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Research Article

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Ligand Bridging Mediates Integrin $\alpha_{IIb}\beta_3$ (Platelet GPIIB-IIIA) Dependent Homotypic and Heterotypic Cell–Cell Interactions

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Abstract

The aggregation of cells bearing recombinant integrin $\alpha_{IIb}\beta_3$ (platelet GPIIb-IIIa) has been analyzed by two-color flow cytometry. As in normal platelets, aggregation requires functional $\alpha_{IIb}\beta_3$, "activation" of $\alpha_{IIb}\beta_3$, and fibrinogen (fg) binding to $\alpha_{IIb}\beta_3$. Cellular aggregation required that both interacting cells express functional $\alpha_{IIb}\beta_3$, because a binding defective mutant, $\alpha_{IIb}\beta_3$ (D119 → Y), failed to support interaction with wild type $\alpha_{IIb}\beta_3$-bearing cells. In addition, cells bearing resting $\alpha_{IIb}\beta_3$ were incorporated into aggregates formed by cells bearing a constitutively active mutant, $\alpha_{IIb}\beta_3$ (β_1-2), indicating that only one of the cells in an interacting pair must be activated. Finally, heterotypic interactions occurred between cells bearing activated $\alpha_{IIb}\beta_3$ and cells bearing $\alpha_v\beta_3$, a fg-binding integrin present on endothelial and tumor cells. Thus, ligand bridging between fg-binding integrins represents a mechanism of cell–cell interaction, cells bearing resting $\alpha_{IIb}\beta_3$ (e.g., resting platelets) may be incorporated into aggregates formed by cells bearing activated $\alpha_{IIb}\beta_3$, and $\alpha_{IIb}\beta_3$ mediates heterotypic interactions with cells bearing other fg receptors. (*J. Clin. Invest.* 1991. 88:1128–1134.) Key words: thrombosis • hemostasis • platelet aggregation • fibrinogen • RGD

Introduction

The integrins comprise a family of heterodimeric cell surface receptors composed of α and β subunits which mediate cell–cell and cell-extracellular matrix interactions (1, 2). The integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) is obligatory for physiologic platelet aggregation (3, 4). The binding of fibrinogen (fg)[1] to $\alpha_{IIb}\beta_3$ is necessary for normal aggregation, and fg binding requires cellular activation by agonists such as ADP, epinephrine, or thrombin (3, 4). A closely related but more widely distributed integrin, $\alpha_v\beta_3$ (vitronectin receptor), is found on platelets (5) but is also present on a variety of other cell types such as endothelial, smooth muscle, and tumor cells (1, 6, 7). Because $\alpha_v\beta_3$ also binds adhesive proteins such as fg (7–9), the presence of this integrin on endothelial and tumor cells may mediate interactions involved in events such as tumor attachment and invasion through the endothelium.

Several integrins mediate cell–cell interactions by binding to integral membrane protein counter-receptors on other cells. In the case of leukocyte integrins, at least three such counter-receptors (ICAM-1 [10], ICAM-2 [11], and VCAM-1 [12]) are members of the immunoglobulin supergene family. Members of the immunoglobulin family are present on the platelet surface as well (13). Moreover, the binding of fg to $\alpha_{IIb}\beta_3$ provokes changes in the conformation of both the $\alpha_{IIb}\beta_3$ (14) and the fg (15). Thus, it is possible that the conformational changes in $\alpha_{IIb}\beta_3$ which follow fg binding enable it to mediate aggregation by binding to a cellular counter-receptor. In the present work, we have evaluated the requirement for an $\alpha_{IIb}\beta_3$ counter-receptor by analysis of fg-mediated aggregation of Chinese hamster ovary (CHO) cells bearing recombinant $\alpha_{IIb}\beta_3$. The $\alpha_{IIb}\beta_3$ in these cells, once activated, binds fg at 1:1 stoichiometry with an affinity similar to the affinity of platelet $\alpha_{IIb}\beta_3$ (16). Furthermore, the $\alpha_{IIb}\beta_3$-expressing transfectants undergo fg-mediated aggregation which mimics features of physiologic platelet aggregation (17). In the present study, the molecular requirements for β_3-mediated cell–cell interaction have been analyzed using two-color flow cytometry. We found that (*a*) aggregation requires functional β_3 receptors on both apposing cells, but activation of $\alpha_{IIb}\beta_3$ is not absolutely required for cellular incorporation into existing aggregates, and (*b*) $\alpha_v\beta_3$-bearing cells undergo fg-mediated binding to cells bearing activated $\alpha_{IIb}\beta_3$, indicating a potential mechanism of platelet interaction with tumor or endothelial cells.

Methods

Stable cell lines expressing recombinant human β_3 integrins. Stable CHO cell lines, cotransfected with human β_3 and α_{IIb} or α_v cDNAs were prepared and characterized as described (16, 18). Cell lines bearing either α_{IIb} or α_v together with β_3 (D119 → Y), which fail to bind ligands were also employed (18). A six amino acid substitution mutant, β_3 (β_{1-2}), was cotransfected with α_{IIb} to establish stable cell lines which spontaneously bind fg as described (19). All transfectants expressed approximately equal quantities of recombinant integrins on the cell surface as judged by flow cytometric immunofluorescence with α_{IIb}, β_3, and $\alpha_{IIb}\beta_3$ monoclonal antibodies as described (16–18).

