protease inhibitors leupeptin (2 $\times 10^{-5}$ M), phenylmethylsulfonyl fluoride (0.2 mM), benzamidin HCl (0.2 mM), pepstatin A (2 ng/ml), and 1,10-phenanthroline (2 ng/ml) (Sigma Chem. Co.) in Tyrode's buffer. To inhibit thrombin activity, hirudin (final concentration, 0.2 U/ml) was added immediately before lysis. After detergent treatment, the samples were spun in an Eppendorf microcentrifuge (Brinkmann Instrs. Co., Westbury, NY) for 4 min at 8,730 g (Phillips et al., 1980). The detergent-soluble fraction (supernatant) was mixed at a ratio of 2:1 with 3 \times Laemmli sample buffer (Laemmli, 1970) and boiled for 4 min. The detergent-insoluble pellets were washed without resuspension in 1 \times detergent lysis buffer and recentrifuged. The final pellets were resuspended in 200 μ L of 1 \times lysis buffer plus 100 μ L of 3 \times Laemmli sample buffer and boiled for 4 min. As

a result of this preparation, the final detergent-insoluble samples were concentrated five times relative to the detergent-soluble samples. The proteins present in each fraction were resolved by 10% SDS-PAGE as described by Laemmli (1970) except that the bisacrylamide concentration was 0.13%. Molecular mass markers were purchased from Bio-Rad Labs. (Hercules, CA). Polypeptide bands were detected by staining the gels with Coomassie brilliant blue (Sigma Chem. Co.) Western immunoblot analysis was performed according to the method of Towbin et al. (1979) with the modifications described by Beckerle (1986). The ^{125}I -protein A used in immunoblots was purchased from ICN Biomedicals, Inc. (Irvine, CA). Densitometry was performed on Coomassie blue-stained gels and autoradiograms using either a Sony CCD (monochrome-charged couple device) video camera attached to a Macintosh IICI computer with an NIH Image 1.36 software package or the Phosphor Imager (model 400E) with an Image Quant 3.2 software package (Molecular Dynamics). Because of the large amounts of actin present in cytoskeletal pellets isolated from both resting and thrombin-activated platelets, differences in actin content between these samples could only be detected by video densitometry of Coomassie blue-stained gels on which smaller amounts of protein were loaded.

Iodination of Platelets

Aliquots of platelets (10^9 cells/ml) were radiolabeled at room temperature by adding 1 mCi $\text{Na-}^{125}\text{I}$ (Amersham Corp., Arlington Heights, IL) per ml platelets, 4.2 U/ml lactoperoxidase (Sigma Chem. Co.) in PBS (2.7 mM KCl, 1.1 mM KH_2PO_4 , 138 mM NaCl, 8.1 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mM CaCl_2 , and 1 mM MgCl_2), 20 mM β -D-glucose (Sigma Chem. Co.), and 0.025 U/ml glucose oxidase (Sigma Chem. Co.) which catalyzes the generation of H_2O_2 from the β -D-glucose substrate (Schenkein et al., 1972). The samples were incubated for 10 min with frequent stirring. Saturated tyrosine solution was then added and samples were incubated an additional 5 min. The reaction volume was then raised to 10 ml with a washing buffer containing 0.154 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.4 (Phillips and Agin, 1977). The radiolabeled platelets were sedimented by centrifugation, washed once more, and resuspended to the original volume in Tyrode's buffer. Control samples were subjected to the same treatment, with the exception that unlabeled NaI was used in place of $\text{Na-}^{125}\text{I}$, and were analyzed by Western immunoblot. Activation of iodinated platelets and isolation of actin cytoskeletons were performed as described above. Similar results in terms of cytoskeletal composition and the amount of iodinated GPIIb-IIIa associated with the cytoskeletons were obtained for platelets activated in the presence of divalent cations and those activated in the presence of divalent cation chelators. Radiolabeled proteins were resolved by 7.5% SDS-PAGE and visualized by autoradiography.

Antibody Preparation and Characterization

B11 and B5 rabbit polyclonal antisera were raised against human platelet talin and GPIIb-IIIa, respectively (Beckerle et al., 1989). When used for Western blots of two-dimensional gels (O'Farrell, 1975) of total human platelet protein, the B5 antibody recognized a single pair of spots corresponding to authentic GPIIb-IIIa (data not shown). Additionally, no detectable bands were present when B5 antibody was used for Western immunoblots of total platelet protein from Glanzmann thrombasthenic platelets (our unpublished observations) which lack detectable GPIIb-IIIa (Glanzmann thrombasthenic platelets were provided by Dr. Paul Bray, Johns Hopkins University, Baltimore, MD). B15 antiserum was raised against human platelet vinculin. Anti- α -actinin antibody was the generous gift of Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC) and was raised in rabbits against bovine cardiac α -actinin. These antibodies were characterized by Western immunoblot analysis as described in the text. S12 monoclonal antibody against the α -granule membrane glycoprotein, P-selectin, and a goat anti-human P-selectin polyclonal antibody were the generous gifts of Dr. Rodger P. McEver (University of Oklahoma, Oklahoma City, OK) and have been extensively characterized (McEver and Martin, 1984; Stenberg et al., 1985). The rabbit polyclonal antibody against human fibrinogen was obtained from Cappel Labs., (Durham, N.C.).

Fluorescence Microscopy

Rhodamine Phalloidin Labeling of Intact Cells and Actin Cytoskeletons. Platelet lysates were prepared from samples of resting and thrombin-activated platelets as described above. Samples of detergent-lysed platelets (400 μL) were fixed in 3 vol of 3.7% formaldehyde in PBS. These samples were then diluted with Tyrode's buffer (total volume, 5 ml), and the

detergent-insoluble material was centrifuged (130 g) onto Alcian blue-coated coverslips placed on inserts in the bottom of 15 mL Corex tubes. The coverslips were then rinsed in TBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 0.1% sodium azide) for 5 min at room temperature, incubated in 1% BSA in TBS for 30 min at 37°C, rinsed for 10 min in TBS, and incubated in rhodamine-labeled phalloidin (Molecular Probes Inc., Eugene, OR) at a dilution of 1:50 in TBS for 1 h at 37°C. The coverslips were then rinsed in TBS for 10 min and mounted on slides using Gelvatol (Air Prods. & Chem. Inc., Allentown, PA).

Indirect Immunofluorescence. Fixed samples of resting and activated platelets were prepared for indirect immunofluorescence as described previously (Beckerle et al., 1989). The S12 antibody against P-selectin was used at a concentration of 2 $\mu\text{g}/\text{ml}$ to stain unpermeabilized cells. The secondary antibody, rhodamine-labeled goat anti-mouse IgG, was purchased from Cappel Labs. Photomicrographs were taken on a fluorescence microscope (Axiophot; Carl Zeiss Inc., Thornwood, NY) equipped with a 100 \times Plan-Neofluor objective. Quantitative analysis of P-selectin expression on platelet surfaces was performed as follows: the platelets were observed by differential interference contrast microscopy and fields containing 25 or more platelets were selected at random and photographed. The same fields were then photographed for fluorescent staining. The percentage of cells exhibiting a detectable ring of fluorescence, representative of significant S12 binding, was then determined for 300–600 cells under each condition in independent experiments using a different blood donor for each experiment.

Inhibition of α -Granule Secretion

The α -granule release response of thrombin-stimulated platelets was prevented by adding the metabolic inhibitors 2-deoxyglucose (15 mM in 0.15 M NaCl) and gluconolactone (20 mM in 0.15 M NaCl) to samples of resting platelets 5 min before thrombin addition (Holmsen et al., 1974; Tuszynski et al., 1982). To verify that these metabolic inhibitors did indeed block α -granule secretion, we performed indirect immunofluorescence on fixed, unpermeabilized samples of inhibitor-treated platelets using the S12 antibody against P-selectin. Although α -granule secretion was blocked, activation-dependent shape change still occurred, as observed by phase contrast microscopy. Twofold higher concentrations of the inhibitors blocked shape change as well as secretion, while twofold lower concentrations did not have a significant effect on the secretion response. Because of concerns regarding the toxicity of these metabolic inhibitors on platelets, individual experiments in which normal, resting platelet morphology was altered or thrombin-stimulated shape change failed to occur, were excluded from further analysis. The addition of 0.15 M NaCl alone had no effect on either the platelet activation response or the activation-dependent incorporation of GPIIb-IIIa into the actin cytoskeleton.

