INTEGRIN αIIbβ3 ACTIVATION IN CHO CELLS AND PLATELETS INCREASES CLUSTERING NOT AFFINITY

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Running head: αIIbβ3 integrin ligand affinity

Integrin αIIbβ3 affinity regulation by talin binding to the cytoplasmic tails of β3 is a generally accepted model for explaining activation of this integrin in CHO cells and human platelets. Most of the evidence for this model comes from the use of multivalent ligands. This raises the possibility that the activation being measured is that of increased clustering of the integrin rather than affinity. Using a newly developed assay that probes integrins on the surface of cells with only monovalent ligands prior to fixation, we do not find increases in affinity of αIIbβ3 integrins by talin head fragments in CHO cells nor do we observe affinity increases in human platelets stimulated with thrombin. Binding to a multivalent ligand does increase in both of these cases. Our assay does report affinity increases induced by either Mn2+, a cytoplasmic domain mutant (D723>R) in the cytoplasmic domain of β3, or preincubation with a peptide ligand. These results reconcile the previously observed differences between talin effects on integrin activation in Drosophila and vertebrate systems and suggest new models for talin regulation of integrin activity in human platelets.

Integrins are adhesive heterodimeric transmembrane proteins that bind to extracellular matrix (ECM) ligands or to cell surface proteins on adjacent cells. The cytoplasmic tails of the integrins are linked directly, or via adaptors, to numerous cytoskeletal and signaling proteins and transmit signals from the outside of the cell to the inside. The adhesive properties of integrins are dynamically regulated as these receptors shift between different conformations upon binding to extracellular ligands or cytoplasmic proteins. Thus, integrins are present in high or low affinity states on the surface of cells depending on the cellular environment. Regulation of integrin activation is critical in controlling cell adhesion, migration, and extracellular matrix-assembly. This regulation is therefore important in normal development, hemostasis, inflammation, angiogenesis, tumor cell metastasis, and immune responses (1-4).

Talin is one of the most intensively studied cytoplasmic activators of integrin activity (5,6). The N-terminal globular head region of talin contains a FERM (band four-point-one, ezrin, radixin, moesin homology) domain that has the ability to bind to β3 integrin cytoplasmic tails and this results in the activation of αIIbβ3 integrins. Talin’s ability to interact with integrins is itself regulated, as the FERM domain is autoinhibited by binding to its C-terminal tail in an intra- or intermolecular manner (2,3,7,8). Autoinhibition of talin can be removed by the Rap1 effector molecule RIAM in response to extracellular cues (9). Experimentally the autoinhibition can be removed by expressing the talin head (or FERM) domain in the absence of the inhibitory tail. Overexpression of the talin head or FERM domain activates the β3 and β1 integrins and inhibition of talin expression reduces integrin activity in mammalian cell culture (5,10).

Intriguingly, recent experiments in our lab and others have not been able to detect an effect of talin FERM domain expression or reduction in talin expression on the activation state of the Drosophila PS2 integrins. This has lead to speculation of a fundamental difference between Drosophila talin-PS2 integrin interactions and those observed for talin-αIIbβ3 in vertebrates (11). However, the methodologies used to measure integrin affinity for ligand in these two studies...
were fundamentally different. In this report, we find that when identical binding assays are conducted for \( \alpha_{IIb}\beta_3 \) integrins as were used for the *Drosophila* PS2 integrins talin has no effect on the affinity of \( \alpha_{IIb}\beta_3 \) for monovalent ligand. Thus, we find no difference between the *Drosophila* talin-PS2 integrin interactions and those seen for vertebrate talin-\( \alpha_{IIb}\beta_3 \) integrin.

We do find that talin FERM domain increases the ability of \( \alpha_{IIb}\beta_3 \) to bind to multivalent ligands and this appears to be the source of discrepancy between the binding assays. Numerous studies have shown that a clustering mechanism activates \( \beta_3 \) integrins. In platelets, avidity is an important component of \( \alpha_{IIb}\beta_3 \) binding to ligand as PAC-1 IgM showed 60 fold greater binding capacity as compared with the PAC-1 Fab even in the presence of secondary antibodies (12). Ligand binding, talin head expression, and agonist-induced activation in CHO cells all result in integrin clustering (13-15).

