DETECTION OF INTEGRIN αIIβ3 CLUSTERING IN LIVING CELLS

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SUMMARY

In platelets, bidirectional signaling across integrin αIIbβ3 regulates fibrinogen binding, cytoskeletal reorganization, cell aggregation and spreading. Since these responses may be influenced by clustering of αIIbβ3 heterodimers into larger oligomers, we established two independent methods to detect integrin clustering and to evaluate factors that regulate this process. In the first, weakly complementing β-galactosidase mutants were fused to the C-terminus of individual αIIb subunits, and the chimeras were stably expressed with β3 in CHO cells. Clustering of αIIbβ3 should bring the mutants into proximity and reconstitute β-galactosidase activity. In the second method, αIIb was fused to either GFP or Renilla luciferase and transiently expressed with β3. Here, integrin clustering should stimulate bioluminescence resonance energy transfer between a cell-permeable luciferase substrate and GFP. These methods successfully detected integrin clustering induced by anti-αIIbβ3 antibodies. Significantly, they also detected clustering upon soluble fibrinogen binding to αIIbβ3. In contrast, no clustering was observed following direct activation of αIIbβ3 by MnCl2 or an anti-αIIbβ3 activating antibody Fab in the absence of fibrinogen. Intracellular events also influenced αIIbβ3 clustering. For example, a cell-permeable, bivalent FKBP ligand stimulated clustering when added to cells expressing an αIIb(FKBP)2 chimera complexed with β3. Furthermore, αIIbβ3 clustering occurred in the presence of latrunculin A or cytochalasin D, inhibitors of actin polymerization. These effects were enhanced by fibrinogen, suggesting that actin-regulated clustering modulates αIIbβ3 interaction with ligands. These studies in living cells establish that αIIbβ3 clustering is modulated by fibrinogen and actin dynamics. More broadly, they should facilitate investigations of the mechanisms and consequences integrin clustering.
INTRODUCTION

For most cell surface receptors, clustering into dimers or larger oligomers is thought to represent an important regulatory event (1-4). Receptor clustering may be triggered in several ways, for example through interactions with multivalent ligands, apposition of dimerization interfaces, relief of cytoskeletal constraints, and partitioning into membrane domains, such as lipid rafts.

Integrins are a family of transmembrane αβ heterodimers that function as cell adhesion and signaling receptors (5). There is substantial indirect evidence that lateral diffusion and clustering influences integrin functions (6,7). When clustering of recombinant integrins is induced by chemical dimerizers or is abrogated by mutagenesis, their avidity for ligands is affected (8,9). Indeed, in such systems integrin clustering operates in concert with receptor conformational changes and affinity modulation to determine ligand binding (5,10-12). When cells attach to extracellular matrices, integrin clustering promotes the assembly of a range of actin-based complexes, including initial adhesions, focal complexes and focal adhesions (13-17). These structures, particularly the larger ones, are visible by light microscopy, and they function as signaling centers to help regulate cell motility and gene expression (5,18). Focal adhesions can be visualized in real time using green fluorescent protein (GFP) chimeras containing focal adhesion targeting sequences (19). On the other hand, smaller nascent integrin clusters containing perhaps a few heterodimers are not visible by routine light microscopy; yet they are likely to function as signaling centers in their own right and to represent precursors of the larger adhesion structures (9,17). In some cases, nascent integrin clusters may recruit functionally important signaling molecules that do not partition to the larger focal adhesions (20,21). Consequently, the precise roles of integrin clusters might be better understood if there were a means to detect their initial formation and maturation in living cells.

Integrin αIIbβ3 mediates platelet adhesion and aggregation in hemostasis by engaging fibrinogen or von Willebrand factor (22,23). As with other integrins, clustering may modulate αIIbβ3 function. Thus, in platelets and a CHO cell model system, binding of multivalent but not monovalent ligands to αIIbβ3 leads to activation of the integrin-associated tyrosine kinases, Src and Syk (21,24,25). These kinases also become activated when an αIIb(FKBP) chimeric subunit is expressed with β3 in CHO cells and the integrin is conditionally clustered with a cell-permeable, bivalent FKBP ligand (9). Therefore, in order to shed more light on clustering of αIIbβ3 and other integrins, we sought here to develop new means to assess this process in living cells. Two complementary techniques were utilized for this purpose, β-galactosidase (β-gal) complementation and bioluminescence resonance energy transfer (BRET). Recently, each of these has been used to study proximity and clustering of other types of cell surface receptors (26-30). The results establish that αIIbβ3 clustering can be detected in living cells and is modulated by extracellular and intracellular factors.
EXPERIMENTAL PROCEDURES

