

Effect of Platelet Activation on the Conformation of the Plasma Membrane Glycoprotein IIb-IIIa Complex*

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Peter J. Sims†§, Mark H. Ginsberg¶, Edward F. Plow¶, and Sanford J. Shattil**

From the ‡Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104, the ¶Committee on Vascular Biology, Scripps Clinic and Research Foundation, La Jolla, California 92037, and the ||Hematology-Oncology Section, Departments of Medicine and Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Platelet activation converts the membrane GP IIb-IIIa complex into a functional receptor for fibrinogen, but the mechanism is poorly understood. We asked whether induction of receptor competency coincides with a conformational change affecting the spatial arrangement of exoplasmic domains of the IIb and IIIa subunits. Epitopes on these subunits were labeled with monoclonal antibodies conjugated to either a donor fluorescein (FITC) or an acceptor tetramethylrhodamine (TR) chromophore. Then, fluorescence resonance energy transfer (RET) between platelet-bound FITC and TR was measured by flow cytometry. In unstimulated platelets, 6–8% RET efficiency was detected between antibody B1B5, bound to GP IIb, and antibody SSA6, bound to GP IIIa, regardless of which antibody served as RET donor. RET was also observed between these antibodies and A2A9, an antibody specific for the GP IIb-IIIa complex. Cell stimulation by thrombin, ADP plus epinephrine or phorbol-ester caused up to a 2-fold increase in RET between chromophore-labeled, platelet-bound B1B5, SSA6, and A2A9 ($p \leq 0.05$), suggesting a change in the separation or orientation of these epitopes within the GP IIb-IIIa complex. The activation-related conformational change detected by the increase in RET between antibody B1B5 and SSA6 was independent of receptor occupancy since it was unaffected by the addition of fibrinogen or by the inhibition of fibrinogen binding by the antibody, A2A9, or the peptide, RGDS. In contrast to these results with antibodies bound to different epitopes within GP IIb-IIIa, no RET was observed between FITC-A2A9 and TR-A2A9 bound to different GP IIb-IIIa complexes or between a TR-labeled GP IIb antibody and FITC-labeled GP IIb-IIIa antibodies. These studies demonstrate that platelet activation causes a change in the spatial separation or orientation of exoplasmic domains within GP IIb and IIIa, which may serve to convert this integrin into a functional adhesion receptor.

Glycoproteins (GP)¹ IIb and IIIa are members of the integrin family of adhesion receptors (1). Together these two subunits constitute a platelet receptor for RGD-containing adhesive macromolecules, including fibrinogen, von Willebrand factor, vitronectin, and fibronectin (2, 3). Expression of GP IIb and IIIa on the cell surface is dependent on the coexpression of both subunits (4). Studies using monoclonal antibodies specific for the GP IIb-IIIa complex indicate that GP IIb exists in a calcium-dependent heterodimer complex with GP IIIa within the platelet membrane (5, 6). Unstimulated platelets contain approximately 50,000 surface-expressed GP IIb-IIIa complexes/cell, but these complexes do not bind fibrinogen or other adhesive macromolecules. However, when platelets are stimulated by one or more agonists, GP IIb-IIIa becomes competent to bind fibrinogen in a process that is dependent on extracellular ionized calcium (2, 3, 7). In addition to this modification of existing surface membrane GP IIb-IIIa, platelet activation induces the surface expression and ligand-binding function of an additional pool of GP IIb-IIIa derived from α -granule membranes and/or the surface-connecting open canalicular system (8, 9).

The process by which GP IIb-IIIa is converted into a competent fibrinogen receptor during cell activation is poorly understood. Recently, a monoclonal antibody, PAC1, has been used to study this process. PAC1 binds specifically to GP IIb-IIIa on activated platelets (10). Like the binding of fibrinogen, the binding of PAC1 is dependent on extracellular calcium and is inhibitable by fibrinogen or by small RGD-containing peptides (11–13). This suggests that the neo-epitope for PAC1 comprises part of the binding pocket within GP IIb-IIIa for adhesive ligands. Some workers have suggested that platelet activation might induce a change in the membrane “microenvironment” around GP IIb-IIIa, allowing access of macromolecular ligands, such as fibrinogen or PAC1, to their binding site (14, 15). Alternatively, cell activation might cause a conformational change within GP IIb-IIIa itself, thereby inducing the formation of a ligand-binding pocket or enabling access of ligand to a preformed binding pocket within the exoplasmic domains of these subunits (3, 16, 17). However, there is as yet no direct evidence that such a conformational change in GP IIb-IIIa takes place when platelets are activated.

The purpose of the present study was to determine whether the cell surface-exposed domains of plasma membrane GP IIb-IIIa undergo a conformational change during the process of platelet activation. We were particularly interested in determining whether a conformational change in GP IIb-IIIa

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§ Established Investigator of the American Heart Association.

** To whom correspondence should be addressed: Hematology-Oncology Section, Hospital of the University of Pennsylvania, 3400 Spruce St., Philadelphia, PA 19104.

¹ The abbreviations used are: GP, glycoprotein; FITC, fluorescein isothiocyanate; TR, tetramethylrhodamine; RET, resonance energy transfer; EGTA, [ethylenedis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

