The Effect of Glycoprotein IIb-IIIa Receptor Occupancy on the Cytoskeleton of Resting and Activated Platelets*

(Received for publication, March 21, 1991)

William C. Kounst,§ Carol F. Fox,§ William J. Lamoreaux,*, Lewis B. Coons,†, and Lisa K. Jennings§§

From the Departments of §Medicine and ¶Biochemistry, the University of Tennessee, Memphis, Tennessee 38163 and the ¶Center for Electron Microscopy, Memphis State University, Memphis, Tennessee 38152

The platelet integrin, glycoprotein IIb-IIIa (GPIIb-IIIa), serves as the receptor for fibrinogen. This study examined what effect GPIIb-IIIa receptor occupancy had on the cytoskeleton of resting and activated platelets. Triton X-100-insoluble residues (cytoskeletons) were isolated from resting washed platelets incubated with either 500 μM RGDS or 500 μM RGES and examined for protein content. RGDS did not increase the amount of GPIIb-IIIa associated with the cytoskeletal residues which sedimented at either 15,800 × g or 100,000 × g. To determine the effect of receptor occupancy on the formation of the activated platelet cytoskeleton, stirred and nonstirred RGDS-treated platelets in plasma were activated with ADP. Triton X-100-insoluble residues were isolated and examined for both protein content and retention of GPIIb-IIIa. Further, morphological studies were performed on the RGDS-ADP-stimulated platelets. The results of this study suggest that 1) RGDS peptide receptor occupancy does not lead to GPIIb-IIIa linkage to the cytoskeleton, 2) ADP-stimulated platelet shape change, polymerization of actin, and association of myosin with the cytoskeleton are unaffected by RGDS peptide receptor occupancy, 3) RGDS inhibits an aggregation-dependent incorporation of ABP, α-actinin, talin, and GPIIb-IIIa into the Triton-insoluble residue.

Platelets contain a number of adhesive protein receptors belonging to the integrin superfamily (1, 2). These include the glycoprotein complexes Ia-IIa (VLA-2), Ic-IIa (VLA-5), VLA-6, α-IIIa, and GPIIb-IIIa (3-9). As members of the integrin superfamily, these receptors are proposed to bind extracellular ligands and to interact with the cytoskeleton. Interestingly, GPIa-IIa has been shown to be linked to the resting platelet cytoskeleton via actin-binding protein while GPIIb-IIIa has been suggested to become associated with the cytoskeleton following platelet aggregation (10, 11). Therefore, it is of considerable interest to determine what regulates the interactions of these receptors with the cytoskeleton.

On the activated platelet, GPIIb-IIIa serves as the receptor for fibrinogen (12-16). GPIIb-IIIa can also bind fibronectin, von Willebrand factor, and vitronectin (17-24). The binding of these adhesive proteins is due, in part, to an RGD(X) sequence specificity since synthetic RGDS peptides are able to inhibit the binding of these ligands to GPIIb-IIIa (25-28). In contrast to the intact ligand, these peptides are able to bind to both resting and activated platelets (29). RGDS peptide binding to GPIIb-IIIa leads to conformational changes in both GPIIb and GPIIIa (30-32) and causes clustering of the receptors within the plane of the membrane (33). This study examined whether RGDS peptide receptor occupancy led to an increase in the amount of GPIIb-IIIa associated with the cytoskeletal elements which are Triton X-100-insoluble and which sediment at either 15,800 × g or 100,000 × g.

GPIIb-IIIa functions not only as an adhesive protein receptor, but it has recently been proposed that GPIIb-IIIa may either serve as or regulate a calcium channel(s). Evidence for GPIIb-IIIa participation in the regulation of calcium flux comes from the following findings. First, purified GPIIb-IIIa can act as a calcium channel in liposomes (34, 35). Second, receptor occupancy by RGDS peptides or fibrinogen can inhibit calcium influx in platelets stimulated with low dose agonist (36, 37). Third, platelets from patients with Glanzmann's thrombasthenia (GT) have a defect in calcium influx in response to low dose agonist (38). Fourth, resting GT platelets have a defect in calcium distribution and transport (39). Since fibrinogen receptor occupancy appears able to inhibit calcium influx in response to platelet stimulation with weak agonists, this study further examined whether receptor occupancy by RGDS peptides could modulate the formation of the activated platelet cytoskeleton.

Previous studies have shown that the platelet is a useful model to study the alterations in cytoskeletal protein interactions which result from cellular activation. Following thrombin stimulation, alterations in the platelet cytoskeleton were shown to include a net polymerization of monomeric actin into filaments and an increased association of these filaments with other cytoskeletal proteins (40, 41). Later studies suggested that the platelet cytoskeleton consisted of separable pseudopodial and contractile cytoskeletal assemblies (42, 43) and a membrane skeleton which may serve to stabilize the plasma membrane of unactivated platelets (44). The pseudopodial cytoskeleton was suggested to be composed primarily of ABP and α-actinin in association with actin filaments while the contractile gel was composed of myosin associated with filamentous actin (42).