A clear understanding of \( \alpha_{IIb}\beta_3 \) activity regulation that distinguishes affinity from clustering effects is important as it may impact the development of therapeutic agents designed to modulate integrin activity to treat pathologies involving inflammation, thrombosis, angiogenesis, and tumor progression. If “integrin activation” or “integrin activity” is a general term that encompasses clustering and/or affinity changes, then clearly talin activates \( \alpha_{IIb}\beta_3 \) integrins in CHO cells. However, “integrin activation” is commonly interpreted to mean an increase in the affinity of an individual integrin heterodimer for ligand prior to encountering ligand (3). It is this latter definition of integrin regulation by talin that is found in at least one cell biology textbook that states “Thus when talin binds to the \( \beta \) chain it undoes the intracellular \( \alpha-\beta \) linkage, allowing the two legs of the integrin molecule to spring apart. This drives the extracellular portion of the integrin into its extended, active conformation.”(16) Our results in CHO cells and additional experiments in human platelets contradict this view of integrin affinity regulation by talin and point instead to talin’s role in integrin clustering.

**EXPERIMENTAL PROCEDURES**

*Antibodies*- PAC-1 IgM (17) was purchased from Becton Dickinson (# 340535). PAC-1 Fab was expressed as a his-tagged fusion protein expressed by cultured *Drosophila* cells (18) and prepared as described for TWOW-1 (19). R-phycoerythrin labeled or unlabeled HIP8 (BD Pharmingen # 555467 and #555465) were used to quantify \( \alpha_{IIb}\beta_3 \) levels in CHO cells or platelets. This antibody gave similar results in CHO cells and platelets as the conformation insensitive monoclonal antibody SSA6 (a generous gift from Sandy Shattil). The secondary antibody used to detect PAC-1 and unlabelled HIP8 was R-phycoerythrin conjugated goat anti-mouse IgG (H+L) (Invitrogen P852). Alexa Fluor 568 goat anti-mouse IgG (Invitrogen A11031) was used for microscopy of PAC-1 bound to CHO cells.

*Cell Culture*- CHO cells stably transformed to express human integrin \( \alpha_{IIb}\beta_3 \) (A5 cells) or constitutively active \( \alpha_{IIb}\beta_3(D723>R) \) have been described (20,21). CHO cells were grown in DMEM (cellgro #15-013-CV) supplemented with essential amino acids (Gibco #11140), penicillin-streptomycin-glutamine (Gibco #10378-016), and 10% fetal bovine serum (FBS; Sigma #F6178). For PAC-1 binding experiments, cells were thawed and used within 3 passages. Outdated platelets were obtained from the University of Arizona blood bank and were used within 2 days.

*Transfections*- Transfections were done using lipofectamine 2000 reagent (Invitrogen) as recommended by the supplier. Cells were seeded at 2.5 x 10^5 cells per ml in 60 mm tissue culture plates (4.5 ml/well). On the following day, the media was replaced with the same volume of media containing FBS but lacking antibiotics. 30 µl lipofectamine-2000 was mixed with 500 µl serum free medium and allowed to incubate for 5 minutes and then mixed with 500 µl serum free medium containing 6 µg plasmid DNA. After 20 minutes this was added directly to the cells. On the following day the transformed cells were rinsed with phosphate buffered saline (PBS; 0.8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 154 mM NaCl, pH 7.4), trypsinized (Cellgro Trypsin EDTA #25-052-CI) for 2-3 minutes. Trypsinization was stopped with the addition of media containing FBS. Following one wash, the cells were diluted...
in 10 ml of fresh medium containing FBS and antibiotics in a 100 mm tissue culture plate. Cells were used in binding experiments 48 hours after transfection. Plasmids used for transfections were either a GFP-murine talin head F2-F3 domain (amino acids 206-405) chimera, or an empty vector expressing only GFP (10,22).