Construction of recombinant integrins. For β-gal complementation assays, a pWZL plasmid containing the alpha deletion mutant (Δα) of β-gal (a gift from Dr. Helen Blau, Stanford University (27)), was subjected to PCR using Platinum Pfx polymerase (Life Technologies, Gaithersburg, MD) to place Spe I restriction sites at the 5’ and 3’ ends of Δα. Similarly, the β-gal omega deletion mutant (Δω) was subjected to PCR to place a Spe I site at the 5’ end and a FLAG epitope tag/Spe I site at the 3’ end. PCR products were digested with Spe I and ligated into a Spe I-cut, dephosphorylated mammalian expression vector (pCF2E) containing full-length human integrin αIIb fused at its cytoplasmic tail to tandem FK506-binding protein (FKBP) repeats and a hemagglutinin (HA) epitope tag (9). This resulted in two constructs, one encoding αIIb/(FKBP)$_2$/Δα β-gal/HA and the other αIIb/(FKBP)$_2$/Δω β-gal/FLAG. To obtain the same expression constructs without FKBP, (FKBP)$_2$ was removed by deletion mutagenesis (Exsite Site-directed Mutagenesis Kit; Stratagene, La Jolla, CA). For BRET assays, plasmid templates encoding αIIb/(FKBP)$_2$/HA or αIIb/HA (Hato et al, 1998) were subjected to PCR to place 5’ Mlu I and 3’ Hind III restriction sites. PCR products were sub-cloned into the Mlu I and Hind III sites of the mammalian expression vectors, pGFPN3 (which encodes a GFP optimized for BRET) and pRLUCN3 (which encodes Renilla luciferase (Rluc) (Packard Biosciences, Meriden, CT). Transformed colonies were screened by colony PCR, coding sequences were verified by DNA sequencing, and plasmids were amplified and purified (QIAfilter Plasmid Midi Kit; Qiagen, Inc., Chatsworth, CA). Full-length human β3 was cloned into pCDM8 (31).

Cell culture, transfections, and establishment of stable cell lines. CHO cells were maintained in culture as described (31). Transfections were performed at 70-80% cell confluency using Lipofectamine (Life Technologies) according to the manufacturer’s recommendations. For β-gal complementation assays, CHO cells stably expressing recombinant integrins were obtained by co-transfecting the appropriate pair of αIIb/β-gal plasmids along with pCDM8/β3 and pCDM8/neomycin (31). After 48 h, cells were subjected to selection with 0.8 mg/ml G418 (Life Technologies). Two weeks later, cells were stained with anti-αIIbβ3 antibody D57 (a gift from Mark Ginsberg, Scripps), and single cell clones expressing αIIbβ3 were isolated by FACS sorting (31). Preliminary studies with the β-gal complementation assay showed that dynamic integrin clustering was best detected at relatively low levels of αIIbβ3 expression. Consequently at least two independent clones expressing low but easily detectable levels of αIIbβ3 were isolated and used in the studies reported below.

Characterization of recombinant αIIbβ3. Surface expression of αIIbβ3 was analyzed by flow cytometry using antibody D57. The affinity/avidity state of αIIbβ3 was assessed with PAC-1, a fibrinogen-mimetic monoclonal antibody (31,32). αIIbβ3 expression was evaluated by immunoprecipitation and/or Western blotting. CHO cells were solubilized for 10 min on ice in a buffer containing 0.5% NP-40, 50 mM NaCl,
a protease inhibitor cocktail (Complete, Life Technologies), and 50 mM Tris-HCl, pH 7.4. After clarification at 10,000 rpm for 10 min at 4°C in a microcentrifuge, 400 µg of lysate protein were immunoprecipitated with D57 (or an isotype-matched control IgG) and protein A Sepharose (24,33). After washing, immunoprecipitates were subjected to electrophoresis in 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose. Immuno-reactive bands were detected by enhanced chemiluminescence, with reaction times varying from 0.1-1 min (SuperSignal WestPico reagent; Pierce, Rockford, IL).