In addition, platelet membrane glycoproteins have been demonstrated to interact with the platelet cytoskeleton. Studies by Painter et al. (45) suggested that a fraction of GPIIb-IIIa (≈15%) was linked directly to the cytoskeleton via interaction with actin. Studies by Phillips et al. (11) demonstrated that following thrombin-stimulated platelet aggregation, a population of GPIIb-IIIa (≈25%) became associated with the...
Triton X-100-insoluble cytoskeleton. Further studies suggested that GPIIb-IIIa association with the cytoskeleton was dependent on both the degree of aggregation and the formation of the pseudodoplidal cytoskeletal assembly (46). The studies of Painter and Ginsberg (47) confirmed that aggregation was required for GPIIb-IIIa retention in the insoluble residue in thrombin-stimulated platelets and demonstrated that concanavalin A could induce interaction of GPIIb-IIIa with the cytoskeleton in the absence of aggregation. The exact mechanism of GPIIb-IIIa association with the cytoskeletal components remains unclear. It has been proposed that GPIIb-IIIa linkage to the cytoskeleton may be mediated by interaction with specific linking proteins such as vinculin (48, 49) or talin (50, 51). Similarly, GPIb and glycoproteins Ia-IIa have been demonstrated to be directly linked to the resting platelet membrane skeleton via interaction with ABP (10, 52).

The results of this study suggest that 1) the receptor clustering and conformational changes induced by RGDS peptide receptor occupancy are insufficient to induce the linkage of GPIIb-IIIa to the platelet cytoskeleton; 2) RGDS peptide receptor occupancy prior to ADP stimulation does not inhibit ADP-stimulated shape change, polymerization of actin, or the association of myosin with the platelet cytoskeleton; 3) platelet aggregation leads to an increased retention of ABP, a-actinin, GPIIb-IIIa, and talin in the Triton X-100-insoluble residue.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tris-HCl, glycine, SDS, Coomassie Brilliant Blue, 2-mercaptoethanol, Triton X-100, goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate, horseradish peroxidase color development reagent, and nitrocellulose were purchased from Bio-Rad. ACS reagent grade ethyl alcohol, sodium chloride, potassium chloride, magnesium chloride, sodium citrate dihydrate, and EDTA were obtained from Mallinckrodt Chemical Works. BSA, EGTA, and cytochalasin B were purchased from Sigma. Sodium phosphate monobasic was purchased from Fisher Scientific Co. Leupeptin was obtained from Vega Biochemicals, Tucson, AZ. Prestained molecular weight standards were obtained from Diversified Biotech, Newton Center, MA. Sequester-Sol was purchased from Cambridge Chemical Products, Inc., Detroit, MI.

**Monoclonal Antibodies**—Polyclonal anti-GPIIb-IIIa was a generous gift of Dr. D. R. Phillips (Cor Therapeutics, San Francisco, CA). Polyclonal anti-glycocalcin and anti-talin were a kind gift of Dr. J. E. B. Fox (Gladstone Foundation, University of California, San Francisco, CA). The A2AB monoclonal antibody was a generously supplied by Dr. Joel Bennett (University of Pennsylvania, Philadelphia, PA).

**Platelet Preparation**—After informed consent was obtained, venous blood was collected into sodium citrate (1 part 3.8% sodium citrate and 9 parts whole blood). Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 135 × g for 20 min. Platelet-poor plasma was obtained by centrifugation of whole blood at 1600 × g for 10 min. Platelets in PRP were counted on a hemacytometer and adjusted to a count of 3 × 10^9/ml with autologous platelet-poor plasma.

**Aggregation**—Samples (0.5 ml) were placed in siliconized cuvettes, and aggregation was monitored by a Payton Aggregometer with constant stirring at 1000 rpm and at a constant temperature of 37°C.

**Cytoskeleton Preparation**—Triton X-100-insoluble cytoskeletons were isolated by the method of Fox et al. (10) with minor modification. Briefly, washed platelets (3 × 10^9 platelets/ml) were incubated for 10 min at 37°C with either 500 μM RGDS or 500 μM RGES peptides. Platelets were then lysed by addition of an equal volume of 2% Triton X-100 extraction buffer containing 10 mM EGTA, 100 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, leupeptin, and 100 μM Tris-HCl, pH 7.4. The Triton-insoluble material was isolated by centrifugation at 15,600 × g for 5 min. The supernatant was withdrawn and recentrifuged at 100,000 × g for 2.5 h at 4°C. The pellets were resuspended in sample buffer containing 2% SDS, 2% mercaptoethanol, 10% glycerol, 0.01% bromphenol blue, 1 mM EGTA, 4 mM EDTA, and 50 mM Tris-HCl, pH 6.8.