Preparation of cell suspensions and fluorescent labeling of cells. Monolayer cultures of wild-type and the transfectant cells were removed from culture flasks using 3.5 mM EDTA and 0.01% TPCK-trypsin (Worthington Biochemicals, St. Louis, MO) in PBS, pH 7.4. This dissociates cells into a single cell suspension without affecting gross $\alpha_{IIb}\beta_3$ structure (17). Cells were washed twice in the presence of 0.05% soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) resuspended in Tyrode's solution (137.5 mM NaCl, 12 mM $NaHCO_3$, 2.6 mM KCl, and 1 mM $MgCl_2$, pH 7.4) containing 0.1% BSA and 0.1% dextrose. The cell count was adjusted to 10^7 cells/ml. Stock solutions of sulfo-fluorescein diacetate (SFDA) (Molecular Probes, Inc., Junction City, OR) and hydroethidine (HE) (Polysciences, Inc., Warrington, PA) were prepared by dissolving the fluorochromes in DMSO at a concentration of 10 mM or 80 mg/ml, respectively. These stock solutions were stored at −20°C for a maximum of 4 wk and thawed just before use. Working solutions were prepared by diluting

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[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; fg, fibrinogen; HE, hydroethidine; SFDA, sulfo-fluorescein diacetate.

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stock with Tyrode's buffer to 200 μM SFDA and 20 μg/ml HE and filtering through a 0.22-μm filter. Homogenous cell fluorescence was achieved by adding equal volumes of dyes to the cell suspensions and incubation for 45 min at 22°C under gentle agitation. Labeled cells were washed thrice in cation-free Tyrode's buffer and resuspended in divalent cation-containing Tyrode's buffer (2 mM $CaCl_2$, 1 mM $MgCl_2$), pH 7.4. The assay mixture contained 4×10^6 cells/ml. The viability of labeled cells was always > 96% based on Trypan Blue exclusion.

Aggregation assay. Aggregation experiments were performed on a gyrorotatory shaker (American Rotator V; American Dade, Miami, FL) in with 1% BSA precoated 24-well tissue culture plates (NUNC, Denmark) at room temperature, as described previously (17). Equal volumes of labeled cells were mixed and 100 μl were added to the wells. When indicated, cells were incubated with monoclonal antibodies (20 nM of 4F10, 1 μM LM609, 8 μM anti-LIBS2) or with GRGDSP or H12 peptides (1 mM) for 20 min. Aggregation was initiated by addition of 300 μg/ml fg (10 μl) and incubation for 15 min at 100 rpm. The cells were fixed by addition of 100 μl of 0.5% paraformaldehyde and samples were held on ice for 30 min before subsequent FACS analysis. The concentrations of SFDA or HE used did not reduce cell viability or aggregation.

Two-color flow cytometry and fluorescence microscopy. Data was obtained in a FACStar 440 and analyzed by Consort 30 software (Becton Dickinson Co., Mountain View, CA). The optics were configured to excite fluorescence of both dyes using the 488-nm peak of an Argon laser, filtered through band pass 488±5 nm filter. Emissions were split and filtered to include 515–545 nm as the green SFDA signal (BP 530±15 nm), and 607–643 nm (BP 625±17 nm) as the red HE signal. A dichroic mirror (DM 570) was used for side scatter adjustment. Particles were passed with a flow rate of 300–700 particles/s through a 70-μm orifice, which was found in preliminary experiments not to dissociate aggregates (data not shown). Aggregates were detected as two-color particles in the green (FL1) versus red fluorescence (FL2) contour plots using five log scales. The stained cells or aggregates, were also observed through a Zeiss universal epifluorescence microscope and photographed on Ektachrome 400 film. To determine the exact composition of aggregates, incorporated red (HE) and green (SFDA) cells were counted visually.

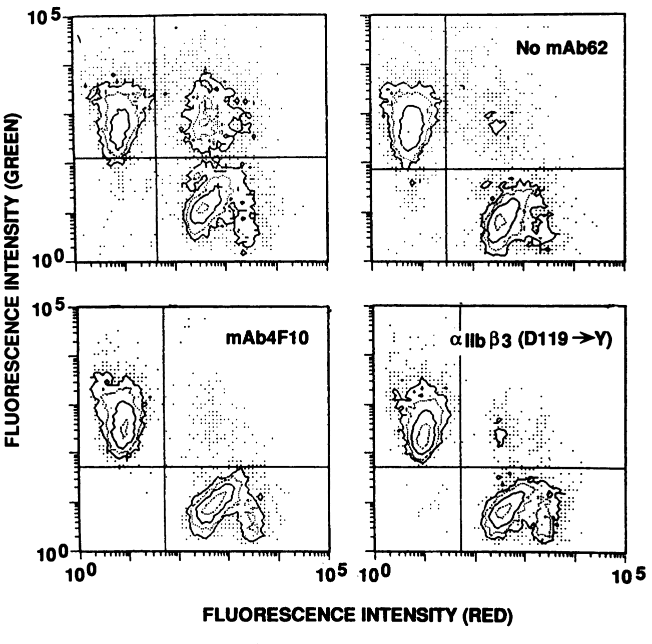

Figure 1. FACS analysis of cellular coaggregation. Contour plots illustrate the two-color FACS analysis of aggregation of SFDA-(green) and HE-(red)-labeled $\alpha_{IIb}\beta_3$-bearing cells. Cells (10^7 cells/ml) were mixed and incubated in the presence of an activating monoclonal antibody, mAb62 (8 μM). After 20 min, fg was added (1 μM) and the mixture was rotated at 100 rpm at room temperature for 30 min. Aggregates were identified as two-color particles (*top left*). The effect of omission of MAb 62 (*top right*) or addition of an anti-$\alpha_{IIb}\beta_3$ monoclonal antibody, mAb4F10 (20 nM), which inhibits fg binding is shown (*bottom left*). Aggregation experiments were also performed using a transfectant expressing a point mutation of β_3, $\alpha_{IIb}\beta_3$ (D119 → Y) which lacks fg binding function (*bottom right*). Each panel represents the analysis of 10,000 particles. The contour lines are 3, 9, 27, and 81. The percentage of total events lying in the aggregate (two-color particle) area are 22.4% (*top left*), 2.8% (*top right*), 1.3% (*bottom left*), and 1.9% (*bottom right*).