occurs prior to fibrinogen binding, suggesting a mechanism for its conversion into a functional receptor. To directly examine the spatial relationships among specific epitopes on platelet GP IIb and IIIa, platelets were labeled with anti-GP IIb and IIIa monoclonal antibodies that had been conjugated with either fluorescein or tetramethylrhodamine. Activation-induced changes in the spatial arrangement of these epitopes within the GP IIb-IIIa heterodimer were monitored by fluorescence resonance energy transfer (RET) between the donor (fluorescein) and acceptor (tetramethylrhodamine) chromophores on these cell surface-bound antibodies, measured by flow cytometry. This approach has been used to study the molecular dynamics of surface membrane proteins in other cells (18–22). We demonstrate that platelet activation results in a conformational change that affects the topology of surface-exposed domains of GP IIb-IIIa and that this event is independent of fibrinogen binding. This suggests that fibrinogen receptor expression arises through a structural rearrangement of the subunits of this integrin.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin (globulin and fatty acid free), prostaglandin E_1 , PGI_2 apyrase, 2-deoxyglucose, antimycin A, *N*-hydroxysuccinimide biotin ester, and diatomaceous earth (Celite) were obtained from Sigma; fluorescein-5-isothiocyanate-Celite, and 5-(and-6)carboxytetramethylrhodamine succinimidyl ester were from Molecular Probes; phycoerythrin-streptavidin conjugate was from Southern Biotechnology Associates (Birmingham, AL). Human fibrinogen and the peptide, RGDS, were prepared as described previously (23). Several murine monoclonal antibodies recognizing exoplasmic epitopes of platelet membrane glycoproteins were used in these studies. AP1, specific for GPIb, was from Dr. Thomas J. Kunicki (Blood Center of Southeastern Wisconsin, Milwaukee, WI) (24). Antibody B1B5 recognizes GP IIb, and antibody SSA6 recognizes GP IIIa (25, 26). A2A9 recognizes an epitope expressed by the heterodimer complex of GP IIb-IIIa (27); PAC1 recognizes a neo-epitope in the GP IIb-IIIa complex that is expressed upon fibrinogen receptor exposure (10). LIBS1 is an antibody that recognizes GP IIIa only when GP IIb-IIIa is in the ligand-bound state (28). 9F9 is an antibody specific for fibrinogen bound to GP IIb-IIIa (26). All other chemicals were of reagent or analytical grade.

Fluorescence Labeling of Antibodies—All monoclonal antibodies were labeled with either fluorescein-5-isothiocyanate (FITC) or 5-(and-6)carboxytetramethylrhodamine succinimidyl ester (TR) using Celite carrier as described previously (29). TR-Celite was prepared by vacuum evaporation of a 10% (w/w) mixture of TR and Celite in methanol. In each case, unconjugated label was removed by gel filtration followed by dialysis. Final dye-to-protein molar ratios of 1–5 were achieved. The concentrations of labeled antibodies were determined by dye binding assay (Bio-Rad), using the respective unlabeled protein as standard. FITC concentration was determined using a molar extinction (492 nm) of 68,000. TR concentration was determined using a molar extinction (544 nm) of 60,000.

Preparation of Platelets for Flow Cytometry—Gel-filtered human platelets were prepared and collected at 1×10^8 /ml into an incubation buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1 mg/ml bovine serum albumin, 3.3 mM NaH_2PO_4 , and 20 mM HEPES (Sigma), pH 7.4 (12). Then, triplicate or quadruplicate aliquots of platelets (1.5×10^6 cells in 25- μ l final volumes) were incubated in the dark and without stirring for 10 min at room temperature with saturating concentrations of one or more of the following FITC- or TR-labeled antibodies as indicated for each experiment: SSA6 (10 μ g/ml), B1B5 (30 μ g/ml), A2A9 (10 μ g/ml), PAC1 (40 μ g/ml), AP-1 (1 μ g/ml), 9F9 (50 μ g/ml), LIBS1 (50 μ g/ml). During this incubation, the platelets were either maintained in a nonactivated state with PGI_2 (0.1 μ M) or activated with thrombin (0.5 unit/ml), phorbol 12-myristate 13-acetate (0.2 μ M), or ADP plus epinephrine (10 μ M each). Following the incubation, cells were diluted to 0.15 ml in incubation buffer and analyzed immediately by flow cytometry. All analyses were complete within 30 min. Under these conditions, dilution of the samples for flow cytometry did not affect the amount of labeled antibody associated with the platelets. In some experiments, platelet activation was performed in the presence of 100 μ g/ml fibrinogen or 2 mM RGDS.

Flow Cytometry—Samples were analyzed using Becton Dickinson FACStar and FACScan flow cytometers each formatted for two-color analysis as described previously (29). The light scatter and fluorescence channels were set at logarithmic gain. A two-dimensional analysis gate of forward and right angle light scatter was drawn so as to include single platelets and exclude platelet aggregates and platelet microparticles, and the mean fluorescence intensity was determined. After correction for cell autofluorescence (8–10 arbitrary fluorescence units), mean fluorescence detected in the FL1 (530 nm) channel was used to calculate efficiency of RET from FITC to TR according to the following relationship:

$$RET (\% EFF) = 100 \times (1 - F_{DA}/F_D)$$

where F_{DA} is the mean FL1 fluorescence at the fluorescein emission wavelength of 530 nm measured for cells stained with both a FITC-labeled antibody (RET donor) and a TR-labeled antibody (RET acceptor). F_D refers to the mean FL1 fluorescence (530 nm) measured for these same cells stained with the FITC-labeled antibody, but with unlabeled antibody substituting for the TR-labeled RET acceptor antibody. RET antibody pairs included the FITC and TR derivatives of B1B5 and SSA6, B1B5 and A2A9; SSA6 and A2A9, A2A9 and A2A9, SSA6 and AP1, B1B5 and AP1, and A2A9 and AP1.

Fluorescence Spectroscopy—In order to monitor activation-related changes in the fluorescence emission spectra of the cell-bound FITC-B1B5/TR-SSA6 RET pair, platelets were incubated (20 min, 23 °C) with saturating concentrations of FITC-B1B5 and TR-SSA6 before gel filtration over Sepharose CL-2B (Pharmacia LKB Biotechnology Inc.) into incubation buffer. The eluted platelets, now free of unbound antibody, were diluted to 5×10^7 /ml in incubation buffer, and 2-ml aliquots were incubated in the dark without stirring for 10 min at 37 °C with either PGE_1 (1 μ M), ADP (10 μ M), or thrombin (0.5 unit/ml). Samples were then transferred to a fluorescence cuvette, and emission spectra (500–600 nm; 8-nm slit width) were obtained with excitation at 470 nm (8 nm slit) using a SLM-Aminco 8000 Spectrofluorometer in the photon counting mode. The output was automatically integrated at 5-s intervals and corrected for instrument response using correction factors supplied by the manufacturer.