Protein Microsequencing

Polyacrylamide gels (10%) were made with recrystallized SDS (Hunkapiller et al., 1983) and pre-run for 2 h at 25 V in running buffer containing 50 μM glutathione (Sigma Chem. Co.) (Yuen et al., 1986; Moos et al., 1988). The running buffer was changed before sample electrophoresis, and 100 mM sodium thioglycolate (Sigma Chem. Co.) was added to a final concentration of 0.1 mM (Hunkapiller et al., 1983; Yuen et al., 1986; Moos et al., 1988). These free radical scavengers were added to prevent destruction of amino acid side chains by oxidants and free radicals in the gel (Hunkapiller et al., 1983). After sample electrophoresis, the proteins were electroeluted onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA) (Matsudaira, 1987; Yuen et al., 1988). The PVDF membranes were then stained with Coomassie blue (Matsudaira, 1987) and the 110-kD band was excised and stored at -20°C . The protein was sequenced on a model 477A protein sequencer (Applied Biosystems Inc., Foster City, CA). The amino acid sequence was analyzed using the University of Wisconsin GCG software package (University of Wisconsin Biotechnology Center, Madison, WI).

Results

Platelets can be isolated in the resting state and then activated *in vitro* by exposure to a variety of agonists. The activation process can be monitored by light microscopy as well as aggregometry. Resting platelets exhibit a characteristic discoid shape by light microscopy and do not aggregate spontaneously

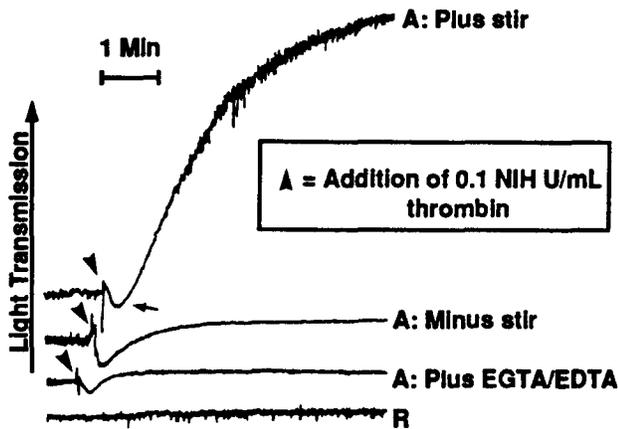


Figure 1. Aggregometer tracings of resting, activated, and aggregated platelets. Suspensions of resting platelets (*R*) are turbid, allowing little light to transmit through the sample. In response to thrombin activation (*arrowheads*), platelets undergo a shape change as marked by a decrease in light transmission (*arrow*). When activated platelets contact each other (*A: Plus stir*) they form aggregates allowing an increasing amount of light to transmit through the sample. The addition of divalent cation chelators (*A: Plus EGTA/EDTA*) or the absence of stirring (*A: Minus stir*) prohibits platelet aggregation; however, shape change still occurs.

as shown by aggregometer tracings (Fig. 1, *R*). Activation-dependent shape change (*arrow*) occurs almost immediately after the addition of 0.1 NIH U/ml thrombin (*arrowheads*). When suspensions of thrombin-activated platelets are stirred in the presence of divalent cations, such as Mg^{2+} and Ca^{2+} , the platelets aggregate as demonstrated by an increase in light transmitted through the sample (Fig. 1 *A: Plus stir*). It is possible to prevent platelet aggregation by incubating the activated cells without stirring to minimize platelet-platelet contact (Fig. 1 *A: Minus stir*) or by adding divalent cation chelators before activation (Fig. 1 *A: Plus EGTA/EDTA*). Incubation of platelets at 37°C in the presence of divalent cation chelators results in dissociation of plasma membrane-associated GPIIb-IIIa complexes which prevents fibrinogen binding and completely inhibits platelet aggregation (Zucker and Grant, 1978; Jennings and Phillips, 1982; Carrell et al., 1985; Fitzgerald and Phillips, 1985; Shattil et al., 1985a; Pidard et al., 1986).

Platelets exhibit elaborate actin cytoskeletons which are thought to be important in the contractile response of these cells (Fox, 1985; Fox et al., 1988). Results of a variety of morphological and biochemical studies have demonstrated that the cytoplasmic actin filament network undergoes a dramatic reorganization upon platelet activation, enabling platelets to participate in blood clot formation and retraction (Nachmias, 1980; Jennings et al., 1981; Fox and Phillips, 1983; Fox et al., 1984). It is possible to isolate a complex containing filamentous actin and associated proteins from resting or activated, nonaggregated platelets by detergent lysis of the cells in the presence of divalent cation chelators and protease inhibitors followed by centrifugation at low *g* forces. We refer to this detergent-insoluble complex as the actin cytoskeleton or platelet cytoskeleton throughout the text. As can be seen in Fig. 2 *B*, actin cytoskeletons isolated from activated platelets by this method are brightly labeled with rhodamine phalloidin which reveals the presence of

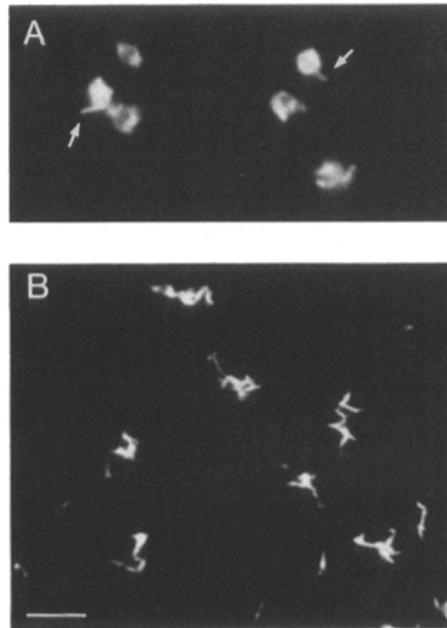


Figure 2. Actin-rich cytoskeletal structures are present in the isolated detergent-insoluble material. The distribution of filamentous actin in intact, thrombin-activated platelets is compared to that in the isolated detergent-insoluble cytoskeletons by rhodamine phalloidin labeling. *A* shows the organization of filamentous actin in activated platelets. Actin-rich filopodia extend from the surfaces of activated platelets (*arrows*). The cytoskeletal structures in *B* are similar in size to intact cells and are rich in filamentous actin. Bar, 5 μ m.

filamentous actin. These structures exhibit a stellate, platelet-sized morphology (see Fig. 2 *A* for comparison to intact platelets). As will be shown in later figures, actin and myosin are the two most prominent proteins present in these preparations which supports our contention that the isolated material does indeed represent a cytoskeletal fraction. Our ability to isolate significant amounts of cytoskeletal material was dependent on the presence of divalent cation chelators at least 1 min before detergent lysis (not shown). We believe that the chelators stabilize the cytoskeletons during cell lysis and centrifugation, as has been suggested by others (Rosenberg et al., 1982; Fox et al., 1988).