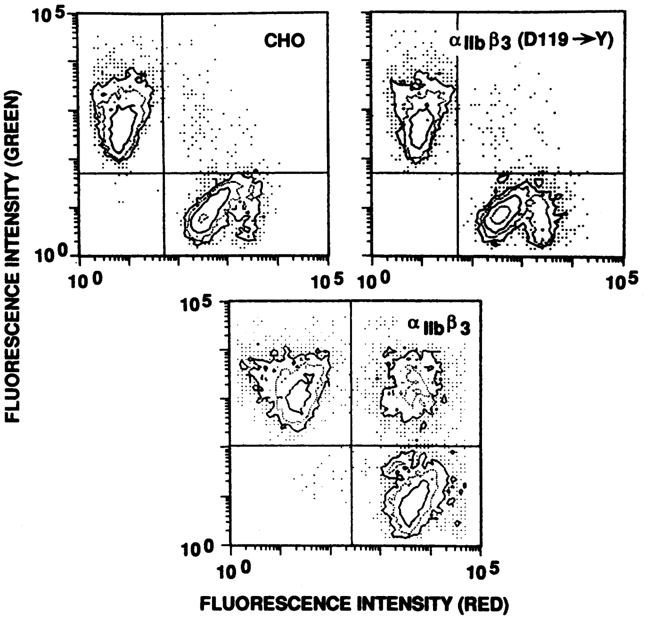

Figure 2. Coaggregation requires functional fg receptors on both cell types. Equal amounts of SFDA-labeled $\alpha_{IIb}\beta_3$ bearing cells and HE-labeled CHO (*top left*), $\alpha_{IIb}\beta_3$ (D119 → Y) (*top right*) or $\alpha_{IIb}\beta_3$ (*bottom*)–expressing cells were mixed and incubated with activating antibody mAb62 for 20 min. fg (1 μM) was added and aggregation was performed in gyrotation at 100 rpm. The percentage of particles lying within the aggregate (two-color particle) region were: 1.1% (*left*), 0.9% (*right*), and 21.6% (*bottom*).

Monoclonal antibodies and reagents. Monoclonal antibody mAb62 recognizes an epitope on β_3 and stimulates fg binding and aggregation as either intact antibody or Fab fragments (16, 17). 4F10 (20) and LM609 (7) are inhibitory anti-$\alpha_{IIb}\beta_3$ and anti-$\alpha_v\beta_3$ antibodies, respectively, which were generously provided by Dr. Virgil Woods (University of California San Diego) and Dr. David Cheresh (Research Institute of Scripps Clinic, La Jolla, CA). The peptides GRGDSP and HHLGGAKQAGDV (H12) were prepared as described (21, 22). Fibronectin-depleted fg was purified according to described methods (21). All other reagents or chemicals used were of the highest grade available.

Results

Validation of two color FACS coaggregation assay. CHO cells expressing human $\alpha_{IIb}\beta_3$ undergo activation and fg-dependent aggregation as assessed microscopically or by particle counting (17). To analyze heterotypic aggregation, we labeled cells with either sulfofluorescein (green fluorescence) or hydroethidine (red fluorescence), and then coaggregation was examined by use of two-color flow cytometry. With this method, aggregation