**PAC-1 Binding-** CHO cells expressing integrins were rinsed with PBS, trypsinized (Cellgro Trypsin EDTA #25-052-C1) for 2-3 minutes. Trypsinization was stopped with the addition of media containing FBS. Cells were centrifuged, washed with medium and then with PBS. 5 x 10^5 cells were then resuspended in 30 µl Tyrode’s (12.1 mM NaHCO3, 5 mM HEPES, 137 mM NaCl, 2.6 mM KCl, and 5.6 mM Glucose), containing 1 mg/ml bovine serum albumin (BSA) and either; 1.66 mM CaCl2 and MgCl2; 1.66 mM CaCl2, MgCl2 and MnCl2; or 8.3 mM EDTA. 20 µl of PAC-1 IgM or PAC-1 Fab was added, yielding the final concentrations of 1 mM Ca^2+, 1 mM Mg^2+, 1 mM Mn^2+, and 5 mM EDTA. PAC-1 IgM binding was performed using a standard protocol (23). PAC-1 IgM was incubated with cells for 30 minutes at room temperature. Cells were washed by adding 1.5 ml of Tyrode’s with appropriate divalent cations or EDTA followed by centrifugation. Cells were then resuspended in 50 µl R-phycoerythrin (PE) conjugated secondary antibody (10 µg/ml) in Tyrode’s containing 1 mg/ml BSA. After 25 minutes incubation on ice, cells were diluted with ice cold PBS and analyzed immediately by flow cytometry. PAC-1 Fab binding was done basically as described (19) with slight modifications of the buffer so that it was identical to that used for PAC-1 IgM binding. A similar protocol has been used to measure the affinity of LFA-1 and its ligand ICAM-1 (24). Cells were incubated as above with PAC-1 Fab for 10 minutes followed by the addition of 50 µl 4% formaldehyde in Tyrode’s to fix bound PAC-1 to the cells. Following a 5-minute fixation, cells were collected by centrifugation and resuspended in 50 µl R-phycoerythrin conjugated secondary antibody (10 µg/ml) in Tyrode’s containing 1 mg/ml BSA. After a 25-minute incubation, cells were collected by centrifugation and resuspended in 0.5 ml PBS containing 2% formaldehyde. To determine αIIbβ3 integrin expression levels, 5 x 10^5 cells were also incubated for 30 minutes with 50 µl R-phycoerythrin labeled HIP8 (diluted 1:1 with PBS). All centrifugations for CHO cells were done for 2 minutes at 1000 xg.

For each experiment PE fluorescence levels for 1,000-5,000 strongly GFP-positive cells were analyzed by flow cytometry. To determine integrin-dependent binding, we subtracted nonspecific binding—the amount observed when divalent cations had been removed (by EDTA). Integrin-dependent mean fluorescence intensity (MFI) of PAC-1 binding was divided by MFI of HIP8 binding, in the GFP strongly positive cells, thereby allowing us to adjust for differences in expression levels between samples, which was 25% or less.

Significant differences in binding is given as p-values using Student’s t-test.

**Platelet Activation and PAC-1 Binding-** For thrombin activation, platelets were collected by centrifugation and resuspended at 2.9 x 10^8 cells/ml in Tyrode’s containing 1 mM MgCl2, 1 mg/ml BSA. Thrombin (Sigma T7513) from a 100 units/ml frozen stock was added to a final concentration of 0.5 units/ml to activate the platelets. Platelets were incubated for 30 minutes at room temperature with intermittent vigorous pipetting to reduce aggregation. 35 µl of cells (1 x 10^7) were added to tubes containing 5 µl Tyrode’s with 1 mg/ml BSA and either; 10 mM CaCl2, 3 mM MgCl2; 10 mM CaCl2, 3 mM MgCl2, 10 mM MnCl2; or 50 mM EDTA. Control platelets were treated identically but without the addition of thrombin. The vigorous pipetting was necessary for only the thrombin activated platelets to reduce their aggregation. This treatment did not inadvertently activate the platelets in the absence of thrombin as determined by microscopic and flow cytometry analysis or the levels of surface fibrinogen (Fig. S2).

For RGD activation of integrins, platelets were collected by centrifugation and resuspended at 2 x 10^8 cells/ml in Tyrode’s containing 1 mg/ml BSA, 1 mM MgCl2 and 1 mM CaCl2. GRGDSP, GRGESP (AnaSpec, Inc. 22945 and 22949), or no peptide, were added to a final concentration of 1 mM and incubated for 5 minutes at room temperature. Cells were then fixed by the addition of an equal volume of Tyrode’s containing 1 mM MgCl2, 1 mM CaCl2, and 4% formaldehyde. After 5 minutes of fixation, the fix was diluted by the addition of 30 volumes of Tyrode’s (containing 1
mM MgCl₂ and 1 mM CaCl₂ or 5 mM EDTA). 1.5 ml (5 x 10^6 cells) were centrifuged and resuspended in Tyrode’s containing 1 mg/ml BSA and either; 1.25 mM CaCl₂, 1.25 mM MgCl₂; 1.25 mM CaCl₂, 1.25 mM MgCl₂, 1.25 mM MnCl₂; or 6.25 mM EDTA.