We have used SDS-PAGE and Western immunoblot analysis to study the molecular composition of the isolated cytoskeletons. In particular we have examined whether GPIIb-IIIa, talin, α -actinin, and vinculin become incorporated into the actin cytoskeleton of platelets in an activation-dependent, aggregation-independent manner. All of these proteins have been postulated to play pivotal roles in forming membrane-actin cytoskeleton linkages (Luna et al., 1992). The specificity of the polyclonal antibodies against GPIIb-IIIa, talin, α -actinin, and vinculin is demonstrated in the Western immunoblots shown in Fig. 3. We examined the polypeptide composition of the actin cytoskeletons isolated from resting and thrombin-activated platelets (Fig. 4 *A*). Actin (*A*, 43 kD) and myosin (*M*, 200 kD) are the primary constituents of the isolated cytoskeletons. Some actin-binding protein (*ABP*, 250 kD), or filamin, is also associated with the cytoskeletal pellets. As has been noted by previous investigators (Phillips et al., 1980; Pribluda and Rotman, 1982; Fox

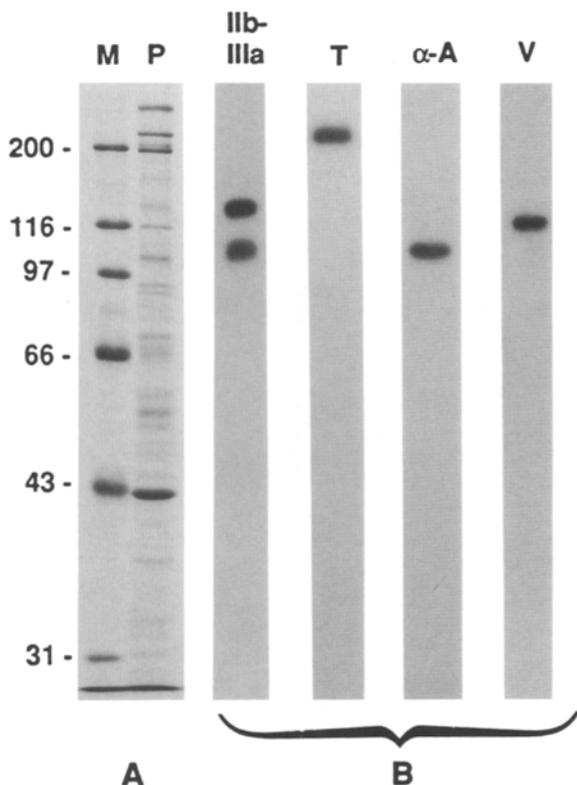


Figure 3. Characterization of antibodies directed against GPIIb-IIIa, talin, α -actinin, and vinculin. The specificity of the antibodies used in our studies is shown here by Western immunoblot analysis. (A) A Coomassie blue-stained gel of molecular mass standards (M) and total human platelet protein (P). (B) Parallel Western immunoblots of human platelet proteins probed with the following antibodies: anti-GPIIb-IIIa (Iib-IIIa), anti-talin (T), anti- α -actinin (α -A), and anti-vinculin (V).

and Phillips, 1983; Tuszyński et al., 1985), we detect the association of several new proteins cosedimenting with the actin cytoskeleton upon platelet activation (compare lanes Rp and Ap in Fig. 4 A). For example, we detect a polypeptide at ~110 kD in actin cytoskeletons isolated from platelets activated in the presence of Ca^{2+} and Mg^{2+} (Fig. 4 A, lane Ap). Although this protein migrates close to the position of α -actinin on 10% polyacrylamide gels, by Western immunoblot analysis we have determined that this 110-kD polypeptide is not α -actinin (not shown). To identify this protein, the electrophoretically isolated protein was subjected to direct microsequence analysis. We obtained 15 amino acids of NH_2 -terminal sequence and determined that the sole sequenceable protein migrating with an apparent mass of 110 kD is γ -fibrinogen (Fig. 5). The 50-kD γ -fibrinogen subunit self-associates to generate covalently linked dimers when platelets are activated with thrombin in the presence of Ca^{2+} (McDonagh and McDonagh, 1972; Fox et al., 1983). It is formally possible that other proteins are also present at this molecular weight but are NH_2 -terminally blocked and therefore failed to be sequenced.

Western immunoblot analysis was used to examine the cytoskeletons of resting and thrombin-activated platelets for the presence of the major platelet integrin, GPIIb-IIIa. As can be seen in Fig. 4 B, the amount of GPIIb-IIIa incorpo-

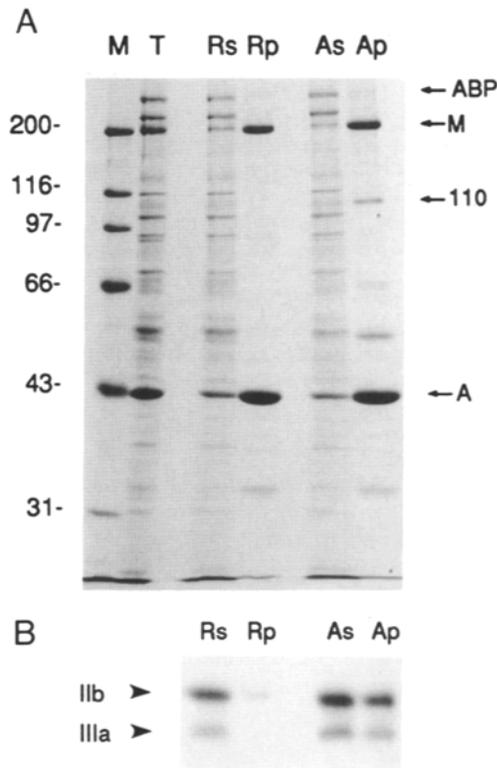


Figure 4. An activation-dependent increase in the amount of GPIIb-IIIa associated with the platelet cytoskeleton. Resting or thrombin-activated platelets that had been incubated in the presence of $\text{Ca}^{2+}/\text{Mg}^{2+}$ were treated with EGTA/EDTA for 1 min, lysed with Triton X-100-containing buffer, and centrifuged to separate the detergent-soluble (supernatant) and detergent-insoluble (pellet) fractions. (A) A Coomassie blue-stained gel of molecular mass standards (M), total human platelet protein (T), resting platelet supernatant (Rs), resting platelet pellet (Rp), activated platelet supernatant (As), and activated platelet pellet (Ap). As loaded on the gels, pellet samples are five times more concentrated than supernatant samples. Arrows point to actin-binding protein (ABP), myosin (M), a 110-kD protein (110), and actin (A). (B) A corresponding Western immunoblot probed with an antibody against GPIIb-IIIa. The amount of GPIIb-IIIa associated with the detergent-insoluble, cytoskeletal fraction increases in an activation-dependent manner.

rated into the detergent-insoluble actin cytoskeleton increases upon platelet activation. We observed that ~22% of the total GPIIb-IIIa pool is associated with actin cytoskeletons isolated from thrombin-activated, nonaggregated platelets (Table I). In the experiment shown here (Fig. 4), resting and activated platelets were maintained at 37°C without stirring in a physiological buffer containing the divalent cations Ca^{2+} and Mg^{2+} , and divalent cation chelators were added to the platelet suspensions 1 min before lysis, a step required for the effective isolation of the cytoskeletons. Immediately before lysis, small aliquots were removed from each platelet sample and fixed for light microscopic evaluation of cell morphology; we determined directly that no platelet aggregation had occurred in the thrombin-activated samples we analyzed. Parallel samples were evaluated for the extent of platelet aggregation using a lumiaggregometer and, consistent with our light microscopic analysis, no aggregation was detected. Similar results in terms of both cytoskeletal com-

Protein Amino Acid Sequence

110 kDa NH₂-YVATRDNXXILDERF-
 ||||| |||||
 γ-Fbg NH₂-YVATRDNCCILDERF-

Figure 5. Comparison of the first fifteen amino acids of γ-fibrinogen and a 100-kD protein associated with cytoskeletons isolated from thrombin-activated platelets. We determined the NH₂-terminal sequence of a prominent 110-kD protein that becomes associated with cytoskeletons of thrombin-activated platelets. With the exception of two cysteine residues at amino acids 8 and 9 which could not be identified because the protein was not derivatized before sequencing, the first fifteen amino acids of the 110-kD protein are identical to the amino terminal sequence of γ-fibrinogen (γ-Fbg).

position and platelet aggregation were obtained for platelets activated in the presence of divalent cation chelators at 37°C (see Materials and Methods for details), a condition which has repeatedly been shown to completely prohibit platelet aggregation (Fitzgerald and Phillips, 1985; Shattil et al., 1985a; Pidard et al., 1986). Platelet aggregation is therefore not required to yield the level of GPIIb-IIIa-cytoskeleton association that we observe by Western immunoblot analysis.