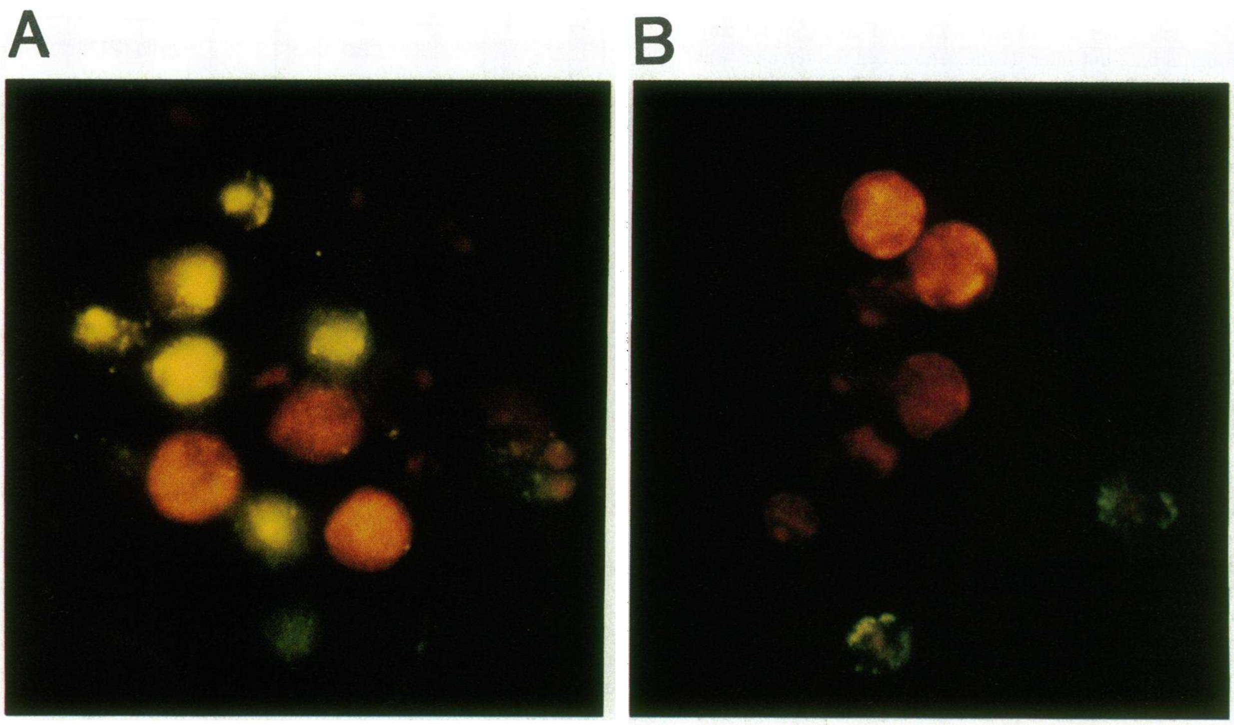

Figure 3. Evaluation of coaggregation using fluorescence microscopy. A representative two-color aggregate is shown composed of $\alpha_{IIb}\beta_3$-bearing cells, labeled separately with SFDA (*green*) and HE (*red*) (*A*). In *B*, $\alpha_{IIb}\beta_3$-expressing cells (*red*) were mixed with transfectants bearing the nonfunctional $\alpha_{IIb}\beta_3$ (D119 → Y) (*green*). Aggregation experiments were performed in presence of 8 μM mAb62 and 1 μM fg. Note that aggregation occurred between $\alpha_{IIb}\beta_3$-bearing cells (*red*), whereas $\alpha_{IIb}\beta_3$ (D119 → Y)-expressing cells (*green*) were not incorporated into aggregates.

of activated $\alpha_{IIb}\beta_3$ bearing cells was readily observed (Fig. 1), as indicated by the detection of particles bearing both fluorochromes. This aggregation required the activating mAb 62 and was blocked by inhibitors of fg binding such as mAb 4F10 (Fig. 1). Aggregation did not occur with untransfected cells (not shown), or cells transfected with nonfunctional fg-receptor, $\alpha_{IIb}\beta_3$ (D119 → Y) (Fig. 1). The two-color FACS analysis thus corresponded well with previous results obtained by direct microscopic evaluation.

Coaggregation requires functional $\alpha_{IIb}\beta_3$ on both cells. The interaction of cells with other cells bearing occupied fg receptors required the presence of $\alpha_{IIb}\beta_3$, because wild-type CHO cells did not form coaggregates with $\alpha_{IIb}\beta_3$-bearing cells in the presence of activating antibody and fg (Fig. 2). Moreover, functional $\alpha_{IIb}\beta_3$ was necessary for cells to coaggregate, because the $\alpha_{IIb}\beta_3$ (D119 → Y) mutant, which lacks fg binding function, also failed to coaggregate with $\alpha_{IIb}\beta_3$-expressing cells (Fig. 2). The results of these two-color FACS analyses were confirmed by direct observation of mixed cell aggregates by fluorescence microscopy (Fig. 3). Thus, when two populations of $\alpha_{IIb}\beta_3$-bearing cells were each labeled with a different fluorochrome, mixed aggregates between these two were readily observed (Fig. 3 *A*). In contrast, when $\alpha_{IIb}\beta_3$-bearing cells, labeled with hydroethidine (red), were coaggregated with sulfofluorescein (green) labeled $\alpha_{IIb}\beta_3$ (D119 → Y)-bearing or wild-type CHO cells, red aggregates were observed surrounded by green single cells (Fig. 3 *B*). Indeed, direct counting of cells incorporated in aggregates revealed that these aggregates were comprised almost exclusively of red cells (not shown, but cf. Fig. 6). In reverse experiments, when the $\alpha_{IIb}\beta_3$-expressing cells were labeled with sulfofluorescein, green aggregates excluding red single cells were observed when the mutant $\alpha_{IIb}\beta_3$ (D119 → Y) or CHO cells were labeled with hydroethidine (data not shown). Thus, in order for $\alpha_{IIb}\beta_3$-mediated cell–cell interaction to proceed, functional receptors must be present on both cell types.