Triton X-100-insoluble cytoskeletons of control and activated platelets in plasma were prepared according to the method of Carroll et al. (42). Briefly, PRP (0.5 ml) was withdrawn directly from aggregometer cuvettes and rapidly mixed with 0.5 ml of ice-cold 2% Triton X-100 extraction buffer containing 100 mM Tris-HCl, 10 mM EGTA, and 2 mM 2-mercaptoethanol and 2 × 10^-6 M leupeptin, pH 7.4. The sample was vortexed for 10 s and centrifuged at 10,000 × g for 5 min. The supernatant was discarded and the pellet washed twice in 1 × extraction buffer. Sample buffer (0.5 ml) was added to the final pellet. The sample was then heated for 10 min at 100°C. Whole platelet lysates were prepared by adding two drops of Sequester-Sol to citrated PRP and then centrifuging at 1600 × g for 10 min. The supernatant was removed, and the pellet was washed once in buffer containing 140 mM NaCl, 20 mM HEPES, and 1 mM EDTA, pH 7.1, and resuspended in buffer containing 157 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 3.3 mM NaH2PO4, and 20 mM HEPES, pH 7.4, to half the original volume. An equal volume of sample buffer was then added for SDS-PAGE analysis.

**Gel Electrophoresis**—Samples were denatured with sample buffer and electrophoresed on 5–20% SDS-polyacrylamide exponential gradient gels (53) overnight at a constant voltage of 25 V. Proteins were visualized by staining with Coomassie Brilliant Blue. Scanning laser densitometry was performed on an LKB Ultrascan II Laser Densitometer.

**Immunoblotting**—An LKB 2005 Transfer Electroblotter was used to transfer polypeptides from SDS-polyacrylamide gels to nitrocellulose sheets. The transfer buffer consisted of 25 mM Tris, 190 mM glycine, and 20% methanol, pH 8.3. Proteins were electrotransferred for 1 h at 85 V (54). Following transfer, the nitrocellulose sheets were incubated overnight with 10 mM Tris, 0.9% NaCl, and 5% BSA, pH 7.4, with 1:500 dilution of anti-GPIIb-IIIa antibody. Primary antibody binding was blocked with 1% BSA in PBS for 1 h. The sheets were washed four times with 10 mM Tris, 0.9% NaCl, and 1% BSA, pH 7.4 (immune incubation buffer). Visualization of bound antibody was achieved by incubating the membrane with 50 ml of immune incubation buffer containing the species specific anti- IgG horseradish peroxidase conjugate for 2 h. The sheets were washed four times in immune incubation buffer and developed by 4-chloro-1-naphthol color development reagent. In some instances the nitrocellulose was then washed in distilled H2O and reincubated with a separate primary antibody in immune stain buffer. The blot was again washed and bound antibody visualized as above.

**Scanning Electron Microscopy**—Platelets were fixed according to the method of Gear et al. (55) with minor modification. Briefly, PRP was diluted 1:5 with 0.15 M NaCl. Aliquots (1 ml) were warmed to 37°C for 30 min and incubated for 2 min at 30°C before addition of LPA and fixed for 2 min. The samples were then fixed with a glutaraldehyde solution containing 2% glutaraldehyde in 0.15 M NaCl for 60 min at ambient temperature. The fixed platelets were washed once with distilled H2O, then once with distilled H2O, and then fixed with a solution containing 20 mM NaCl, 0.1 M NaCl, and distilled H2O, then once with distilled H2O, and finally fixed with a solution containing 20 mM NaCl, 0.1 M NaCl, and distilled H2O.

**RESULTS**

The Effect of RGDS Peptide Receptor Occupancy on the Cytoskeleton of Resting Platelets—To examine the effect of RGDS peptide receptor occupancy on the association of

---

1 The abbreviations used are: SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; EGTA, (ethylenebis(oxyethylenetricarbonyl)tetrasodium acid; PRP, platelet-rich plasma; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; CB, cytochalasin B; mAb, monoclonal antibody.
GPIIb-IIIa with the cytoskeleton, washed resting platelets were incubated for 10 min with either 500 μM RGDS or 500 μM RGES peptides. Cytoskeletons were then isolated by platelet solubilization with Triton X-100 and centrifugation at 15,600 × g (low speed pellet) and 100,000 × g (high speed pellet). Following SDS-PAGE, the proteins were electrotransferred to nitrocellulose sheets and reacted with anti-GPIIb-IIIa and anti-GPIb (anti-glycocalcin) polyclonal antibodies. Cytoskeletons were then isolated by platelet solubilization with Triton X-100 and centrifugation at 100,000 × g. In agreement with the studies of Fox et al. (10), this pellet contained the majority of the actin filaments. The high speed pellet has been suggested to contain the shorter actin filaments of the membrane skeleton. Again, in agreement with previous findings (10), GPIb was associated with the Triton-insoluble residue of the high speed pellet (Fig. 1B, lanes 1 and 4). Some GPIb-IIIa-II was also associated with this residue; however, RGDS (Fig. 1B, lane 4) did not increase the amount of GPIb-IIIa-II above that observed with the RGES-treated platelets (Fig. 1B, lane 5). The experiment was shown to be representative of six experiments. These data suggest that RGDS-receptor occupancy does not lead to the association of GPIIb-IIIa-II with the platelet cytoskeleton. The fact that a fraction of the GPIb-IIIa-II was present in the high speed pellet suggests the findings of Painter et al. (45) which suggested that a small portion of GPIb-IIIa-II is associated with the cytoskeleton.