Detection of αIIbβ3 clustering. CHO cells transiently or stably expressing the appropriate αIIbβ3 chimeras were harvested by trypsinization, washed once and resuspended to 0.5-1.0 x 10⁶ cells/ml in Tyrode’s buffer supplemented with 2 mM CaCl₂ and MgCl₂ (31). The cells were then subjected to various treatments designed to assess αIIbβ3 clustering, as indicated below for each experiment. To induce clustering by antibodies, 100 µl of cells were added to U-bottom, 96-well non-tissue culture plates pre-blocked with 10 mg/ml heat-denatured bovine serum albumin. After incubation with 10 µg/ml antibody D57 for 30 min, cells were washed and incubated another 30 min with 20 µg/ml goat anti-mouse IgG (Biosource International, Camarillo, CA). To induce clustering of αIIbβ3 constructs containing (FKBP)₂, cells were incubated for 30 min at room temperature with 1 µM AP1510, a cell-permeable bivalent FKBP ligand (Ariad Pharmaceuticals, Inc.), or an equivalent vol of vehicle (9). The effects of fibrinogen on αIIbβ3 clustering were evaluated by incubating cells for 30 min at room temperature with purified fibrinogen (Enzyme Research Laboratories, South Bend, IN). In some cases, fibrinogen binding was stimulated with 0.5 mM MnCl₂ or 150 µg/ml anti-LIBS6 Fab, which directly activate αIIbβ3 (10,34). The effects of cytochalasin D or latrunculin A (Calbiochem, San Diego, CA) on αIIbβ3 clustering were assessed by incubating cells with the compound or vehicle for 10 min at room temperature.

αIIbβ3 clustering was detected in stably-transfected cells by measuring β-gal complementation, as described (28,35). Briefly, 100,000 cells in 100 µl were deposited in solid white U-bottom microtiter wells (#3912, Costar, Corning Life Sciences, Acton, MA). Cells were lysed by addition of an equal vol of Gal-Screen substrate (Buffer B formulation; Tropix PE Biosystems, Bedford, MA) and incubated for 30 min at room temperature. Chemiluminescence was measured in a Rosys Lucy2 luminometer (Anthos Labtec Instruments, Austria) and expressed in arbitrary units. The absolute mean baseline value for untreated cells in the experiments reported here was 1995 units.

αIIbβ3 clustering was detected in transiently-transfected cells by measuring BRET using the BRET² system (Packard Biosciences, Meriden, CT) (36). Briefly, 50,000 cells in 15 µl of BRET² buffer (phosphate-buffered saline supplemented with 0.68 mM CaCl₂, 0.5 mM MgCl₂, 1.0 g/L glucose, and 2 µg/ml aprotinin) were added to microtiter wells. Immediately after addition of 50 µl of a luciferase
substrate (coelenterazine; Deep Blue C, Packard Biosciences, final concentration of 10 µM), BRET was analyzed by luminometry using a 410 nm/80 nm bandpass filter for Rluc and a 515 nm/30 nm bandpass filter for GFP. Results are expressed as the BRET ratio, calculated as follows: (Emission at 515 nm - BG_{515})/(Emission at 410 nm - BG_{410}), where BG_{515} is the emission at 515 nm and BG_{410} the emission at 410 nm of a 5 µM solution of coelenterazine prepared in BRET$^2$ buffer (26,29,36). The absolute mean baseline BRET ratio for untreated cells in the experiments reported here was 0.202.
RESULTS

Systems to detect αIIbβ3 clustering. Two methods potentially capable of detecting integrin clustering were evaluated in CHO cells. In the first, weakly complementing β-gal deletion mutants (Δα and Δω) were fused to the C-termini of separate αIIb subunits (Figure 1A). Epitope tags were included to distinguish between chimeric subunits. The chimeric αIIb subunits were stably expressed along with β3, with the goal of achieving simultaneous surface expression of two distinct αIIbβ3 species, αIIb/Δα β-galβ3 and αIIb/Δω β-galβ3. In some cases, additional stable cell lines were developed in which tandem FKBP repeats were interposed between αIIb and β-gal, resulting in integrins αIIb/(FKBP)2/Δα β-galβ3 and αIIb/(FKBP)2/Δω β-galβ3 (Figure 1A). The latter constructs were designed to evaluate the effects of conditional integrin clustering upon addition of 1 µM AP1510, a cell-permeable bivalent FKBP ligand (9). Expression of αIIbβ3 constructs was confirmed by immunoprecipitation and Western blotting (Figure 1B), and by analyzing surface expression by flow cytometry (not shown). In all of these cell lines, clustering of αIIbβ3 heterodimers into larger oligomers should bring the Δα and Δω β-gal mutants into proximity such that β-gal enzyme activity is reconstituted (Figure 1C) (28). This approach has been used to detect oligomerization of EGF receptors (27).