RESULTS

Resonance Energy Transfer Studies of GP IIb-IIIa on Unstimulated Platelets—The main goal of these studies was to examine whether GP IIb-IIIa undergoes a conformational change upon agonist-mediated platelet activation. To address this issue, we used the following three monoclonal antibodies specific for distinct exoplasmic epitopes on platelet GP IIb-IIIa: B1B5, (specific for GP IIb), SSA6 (specific for GP IIIa), and A2A9 (specific for the GP IIb-IIIa complex). Aliquots of gel-filtered platelets were incubated simultaneously with saturating concentrations of pairs of these antibodies. One antibody of each pair was FITC labeled to serve as a fluorescence donor, and the other was TR labeled to serve as a fluorescence acceptor. Then, platelet fluorescence due to the presence of cell-bound FITC-labeled antibody was measured in a flow cytometer. We hypothesized that 1) the epitopes for the two antibodies in each pair might be close enough to each other within the GP IIb-IIIa heterodimer so that we could detect fluorescence resonance energy transfer between the FITC and TR chromophores covalently bound to each of the antibodies, and 2) during platelet activation, a change in the spatial relationship between the two epitopes within GP IIb-IIIa might occur, resulting in a detectable change in RET. As a first step in validating this approach, we studied RET between these GP IIb-IIIa antibodies in unstimulated platelets. These experiments were carried out in the presence of 0.1 μ M PGI_2 to insure that the platelets remained in a nonactivated state. Although not shown, similar results were obtained when PGI_2 was omitted.

When unstimulated platelets were labeled simultaneously with saturating concentrations of FITC-B1B5 and TR-SSA6, there was a small but consistent reduction in platelet-associated FITC-B1B5 fluorescence compared to platelet samples

stained with FITC-B1B5 and unlabeled SSA6 (Fig. 1). In eight separate experiments, the reduction in FITC-B1B5 fluorescence averaged $5.7 \pm 1.8\%$ (S.E.; $p < 0.03$). This apparent energy transfer between these chromophore-labeled antibodies specific for GP IIb and IIIa was also observed when FITC-SSA6 was used as the fluorescence donor and TR-B1B5 was used as the fluorescence acceptor (Fig. 1). In this case, the reduction in FITC-SSA6 fluorescence due to the simultaneous platelet binding of TR-B1B5 averaged $8.0 \pm 1.0\%$ ($n = 8$; $p < 0.001$). Similar experiments were conducted with either B1B5 or SSA6 paired with the anti-GP IIb-IIIa antibody, A2A9. In each case, RET was always observed when the fluorescence of FITC-SSA6, FITC-B1B5, or FITC-A2A9 was measured in the presence of a second GP IIb-IIIa-reactive antibody labeled with the chromophore, TR (Table I). On the other hand, when cell-associated fluorescence from FITC-A2A9 was measured

in the presence of equimolar concentrations of either unlabeled or TR-labeled A2A9, the signal from FITC-A2A9 on platelets was unaffected by the presence of TR-A2A9 (Table I). In this case, the potential RET donor and acceptor antibodies are bound to separate GP IIb-IIIa complexes because each heterodimer expresses only one A2A9 epitope.

The results of Fig. 1 and Table I suggest that the TR-dependent quenching of the fluorescein signal is due to resonance energy transfer from FITC to TR when these chromophores are brought into close proximity upon binding of FITC- and TR-labeled antibodies to different epitopes within the same GPIIb-IIIa heterodimer. Alternatively, the apparent quenching of platelet-associated FITC fluorescence when measured in the presence of a second TR-labeled antibody to GP IIb-IIIa might also reflect 1) reduced cell-surface binding of the FITC-labeled antibody due to steric hindrance arising from antibody bound to a second epitope in GP IIb-IIIa, 2) a change in the environment of the FITC chromophore upon binding of a second antibody that might affect either the extinction coefficient or quantum yield of fluorescein, or 3) inner filter effects due to TR (which absorbs maximally at 544 nm), resulting in the recapture of photons originally emitted by the FITC fluorophore (a process unrelated to the proximity of the FITC- and TR-labeled antibodies on the cell surface).

The possibility that the three anti-GP IIb-IIIa antibodies used in this study competed with one another for binding to their respective epitopes on GPIIb-IIIa was examined by comparing the fluorescein signal of platelets stained with FITC-labeled antibody in the presence and absence of saturating concentrations of each of the other two (unlabeled) antibodies. In all cases, the cell-associated fluorescence arising from FITC-SSA6, FITC-B1B5, or FITC-A2A9 was unaffected by the presence of either of the other unlabeled antibodies, indicating that these antibodies do not cross-compete for binding to their respective epitopes (data not shown). This absence of a change in the FITC signal when measured in the presence of unlabeled antibody against another epitope in GPIIb-IIIa also suggests that the simultaneous binding of these antibody pairs to their respective epitopes in GPIIb-IIIa does not in itself alter the environment of the FITC chromophores so as to quench the fluorescein emission. To determine whether inner filter effects contributed to the apparent quenching of the fluorescein signal observed in the presence of TR-labeled antibodies, the signal from platelet-bound FITC-AP1, an antibody specific for an epitope on cell surface GP Ib (24), was measured in the presence of a saturating concentration of either TR-B1B5, TR-SSA6 or TR-A2A9. As shown by the data of Table I, in this circumstance, no TR-dependent change in fluorescein signal was observed, suggesting that the presence of a FITC-labeled antibody at a site distant from GP IIb-IIIa does not give rise to the apparent quenching at fluorescein emission that is seen when both antibodies are directed to epitopes located on the same GP IIb-IIIa complex.