Inhibition of Aggregation by RGDS Peptides—To examine the effect of RGDS-receptor occupancy on the formation of the activated platelet cytoskeleton, we first determined the maximal concentration of RGDS peptides required to inhibit aggregation. Platelets in plasma (3 × 10^7/ml) stimulated with 10^−7 M ADP had an 85% aggregation response (Fig. 2A). This response was completely inhibited by platelet preincubation (2 min) with 1.8 mM RGDS prior to the addition of ADP (Fig. 2B). While additions of 0.45 mM (Fig. 2B), 0.9 mM (Fig. 2C), and 1.35 mM (Fig. 2D) were effective in inhibiting aggregation, a concentration of 1.8 mM maximally suppressed aggregation and was therefore chosen for all experiments. The concentrations of RGDS peptides required to maximally inhibit the aggregation of platelets in plasma were higher than those previously reported for the inhibition of washed platelets stimulated with ADP (28). Isenberg et al. (33) found that 200 μM RGDS peptides could effectively inhibit the aggregation of washed platelets. However, if the concentration of BSA in the suspending media was reduced from 2 to 0.35%, 50 μM RGDS peptides were effective in inhibiting aggregation.

Therefore, the higher concentration of RGDS peptides required to inhibit ADP-stimulated platelet aggregation may be due to the higher concentration of albumin and fibrinogen in the plasma. Alternatively, the higher concentration of RGDS peptides required to inhibit aggregation could be due to the higher dosage (100 versus 10 μM) of ADP used in these experiments compared with those used in the earlier experiments of Gartner et al. (28).

RGDS-mediated Inhibition of the Incorporation of ABP, α-Actinin, and Talin into the Triton X-100-insoluble Residue—Fig. 3 shows that platelets stimulated with 10^−7 M ADP (Fig. 3A, lane 4) had an increased incorporation of ABP, talin, myosin, α-actinin, and f-actin into the Triton-insoluble residue when compared with unstimulated platelets which had been incubated with either PBS (Fig. 3A, lane 1) or 1.8 mM RGDS alone (Fig. 3A, lane 2). The identity of talin was confirmed by immunoblotting (data not shown). Filamentous actin is considered to be the Triton X-100-insoluble actin as studies comparing the amount of insoluble actin with lack of DNase I inhibitory activity correlate very closely (40). Incubation of platelets with 1.8 mM RGDS prior to their stimulation with 10^−7 M ADP (Fig. 3A, lane 3) inhibited the incorporation of ABP, talin, and α-actinin into the Triton-insoluble residues. As there was some variation in the amount of retained protein (i.e. Fig. 3A, myosin in lanes 3 and 4) from different donors, these experiments were repeated four times, and the gels were scanned using a laser densitometer. The
RGDS Peptide Effects on the Cytoskeleton of Resting and Activated Platelets

Panel A: electrophoretic analysis of 1% Triton X-100-insoluble residues isolated from stirred PRP. Platelets (3–4 × 10⁶ platelets/ml) in plasma were removed directly from aggregometer cuvettes and rapidly lysed with an equal volume of buffer containing 2% Triton X-100 and leupeptin. The Triton X-100-insoluble residues were electrophoresed through 5-20% exponential SDS-PAGE gels and visualized by staining with Coomassie Brilliant Blue. Lane assignments as follows: Triton X-100-insoluble residue from platelets preincubated with PBS for 4 min (lane 1), 1.8 mM RGDS for 4 min (lane 2), 1.8 mM RGDS for 2 min followed by stimulation with 10⁻⁴ M ADP for 2 min (lane 3), or preincubated with PBS for 2 min followed by stimulation with 10⁻⁴ M ADP for 2 min (lane 4). An equivalent aliquot of solubilized whole platelets (lane 5). Preincubation of platelets with RGDS peptides prior to stimulation with 10⁻⁴ M ADP inhibited incorporation of ABP, α-actinin, and talin into the Triton-insoluble residue. Preincubation of platelets with RGDS peptides prior to stimulation with 10⁻⁴ M ADP inhibited incorporation of α-actinin and talin into the Triton-insoluble residue. Sample results are expressed quantitatively in Fig. 4. RGDS treatment prior to ADP stimulation inhibited the incorporation of ABP (from 77 to 29%), talin (from 13 to 4%), and α-actinin (from 54 to 14%) into the Triton-insoluble residue but did not inhibit incorporation of myosin (41%) or F-actin (72%) (Fig. 3). Decreased ABP and talin incorporation was not due to proteolysis by Ca²⁺-dependent proteases since: 1) GRGDS peptides do not stimulate platelet Ca²⁺ flux (37), and 2) the ABP and talin in whole platelets was not degraded by these proteases after a 5-min incubation period with these peptides (data not shown).