In the second method, αIIb (or αIIb/(FKBP)2) was fused to GFP or Renilla luciferase and transiently expressed with β3 in CHO cells (Figure 2A). Expression of these αIIbβ3 constructs was confirmed by Western blotting of cell lysates (Figure 2B) and flow cytometry. In this system, if integrin clustering results in appropriately oriented GFP and Rluc moieties coming within ~80 Å of each other, BRET should occur between a cell-permeable luciferase substrate (coelenterazine) and GFP (Figure 2C). This technique has been used to detect oligomerization of G protein-coupled and insulin receptors (29,30,36). Though not shown, the chimeric integrins used for BRET and β-gal assays were functional in that they bound the ligand-mimetic antibody, PAC-1, after direct integrin activation with 0.5 mM MnCl2 or 150 µg/ml anti-LIBS-6 Fab. Moreover, they supported specific cell adhesion and spreading on immobilized fibrinogen. Overall, these results indicate that β-gal and BRET reporter domains can be fused to αIIb without disrupting expression of a functional αIIbβ3 complex.

Detection of αIIbβ3 clustering induced by extracellular or intracellular means. Since integrin clustering may be triggered by the binding of ligands to the extracellular or intracellular portions of αIIbβ3, we set out to determine whether clustering could be detected under such conditions. Anti-αIIbβ3 IgG antibodies are bivalent and at appropriate concentrations should mediate apposition of two αIIbβ3 complexes. Indeed, when CHO cells expressing αIIb/Δα β-galβ3 and αIIb/Δω β-galβ3 were examined by the β-gal complementation assay, a significant increase in β-gal signal was detected in response to 10 µg/ml D57, an IgG1 anti-αIIbβ3 monoclonal antibody (P < 0.05) (Figure 3A). However, no increase in β-gal signal was observed at higher concentrations of D57, a result that would be expected if the pool of
αIIbβ3 available for cross-linking had become saturated with D57 (not shown). At 10 µg/ml D57, addition of a second antibody to cross-link the primary antibody caused a further increase in β-gal signal, consistent with formation of even larger αIIbβ3 oligomers (Figure 3A). Similar results were obtained in the presence of D57 and secondary antibodies when αIIbβ3 clustering was analyzed by BRET. In this case, however, no response was seen with D57 alone, suggesting that BRET in the present configuration may be less sensitive than β-gal complementation for detection of relatively small αIIbβ3 oligomers (Figure 4A). The interpretation that the β-gal and BRET responses reflected antibody-mediated clustering of αIIbβ3 was supported by the fact that no increase in β-gal or BRET signal was obtained if cells expressed only one of the two complementary αIIb reporter constructs, or if the reporter constructs were each expressed in separate cells (not shown).

To determine whether clustering of αIIbβ3 could be detected if triggered from within the cell, CHO cells expressing αIIbβ3 with tandem FKBP repeats fused to the αIIb chimeras were evaluated. Addition of the FKBP ligand, AP1510, at a saturating concentration of 1 µM should cause rapid clustering of these αIIbβ3 species into oligomers larger than dimers (9). Indeed, addition of AP1510 triggered β-gal complementation (Figure 3B) and BRET (Figure 4B). Thus, the β-gal complementation and BRET assays are sufficiently sensitive to detect αIIbβ3 clustering, whether this process is initiated from outside or inside living cells.

Modulation of αIIbβ3 clustering by fibrinogen and the actin cytoskeleton. Fibrinogen mediates platelet aggregation by bridging αIIbβ3 receptors on adjacent cells. However, fibrinogen binding also triggers outside-in signaling, which may require clustering of αIIbβ3 complexes within the same cell (9,21,24). Since fibrinogen is a dimer with several potential sites for interaction with αIIbβ3 (37,38), a single fibrinogen molecule might be able to support both cell aggregation and αIIbβ3 clustering. Alternatively, individual fibrinogen molecules may promote each response separately. To determine whether fibrinogen binding can trigger αIIbβ3 clustering, β-gal complementation and BRET assays were performed at a sub-saturating concentration of fibrinogen (150 µg/ml) (39). Ligand binding was induced with 0.5 mM MnCl2 or anti-LIBS6 Fab. Under these conditions, fibrinogen induced αIIbβ3 clustering (Figure 5). This response was fibrinogen-dependent because it was inhibited by EDTA, and was not observed with MnCl2 or anti-LIBS6 Fab in the absence of fibrinogen. Moreover, no significant fibrinogen-dependent clustering was observed in cells expressing an integrin mutant that is incapable of binding fibrinogen (αIIbβ3 (D119A)) (Figure 6A) (40). As might be expected for interaction with a multivalent ligand, the extent of αIIbβ3 clustering was reduced at concentrations of fibrinogen ≥ 250 µg/ml (Figure 6B). These results demonstrate that fibrinogen binding to αIIbβ3 causes integrin clustering. Furthermore, this response may require ligand multivalency because another bivalent anti-
αIIb3 ligand, antibody D57, also induced αIIb3 clustering (Figure 3A), but a monovalent ligand, anti-LIBS6 Fab, did not (Figure 5A).