The cytoskeletons of resting and thrombin-activated platelets were also analyzed by Western immunoblot for the presence of talin, α-actinin, and vinculin, proteins postulated to be involved in membrane-cytoskeleton interactions. In contrast to our findings for GPIIb-IIIa (Fig. 6 A), talin, α-actinin, and vinculin are detected exclusively in supernatants isolated from both resting and thrombin-activated platelets (Fig. 6, B-D). It is possible that very small, undetectable amounts of talin, vinculin, and/or α-actinin are associated with the actin cytoskeleton; however, by characterizing the detection limits of our Western immunoblot assays, we have determined that no greater than 1% of the total platelet complement of each of these proteins is associated with our isolated cytoskeletal pellets.

In previous studies designed to examine the incorporation of GPIIb-IIIa into the actin cytoskeleton, researchers observed GPIIb-IIIa associated with cytoskeletons only after platelet aggregation (see for example Phillips et al., 1980; Jennings et al., 1981; Painter and Ginsberg, 1982; Wheeler et al., 1984). However, in these studies, lactoperoxidase-catalyzed radioiodination of surface glycoproteins was used to monitor the cytoskeletal association of GPIIb-IIIa. Interestingly, in addition to plasma membrane-associated GPIIb-IIIa, resting platelets also contain an internal pool of GPIIb-IIIa associated with the membranes of α-granules and the deeper regions of the surface-connected canalicular system (SCCS) that is not available for surface labeling (Gogstad et al., 1981; Stenberg et al., 1984; Wencel-Drake et al., 1986; Woods et al., 1986). Consequently, we hypothesized that the cytoskeleton-associated GPIIb-IIIa that we detect by Western immunoblot could be derived from the internal pool

Table 1. Analysis of Cytoskeleton-Associated GPIIb-IIIa

Platelet samples (Cytoskeletal pellets)	GPIIb-IIIa (% of total)	(n)	(SEM)
Resting	2.4	10	0.6
Thrombin-activated	21.9	11	1.7

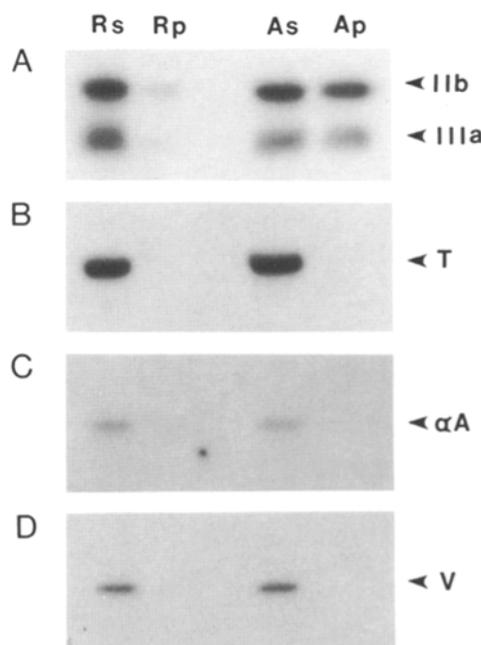
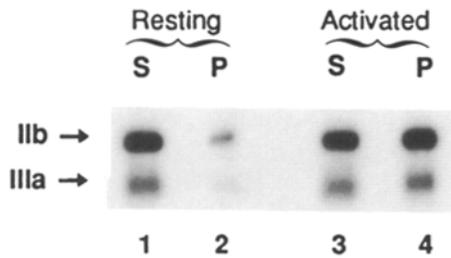


Figure 6. GPIIb-IIIa can associate with the platelet cytoskeleton in a talin-, α-actinin-, and vinculin-independent manner. Resting platelet supernatants (Rs), resting platelet pellets (Rp), activated platelet supernatants (As), and activated platelet pellets (Ap) were examined by Western immunoblot analysis for the presence of GPIIb-IIIa (A; Ib, IIIa), talin (B; T), α-actinin (C; αA), and vinculin (D; V). As loaded on the gels, pellet samples are five times more concentrated than supernatant samples. In contrast to our finding that the amount of GPIIb-IIIa associated with the actin cytoskeleton pellet increases upon thrombin-activation of platelets (A), talin (B), α-actinin (C), and vinculin (D) are detected exclusively in the detergent-soluble fractions from both resting and thrombin-activated platelets suggesting that these proteins are not required for the maintenance of the association between GPIIb-IIIa and the actin cytoskeleton of thrombin-activated, nonaggregated platelets. In the experiment shown in this figure, platelets were activated in the presence of EGTA/EDTA to ensure that no aggregation took place in the thrombin-activated samples; however, as noted in the text, the same results are obtained if platelets are activated in the presence of divalent cations, and chelators are added 1 min before cell lysis.

of GPIIb-IIIa, an interaction which would not be detected by examination of surface-labeled GPIIb-IIIa. To investigate this possibility more rigorously, we compared the two different approaches for monitoring GPIIb-IIIa in parallel experiments (Fig. 7). Consistent with the results described above (Fig. 4), we observed a significant increase in cytoskeleton-associated GPIIb-IIIa in response to thrombin activation when we used an immunological method for detecting GPIIb-IIIa (Fig. 7 A); in contrast, we observed only a negligible increase in GPIIb-IIIa associated with the actin cytoskeleton upon platelet activation when the protein was tagged by surface iodination (Fig. 7 B). Specifically, we found that the amount of surface-iodinated GPIIb-IIIa that becomes associated with the actin cytoskeleton increases by less than twofold upon thrombin activation in contrast to the ninefold increase in cytoskeleton-associated GPIIb-IIIa that we detect by Western immunoblot. The experiment shown in Fig. 7 was performed in the presence of EGTA/EDTA to ensure that no aggregation took place in the thrombin-activated

A Western Immunoblot



B Lactoperoxidase-Catalyzed Radioiodination

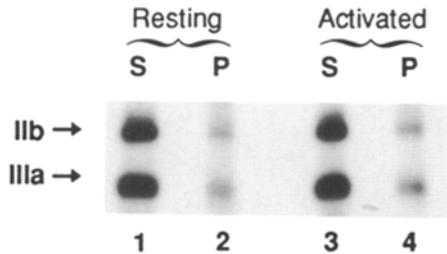


Figure 7. Intracellular GPIIb-IIIa becomes incorporated into the actin cytoskeleton of thrombin-activated platelets. Actin cytoskeletons were isolated from unlabeled platelets and platelets whose surface proteins had been iodinated using a lactoperoxidase-catalyzed radioiodination procedure. *S*, detergent-soluble supernatant (lanes 1 and 3); *P*, detergent-insoluble pellet (lanes 2 and 4). By Western immunoblot (*A*) we detect a significant increase in the amount of GPIIb-IIIa associated with actin cytoskeletons upon thrombin activation (compare lane 2 to lane 4). However, the amount of surface-iodinated GPIIb-IIIa associated with activated cytoskeletons does not increase significantly from the resting level (*B*, compare lane 2 to lane 4). In this representative experiment, the percentage of the total amount of radiolabeled GPIIb-IIIa present in cytoskeletal pellets (*B*) increased from 3% in the resting sample (lane 2) to 4% in the activated sample (lane 4). These results suggest that the majority of cytoskeleton-associated GPIIb-IIIa detected by Western immunoblot is derived from an intracellular pool of GPIIb-IIIa that is unavailable for surface-labeling.

samples. However, similar results were obtained when platelets were activated in the presence of divalent cations and the absence of stirring with the addition of chelators only 1 min before lysis (not shown). Based on these results, we believe that the use of Western immunoblot analysis has enabled us to detect the cytoskeletal incorporation of an intracellular GPIIb-IIIa pool which is inaccessible to lactoperoxidase-catalyzed radioiodination and therefore was not detected in previous studies in which only the surface-iodinatable pool of GPIIb-IIIa was examined.