The Effect of RGDS Peptides on GPIIb-IIIa Incorporation into the Triton X-100-insoluble Residues—Immunoblotting was used to demonstrate that GPIIb-IIIa was incorporated into the Triton X-100-insoluble cytoskeletons of ADP-aggregated platelets (Fig. 3B, lane 5). In contrast, GPIIb-IIIa was not detected in the insoluble cytoskeletons of platelets which had been preincubated with PBS (Fig. 3B, lane 2), 1.8 mM RGDS (Fig. 3B, lane 3), or preincubated with 1.8 mM RGDS prior to stimulation with 10⁻⁴ M ADP (Fig. 3B, lane 4). The polyclonal GPIIb-IIIa antibody was also reactive with a 55-kDa band in the insoluble residues of both resting and stimulated platelets. However, the identity of this band is unknown.
The Effect of RGDS on Cytoskeletal Reorganization of Non-aggregated Platelets in Plasma—To determine whether the RGDS effect was caused directly by receptor occupancy or secondarily by an inhibition of aggregation, identical experiments were performed on nonstirred PRP. Nonstirred ADP-stimulated platelets do not aggregate. This was confirmed in our S.E. of nonstirred ADP-stimulated platelets (Figs. 8, C and c, and 9C). Fig. 5, lane 5, represents the Triton-insoluble residue from platelets preincubated with PBS for 2 min followed by stimulation with \(10^{-4}\) M ADP for 2 min. As compared with control platelets incubated with either PBS for 4 min (Fig. 5, lane 2) or RGDS peptides for 4 min (Fig. 5, lane 3), there was an increase in the incorporation of myosin and F-actin but only a slight increase in the incorporation of ABP (approximately 21% incorporation, \(n = 5\)). Little difference was observed between the ADP-stimulated platelet Triton-insoluble residues (Fig. 5, lane 5) and the residues of platelets preincubated with RGDS peptides for 2 min prior to ADP stimulation for 2 min (Fig. 5, lane 4). Thus, the increase in ABP, \(\alpha\)-actinin, and talin seen in the cytoskeletal residues isolated from stirred platelets was dependent on aggregation. A similar increase in the incorporation of ABP and \(\alpha\)-actinin into the Triton-insoluble cytoskeletons following either thrombin or ADP-induced aggregation has been reported using washed platelets (66). Consistent with the results from the ADP-aggregated platelets, RGDS receptor occupancy did not decrease the incorporation of myosin or F-actin into the insoluble residue of the ADP-activated platelets.

Immunoblots of Triton X-100-insoluble residues isolated from RGDS-ADP and ADP-stimulated platelets were reacted with anti-GPIIb-IIIa to determine if GPIIb-IIIa was retained in these residues. The results from these studies confirmed the previous findings of Phillips et al. (11) that aggregation was required for the retention of GPIIb-IIIa in the 15,800 \(x\) g sedimentable Triton-insoluble residues.

The Effect of Cytochalasin B (CB) on the Aggregation-dependent Retention of ABP, \(\alpha\)-Actinin, and Talin—To determine whether the increased retention of ABP, talin, and \(\alpha\)-actinin following aggregation was dependent on the formation of filamentous actin, platelets in plasma were incubated with 0.9 mg/ml of CB for 10–15 min prior to stimulation with \(10^{-4}\) M ADP. CB has previously been shown to inhibit the polymerization of actin induced by platelet activation but does not inhibit platelet aggregation (42, 57). PRP pretreated with dimethyl sulfoxide (Fig. 6, lane 2) and cytoskeletons isolated from platelets pretreated with CB followed by stimulation with \(10^{-4}\) M ADP (Fig. 6, lane 3) had comparable levels of F-actin, 45 and 44%, respectively. In contrast, the Triton-insoluble cytoskeletal residue from PRP pretreated with dimethyl sulfoxide followed by stimulation with \(10^{-4}\) M ADP (Fig. 6, lane 4) retained 68% F-actin. Thus, CB effectively reduced the incorporation of F-actin into the ADP-stimulated platelet cytoskeletons. CB also partially inhibited the incorporation of \(\alpha\)-actinin (from 44 to 19%) and ABP (from 68 to 44%). Talin retention was unaffected by the CB pretreatment. These data suggest that, while there is a pool of ABP (approximately 20%) which is dependent on CB-sensitive F-actin formation for retention in the Triton-insoluble residue, there is also a pool (approximately 40%) which is not dependent on the CB-sensitive F-actin formation for retention in the residue.

To determine if the increased ABP, \(\alpha\)-actinin, and talin in the Triton-insoluble residue might reflect membranous cytoskeletal structure, immunoblots of residues from ADP-aggregated samples were reacted with anti-glycocalcin (GPIb) and GPIIb-IIIa polyclonal antibodies. Glycocalcin and GPIIb-IIIa were detected in the residue of the ADP-aggregated platelets (Fig. 6, lane 3). This suggested that submembranous microfilaments or portions of the membrane were retained in the insoluble residue isolated from the aggregated platelets.