Cells bearing "resting" $\alpha_{IIb}\beta_3$ coaggregate with cells bearing "activated" $\alpha IIb\beta 3$. Because $\alpha_{IIb}\beta_3$ must be activated to bind fg (3, 4) and to initiate cell–cell interaction, we asked whether both partners in a cellular aggregate must bear activated receptors. To do this, we made use of a mutant, $\alpha_{IIb}\beta_3$ (β_1-2), which results in $\alpha_{IIb}\beta_3$ which constitutively binds fg (19). When these cells were labeled with both green and red dyes, they formed coaggregates in the absence or presence (Fig. 4) of the activating antibody. This aggregation was completely inhibitable by a complex specific anti-$\alpha_{IIb}\beta_3$ monoclonal antibody. When resting $\alpha_{IIb}\beta_3$-bearing cells were mixed with those bearing this spontaneously active mutant in the presence of fg, coaggregates were observed (Fig. 5) which were increased further by the presence of the activating monoclonal antibody mAb62. These coaggregates were completely inhibitable with the $\alpha_{IIb}\beta_3$ specific monoclonal antibody mAb4F10 (Fig. 5) or GRGDSP peptide (not shown). The appearance of two-color particles in the FACS analysis suggested that the resting $\alpha_{IIb}\beta_3$-bearing cells could be incorporated into aggregates formed by cells expressing constitutively active $\alpha_{IIb}\beta_3$ (β_1-2), although they appear to do less efficiently than cells bearing activated $\alpha_{IIb}\beta_3$. To assess this more directly, microscopic analysis was undertaken (Fig. 6). When both cells in a red/green pair were activated, aggregates were formed of equal numbers of red and green cells (Figs. 3 *A* and 6). In contrast, when only one of the cells in a pair were activated $> 80\%$ of the cells in each aggregate were of the activated phenotype (Fig. 6) and a lesser number of cells

(~ 20%) expressed the "resting" $\alpha_{IIb}\beta_3$. Incorporation of the "unactivated" cells into aggregates was specific in that cells bearing $\alpha_{IIb}\beta_3$ (D119 → Y) failed to incorporate into aggregates with the spontaneously active mutants (Fig. 5). Finally, when an activating antibody was added to the mixture of "resting" ($\alpha_{IIb}\beta_3$) and "activated" ($\alpha_{IIb}\beta_3[\beta_1\text{-}2]$) cells, equal quantities of both cells were incorporated into the aggregates (Fig. 6). Thus, activation of $\alpha_{IIb}\beta_3$ is not an absolute requirement for cellular incorporation into aggregates as long as other cells in the mixture contain activated receptors.

Activated $\alpha_{IIb}\beta_3$ mediates coaggregation with cells bearing $\alpha_v\beta_3$. Cells bearing other fg binding integrins (e.g., the $\alpha_v\beta_3$ vitronectin receptor [23] or CD11b/CD18 [24]) may readily come into contact with activated platelets. Thus, we asked whether cells bearing $\alpha_v\beta_3$ receptor could coaggregate with cells bearing activated $\alpha_{IIb}\beta_3$ in the presence of fg. To do this, we examined CHO cells expressing recombinant $\alpha_v\beta_3$ for their capacity to coaggregate with cells bearing recombinant $\alpha_{IIb}\beta_3$. When $\alpha_{IIb}\beta_3$-bearing cells were mixed with $\alpha_v\beta_3$-expressing cells in the presence of an activating antibody and fg, both cell types were incorporated into aggregates (Fig. 7). Coaggregation was specific, because it was inhibitable by a complex-specific anti-$\alpha_v\beta_3$ monoclonal antibody, LM609, (Fig. 7). Moreover, the mutant form $\alpha_v\beta_3$ (D119 → Y) did not coaggregate with activated $\alpha_{IIb}\beta_3$-bearing cells (data not shown). Microscopic analysis of the composition of mixed aggregates formed with $\alpha_{IIb}\beta_3$-bearing cells revealed that 14.0±5.7% of cells in aggregates bore $\alpha_v\beta_3$. In contrast, cells carrying $\alpha_v\beta_3$ (D119 → Y) failed to coaggregate with $\alpha_{IIb}\beta_3$-bearing cells (3.9±4.6% of cells in aggregates bore $\alpha_v\beta_3$ [D119 → Y]).

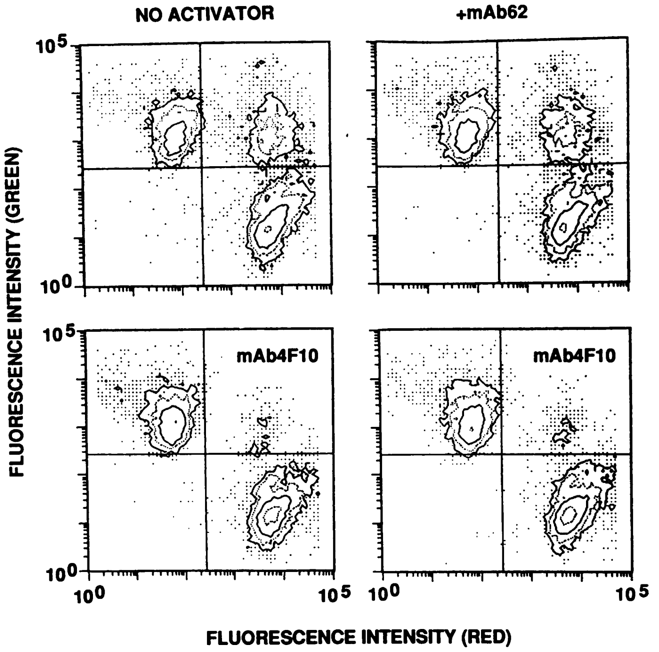

Figure 4. Activation-independent aggregation of $\alpha_{IIb}\beta_3$ (β_1-2) transfectants. Cells expressing constitutively active $\alpha_{IIb}\beta_3$ (β_1-2) (see Methods) were labeled with either SFDA or HE. Aggregation was initiated by addition of fg in the absence (*left*) or presence (*right*) of mAb62. The effect of an anti-$\alpha_{IIb}\beta_3$, 4F10, is shown in lower panels. Percent of particles in the aggregate region are 15.1% (*top left*), 21.2% (*top right*), 2.9% (*bottom left*), and 3.9% (*bottom right*).