Although we found an increase in GPIIb-IIIa-cytoskeleton association upon thrombin activation of platelets, it was surprising that GPIIb-IIIa appears to be associated with the platelet cytoskeleton in the absence of a number of other proteins postulated to be essential for integrin-actin interactions. It was therefore critical to ascertain whether GPIIb-IIIa is specifically associated with the actin cytoskeleton or if it is merely cosedimenting with actin. Zucker and Masiello's observation (1983) that significant amounts of phospholipids are present in detergent-insoluble cytoskeletons

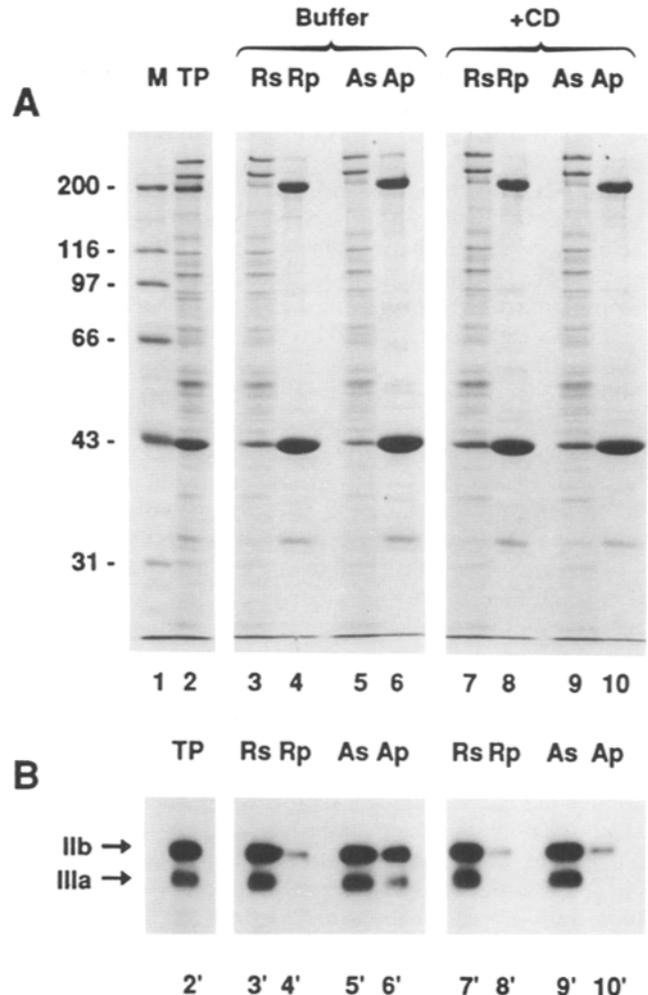


Figure 8. Inhibition of actin polymerization by cytochalasin D blocks the incorporation of GPIIb-IIIa into the activated platelet cytoskeleton. Platelets were incubated in buffer plus or minus cytochalasin D for one hour before platelet activation. 15 min after thrombin addition, detergent-soluble and insoluble fractions were isolated from resting and activated samples. (*A*) A Coomassie blue-stained gel of molecular mass standards (*M*; lane 1); total human platelet protein (*TP*; lane 2); resting platelet supernatants (*Rs*; lanes 3 and 7); resting platelet pellets (*Rp*; lanes 4 and 8); activated platelet supernatants (*As*; lanes 5 and 9); and activated platelet pellets (*Ap*; lanes 6 and 10). (*B*) A corresponding Western immunoblot probed with antibody directed against GPIIb-IIIa reveals that cytochalasin D blocks the activation-dependent increase in GPIIb-IIIa associated with the actin cytoskeleton (compare lane 6' with lane 10'). In this example, pellets are five times more concentrated than supernatants in order to detect changes in the amount of GPIIb-IIIa associated with the actin cytoskeleton. Densitometric analysis of gels in which smaller amounts of sample were loaded revealed that cytochalasin D-treated activated platelets contain 20% less sedimentable actin than control samples. The cytochalasin D carrier, ethanol, had no detectable effect on either actin content or the cytoskeletal incorporation of GPIIb-IIIa (not shown).

isolated from aggregated platelets raised the possibility that nonspecific trapping of GPIIb-IIIa-containing membrane vesicles by actin filaments could occur during the cytoskeleton isolation procedure and lead to the recovery of GPIIb-IIIa in the detergent-insoluble pellet. We do not believe that this is the case in our experiments for three reasons. First,

we did not observe a direct relationship between the amount of pelleted filamentous actin and the amount of GPIIb-IIIa recovered in the cytoskeletal pellet. For example, as can be seen in Fig. 4 A, there is a significant amount of filamentous actin present in cytoskeletons isolated from resting platelets but relatively little GPIIb-IIIa present in these samples (Table I). Moreover, quantitative analysis of the amounts of both actin and GPIIb-IIIa in the isolated cytoskeletons revealed no direct correlation between the amount of sedimented actin and integrin (data not shown). Second, the fact that the incorporation of GPIIb-IIIa into the actin cytoskeleton is selective, and results primarily from an internal pool of GPIIb-IIIa, argues against the general, nonspecific association of GPIIb-IIIa with the cytoskeleton. Third, although intracellular GPIIb-IIIa is detected in the activated platelet cytoskeleton, this GPIIb-IIIa cannot result from trapping of α -granule membranes since another α -granule membrane-associated protein, P-selectin (McEver, 1990), is not detected in our isolated actin cytoskeletons (data not shown). Collectively, these observations suggest that the incorporation of GPIIb-IIIa into the actin cytoskeleton occurs via a selective, activation-dependent mechanism and is not the result of nonspecific trapping of GPIIb-IIIa-containing membrane vesicles during the isolation of the detergent-insoluble material.

To test further whether GPIIb-IIIa is sedimenting by virtue of a specific association with the actin cytoskeleton, we examined the effect of cytochalasin D on the incorporation of GPIIb-IIIa into the detergent-insoluble pellet. Incubation of platelets in cytochalasin D before thrombin addition blocks the activation-dependent increase in the amount of filamentous actin present in the actin cytoskeleton, but does not cause appreciable disassembly of actin filaments already present in resting platelets (Fox and Phillips, 1981; Casella et al., 1981). Treatment of platelets with cytochalasin D blocks the incorporation of GPIIb-IIIa into the activated platelet cytoskeleton (Fig. 8 B, compare lanes 6 and 10). This result suggests that the sedimentation of GPIIb-IIIa with the actin cytoskeleton is dependent on the polymerization of filamentous actin and/or the assembly of these filaments into the actin cytoskeleton.

We also addressed the possibility that GPIIb-IIIa was sedimenting by virtue of an association with fibrin polymers and not with the actin cytoskeleton. Fibrinogen consists of α , β , and γ chains of ~ 68 kD, 55 kD, and 50 kD, respectively (Fig. 9 A, lane 2). In a multi-step process thrombin-mediated cleavage of fibrinogen generates fibrin monomers which further associate into polymers (for review see Doolittle, 1984). The rate at which fibrin monomers polymerize is Ca^{2+} -dependent (Boyer et al., 1972; Brass et al., 1978). Because fibrin polymers are detergent-insoluble (Casella et al., 1983), it is feasible that sedimentable fibrin could mediate the association of GPIIb-IIIa with the detergent-insoluble pellet in the absence of any interaction between GPIIb-IIIa and the actin cytoskeleton. Using Western immunoblot analysis, we compared the amount of GPIIb-IIIa with the amount of fibrin polymer in actin cytoskeletons isolated from resting and thrombin-activated platelets in the presence or absence of Ca^{2+} and Mg^{2+} (Fig. 9 B). Anti-fibrinogen antibody recognizes fibrinogen subunits (Fig. 9 B, lane 2) as well as covalently associated high molecular weight fibrin species in activated platelets maintained in the presence of Ca^{2+} and