A2A9-mediated Inhibition of the Aggregation-dependent Retention of ABP, \(\alpha\)-Actinin, and Talin—To determine if alternate methods of inhibiting aggregation also inhibited retention of ABP, \(\alpha\)-actinin, and talin following ADP-stimulation, platelets were preincubated with a mAb, A2A9, which has previously been shown to bind to the GPIIb-IIIa complex and inhibit agonist-induced fibrinogen binding (13). Platelets were incubated with either PBS for 4 min (Fig. 7, lane 2), 9 mg of A2A9 for 2 min followed by ADP (\(10^{-4}\) M) stimulation for 2 min (Fig. 7, lane 3) or PBS for 2 min followed by addition of \(10^{-3}\) M ADP for 2 min (Fig. 7, lane 4), and the Triton-insoluble residues were isolated. Similar to the RGDS-pre-treated platelets, platelets preincubated with A2A9 prior to stimulation with ADP showed decreased incorporation of ABP, \(\alpha\)-actinin, and talin when compared with those platelets stimulated with ADP.

Scanning Electron Microscopy of RGDS-ADP- and A2A9-ADP-stimulated Platelets—Figs. 8, A and a, show that platelets incubated with PBS for 4 min maintained a discoid shape with few pseudopod-like protrusions. The few pseudopodia present on the resting platelets may have been due to the preparative procedures. Platelets incubated with RGDS peptides (0.9 or 1.8 mM) were discoid and showed no morphological alterations due to peptide binding (data not shown). When platelets were preincubated with RGDS peptides (0.9 mM) for 2 min and then stimulated with \(10^{-3}\) M ADP for 2 min, they changed from discs to spheres (Fig. 8, B and b). Pseudopodial development in the RGDS-ADP-treated platelets appeared to be slightly inhibited. However, the degree of inhibition varied among platelet donors. This variability could be related to
insoluble residues isolated from stirred CB-pretreated PRP. Stirred electrophoresed through 5-20% exponential SDS-PAGE gels and whole platelets were then stimulated with PBS (lane 3), Triton-insoluble residue isolated from platelets incubated with PBS for 4 min (lane 2), 10 μg A2A9 for 2 min followed by stimulation with 10^{-4} M ADP for 2 min (lane 3) or PBS for 2 min followed by stimulation with 10^{-4} M ADP (lane 4).

**Fig. 7.** Electrophoretic analysis of 1% Triton X-100-insoluble residues isolated from stirred PRP preincubated with A2A9. Platelets (3-4 × 10^9 platelets/ml) in plasma were removed directly from aggregometer cuvettes and rapidly lysed with an equal volume of buffer containing 2% Triton X-100 and leupeptin. The Triton X-100-insoluble residues were electrophoresed through 5-20% exponential SDS-PAGE gels and visualized by staining with Coomassie Blue. Lane assignments as follows: whole platelets (lane 1), Triton-insoluble residue isolated from platelets incubated with PBS for 4 min (lane 2), 10 μg A2A9 for 2 min followed by stimulation with 10^{-4} M ADP for 2 min (lane 3) or PBS for 2 min followed by stimulation with 10^{-4} M ADP (lane 4).

**FIG. 6.** Panel A electrophoretic analysis of 1% Triton X-100-insoluble residues isolated from stirred CB-pretreated PRP. Stirred PRP was pretreated for 10–15 min at 37 °C with 10 μg/ml of CB or dimethyl sulfoxide as the control vehicle. Samples were then stimulated with 10^{-3} M ADP, and the Triton X-100-insoluble residues were electrophoresed through 5-20% exponential SDS-PAGE gels and visualized by staining with Coomassie Blue. Lane assignments as follows: molecular weight standards (lane 1), Triton X-100-insoluble residues from dimethyl sulfoxide-treated platelets stirred for 2 min with PBS (lane 2), CB-treated platelets stimulated for 2 min with 10^{-3} M ADP (lane 3), dimethyl sulfoxide-treated platelets stimulated with 10^{-4} M ADP for 2 min (lane 4), equivalent aliquot of whole platelets (lane 5). Panel B retention of GPIIb-IIIa and glyco-calcin (GPIb) in 1% Triton X-100-insoluble residue of CB-pretreated platelets. Following electrophoresis of the identical samples presented in Fig. 2A, the proteins were electrophoresed to nitrocellulose and reacted with anti-GPIIb-IIIa and anti-glycoprotein polyclonal antibodies. Visualization of bound antibody was achieved by the horse-radish peroxidase method. Lane assignments were as follows: molecular weight standards (lane 1), Triton X-100-insoluble residues from dimethyl sulfoxide-treated platelets stirred for 2 min with PBS (lane 2), CB-treated platelets stimulated for 2 min with 10^{-3} M ADP (lane 3), dimethyl sulfoxide-treated platelets stimulated with 10^{-4} M ADP for 2 min (lane 4), equivalent aliquot of whole platelets (lane 5).