In addition to a decrease in fluorescence of the RET donor chromophore, RET can be accompanied by an increase in emission from the acceptor fluorophore. However, due to loss of resonance energy by nonradiative decay of the excited state of the acceptor chromophore, this sensitized fluorescence of the RET acceptor is less readily detected than the quenching of donor fluorescence. Because of the low range of RET efficiencies detected for the various GP IIb-IIIa-reactive antibody pairs employed in this study (2.8–8.0% based on donor quenching) and the low quantum yield of the TR acceptor (approximately 0.28), we did not attempt to analyze RET

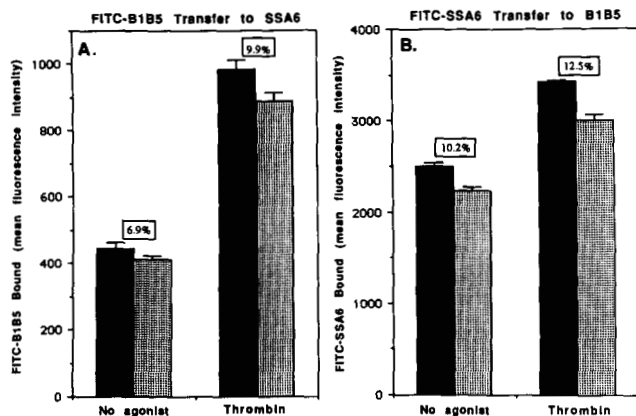


FIG. 1. RET between antibodies bound to GP IIb (B1B5) and GP IIIa (SSA6). In this representative experiment, gel-filtered platelets were incubated for 10 min with either $0.1 \mu\text{M}$ PGI₂ (No agonist) or 0.5 unit/ml thrombin in the presence of saturating concentrations of antibodies B1B5 and SSA6. In A, FITC-B1B5 was used along with either unlabeled SSA6 (black bars) or TR-labeled SSA6 (hatched bars). In B, FITC-SSA6 was used in combination with either unlabeled B1B5 (black bars) or TR-B1B5 (hatched bars). Then platelet-bound FITC fluorescence was quantitated by flow cytometry as described under "Experimental Procedures." RET was calculated for each combination of donor (FITC) and acceptor (TR)-labeled antibody from the difference in FITC fluorescence obtained in the presence of TR-labeled acceptor antibody and that obtained with the same unlabeled acceptor antibody (see "Experimental Procedures" details). Values for RET are shown above the bars. The data represent the mean $\pm$ S.D. of triplicate samples.

TABLE I

Fluorescence resonance energy transfer between chromophore-labeled monoclonal antibodies bound to platelets

Donor antibody	Donor specificity	Acceptor antibody	Acceptor specificity	RET ^a	No. of experiments
				%	
FITC-A2A9	IIb-IIIa	TR-B1B5	IIb	3.2 ± 0.9^b	5
FITC-A2A9	IIb-IIIa	TR-SSA6	IIIa	4.5 ± 1.1^b	5
FITC-SSA6	IIIa	TR-A2A9	IIb-IIIa	3.2 ± 0.7^b	8
FITC-B1B5	IIb	TR-A2A9	IIb-IIIa	2.8 ± 2.0	6
FITC-A2A9	IIb-IIIa	TR-A2A9	IIb-IIIa	0.1 ± 0.8	4
FITC-AP1	Ib	TR-B1B5	IIb	0.6 ± 0.5	3
FITC-AP1	Ib	TR-SSA6	IIIa	0.4	1
FITC-AP1	Ib	TR-A2A9	IIb-IIIa	1.0 ± 0.2	2

^a Resonance energy transfer efficiency between fluorescein (FITC)-labeled donor antibodies and tetramethylrhodamine (TR)-labeled acceptor antibodies. RET was derived as described under "Materials and Methods." Values are means $\pm$ S.E. of the indicated number of experiments.

^b Indicates that the values are statistically greater than 0% RET at $p \leq 0.01$ (Student's *t* test for paired samples).

efficiency from the red fluorescence of the TR-labeled antibodies. However, it could be demonstrated that the reduction in FITC fluorescence of an antibody bound to platelet GP IIb-IIIa was related to the amount of a second TR-labeled acceptor antibody bound to the platelets, consistent with RET arising through localization of both the FITC- and the TR-labeled antibody within the same GP IIb-IIIa heterodimer (Fig. 2). On the basis of these observations, we conclude that the observed quenching of donor fluorescence arises by RET between donor and acceptor chromophores on platelet-bound B1B5, SSA6, and A2A9, reflecting the proximity of these antibodies to each other when they are bound to their specific epitopes on a GP IIb-IIIa complex.

Resonance Energy Transfer Studies of GP IIb-IIIa on Activated Platelets—Platelet activation causes GP IIb-IIIa to express binding activity for RGD-containing adhesive macromolecules, such as fibrinogen, and for the monoclonal antibody, PAC1 (2, 3, 7, 10). To see whether induction of binding competency might relate to a change in the conformation of the GP IIb-IIIa complex, platelets were activated by thrombin and resonance energy transfer between chromophore-labeled, platelet-bound B1B5 and SSA6 was examined. Thrombin at 0.5 unit/ml induced the expression of the ligand binding function of platelet GP IIb-IIIa as evidenced by 1) FITC-PAC1 binding to platelets (Fig. 3A), 2) fibrinogen binding to platelets (determined with FITC-9F9, an antibody specific for platelet-bound fibrinogen (26) (Fig. 3B), and 3) platelet binding of FITC-LIBS1, an antibody specific for a neo-epitope in GP IIIa expressed upon the binding of fibrinogen to GP IIb-IIIa (28) (Fig. 3C). RET between chromophore-labeled B1B5 and SSA6 increased as a result of thrombin stimulation (Fig. 1). In eight experiments, the efficiency of RET between FITC-B1B5 and TR-SSA6 averaged $9.9 \pm 2.1\%$ for thrombin-stimulated platelets, a value 72% higher than that for unstimulated platelets ($p < 0.02$) (Fig. 4). An increase in RET with thrombin was also observed when FITC-SSA6 was used as