Integrins in unstimulated platelets are maintained in a low affinity/avidity state and are tethered to components of the actin cytoskeleton (41,42). Inhibitors of actin polymerization, such as latrunculin A or cytochalasin D, promote fibrinogen binding to platelets, an effect that has been attributed to perturbation of actin dynamics, relief of cytoskeletal constraints and αIIb3 activation (43). Were these relationships to be preserved in CHO cells, we would hypothesize that inhibition of actin polymerization would promote lateral mobility and clustering of αIIb3. To evaluate this, CHO cells were incubated with latrunculin A or cytochalasin D and αIIb3 clustering was monitored by the β-gal complementation assay. Latrunculin A, which inhibits actin polymerization by sequestering G actin monomers (44), caused a small but significant increase in αIIb3 clustering at a concentration of 3 µM (P < 0.05). Interestingly, addition of 150 µg/ml fibrinogen increased the β-gal signal further, an effect observed over a range of latrunculin A concentrations and without the need for MnCl2 or anti-LIBS6 Fab (Figure 7A). αIIb3 clustering and its enhancement by fibrinogen were also observed at µM concentrations of cytochalasin D, which inhibits actin polymerization by binding to barbed actin filament ends (45) (Figure 7B). Although the dose-response curves for latrunculin A and cytochalasin D differed, these results indicate that inhibition of actin polymerization can influence the oligomerization state of αIIb3. Furthermore, the enhanced β-gal responses observed in the presence of fibrinogen suggest that the actin cytoskeleton may influence αIIb3/fibrinogen interactions, in part, by regulating integrin clustering.
αIIb and β3 are expressed in plasma membranes as an obligate heterodimer (46), and αIIbβ3 is usually depicted in models as a heterodimer in platelets. However, αIIbβ3 heterodimers probably exist in equilibrium with larger oligomers, and this equilibrium may be regulated, with implications for integrin signaling. Indeed, when platelets are stimulated to bind fibrinogen in response to ADP, clusters of αIIbβ3 can be visualized on the platelet surface by electron microscopy (47,48). In addition, electron microscopic and biochemical techniques have detected oligomers of detergent-solubilized αIIbβ3 under certain experimental conditions, including after fibrinogen binding (49-51). One possible structural basis for αIIbβ3 oligomerization comes from recent in vitro studies with transmembrane/cytoplasmic domain fusions of αIIb and β3, which indicate that homomeric contacts can occur between transmembrane domains (52). Despite these observations and experimental evidence in CHO cells that forced clustering of αIIbβ3 by a chemical dimerizer can promote ligand binding and outside-in signaling (9), the factors that regulate αIIbβ3 clustering remain to be characterized.

An impediment to progress in this area has been the inability to monitor αIIbβ3 clustering in living cells. To overcome this, we employed two complementary methods, β-galactosidase complementation and BRET, each of which is capable of reporting the proximity between two proteins (28,30). We reasoned that any stimulus that caused oligomerization of αIIbβ3 would lead to an increase in homomeric interactions between the αIIb cytoplasmic tails on adjacent αIIbβ3 complexes. Therefore, reporter groups were fused to the C-terminus of individual αIIb subunits and clustering was monitored by β-gal complementation and BRET assays. αIIb tails were used here instead of β3 tails as fusion partners because our previous experience indicates that αIIb fusions are tolerated, while β3 fusions may impair integrin function (9). The following conclusions can be drawn from these studies: 1) αIIbβ3 clustering can be detected in living cells, whether triggered from outside or inside the cell. 2) Fibrinogen binding to αIIbβ3 induces integrin clustering, whereas direct activation of αIIbβ3 in the absence of fibrinogen does not. 3) Inhibition of actin polymerization causes dose-dependent clustering of αIIbβ3, a response that is enhanced by fibrinogen.