**DISCUSSION**

GPIIb-IIIa extracellular ligand interactions have been well characterized (12–25, 58–61). Much less is understood about GPIIb-IIIa interaction with the cytoskeleton. To further probe GPIIb-IIIa-cytoskeleton interactions, we examined the effect of fibrinogen receptor occupancy on the cytoskeleton of resting and activated platelets. Our data support the following conclusions: (a) occupancy of the fibrinogen receptor by RGDS peptides does not lead to association of GPIIb-IIIa with the Triton X-100-insoluble elements of the cytoskeleton, (b) while RGDS peptides may inhibit Ca^{2+} influx, this inhibition is insufficient to affect the ability of the platelet to change shape, (c) following aggregation, there is an increased association of ABP, α-actinin, and talin with the Triton X-100-insoluble residue, (d) the increased association of ABP, α-actinin, and talin with the Triton X-100-insoluble residue is not dependent on the formation of pseudopodia.

RGDS peptides have been shown to induce conformational changes in the GPIIb-IIIa complex (30–32) and clustering of
FIG. 8. Scanning electron microscopy of RGDS-ADP-stimulated platelets. PRP was diluted 1:5 with 0.15 M NaCl. The diluted platelets were incubated with PBS for 4 min (A, a), 0.9 mM RGDS for 2 min followed by stimulation with 10⁻⁴ M ADP for 2 min (B, b) or PBS for 2 min followed by stimulation with 10⁻⁴ M ADP for 2 min (C, c). Platelets were then fixed with an equal volume of 2% glutaraldehyde in 0.15 M NaCl for 60 min and filtered onto polylysine-treated polycarbonate filters. Following dehydration, critical point drying and sputter coating, the platelets were imaged on a JSM-840A scanning electron microscope at 20 KU accelerating voltage. Random fields were selected for photographs.

the complex within the plane of the membrane (33). As a number of studies have suggested that GPIIb-IIIa can interact with the platelet cytoskeleton, we questioned whether RGDS-peptide receptor occupancy could serve as a signal to induce this linkage and whether such interactions could serve as a mechanism to drive receptor clustering. At RGDS peptide concentrations (0.5 mM) shown to induce GPIIb-IIIa receptor clustering (33), we were unable to find an increase in association of GPIIb-IIIa with the Triton X-100-insoluble cytoskeletal elements which sedimented at either 15,800 or 100,000 × g. While it is possible that GPIIb-IIIa-cytoskeletal interactions are weak and lost in the solubilization process, our data suggest that RGDS receptor occupancy does not lead to increased association of GPIIb-IIIa with the cytoskeleton. As an alternative hypothesis to cytoskeletal driven clustering, exposure of neoantigenic sites following receptor occupancy could serve to provide the attractive forces which induce the RGDS clustering response. Thus, the RGDS-initiated GPIIb-IIIa clustering observed by Isenberg et al. (33) could be the reaction of a population of GPIIb-IIIa which was linked to the cytoskeleton prior to receptor occupancy.

Interestingly, White and Escolar (62) have recently shown that mobility of GPIIb-IIIa receptors within the membranes of surface- and suspension-activated platelets did not depend on assembly and contraction of actin. White and Escolar (62) propose that the short actin filaments of the membrane skeleton, which are resistant to CB-induced depolymerization, may drive the process of receptor ligand clearance. Since fibrinogen and RGDS peptides can induce similar conformational changes in the GPIIb-IIIa complex (31, 32), and we were unable to find an increased association of GPIIb-IIIa with the membrane skeletons of RGDS-treated platelets, our data would suggest that receptor occupancy must be accompanied by platelet activation for receptor clearance to occur.

Recently, it has been proposed that GPIIb-IIIa may either regulate Ca²⁺ influx or be an important component of a Ca²⁺ channel (34–38). Sinigaglia et al. (37) have suggested that fibrinogen receptor occupancy by GRGDS peptides inhibits Ca²⁺ influx in whole platelets. However, the mechanism of such inhibition remains unclear as Rybak et al. (35) have shown that RGDS peptides do not inhibit Ca²⁺ flux through purified GPIIb-IIIa in liposomes. Sinaglia et al. (37) showed that incubation of platelets with GRGDS peptides prior to stimulation with ADP (10 μM) or low dose thrombin (0.1 unit/ml) led to decreased cytoskeletal organization as determined by the decreased incorporation of cytoskeletal proteins into the Triton-insoluble residue. They suggested that the decreased cytoskeletal organization may be attributed to the decreased Ca²⁺ influx (37). These studies were done in a washed platelet system, and in this system we had comparable
Further evidence of this was provided when it was demonstrated that the mAb A2A9, which does not appear to inhibit Ca\(^{2+}\) influx (data not shown), was equally efficient at inhibiting the aggregation-dependent incorporation of ABP, \(\alpha\)-actinin, and talin. While evidence suggests that GPIIb-IIIa may be involved in the regulation of Ca\(^{2+}\) influx, the importance of GPIIb-IIIa mediated Ca\(^{2+}\) influx in inducing cytoskeletal reorganization must be critically evaluated. For example, we have shown that platelet Ca\(^{2+}\) influx may be defective in patients with Type I Glanzmann’s thrombasthenia (38), yet platelets from these patients undergo shape change and extend pseudopodia (data not shown). Further, platelets suspended in EGTA-containing buffers are able to change shape in response to agonists (11, 63). Therefore, while Ca\(^{2+}\) influx may be important in sustaining activation-mediated events it would appear that release of Ca\(^{2+}\) from internal stores is sufficient for shape change.