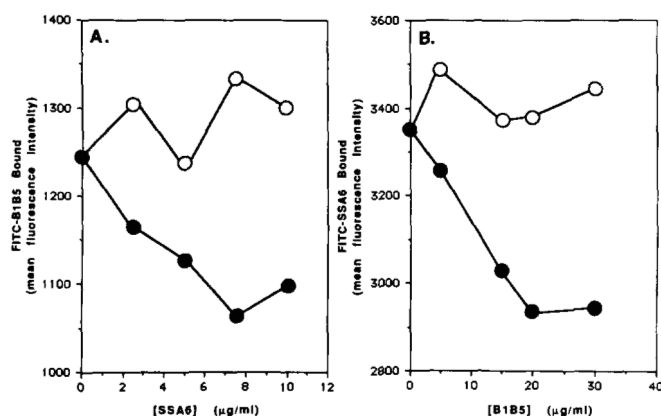


FIG. 2. Effect of a TR-labeled anti-GP IIb-IIIa antibody on platelet-bound fluorescence of a second, FITC-labeled anti-GP IIb-IIIa antibody. Thrombin-stimulated platelets were incubated with a saturating concentration of FITC-B1B5 (30 $\mu\text{g/ml}$) (A) or FITC-SSA6 (10 $\mu\text{g/ml}$) (B) in the presence of increasing concentrations of either unlabeled (open circles) or TR-labeled (closed circles) SSA6 (A) or B1B5 (B). Then platelet-bound FITC-fluorescence was measured by flow cytometry. Note that in each case, TR-labeled antibody caused a dose-dependent decrease in FITC fluorescence while the same unlabeled antibody did not. Data are the means of triplicate determinations. At each antibody concentration, the values obtained in the presence of the TR-labeled antibody were significantly less than those obtained with the same unlabeled antibody ($p \leq 0.05$). In separate studies not shown here, the binding of SSA6 to platelets was half-maximal at about 2 $\mu\text{g/ml}$ and maximal at 8 $\mu\text{g/ml}$. B1B5 binding was half-maximal at about 10 $\mu\text{g/ml}$ and maximal at 25 $\mu\text{g/ml}$.

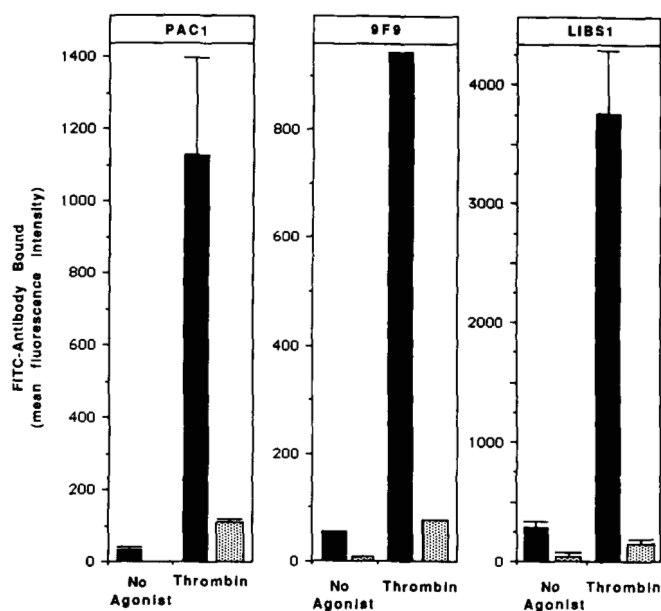


FIG. 3. Effect of antibody A2A9 on binding of PAC1, fibrinogen, and LIBS1 to platelets. Unstimulated or thrombin-stimulated platelets were incubated for 10 min with saturating concentrations of either FITC-PAC1 (40 $\mu\text{g/ml}$), FITC-9F9 (50 $\mu\text{g/ml}$), or FITC-LIBS1 (50 $\mu\text{g/ml}$) in the absence (black bars) or presence of 10 $\mu\text{g/ml}$ A2A9 (hatched bars). Then the amount of FITC-labeled antibody was measured by flow cytometry. Data for PAC1 and LIBS1 represent means $\pm$ S.D. of three experiments. 9F9 binding was studied once. The concentration of A2A9 used here was the same as that used for the RET analyses shown in Table I and Fig. 7.

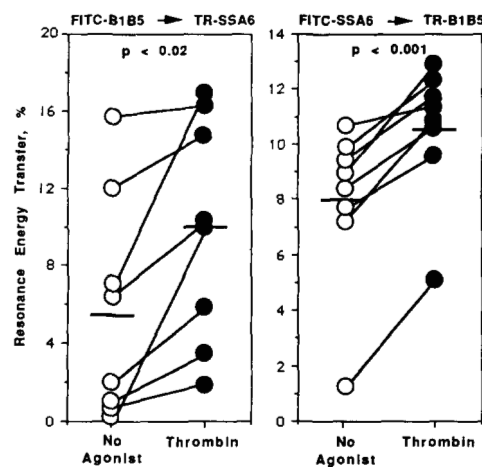


FIG. 4. Effect of 0.5 unit/ml thrombin on RET between platelet-bound antibody B1B5 and antibody SSA6. Platelets were incubated in the presence of chromophore-labeled B1B5 and SSA6 and RET analyses were carried out as described under "Experimental Procedures" and in the legend to Fig. 1. Horizontal bars are the means of eight separate experiments. The p values shown are for the differences between unstimulated and thrombin-stimulated platelets determined by Student's t test for paired samples.

the fluorescence donor and TR-B1B5 as the fluorescence acceptor. In this case, RET for thrombin-stimulated platelets averaged $10.6 \pm 0.9\%$, a value 33% higher than that for unstimulated platelets ($p < 0.0001$) (Fig. 4). In addition, when unstimulated platelets were first incubated with FITC-B1B5 and TR-SSA6 and then gel filtered to remove unbound antibody, thrombin caused an increase in RET between these antibodies (Fig. 5). Since these antibodies had been prebound to the cell surface prior to cell stimulation, these data suggest that platelet activation by thrombin causes a change in the