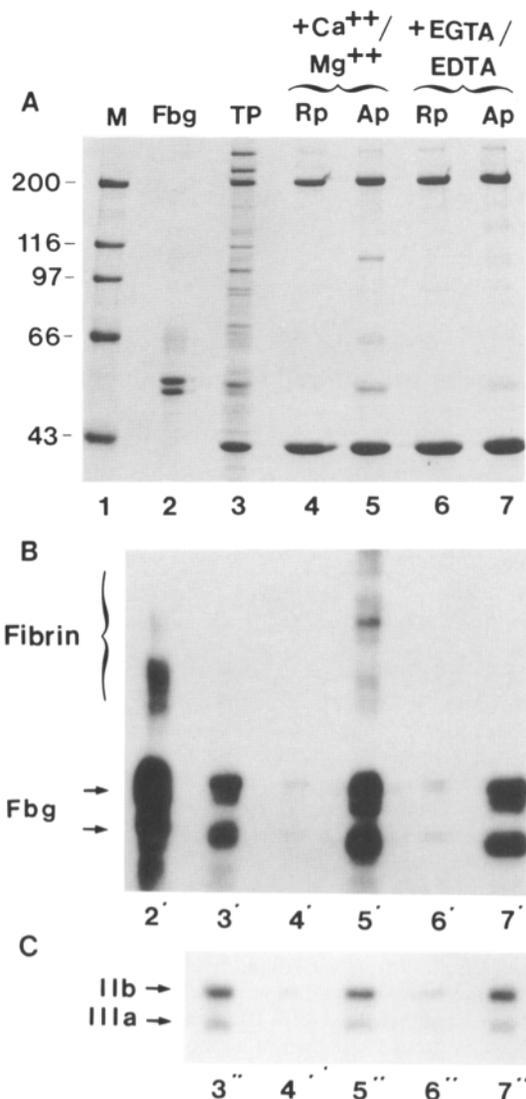


Figure 9. The association of GPIIb-IIIa with the cytoskeletal pellet is independent of fibrin sedimentation. The relevant portion of a Coomassie blue-stained gel is shown in (A) lane 1, molecular mass standards (M); lane 2, human fibrinogen (Fbg); lane 3, total human platelet protein (TP); lanes 4 and 6, resting platelet pellets (Rp); and lanes 5 and 7, activated platelet pellets (Ap). The detergent-insoluble pellets shown in lanes 4 and 5 were isolated from platelets incubated with $\text{Ca}^{++}/\text{Mg}^{++}$ during platelet activation with the addition of EGTA/EDTA 1 min before lysis; the samples in lanes 6 and 7 were isolated from platelets activated in the presence of EGTA/EDTA. (B) The corresponding Western immunoblot probed with anti-fibrinogen antibody which recognizes the α , β , and γ chains of fibrinogen (Fbg, arrows) as well as high molecular weight fibrin species. (C) A parallel Western immunoblot probed with anti-GPIIb-IIIa antibody. High molecular weight fibrin species are prevalent in cytoskeletons derived from platelets activated in the presence of $\text{Ca}^{++}/\text{Mg}^{++}$ (lane 5'). Significantly less fibrin polymer is detected in pellets isolated from samples containing EGTA/EDTA (lane 7'). However, comparable amounts of GPIIb-IIIa are present in both samples (compare lane 5'' to lane 7'').

Mg^{2+} (see for example Fig. 9 B, lane 5'). Detergent-insoluble pellets isolated from platelets activated in the presence of EGTA/EDTA contain significantly fewer high molecular weight fibrin species (Fig. 9 B, lane 7'). We observed

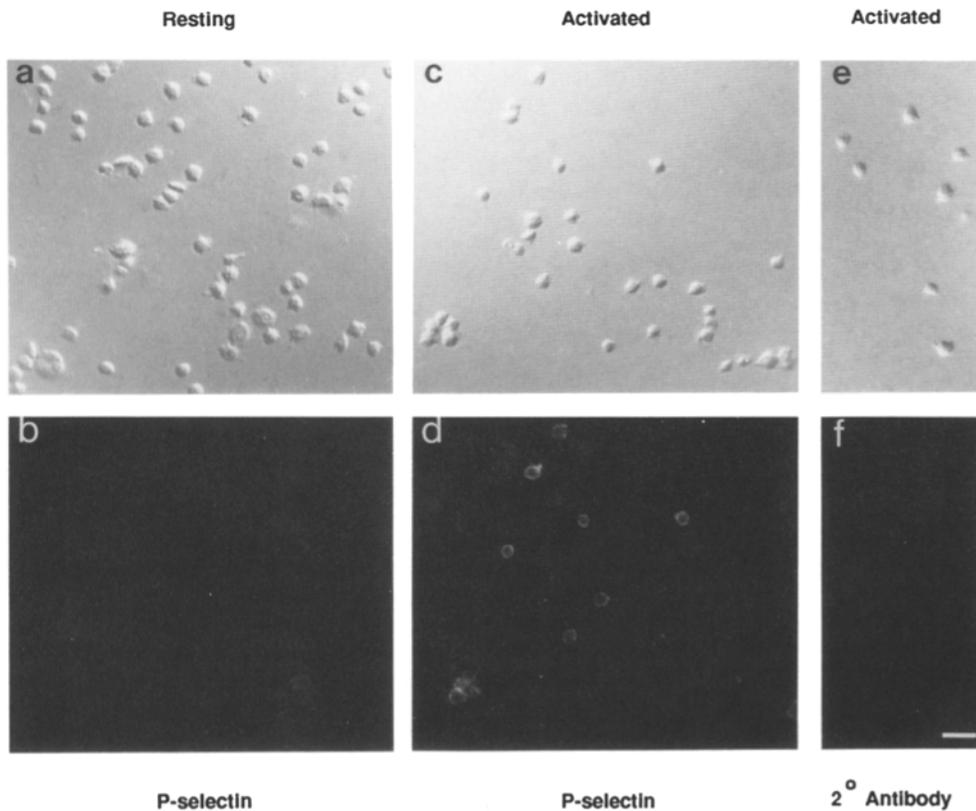


Figure 10. Surface expression of P-selectin in resting and thrombin-activated platelets. (a, c, and e) Differential interference contrast micrographs; (b, d, and f) indirect immunofluorescence. Rhodamine-labeled S12 monoclonal antibody against P-selectin reveals the distribution of P-selectin on the surfaces of nonpermeabilized platelets. Resting platelets (a) have very little P-selectin on their surfaces (b). Upon thrombin activation (c), significantly more cells have surface membrane-associated P-selectin, as seen in d. Thrombin-activated cells (e) labeled with secondary antibody alone are shown in f. Bar, 5 μ m.

comparable amounts of GPIIb-IIIa in cytoskeletons isolated from platelets activated in the presence (Fig. 9 C, lane 5th) or absence (Fig. 9 C, lane 7th) of divalent cations. The lack of correlation between the amount of fibrin and the amount of GPIIb-IIIa present in the activated actin cytoskeletons suggests that the incorporation of GPIIb-IIIa into the detergent-insoluble pellets cannot be explained by an association of GPIIb-IIIa with pelletable fibrin polymers. The isolated integrin-cytoskeleton complexes do exhibit an activation-dependent increase in the level of associated fibrinogen which is presumably bound to GPIIb-IIIa.

Our results suggest that intracellular GPIIb-IIIa becomes selectively incorporated into the actin cytoskeletons of thrombin-activated platelets. Intracellular stores of GPIIb-IIIa are associated with the membranes of the SCCS and α -granules of resting platelets (Wencel-Drake et al., 1986). Upon thrombin activation and subsequent α -granule secretion, intracellular GPIIb-IIIa is translocated to the plasma membrane where it can participate in the adhesive response of platelets to stimuli (Stenberg et al., 1984; Wencel-Drake et al., 1986). It is therefore feasible that the linkage between intracellular GPIIb-IIIa and the reorganizing actin cytoskeleton is dependent on the secretory process. To investigate the possible role of α -granule secretion in the association of intracellular GPIIb-IIIa with the actin cytoskeleton, we examined whether there is a relationship between thrombin-induced α -granule secretion and the incorporation of GPIIb-IIIa into the actin cytoskeleton. We first determined whether α -granule secretion occurs under the conditions used in our studies by monitoring the exposure of the α -granule marker, P-selectin, on the platelet surface upon activation (Hsu-Lin et al., 1984; Stenberg et al., 1985; Berman et al.,

1986). As can be seen in Fig. 10, surface expression of P-selectin is low in resting platelets and increases dramatically in thrombin-activated platelets. These results are expressed quantitatively in Table II. The activation-dependent increase in S12 staining of platelets demonstrates that α -granule secretion does occur during thrombin-induced platelet activation under our buffer conditions.