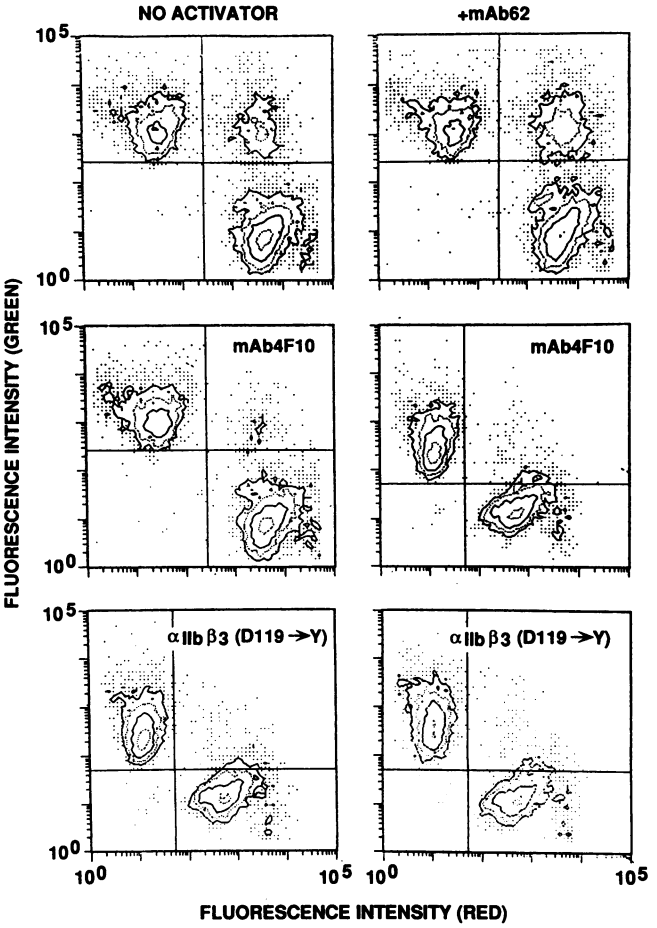

Figure 5. Coaggregation of "resting" and "activated" $\alpha_{IIb}\beta_3$-bearing cells. Cells bearing $\alpha_{IIb}\beta_3$ ($\beta_{1\text{-}2}$), a constitutively active fg receptor, were mixed with cells expressing $\alpha_{IIb}\beta_3$ (*top, middle*) or $\alpha_{IIb}\beta_3$ (D119 → Y) (*bottom*) in the absence (*left*) or presence (*right*) of an activating antibody, mAb62. The effect of mAb4F10 is shown (*middle*). Percentage of particles in aggregate region are 12% (*top left*), 23% (*top right*), 2% (*middle left*), 3.1% (*middle right*), 1.9%, and 0.9% (*bottom, left* and *right*).

It is likely that activated platelets might come into contact with endothelial cells or tumor cells which might not be in an activated state. Therefore, we asked whether the "resting" $\alpha_v\beta_3$-bearing cells could coaggregate with cells bearing active $\alpha_{IIb}\beta_3$ in the absence of activating agents. When cells expressing $\alpha_{IIb}\beta_3$ (β_1-2), were mixed with the $\alpha_v\beta_3$-bearing cells, coaggregation was observed which was inhibitable with LM609 (Fig. 8). This indicates that $\alpha_v\beta_3$-bearing cells do not have to be activated to interact with cells bearing activated $\alpha_{IIb}\beta_3$.

Discussion

The major findings of this work are: (*a*) fg-dependent cellular aggregation may occur when functional β_3 receptors are present on both apposing cells. Thus, $\alpha_{IIb}\beta_3$ appears to require no counter-receptor and fg bridging between two functional β_3 receptors is a likely mechanism of aggregation. (*b*) Although activation of $\alpha_{IIb}\beta_3$ is required for fg binding and platelet aggregation, cells bearing resting $\alpha_{IIb}\beta_3$ may be incorporated into aggregates as long as other cells in the mixture contain activated fg receptors. (*c*) Cells bearing other fg receptors, e.g.,

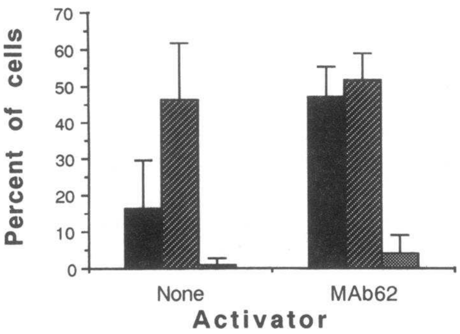

Figure 6. Coaggregation of cells bearing fg receptors in a resting and activated state. The SFDA-labeled, constitutively active mutant, $\alpha_{IIb}\beta_3$ ($\beta_{1\text{-}2}$), was mixed with HE-labeled CHO cells or transfectants bearing $\alpha_{IIb}\beta_3$ or $\alpha_{IIb}\beta_3$ (β_1-2). The cells were incubated with gyrotation in the absence or presence of the activating antibody mAb62. The percentage of cells incorporated in aggregates was determined by counting the numbers of "red" (HE) and "green" (SFDA) cells in aggregates. The HE-labeled CHO cells bore (*solid*) $\alpha_{IIb}\beta_3$, (*hatched*) $\alpha_{IIb}\beta_3$ (β_1-2), or (*stippled*) no transfected β_3 receptor. Results are expressed as mean percentage of HE-labeled cells in 20 aggregates±SD.