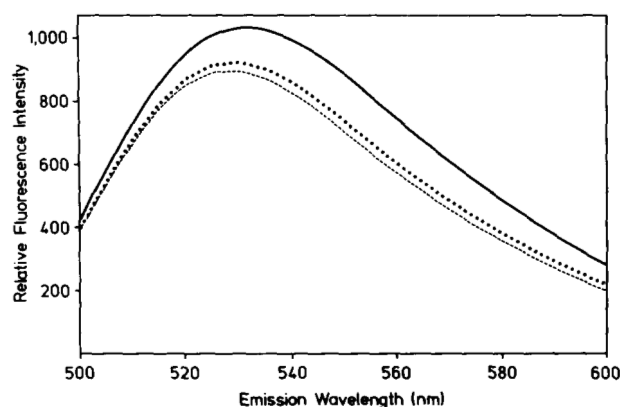


FIG. 5. Fluorescence emission spectra of platelet-bound FITC-B1B5 and TR-SSA6. Platelets were incubated with both FITC-B1B5 and TR-SSA6 and then gel filtered to remove unbound antibody as described under "Experimental Procedures." Fluorescence emission spectra of the gel-filtered cell suspension (5×10^7 /ml) were obtained following incubation in the dark for 10 min at 37°C with either PGE_1 ($1 \mu\text{M}$) (solid line), ADP plus epinephrine ($10 \mu\text{M}$ each) (dotted line), or thrombin (0.5 unit/ml) (dashed line). Excitation was at 470 nm (8-nm slit). This experiment is representative of two so performed.

spatial relationships between the epitopes for B1B5 and SSA6 on GP IIb and IIIa, which arises independently of increased antibody binding due to redistribution of an internal pool of GP IIb-IIIa complexes to the cell surface (see "Discussion").

Other agonists known to activate the fibrinogen receptor function of GP IIb-IIIa also caused an increase in RET efficiency between B1B5 and SSA6. For example, when FITC-SSA6 was the fluorescence donor and TR-B1B5 the acceptor, increases in RET were observed when platelets were stimulated by a combination of epinephrine and ADP (25%, $p < 0.05$; three experiments) or $0.2 \mu\text{M}$ phorbol myristate acetate (40%, $p < 0.05$; four experiments).

Extracellular calcium is required for agonist-mediated activation of GP IIb-IIIa (12) as well as for fibrinogen binding to platelets (7). Therefore, we evaluated the effect of chelation of extracellular calcium on RET between chromophore-labeled B1B5 and SSA6. Addition of 5 mM EGTA to unstimulated platelets at room temperature (an amount of chelator sufficient to decrease the extracellular ionized calcium concentration to $\leq 1 \text{ nM}$ (30)) caused an increase in RET between these two platelet-bound antibodies (Fig. 6). This increase was not related to exposure of the fibrinogen-binding site on GP IIb-IIIa because no increase in FITC-PAC1 or FITC-9F9 (fibrinogen) binding to platelets was observed under these conditions (data not shown). Since the GP IIb-IIIa complex is known to contain saturable binding sites for calcium (30, 31), these results suggest that removal of calcium from GP IIb-IIIa causes a change in its conformation that affects the relative orientation of the epitopes for B1B5 and SSA6. Furthermore, in contrast to the results obtained in the presence of extracellular calcium, thrombin did not cause an increase in the efficiency of RET between these antibodies in the presence of EGTA. Indeed, the values for RET observed with thrombin in the presence of EGTA were lower than those observed for unstimulated platelets in the presence of EGTA, although these differences were not statistically significant (Fig. 6). The inability of thrombin to increase RET in the presence of EGTA is consistent with the inability of this agonist to induce competency of fibrinogen receptors under this condition.

Role of Fibrinogen Binding in Resonance Energy Transfer Studies of GP IIb-IIIa—Several lines of evidence indicate that

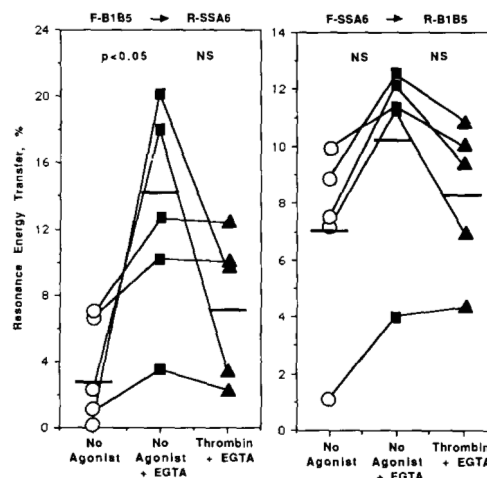


FIG. 6. Effect of EGTA on RET between antibody B1B5 and antibody SSA6. Incubations of platelets with these two chromophore-labeled antibodies were carried out in the presence or absence of 5 mM EGTA, and RET analyses were carried out as described in the legends to Figs. 1 and 4. The p values shown are for the differences between unstimulated and EGTA-treated platelets and between EGTA-treated platelets incubated in the presence or absence of 0.5 unit/ml thrombin.

the conformation of GP IIb-IIIa as well as the distribution of these complexes on the platelet surface can be affected by ligand binding. This raised the possibility that the change in RET we observed upon platelet stimulation occurs secondarily to the binding of fibrinogen to activated receptors and not to a conformational change underlying receptor activation, *per se*. For example, the binding of fibrinogen or small RGD-containing peptides to ADP-stimulated platelets results in the appearance of neo-epitopes on GP IIb and IIIa (32). Furthermore, incubation of solubilized GP IIb-IIIa with peptides derived from fibrinogen causes a slow (hours) change in the conformation of the complex as detected by intrinsic protein fluorescence and density gradient sedimentation (33). Although our experiments were performed in the absence of added fibrinogen, some of the agonists that were used to activate GP IIb-IIIa, particularly thrombin and phorbol myristate acetate, can induce the secretion of fibrinogen from platelet α -granules, providing potential ligand for newly activated cell surface receptors. Therefore, we sought to determine whether the increase in RET observed upon platelet activation was dependent on fibrinogen binding to GP IIb-IIIa.