To validate the β-gal complementation and BRET assays, we conditionally clustered αIIbβ3 using anti-integrin antibodies or the FKBP dimerization system (Figures 3 and 4). The results indicated that integrin clustering by these means can be detected, but they do not establish whether clustering occurs under more biologically relevant conditions. To address the latter, we evaluated the binding of fibrinogen to αIIbβ3, an event that mediates platelet aggregation in vitro and thrombus formation in vivo (23,39). The binding of soluble fibrinogen to platelets requires the agonist-induced conversion of αIIbβ3 from a low- to a high-affinity/avidity state (22,39). In the present experiments, this conversion process was accomplished by activating αIIbβ3 directly with an anti-β3 antibody Fab (anti-LIBS6) or
with MnCl$_2$. Unlike fibrinogen, these reagents by themselves failed to induce detectable clustering of \( \alpha \text{IIb}\beta3 \) (Figure 5). Thus, neither the binding of a monovalent ligand like anti-LIB6 Fab nor the direct activation of the receptor are sufficient to induce clustering of \( \alpha \text{IIb}\beta3 \). Rather, \( \alpha \text{IIb}\beta3 \) clustering in living cells appears to require the binding of a multivalent ligand like fibrinogen. It remains to be determined whether other physiological multivalent ligands, such as von Willebrand factor, also induce \( \alpha \text{IIb}\beta3 \) clustering.

Fibrinogen is a dimer and each half-molecule contains several potential interactions sites with \( \alpha \text{IIb}\beta3 \) (37,38). Consequently, when present at sub-saturating concentrations, fibrinogen might be expected to bring two \( \alpha \text{IIb}\beta3 \) complexes together in cis, an assumption consistent with the present data (Figure 5). However, this may not represent the full extent of \( \alpha \text{IIb}\beta3 \) oligomerization induced by fibrinogen. First, the \( \beta \)-gal complementation and BRET assays were capable of detecting \( \alpha \text{IIb}\beta3 \) clustering by fibrinogen, but neither assay allows precise assessment of the extent of clustering. Second, the BRET assay was not sensitive enough to detect clustering by a bivalent anti-\( \alpha \text{IIb}\beta3 \) antibody (Figure 4A), but it did detect clustering in response to fibrinogen (Figure 5). Finally, ligands like fibrinogen might trigger initial oligomerization of \( \alpha \text{IIb}\beta3 \) through direct binding, a process that likely contributes to the biphasic clustering response observed in Figure 6B. However, further oligomerization may be promoted by fibrinogen/fibrinogen interactions (53), and by post-ligand binding changes in \( \alpha \text{IIb}\beta3 \) that expose or re-orient dimerization interfaces (51,52). Recent progress in the structural analysis of \( \beta3 \) integrins provides a starting point for identifying changes in \( \alpha \text{IIb}\beta3 \) that may affect clustering (11,12,54).

The \( \alpha \text{IIb}\beta3 \) clustering that we observed in CHO cells in response to latrunculin A or cytochalasin D may be relevant to recent platelet studies demonstrating increased ADP-dependent fibrinogen binding in response to these same compounds (43). Those results were interpreted to reflect basal constraints on the fibrinogen binding function of \( \alpha \text{IIb}\beta3 \) imposed by the actin cytoskeleton, and release of these constraints when actin polymerization and filament turnover are prevented. Our results are compatible with this interpretation in the sense that \( \alpha \text{IIb}\beta3 \) clustering due to actin polymerization inhibitors increased further in the presence of fibrinogen (Figure 7). This suggests that actin dynamics may affect fibrinogen binding, at least in part, by regulating \( \alpha \text{IIb}\beta3 \) clustering. Indeed, single particle tracking studies indicate that the actin cytoskeleton exerts prominent effects on integrin lateral diffusion, a possible antecedent to actin-regulated integrin clustering (6).

The methods used here to detect \( \alpha \text{IIb}\beta3 \) clustering have certain limitations for extrapolation of the results to platelets or to other integrins. First, they require the fusion of bulky reporter groups to the \( \alpha \text{IIb} \) subunit and the heterologous expression of \( \alpha \text{IIb}\beta3 \). While \( \alpha \text{IIb}\beta3 \) appeared to function normally under these conditions and to support fibrinogen-dependent cell adhesion and spreading, anomalous behavior could occur in other experimental settings. Second, as already mentioned, these methods can detect
integrin oligomerization but provide no quantitative information as to its extent. Some steady-state αIIbβ3 oligomerization might take place in resting cells, particularly at high receptor densities, but such an event could be difficult to discern by these methods, whose strength is to report changes in receptor oligomerization. Third, integrin activation and clustering may occur preferentially at certain sites in the cell (55), spatial information that is not readily attainable by these methods. Nonetheless, the ability to detect apparently even minor changes in αIIbβ3 clustering, as demonstrated here, should now permit the examination of some unresolved questions in αIIbβ3 biology. Among them, to what extent is αIIbβ3 clustering regulated by talin, an integrin- and actin-binding protein that has been implicated in affinity modulation of αIIbβ3 (56) and clustering of Drosophila integrins (57)? Do mutations in αIIb or β3 that promote affinity modulation also cause integrin clustering (31,58-61)? Is αIIbβ3 ligation, perhaps even by some monovalent ligands, sufficient to induce certain outside-in signaling responses, or is integrin clustering always required?
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ABBREVIATIONS

Abbreviations used: GFP, green fluorescent protein; β-gal, β-galactosidase; BRET, bioluminescence resonance energy transfer; FKBP, FK506-binding protein; HA, hemagglutinin; Rluc, Renilla luciferase.