$\alpha_v\beta_3$, may interact with cells bearing activated $\alpha_{IIb}\beta_3$ via fg bridging. Thus, activated $\alpha_{IIb}\beta_3$ may mediate heterotypic interactions between platelets and cells bearing other fg receptors, e.g., endothelial cells, leukocytes, tumor cells. These findings, which are summarized in Fig. 9, indicate that ligand bridging is a mechanism of integrin-mediated cell–cell interaction, provide insight into the mechanism of platelet aggregation, and suggest molecular mechanisms for platelet interactions with tumor cells and other fg receptor-bearing cells.

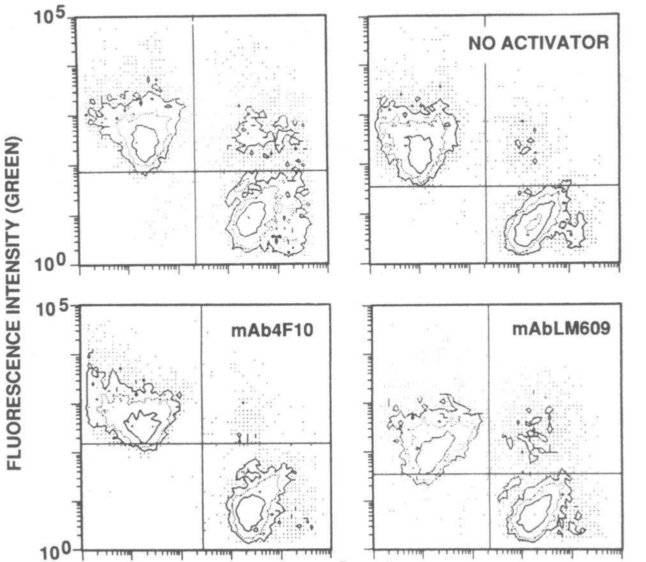

Figure 7. Coaggregation of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ transfectants. SFDA-labeled $\alpha_{IIb}\beta_3$-bearing cells were mixed with HE-labeled vitronectin-receptor ($\alpha_v\beta_3$)-expressing transfectants in the presence or absence of the activating antibody, mAb62. Aggregation was initiated by addition of fg. The effect of anti-$\alpha_{IIb}\beta_3$ (mAb4F10) and anti-$\alpha_v\beta_3$ (mAbLM609) which inhibit ligand binding is shown in lower panels. The percentage of particles in the aggregate region are 9.2% (*top left*), 2.4% (*top right*), 1.8% (*bottom left*), 3.8% (*bottom right*).

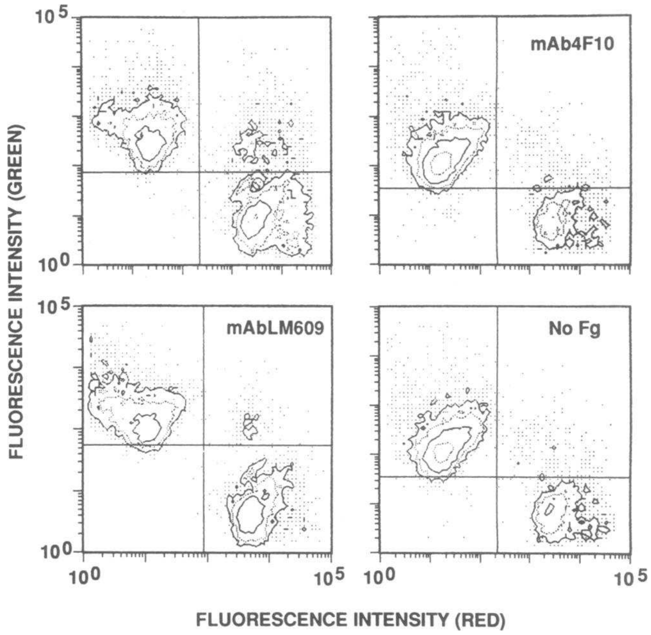

Figure 8. Coaggregation of $\alpha_{IIb}\beta_3$ (β_1-2) and $\alpha_v\beta_3$ transfectants. SFDA-labeled constitutively active mutant $\alpha_{IIb}\beta_3$ (β_1-2) were mixed with HE-labeled $\alpha_v\beta_3$-bearing cells. Aggregation was initiated in the absence of activating antibody mAb62 by addition of fg. The effect of antibodies which inhibit fg binding to $\alpha_{IIb}\beta_3$ (*top right*) or to $\alpha_v\beta_3$ (*bottom left*) are shown. (*Bottom right*) The effect of omission of fg. Percentage of particles in aggregate quadrants are 8.2% (*top left*), 2% (*top right*), 2.2% (*bottom left*), and 1.8% (*bottom right*).