The ability of ADP to stimulate shape change in RGDS-pretreated platelets is apparent in the scanning electron micrographs of these platelets and points to the need for correlative morphological studies when examining Triton X-100-insoluble residues. While RGDS peptides appear to slightly inhibit pseudopodial development, this response was highly variable and not reflected in the protein content of nonstirred RGDS-ADP-stimulated platelet Triton-insoluble residues. More consistent was the A2A9-induced inhibition of ADP-stimulated pseudopodial development. It is possible that inhibition of pseudopodial development in the A2A9-ADP-stimulated platelets is due to Na\(^+/H^+\) antiport inactivation since it has been reported that epinephrine-induced cytoplasmic alkalization is inhibited by platelet preincubation with A2A9 (64). Interestingly, Isenberg et al. (65) and Newman et al. (66) reported that incubation of platelets with both Tab (anti-GPIIb) and AP3 (anti-GPIIIa) monoclonal antibodies prior to stimulation with ADP, inhibited pseudopod formation and aggregation but not fibrinogen binding. Whether inhibition of pseudopodial formation by perturbation of GPIIb-IIIa is a specific effect or simply due to membrane alterations remains to be explored.

To determine if the increased association of ABP, \(\alpha\)-actinin, and talin with the insoluble residue following aggregation was dependent on filamentous actin formation, platelets were preincubated with CB prior to ADP stimulation. Cytochalasin-S shortened filamentous actin (67, 68) and inhibit pseudopod formation in platelets (69). Our studies suggest that there is a pool of ABP (approximately 20%) and \(\alpha\)-actinin (approximately 20%) which is lost from the Triton-insoluble residue of CB-treated platelets. As CB inhibits pseudopod development it is tempting to speculate that this pool of ABP and \(\alpha\)-actinin may represent the ABP and \(\alpha\)-actinin associated with the F-actin of the pseudopodia.

Studies by Zucker and Masiello (70) using washed \(^{125}\)I surface-labeled platelets demonstrated that membrane phospholipids were retained in the Triton X-100-insoluble residue along with \(^{125}\)I-labeled membrane glycoproteins. This suggested that the retention of membrane glycoproteins in the Triton-insoluble residue may be due to poor solubilization of the aggregated platelets and thus nonspecific. However, later studies showed that only two of the membrane lipids, sphingomyelin and phosphatidylcholine, accompanied the retention of GPIIb-IIIa in the Triton-insoluble residue suggesting that the retention was indeed specific (71). Upon immunochromatography examination of the Triton-insoluble residues isolated from ADP-aggregated platelets in plasma, we found retention of both glycostatin (GPIb) and GPIII-IIIa. This suggested that the aggregation-dependent increase in ABP,
α-actinin, and talin, which is insensitive to CB pretreatment, may represent either membrane trapping and/or the collection of submembranous microfilaments resulting from the external cross-linking caused by bound extracellular matrix proteins and cell-cell interactions. In support of this latter possibility, ConA-mediated surface cross-linking of CB-pre-treated platelets is sufficient to retain ABP in the Triton X-100-insoluble residue (72). Further, CB-pre-treated gel-filtered platelets stimulated with thrombin (1 unit/ml) do not retain ABP, while CB-pre-treated platelets stimulated with thrombin (1 unit/ml) in the presence of added fibrinogen (0.1 mg/ml) retain both ABP and a protein of similar mobility as talin (73). In addition, experiments in which iodinated bovine serum albumin was added to PRP demonstrated that there was minimal trapping of plasma proteins (<0.2%) in the Triton insoluble residues isolated from aggregated platelets.

In conclusion, our studies suggest that RGDS peptide receptor occupancy does not induce linkage of GPIIb-IIIa to the cytoskeleton. Thus, RGDS-peptide driven receptor clustering may occur by a mechanism which does not depend on cytoskeletal reorganization. Further, these studies show that neither RGDS peptide nor A2A9 mAb binding to GPIIb-IIIa alters the ability of the platelet to change shape (round up) in response to stimulation with ADP nor does it inhibit the retention of either membrane or F-actin in the Triton X-100-insoluble residues of ADP-stimulated platelets isolated from PRP. Peptide binding and A2A9 binding do, however, inhibit aggregation which leads to inhibition of the aggregation-dependent incorporation of ABP, α-actinin, and talin and may be representative of the submembranous microfilament organization. The mechanism by which A2A9 mAb binding to GPIIb-IIIa may inhibit pseudopodial development remains unclear.

Acknowledgements—We wish to thank Drs. D. R. Phillips, J. E. B. Fox, J. S. Bennett, and R. C. Carroll for their gifts of antibody or their helpful discussion.

REFERENCES


RGDS Peptide Effects on the Cytoskeleton of Resting and Activated Platelets