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**FIGURE LEGENDS**

**Figure 1.** Monitoring αIIbβ3 clustering by β-galactosidase complementation. **A.** The αIIb integrin subunit constructs used in establishing stable CHO cell lines. Shown are αIIb, the Δα β-gal mutant, the Δω β-gal mutant, tandem FKBP domains, and epitope tags. The hashed line denotes the transmembrane domain (TM) of αIIb, with the extracellular domain to the left and intracellular domain to the right. **B.** Co-expression of αIIb chimeras and β3 in CHO cells. CHO cells were stably transfected with the indicated integrin constructs. Cell lysates were subjected to immunoprecipitation with an anti-αIIbβ3 antibody and immunoprecipitates were probed with antibodies to the HA and FLAG tags as described in Experimental Procedures. A5 CHO cells expressing wild type αIIbβ3 served as a negative control. The immunoreactive bands have been aligned for clarity. Based on migration of molecular weight standards, the apparent molecular masses of reduced αIIb/Δα β-gal and αIIb/Δω β-gal were ~140 and ~118 kDa, respectively. The molecular masses of reduced αIIb/(FKBP)2/Δα β-gal and αIIb/(FKBP)2/Δω β-gal were ~165 and ~142 kDa. **C.** Rationale for detection of αIIbβ3 clustering by β-gal complementation. When αIIbβ3 is clustered, for example by fibrinogen, the Δα and Δω β-gal mutants are brought into close proximity, reconstituting enzyme activity and light emission in the presence of a β-gal substrate.

**Figure 2.** Monitoring αIIbβ3 clustering by BRET. **A.** The αIIb constructs used in transient transfections of CHO cells. Shown are αIIb, GFP, Renilla luciferase, tandem FKBP domains, and the epitope tag. **B.** Expression of αIIb/GFP and αIIb/Rluc with β3 in CHO cells. CHO cells were transiently transfected with the indicated integrin constructs. Forty-eight hours later, cell lysates were subjected to Western blotting with antibodies to αIIb or HA. Untransfected CHO cells served as a negative control. The doublet consists of pro-αIIb (upper band) and αIIb (lower band). The apparent molecular masses of non-reduced αIIb/Rluc and αIIb/GFP were ~170 and ~160 kDa, respectively, while reduced αIIb/(FKBP)2/Rluc and αIIb/(FKBP)2/GFP were ~84 and ~76 kDa. **C.** Rationale for detection of αIIbβ3 clustering by BRET. When αIIbβ3 is clustered and GFP and Rluc are brought within ~80Å of each other, BRET occurs between the luciferase substrate (coelenterazine) and GFP, resulting in increased light emission at 515 nm and decreased emission at 410 nm.

**Figure 3.** Detection of αIIbβ3 clustering by β-galactosidase complementation. In **A,** a CHO cell line co-expressing αIIb/Δα β-galβ3 and αIIb/Δω β-galβ3 were incubated with either 10 µg/ml primary anti-αIIbβ3 antibody D57, 20 µg/ml anti-mouse secondary antibody, or D57 followed by secondary antibody. After washing, integrin clustering was monitored by measuring β-gal activity as described in Experimental Procedures. Activity is depicted as % above baseline, defined as enzyme activity in the presence of buffer instead of antibodies. In **B,** a CHO cell line co-expressing αIIb/(FKBP)2/Δα β-galβ3 and αIIb/(FKBP)2/Δω β-galβ3 were incubated for 30 min with 1 µM AP1510 or an equivalent vol of
vehicle as a control. Then β-gal activity was measured. Data are the means ± SEM of three separate experiments.

Figure 4. Detection of αIIbβ3 clustering by BRET. In A, CHO cells transiently transfected with αIIb/GFPβ3 and αIIb/Rlucβ3 were incubated with primary antibody D57 and/or a secondary antibody as in Figure 3. In B, CHO cells transfected with αIIb/(FKBP)2/GFPβ3 and αIIb/(FKBP)2/Rlucβ3 were incubated with AP1510 as in Figure 3. Then the BRET ratio was determined, as described in Experimental Procedures. Data are depicted as % above baseline, defined as the BRET ratio in the presence of buffer. Data are the means ± SEM of three experiments.