For PAC-1 binding to thrombin-activated platelets (or their controls), 10 µl PAC-1 (IgM or Fab) was added to the above tubes and they were incubated for 10 minutes at room temperature. Final concentrations of divalent cations were all 1 mM and EDTA was 5 mM. Bound PAC-1 was fixed to the platelets by addition of 50 µl Tyrode’s containing 4% formaldehyde. Following 5 minutes of fixation at room temperature, the fix was diluted with the addition of 1.5 ml Tyrode’s. Fixed platelets were collected by centrifugation and resuspended in 50 µl R-phycoerythrin conjugated secondary antibody (10 µg/ml) in Tyrode’s containing 1 mg/ml BSA. After a 25-minute incubation, on ice, cells were collected by centrifugation and resuspended in 0.5 ml PBS containing 2% formaldehyde.

10,000 platelets were analyzed by flow cytometry for each experiment. As for CHO cells, nonspecific binding (in the presence of EDTA) was subtracted and PAC-1 values were divided by total integrin expression (as assessed by HIP8 staining) to adjust for any expression differences or increased values due to aggregating platelets. For the platelet experiments, unlabeled HIP8 binding was done identically to PAC-1 binding in the presence Ca^{2+} and Mg^{2+}-- same times, fixations, and detection with the same secondary antibody. HIP8 concentration in these experiments was 2 µg/ml.

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As for CHO cell binding experiments, tests for significant differences in binding is given as p-values using Student’s t-test.

**Immunofluorescence-CHO cell experiments**

CHO cells expressing GFP-Talin F2-F3 were processed as in the PAC-1 IgM binding assay through removal of unbound PAC-1. Bound PAC-1 was then fixed on the cells with formaldehyde for 5 minutes. Following washing to remove the formaldehyde, cells were allowed to attach to glass slides, fixed to the slides with formaldehyde and then PAC-1 was detected using AlexaFluor568 goat anti-mouse IgG secondary antibody (2 µg/ml in Tyrode’s containing 1 mg/ml BSA). Following washing, cells were mounted in Vectashield and examined on a DeltaVision microscope using a 100x, 1.4NA Olympus UPlanSApo objective. The z-series was deconvolved and quick-projected using SoftWoRx (Applied Precision). All exposure times and processing were held constant (with the exception of the number of optical slices). ImageJ software was used to determine particle sizes of fluorescent clusters. Particle sizes for unclustered PAC-1 IgM antibodies were determined by imaging PAC-1 fixed to glass slides in the absence of cells. The threshold intensity for positive pixels was set at approximately 2x background (minimum pixel intensity) and was held constant for all images analyzed. Particle (cluster) areas and intensities are given as the mean ± S.E..

**RESULTS**

**Monovalent-fixation assay reports on αIIbβ3 affinity.** Talin head domain or its F2-F3 subdomain has been demonstrated to increase PAC-1 binding to αIIbβ3 expressed in CHO cells (5,22). The assays used to demonstrate integrin αIIbβ3 binding affinity regulation utilized the multivalent IgM ligand mimetic antibody, PAC-1. This antibody contains the integrin binding motif Arg-Tyr-Asp (RYD) in each of its 10 complementary determining region 3s (H-CDR3s) (25). Alternatively, the binding assays used monovalent ligands, such as PAC-1 Fab (with only one integrin binding motif). In both assays, binding capacity was assessed by the addition of labeled polyclonal secondary antibodies that bind to the PAC-1. Binding levels were then measured by flow cytometry without fixation (6,12,18,23).

One potential artifact in the monovalent binding assay is that PAC-1 Fab and secondary antibodies were present at the same time during binding and this likely produced a clustered multivalent PAC-1 ligand. Using a modified assay which we call the monovalent-fixation assay, that does test integrin affinity for monovalent ligands (19), we were unable to detect any effect of talin on Drosophila PS2 integrin affinity for ligand (11). We therefore set out to test if the monovalent-fixation assay detects an increase in αIIbβ3 affinity by talin F2-F3 in CHO cells.