To address more directly the relationship between the cytoskeletal association of GPIIb-IIIa and α -granule secretion, we monitored the incorporation of GPIIb-IIIa into the cytoskeletons of platelets activated with ADP, a weak platelet agonist. Platelet activation was confirmed by examining fixed samples of ADP-activated platelets for shape change and granule centralization by phase contrast and differential interference contrast microscopy. By following the surface expression of P-selectin, we identified activation conditions under which α -granule secretion is greatly diminished. For

Table II. Analysis of Cell-Surface Expression of P-Selectin

Experimental condition	% of S12-labeled cells*
Resting + buffer	10.8 \pm 3.0 (n = 6)
+ Metabolic inhibitors	4.7 \pm 1.0 (n = 2)
Activated + thrombin	90.4 \pm 2.5 (n = 4)
+ ADP	21.1 \pm 7.6 (n = 2)
+ ADP + thrombin	84.7 \pm 1.4 (n = 2)
+ Metabolic inhibitors + thrombin	5.6 \pm 1.2 (n = 2)

Greater than 300 cells were counted per condition, per experiment.
* These data represent the average of results from independent experiments using different blood donors. Each value represents the mean \pm SEM.

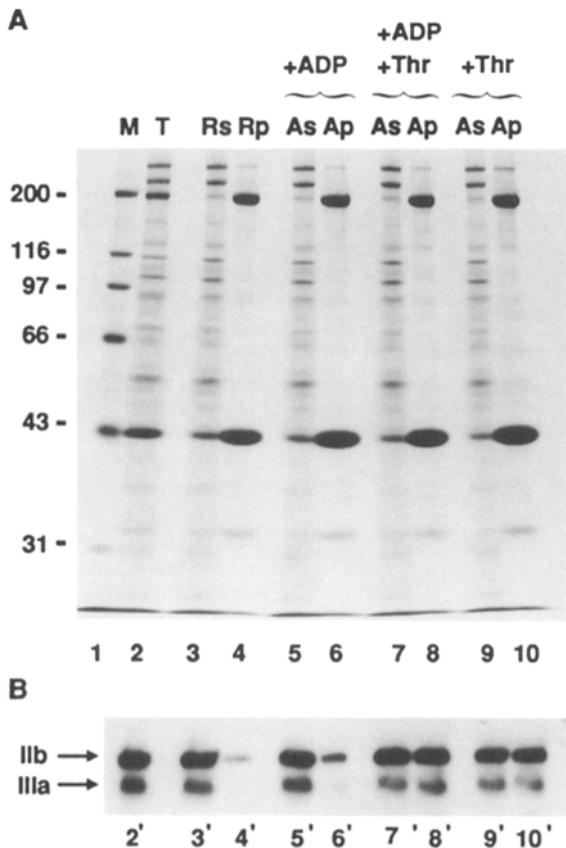


Figure 11. Alpha-granule secretion parallels the incorporation of GPIIb-IIIa into the actin cytoskeleton. (A) A Coomassie blue-stained gel of molecular mass standards (M, lane 1); total human platelet protein (T, lane 2); supernatants from resting platelets (lane 3); ADP-activated platelets (lane 5), ADP + thrombin-activated platelets (lane 7), and thrombin-activated platelets (lane 9); pellets from resting platelets (lane 4), ADP-activated platelets (lane 6), ADP + thrombin-activated platelets (lane 8); and thrombin-activated platelets (lane 10). (B) A corresponding Western immunoblot probed with antibody directed against GPIIb-IIIa. Actin cytoskeletons isolated from ADP-activated platelets (lane 6') (which exhibit minimal α -granule secretion: Table II) contain less GPIIb-IIIa than those isolated from cells activated with ADP followed by thrombin (lane 8') or thrombin alone (lane 10'), conditions that trigger extensive alpha granule secretion.

example, upon activation of washed platelets for 15 min with 100 μ M ADP, only 21% of the cells exhibited surface-exposed P-selectin compared with 90% of cells activated with thrombin (Table II). We also detected less GPIIb-IIIa in cytoskeletal pellets isolated from ADP-activated platelets (Fig. 11 B, lane 6') than from thrombin-activated platelets (Fig. 11 B, lane 10'). The addition of thrombin to platelets that were initially activated with ADP resulted in an increase in the percentage of cells displaying peripheral P-selectin labeling (Table II) and a corresponding increase in the amount of GPIIb-IIIa incorporated into the actin cytoskeleton (Fig. 11 B, lane 8'). Our results demonstrate that there is a positive correlation between α -granule secretion and the incorporation of GPIIb-IIIa into the actin cytoskeleton of activated platelets.

Further evidence that there is a relationship between α -granule secretion and the incorporation of GPIIb-IIIa into

the actin cytoskeleton was obtained by examining the effect of inhibitors of α -granule release on the activation-dependent incorporation of GPIIb-IIIa into the detergent-insoluble material. Incubation of resting platelet samples in 15 mM 2-deoxyglucose and 20 mM gluconolactone (Holmsen et al., 1974; Tuszynski et al., 1982) for 5 min before thrombin addition resulted in nearly complete inhibition of α -granule secretion as evidenced by a lack of S12 binding on the platelet surface (Table II). Under these conditions, we detected only a small activation-dependent increase in the amount of actin cytoskeleton-associated GPIIb-IIIa (data not shown) that was comparable to the increase in the amount of surface-iodinated GPIIb-IIIa detected in the actin cytoskeletons from thrombin-activated platelets (Fig. 7 B).

Discussion

When platelets become activated and adhesive, they establish a transmembrane connection between their actin cytoskeletons and the extracellular matrix. The molecular mechanism by which platelets generate this linkage is not thoroughly understood. Using a biochemical approach we have examined the establishment of integrin-cytoskeleton linkages in thrombin-activated platelets. Here we report that a significant portion (22%) of the total platelet pool of GPIIb-IIIa, the most abundant platelet integrin, becomes selectively and stably associated with the actin cytoskeleton upon platelet activation and α -granule secretion. This association is independent of the platelet aggregation response. The incorporation of GPIIb-IIIa into the detergent-insoluble actin cytoskeleton of thrombin-activated platelets appears to be specific based on the following criteria: (a) It is not an artifact caused by trapping of GPIIb-IIIa with the filamentous actin pellet since there is no direct correlation between the amount of pelletable actin and the amount of GPIIb-IIIa associated with the cytoskeletons; (b) It is cytochalasin D sensitive which suggests that activation dependent actin polymerization and/or cytoskeleton reorganization is essential for the incorporation of GPIIb-IIIa into the actin cytoskeleton; (c) It is detected even in the absence of extensive fibrin polymerization and sedimentation; therefore, the GPIIb-IIIa is not sedimenting by virtue of an association with fibrin; and (d) It does not result from trapping of α -granule membranes since the α -granule membrane marker, P-selectin, is not cytoskeleton-associated. Collectively, these observations suggest that GPIIb-IIIa sediments by virtue of a specific association with the actin cytoskeleton. The interaction between GPIIb-IIIa and the actin cytoskeleton that we observe could mediate a transmembrane link between the platelet contractile machinery and the substratum.

Our ability to detect an aggregation independent interaction between intracellular GPIIb-IIIa and the actin cytoskeleton contrasts with previous findings reported by other groups (Phillips et al., 1980; Painter and Ginsberg, 1982; Wheeler et al., 1984) and absolutely depends on (a) the addition of divalent cation chelators at least 1 min before detergent lysis of the platelets, and (b) the use of Western immunoblot analysis to detect cytoskeleton-associated GPIIb-IIIa. We believe that chelation of divalent cations contributes to the stability of the cytoskeletal complexes by reducing the activity of calcium-dependent proteases (Phillips and Jakobova, 1977; Fox et al., 1985) and actin severing proteins such as