One of the anti-GP IIb-IIIa antibodies used in these studies, A2A9, inhibits the binding of fibrinogen to GP IIb-IIIa in a competitive manner, and its own binding to platelets is poorly reversible (11, 27). Thus, any GP IIb-IIIa complex to which A2A9 has become bound cannot bind fibrinogen simultaneously. We used this feature of A2A9 binding to examine the fibrinogen requirement for thrombin-induced changes in RET. When A2A9 was paired with either B1B5 or SSA6, thrombin caused a significant increase in RET, whether A2A9 was used as the FITC-labeled energy donor or the TR-labeled energy acceptor ($p \leq 0.03$) (Fig. 7). Under the conditions of these experiments, A2A9 effectively prevented thrombin-induced ligand binding: FITC-PAC1 binding was inhibited by 93%, fibrinogen binding by 98%, and FITC-LIBS1 binding by 100% (Fig. 3). Similarly, in two experiments, the activation-related change in RET between labeled B1B5 and SSA6 was unaffected by addition of 2 mM RGDS (to prevent fibrinogen binding) or $100 \mu\text{g/ml}$ fibrinogen (to saturate fibrinogen receptors). Thus, the activation-related conformational

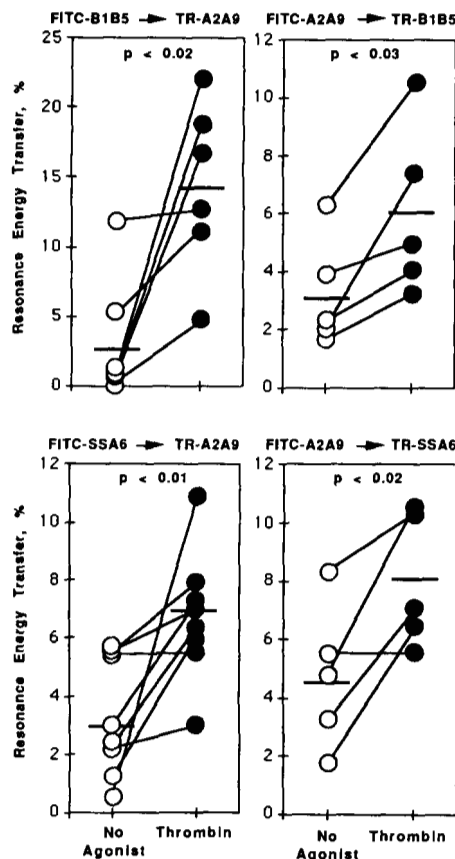


FIG. 7. Effect of thrombin on RET between chromophore-labeled A2A9 and either B1B5 or SSA6. Experiments were carried out as described in the legend to Fig. 1 except that A2A9 was used as one of the antibodies in each pair along with either B1B5 (upper panels) or SSA6 (lower panels). Where indicated, thrombin was present at 0.5 unit/ml. The p values shown are for the differences between unstimulated and thrombin-stimulated platelets.

change that results in increased RET between these antibodies on GP IIb-IIIa does not require fibrinogen binding to the receptor.

One consequence of fibrinogen binding to GP IIb-IIIa is a redistribution of heterodimer complexes within the plane of the plasma membrane resulting in aggregates or clusters of GP IIb-IIIa. Evidence for receptor clustering has been obtained by immunoelectron microscopy and by an NMR technique (34–37). Theoretically, this could result in the apposition of GP IIb-IIIa epitopes on adjacent GP IIb-IIIa complexes and complicate the interpretation of the RET data. However, receptor clustering was considered an unlikely cause of the changes in RET efficiency in our studies. First, when platelets were incubated with equimolar concentrations of FITC-A2A9 and TR-A2A9 in order to label epitopes on separate GP IIb-IIIa complexes, no RET between these antibodies was detected after platelets were maximally stimulated with 0.5 unit/ml thrombin (RET for unstimulated platelets = $0.1 \pm 0.8\%$; thrombin-stimulated platelets = $-1.2 \pm 0.5\%$; $n = 4$, $p > 0.3$). Second, as discussed above, the activation-induced increases in RET (Figs. 4 and 7) were unaffected by addition of fibrinogen and were observed in the virtual absence of fibrinogen binding (Fig. 3), the latter being a prerequisite for receptor clustering (34).

DISCUSSION

These studies demonstrate that 1) the simultaneous binding of two antibodies to distinct cell-surface epitopes within the

platelet GPIIb-IIIa complex can be detected by flow cytometric analysis of (RET) between donor and acceptor fluorophores covalently bound to the antibodies. 2) The efficiency of RET between these labeled antibodies increases upon platelet activation by agonists that induce expression of fibrinogen receptor function. 3) This change in RET observed upon platelet activation does not require fibrinogen binding to GP IIb-IIIa. Taken together, these results suggest that platelet activation leading to expression of cell-surface fibrinogen receptor function is initially accompanied by a conformational change in GP IIb-IIIa that affects the spatial relationship of its exoplasmic domains, potentially accounting for this receptor's interconversion from a ligand-incompetent to a ligand-competent state.

GP IIb and IIIa contain 1,008 and 736 amino acid residues, respectively, and 90–95% of these residues are purportedly exoplasmic (38, 39). Although the precise physical size of the GP IIb-IIIa complex within the platelet surface membrane is unknown, electron microscopic studies of GP IIb-IIIa inserted into artificial lipid vesicles show that individual heterodimers appear as spikes protruding as much as 200 Å from the vesicle surface (40). The present data indicate that when the monoclonal antibodies, B1B5, SSA6, and A2A9 are bound to their respective epitopes on GPIIb-IIIa, they are arrayed in sufficient proximity to allow RET between the chromophores that are linked to each antibody. Considering that the Forster critical distance for RET from FITC to TR is approximately 37 Å, this suggests that the average separation between the FITC and TR chromophores on these antibodies is less than 70 Å (41). Given the apparent size of an IgG molecule and the dimensions of a GP IIb-IIIa complex, this value is consistent with the conclusion that RET is occurring between chromophore-labeled antibodies bound to the same GP IIb-IIIa complex.