Physiologic platelet aggregation requires the presence of platelet $\alpha_{IIb}\beta_3$, cellular activation to expose fg binding sites, and fg binding (3, 4). In the present study, we have exploited unique properties of recombinant $\alpha_{IIb}\beta_3$ in CHO cells to specifically analyze the $\alpha_{IIb}\beta_3$-dependent element of platelet aggregation. (*a*) Because agonist-mediated activation has not been observed in the CHO cell system (16) the potential contribution of numerous "positive feedback" loops in platelet aggregation (25) can be discounted. (*b*) The requirement for maintenance of an active fg receptor through signal transduction pathways was

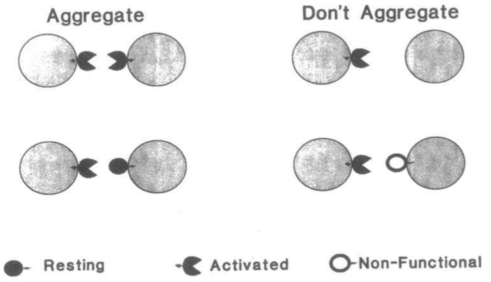

Figure 9. Fg receptors and cell–cell interaction. Illustrated is a summary of the requirements for fg-mediated coaggregation of cells. As shown in the left panel, two cells bearing functional fg receptors coaggregate when at least one of the cells' receptors are "activated." As illustrated in the right panels, coaggregation does not occur when one of the cells lacks functional fg receptors.

obviated by use of monoclonal antibodies which conformationally alter the $\alpha_{IIb}\beta_3$ to produce a stable activated fg receptor (16). (*c*) Because the parent CHO cell lacks the capacity to undergo fg-mediated aggregation, the transfected receptor is the single unique platelet component required for this response. Moreover, because the $\alpha_{IIb}\beta_3$ was recombinant, its ligand-binding state and state of activation could be controlled by introduction of selected mutations. With these tools it was possible, by use of two-color flow cytometry, to directly investigate the possibility of cellular counter-receptors for $\alpha_{IIb}\beta_3$. The aggregation response required that $\alpha_{IIb}\beta_3$ be present on both cell types suggesting that no cellular counter-receptor sufficient to mediate the aggregation response was present. fg undergoes conformational changes (15) after binding to $\alpha_{IIb}\beta_3$ and monoclonal antibodies reactive with neoepitopes on receptor-bound fg inhibit platelet aggregation (26). The epitope for one of these antibodies is distinct from known $\alpha_{IIb}\beta_3$ recognition sequences in fg (26), suggesting the possibility that conformationally altered fg might interact with $\alpha_{IIb}\beta_3$ at a site distinct from its known (18, 27–29) ligand binding pocket. This seems unlikely, because cells transfected with $\alpha_{IIb}\beta_3$ (D119 → Y), which lacks interaction with known $\alpha_{IIb}\beta_3$ binding sequences in fg (18), failed to undergo coaggregation. Thus, because monovalent peptides (30) or fragment D (31) bind to $\alpha_{IIb}\beta_3$ but fail to support aggregation, the simplest explanation of these results is that a single fg molecule interacts with the ligand binding pockets of $\alpha_{IIb}\beta_3$ molecules on adjacent cells thus bridging the two cells.

It is clear that platelets must be activated to initiate the aggregation response (3, 4). In the present work we have employed a spontaneously active mutant of $\alpha_{IIb}\beta_3$ (19) and have found that cells bearing "resting" $\alpha_{IIb}\beta_3$ may be incorporated into aggregates with cells bearing activated $\alpha_{IIb}\beta_3$. Considering the lability of platelet agonists such as ADP and thrombin within the vasculature, these results suggest that activated platelets may nucleate an aggregate, which may contain both activated and resting cells. Because one of the cells in the interacting pair must be activated, it also suggests that platelet aggregates may be "capped" by a layer of resting platelets. It is superficially surprising that "resting" $\alpha_{IIb}\beta_3$ should mediate coaggregation with cells bearing activated $\alpha_{IIb}\beta_3$, because there is an activation requirement for soluble fg binding. Nevertheless, Coller (32) found that "resting" platelets adhered to insolubilized fg, and recombinant $\alpha_{IIb}\beta_3$, which could not be "physiologically" activated in CHO cells, also supported adhesion to insolubilized fg (16). One potential explanation of this would be that fg binds to resting $\alpha_{IIb}\beta_3$ with a low affinity, and receptor bound or surface insolubilized fg, being multivalent with respect to platelets, binds with a higher effective affinity. An alternative possibility is that receptor bound fg undergoes a conformational change (26) resulting in accessibility of the RGDS in the α chain of fg to $\alpha_{IIb}\beta_3$. Because peptides containing the RGD sequence also induce a conformational change in $\alpha_{IIb}\beta_3$ associated with high-affinity fg binding (33), the receptor-bound fg may carry its own internal activator for platelet $\alpha_{IIb}\beta_3$. Evidence in support of this model is provided by the finding that adhesion of resting platelets to fg, in contrast to fg binding to activated platelets, is dependent on the region of the fg α chain containing an RGDS sequence (34).

Since the pioneering studies of Gasic (35–37), it has become clear that platelet-tumor cell interaction may modulate the metastatic behavior of some tumors. In the case of certain tumors, platelet $\alpha_{IIb}\beta_3$ interaction with adhesive ligands plays a role (38, 39). It is also clear that certain tumors have fg receptors such as $\alpha_v\beta_3$ (40) and these receptors may be involved in interaction with platelets. The present studies, by use of recombinant receptors, have reconstructed a potential molecular mechanism of platelet-tumor cell interaction, i.e., a heterophilic bridging interaction involving $\alpha_{IIb}\beta_3$ on the platelet and a resting or activated fg receptor on the tumor cell. $\alpha_v\beta_3$ is also expressed on endothelial cells, thus it seems probable that the ability of $\alpha_v\beta_3$ to support fg-mediated aggregation with cells bearing activated $\alpha_{IIb}\beta_3$ provides a mechanism for platelet-endothelial cell interaction as well.

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