Figure 5. Fibrinogen binding induces αIIbβ3 clustering. In A, CHO cells stably co-expressing αIIb/Δα β-galβ3 and αIIb/Δω β-galβ3 were incubated for 30 min with 150 µg/ml anti-LIBS6 Fab in the absence or presence of 150 µg/ml fibrinogen. Then integrin clustering was monitored by the β-gal complementation assay. In B, CHO cells were transiently transfected with αIIb/GFPβ3 and αIIb/Rlucβ3 and 24 hours later incubated for 30 min with 1 mM MnCl2 in the absence or presence of 150 µg/ml fibrinogen. Where indicated, 10 mM EDTA was added to block fibrinogen binding to αIIbβ3. Then integrin clustering was monitored by BRET. Data are the means ± SEM of three experiments.

Figure 6. Characteristics of αIIbβ3 clustering induced by fibrinogen. In A, CHO cell lines were established that stably co-expressed αIIb/Δα β-galβ3 and αIIb/Δω β-galβ3 (shaded bar). Others were established that co-expressed αIIb/Δα β-galβ3 (D119A) and αIIb/Δω β-galβ3 (D119A) (black bar). Then the effect of anti-LIBS6 Fab-induced fibrinogen binding on integrin clustering was examined by β-gal complementation. Baseline refers to β-gal activity in the presence of anti-LIBS6 Fab alone. Data are the means ± SEM of five experiments, each conducted with an independent pair of CHO cell clones. In B, CHO cells expressing αIIb/Δα β-galβ3 and αIIb/Δω β-galβ3 were incubated for 30 min with fibrinogen and 150 µg/ml anti-LIBS6 Fab. Integrin clustering was then assessed by β-gal complementation. Enzyme activity is depicted as % above baseline, defined as enzyme activity in the presence of buffer instead of fibrinogen. Data are the means ± SEM of triplicate measurements from an experiment that is representative of two so performed.

Figure 7. Effect of latrunculin A and cytochalasin D on integrin clustering. CHO cells stably expressing αIIb/Δα β-galβ3 and αIIb/Δω β-galβ3 were incubated for 10 min with latrunculin A (A) or cytochalasin D (B) in the absence or presence of 150 µg/ml fibrinogen. Integrin clustering was assessed by β-gal complementation. Data are the means ± SEM of three independent experiments, each performed in triplicate.
Figure 1
Figure 2

A. 

| αIIB/GFP | αIIB | GFP |
|----------|------|-----|
| αIIB/Rluc | αIIB | Luciferase |
| αIIB/(FKBP)₂/GFP | αIIB | FKBP | FKBP | HA | GFP |
| αIIB/(FKBP)₂/Rluc | αIIB | FKBP | FKBP | HA | Luciferase |

B. 

| cDNAs       | Anti-αIIB | Anti-HA |
|-------------|-----------|---------|
| αIIB/Rluc:  | -         | +       | -       |
| αIIB/GFP:   | -         | -       | +       | -       | -       | -       | -       |
| αIIB/(FKBP)₂/Rluc: | -   | -     | -     | +     | -       | -       | +       | -       |
| αIIB/(FKBP)₂/GFP: | -   | -     | -     | -     | +       | -       | +       | +       |
| β3:         | -         | +       | +       | +       | +       | +       |

C. 

- αIIBβ3
- αIIBβ3
- Clustering Stimulus
- 410 nm
- Fibrinogen
- Rluc
- Coelenterazine
- Energy Transfer
- GFP
- 515 nm
Beta-Galactosidase Activity (% above baseline)

Primary Ab: - + ++ - +

Secondary Ab: Clustering with Antibodies

Clustering with Dimerizer

Figure 3
Figure 4

Clustering with Antibodies

Clustering with Dimerizer

BRET Ratio (% above baseline)

Primary Ab: - + +
Secondary Ab: + - +

Vehicle

AP1510
A. Integrin Clustering (% above baseline)

- Fibrinogen:  
  - Anti-LIBS6 Fab:  
  - Mn$^{2+}$:  
  - EDTA:  

B. Beta-Gal Complementation

- BRET

![Graph showing Beta-Gal Complementation and BRET results](image)

Figure 5
Figure 6
Figure 7

Latrunculin A, µM

Cytochalasin D, µM

Beta-Galactosidase Activity (% above baseline)

No Fibrinogen
Fibrinogen

A.

B.
Detection of integrin αIIbβ3 clustering in living cells
Charito Buensuceso, Maddalena de Virgilio and Sanford J. Shattil

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