Caution is warranted in the interpretation of the present resonance energy transfer data as they relate to the presumed molecular changes in GP IIb-IIIa during cell activation. First, the segmental mobility of the chromophore-labeled IgG molecules, combined with uncertainty as to the sites within the antibodies that are labeled by FITC or TR, precludes use of these data to deduce the precise spatial separation of the SSA6, B1B5, and A2A9 epitopes within GPIIb-IIIa. Second, the increased RET detected upon platelet activation can reflect a change in separation or alignment of the donor and acceptor chromophores secondary to repositioning of the epitopes to which these antibodies are bound, but does not necessarily imply that these epitopes physically move closer together. Third, the magnitude of the change in RET observed during platelet activation is given by the Forster equation and depends on such factors as the change in distance and orientation of the dipole centers of the donor and acceptor chromophores, as well as the overlap integral of donor emission and acceptor absorbance spectra. Therefore, although we suggest that the conformational change in GP IIb-IIIa detected by RET is related to the conversion of this molecule into a competent fibrinogen receptor, one would not anticipate a strict quantitative relationship between the cell activation-related increase in RET (as seen in Fig. 4) and the increase in fibrinogen binding (as seen Fig. 3).

In addition to an interconversion of GP IIb-IIIa from a ligand-incompetent to ligand-competent state, platelet activation is also accompanied by the redistribution of an internal pool of GPIIb-IIIa complexes to the cell surface, which is detected by increased binding of FITC-SSA6, FITC-B1B5, and FITC-A2A9 to activated *versus* unstimulated platelets (see Fig. 1) (8, 9). This raises the possibility that the enhanced

RET observed for activated platelets reflects an altered conformation unique to this new pool of exposed GPIIb-IIIa, rather than a conformational change that accompanies the interconversion of this receptor to its ligand-competent state. Although this possibility cannot be completely excluded, it is unlikely because RET between chromophore-labeled SSA6 and B1B5 was observed to increase upon platelet activation even when the cells were washed free of unbound antibody before addition of agonist (Fig. 5). In this case, since no free antibody was available to bind to the newly exposed GPIIb-IIIa complexes, the changes in RET presumably reflect the rearrangement of antibody that is bound exclusively to those GPIIb-IIIa complexes that were exposed on the platelet surface prior to cell activation.

As noted above, immunoelectron microscopic data suggest that platelet activation is accompanied by a redistribution of GPIIb-IIIa complexes in the plane of the membrane, resulting in an apparent aggregation or clustering of these heterodimers. However, the activation-related enhancement of RET we observed between antibodies bound to GP IIb-IIIa was unaltered under conditions where the potential binding of secreted fibrinogen to activated receptors was blocked. Since the clustering of multiple GPIIb-IIIa complexes is not observed in the absence of fibrinogen binding (34–36), the weight of evidence suggests that receptor clustering does not account for the increases in RET we observed between these labeled antibodies. Furthermore, when antibody to a single GP IIb-IIIa epitope (A2A9) served as both RET donor and acceptor, no RET was detected for either thrombin-activated or unstimulated platelets. This suggests that RET is only observed between antibodies bound to different epitopes within a single GP IIb-IIIa heterodimer and cannot be accounted for by A2A9 molecules that are bound to separate heterodimer complexes.

Extracellular ionized calcium affects the structure and function of the GP IIb-IIIa complex. Calcium plays a role in maintaining the physical integrity of GP IIb-IIIa heterodimers (5, 6, 42–45) and is required for activation-induced expression of fibrinogen receptors (12) and for fibrinogen binding (7). GP IIb-IIIa has been shown to contain high affinity ($K_D \leq 1 \mu\text{M}$) binding sites for extracellular calcium (31), presumably related to regions in GP IIb that contain amino acid sequences similar to those found in the calcium-binding domains of calmodulin (38). As illustrated by the data of Fig. 6, when unstimulated platelets were incubated in the presence of an amount of EGTA sufficient to decrease extracellular ionized calcium to $\leq 1 \text{ nM}$, RET between SSA6 and B1B5 increased. Furthermore, no additional change in RET was observed in EGTA-treated platelets upon activation by thrombin. Since the EGTA experiments were carried out under conditions in which the heterodimer structure of membrane-associated GP IIb-IIIa remains intact (pH 7.4, room temperature) (6, 44, 45), these results are consistent with the interpretation that removal of calcium from GP IIb-IIIa affects its conformation. The data also suggest that extracellular calcium may be required for a conformational change in GP IIb-IIIa that takes place during cell activation. These conclusions are consistent with previous studies showing that calcium affects the sedimentation properties and enzyme sensitivity of purified GP IIb (43) and that the selective binding of the antibody, PAC1, to activated platelets is calcium-dependent (10, 12).

The relationship of the present findings to the underlying mechanism of agonist-induced fibrinogen receptor exposure must remain speculative until we have a better understanding of the metabolic processes within the platelet that lead to activation of GP IIb-IIIa. Agonists of diverse chemical struc-

ture can induce fibrinogen receptor exposure. In some of these cases, such as thrombin and thromboxane A_2 , fibrinogen receptor expression can be induced by a process involving the interaction of agonist-bound excitatory receptors with one or more G proteins, resulting in the activation of phospholipase C and protein kinase C (46). By contrast, ADP or epinephrine can expose fibrinogen receptors without detectable activation of phospholipase C and protein kinase C (48–50). The metabolic pathways involved in this circumstance as well as the event ultimately responsible for fibrinogen receptor exposure remain to be characterized. Analogous to other transmembrane proteins such as the receptor for platelet-derived growth factor (51), it is conceivable that cell activation induces the physical association of some transducing element, for example a GTP-binding protein, a protein kinase, or a protein phosphatase with the cytoplasmic domains of GP IIb and/or IIIa. As a result of this interaction, a covalent or noncovalent modification of these cytoplasmic domains might then induce a conformational change in the exoplasmic portions of GP IIb-IIIa, thereby resulting in exposure of the fibrinogen receptor. Of note in this regard, thrombin causes increased phosphorylation of the cytoplasmic domain of GP IIIa on serine residues (52). In addition, several platelet proteins become phosphorylated on tyrosine residues following agonist-induced activation of GP IIb-IIIa and platelet aggregation (53). Further characterization of the molecular basis of GP IIb-IIIa activation should be facilitated by the use of reconstitution techniques in which purified GP IIb-IIIa and putative transducing elements are coinserted into liposomes (40, 54) or in which cloned GP IIb-IIIa is inserted into heterologous mammalian expression systems (4).

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