gelsolin (Lind et al., 1982). In the absence of these chelators, our cytoskeletal pellets are smaller and contain significantly less actin, myosin, and GPIIb-IIIa. Moreover, the use of Western immunoblot analysis in our studies allows us to detect all immunoreactive forms of GPIIb-IIIa. Of particular interest, we have determined that a significant amount of the GPIIb-IIIa that we detect in the actin cytoskeletons isolated from activated platelets is derived from a pool of GPIIb-IIIa that is not accessible to lactoperoxidase-catalyzed surface labeling. Lactoperoxidase-catalyzed radiolabeling of surface membrane-associated glycoproteins, the labeling method most commonly used in previous studies to examine the distribution of GPIIb-IIIa in detergent-soluble and insoluble platelet fractions (Phillips et al., 1980; Painter and Ginsberg, 1982; Wheeler et al., 1985), does not label intracellular GPIIb-IIIa pools that are associated with the membranes of α -granules and the deep recesses of the SCCS (Gogstad et al., 1981; Woods et al., 1986). Consequently, interactions between the cytoskeleton and GPIIb-IIIa derived from these intracellular pools would not be detected in studies employing surface iodination to monitor GPIIb-IIIa. Indeed, in agreement with previous workers (Phillips et al., 1980), we found that the amount of surface-labeled GPIIb-IIIa associated with the actin cytoskeletons increases by less than twofold upon thrombin activation. In contrast, by using Western immunoblots to detect GPIIb-IIIa, we observe a much larger ninefold increase in the amount of total platelet GPIIb-IIIa present in actin cytoskeletons after thrombin activation (Table I). The majority of cytoskeleton-associated GPIIb-IIIa that we detect by Western immunoblot must therefore be derived from α -granule or SCCS-derived ("intracellular") pools that are not labeled by surface iodination.

Our finding that a specific subpopulation of the platelet's complement of GPIIb-IIIa preferentially associates with the actin cytoskeleton in activated, nonaggregated platelets represents a clear example of the subcellular heterogeneity of integrins and raises the question of how the selective association of GPIIb-IIIa with the actin cytoskeleton is regulated. It is possible that functionally distinct pools of GPIIb-IIIa are spatially segregated or are specified by ligand binding capacity, posttranslational modifications, or isoform diversity. Isenberg et al. (1987) have shown that ligand binding induces the clustering of GPIIb-IIIa on the platelet surface and have suggested that receptor engagement triggers the formation of integrin-cytoskeleton linkages. In a washed platelet system such as the one we used in our experiments, α -granule and SCCS-derived GPIIb-IIIa would be the first pools of GPIIb-IIIa to be exposed to ligands released by α -granule exocytosis upon thrombin stimulation. Thus, if ligand binding were prerequisite to integrin-cytoskeleton attachment, these so-called intracellular pools of GPIIb-IIIa would be the first to associate with the cytoskeleton. This interpretation is consistent with our finding that fibrinogen is associated with the cytoskeletons from activated platelets as well as our observation that secretion of α -granule contents precedes integrin-cytoskeleton linkage. Interestingly, it has been estimated that α -granules contain enough fibrinogen to engage $\sim 30\%$ of the platelet GPIIb-IIIa receptors (Plow et al., 1986), a value similar to the 22% of the cellular GPIIb-IIIa that we detect associated with the actin cytoskeletons of thrombin-activated platelets after α -granule secretion. The accessibility of granule stores of fibrinogen to intracellular GPIIb-IIIa may there-

fore lead to the immediate engagement of these receptors and the incorporation of the liganded population of GPIIb-IIIa into the actin cytoskeleton upon platelet activation and α -granule secretion.

Intracellular and plasma membrane GPIIb-IIIa could also be distinguished based on differential posttranslational modification. For example, it has been shown that limited phosphorylation of GPIIIa occurs upon platelet activation (Parise et al., 1990; Hillery et al., 1991). The selective phosphorylation of a subpopulation of GPIIIa could lead to differences in the receptor's ability to associate with the cytoskeleton. Likewise, the molecular heterogeneity of GPIIb-IIIa could provide a structural basis for functional diversity. Two forms of GPIIb resulting from alternative mRNA splicing have been identified in human platelets (Bray et al., 1990). Such variation in GPIIb subunits could define the population of GPIIb-IIIa that becomes associated with the actin cytoskeleton of activated, nonaggregated platelets.

It is interesting that the association between intracellular GPIIb-IIIa and the platelet cytoskeleton that we observe is activation-dependent but not aggregation-dependent. Platelet aggregation is coupled to post-receptor signaling pathways including the activation of cellular tyrosine kinases (Ferrell and Martin, 1989; Golden and Brugge, 1989). Activation-dependent responses that occur in the absence of platelet aggregation, such as the cytoskeletal association of intracellular GPIIb-IIIa, must therefore precede and be independent of platelet aggregation and subsequent downstream events in the signal transduction pathway. Indeed, the integrin-cytoskeleton linkage we observe may even be prerequisite to the transmission of transmembrane signals.

Somewhat surprisingly, our isolated integrin-cytoskeleton complexes exhibited no detectable talin, vinculin, or α -actinin. In terms of the molecular mechanisms underlying the integrin-cytoskeleton association, our results are most consistent with the idea that either GPIIb-IIIa can associate directly with actin (Painter et al., 1985a,b) or that some yet unidentified protein(s) mediates this interaction. Because platelets are specialized for adhesion and contain relatively large amounts of talin (Collier and Wang, 1982; O'Halloran et al., 1985; Beckerle et al., 1986), α -actinin (Rosenberg et al., 1981; Langer et al., 1984), and vinculin (Kotliansky et al., 1984; Langer et al., 1984; Rosenfeld et al., 1985), it is probable that these proteins, which are involved in adhesive interactions in other cells, are also essential at some stage of the platelet adhesive response. Other investigators have proposed that these proteins are involved in aggregation-dependent, cytoskeleton-membrane interactions that are essential for clot retraction (Phillips et al., 1980; Langer et al., 1984; Asyee et al., 1987; Kouns et al., 1991). Although from our results, talin, α -actinin, and vinculin do not appear to be essential for the maintenance of the association between GPIIb-IIIa and the actin cytoskeleton of thrombin-activated, nonaggregated platelets, it is possible that these accessory proteins are involved in the initial establishment of actin-membrane interactions or in the stabilization and/or regulation of these associations in vivo. Since differences in experimental conditions, such as extraction buffer and temperature are known to affect the recovery of proteins with the actin cytoskeleton (Rosenberg et al., 1982; Fox et al., 1988), it is possible that talin, α -actinin, and/or vinculin are associated with the actin cytoskeleton in vivo but are not re-

tained during the isolation procedure. Furthermore, we can not exclude the possibility that very small (<1%) but biologically significant amounts of talin, α -actinin, or vinculin may be associated with the cytoskeleton but are not detectable in our assay. However, our ability to recover relatively large amounts of GPIIb-IIIa associated with the actin cytoskeleton in the absence of detectable amounts of talin, α -actinin, and vinculin suggests that these three proteins are not absolutely required to maintain all GPIIb-IIIa-actin interactions in platelets.

In conclusion, we have found that a significant amount of the total GPIIb-IIIa pool becomes incorporated into the actin cytoskeleton of thrombin-activated, nonaggregated platelets. Additionally, we have shown that the majority of cytoskeleton-associated GPIIb-IIIa is derived from intracellular pools of GPIIb-IIIa which could include GPIIb-IIIa associated with the membranes of α -granules and/or the SCCS. It is not possible to distinguish absolutely between GPIIb-IIIa molecules derived from these two internal pools since there is no way to selectively label α -granule membrane-associated GPIIb-IIIa. Moreover, immediately upon platelet activation and α -granule secretion, α -granule membranes fuse with the SCCS and the α -granule and SCCS pools are mixed (Stenberg et al., 1984; Wencel-Drake et al., 1986). Although we have clearly demonstrated the selective incorporation of intracellular GPIIb-IIIa into the actin cytoskeleton before platelet aggregation, the importance of plasma membrane GPIIb-IIIa for hemostasis and thrombosis should not be underestimated. Plasma membrane-derived GPIIb-IIIa is also involved in the adhesive response of platelets and has been shown to be associated with the actin cytoskeletons isolated from aggregated platelets (Phillips et al., 1980). The preferential association of the intracellular population of GPIIb-IIIa with the actin cytoskeleton before platelet aggregation may be important in the establishment of adhesive interactions during the earliest phases of platelet activation. Future studies will be required to determine if the various pools of GPIIb-IIIa in resting platelets differ in structure, conformation, or ligand-binding ability and how these differences influence the function of GPIIb-IIIa in the platelet activation response.

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