In the monovalent-fixation assay, PAC-1 Fab binding to cells is achieved by incubating the cells with PAC-1 Fab for 10 minutes. Bound
monovalent PAC-1 is then formaldehyde fixed to the cells. Unbound PAC-1 is washed away and the bound PAC-1 is detected using a labeled secondary antibody and flow cytometry. Staining for total integrin levels is used to adjust for expression levels that may vary between experiments or cell lines. Using this assay, PAC-1 Fab bound in a dose dependent manner to CHO cells expressing αIIbβ3 integrins (Fig. 1). To determine if our assay reports on previously described activators of integrin affinity, we also did binding curves on cells expressing an integrin containing a β cytoplasmic domain activating mutation, αIIbβ3(D723>R) (21). Also, the divalent cation Mn2+ is a well known integrin activator (26-28) and we asked if its presence in the binding assay increased monovalent ligand binding to wild type αIIb3 and αIIbβ3(D723>R) integrins expressed in CHO cells. αIIbβ3(D723>R) confers a significant increase in affinity in the absence and in the presence of Mn2+ (Fig. 1). The ability of Mn2+ to activate the integrins is much greater than the cytoplasmic domain mutation and is additive with it. This suggests that neither the mutation nor Mn2+ alone fully activates the integrins (Figs. 1 & 3). This is entirely consistent with observations on the PS2 integrins in Drosophila cells (19). Importantly, these results demonstrate the validity of the monovalent-fixation assay for reporting on the affinity state of αIIb3 integrins.

αIIbβ3 binding to multivalent but not monovalent ligand is regulated by talin in CHO cells. To determine the contributions of affinity and clustering on integrin regulation by talin, we measured multivalent ligand binding using a standard PAC-1 IgM binding assay and measured monovalent ligand binding using our monovalent-fixation binding assay. In both cases, CHO cells expressing human αIIbβ3 were transiently transfected to express either the GFP tagged activating talin head fragment (GFP-Talin F2-F3) or GFP alone. Similar to previous reports (10,22), we found that cells expressing GFP-Talin F2-F3 bind more multivalent PAC-1 IgM than cells expressing GFP (Fig. 2A) Surprisingly, we found no difference between cells expressing the GFP-Talin F2-F3 or GFP in their ability to bind PAC-1 Fab (Fig. 2A). The levels of PAC-1 Fab used in this experiment (20 µg/ml) result in binding to approximately 15% of the available integrins (Fig. 1) and were chosen to give similar binding levels when compared to PAC-1 IgM (used at a typical concentration of 10 µg/ml). Preliminary experiments at higher concentrations of PAC-1 Fab (up to 300 µg/ml) also found binding to be unaffected by the presence of GFP-Talin F2-F3 (Fig. S1). Taken together, our results suggest that talin head expression increases the clustering and avidity but not the affinity of αIIbβ3 for PAC-1.

To begin to determine the relative contributions of affinity and clustering on integrin regulation by the artificial activators Mn2+ and the β3(D723>R) activating mutation, we compared their abilities to increase binding to multivalent and monovalent PAC-1. We found that Mn2+ strongly increases binding to both ligands (Fig. 2B) and therefore the increase in binding could largely be explained by increased affinity. αIIbβ3(D723>R) bound significantly more PAC-1 Fab than wild type αIIb3 and this increase was much more pronounced for the multivalent ligand PAC-1 IgM (Fig. 3). This suggests that the β3(D723>R) mutation increases both affinity and the clustering of αIIbβ3.

PAC-1 IgM binding to αIIbβ3 is clustered. To determine if the PAC-1 IgM bound to cells is clustered, we examined PAC-1 IgM on the surface of cells by epifluorescence microscopy (Fig. 4A). Bound PAC-1 IgM appears to be clustered. As a control for fluorescent particles resulting from PAC-1 IgM binding to secondary antibodies in the absence of a cellular context, we examined PAC-1 IgM on glass slides not first incubated with PAC-1 IgM, showed a reduction of 99.6% in particle number (not shown).

The apparent clustered binding is confirmed by quantitative analysis of particle size using NIH ImageJ software. Fluorescent particles from 4 fields (total of 800 particles) of PAC-1 IgM on glass found none greater in area that 0.2 µm2 and only 3±0.6% greater than 0.1 µm2. For PAC-1 bound to cells, 18% of the particles (clusters) examined on 11 cells (total of 1059 particles) were greater in area than 0.2 µm2 . This population of clusters was responsible for an average of 55±6% (range 24-82%) of the total fluorescent area and 59±7% (range 29-89%) of the fluorescence...
intensity in particles on the cell surface. Thus, PAC-1 IgM bound to CHO cells expressing αIIbβ3 and GFP-Talin F2-F3 is clustered in the typical affinity assay.

αIIbβ3 binding to multivalent but not monovalent ligand is increased by thrombin activation of human platelets. To ask if our findings in CHO cells are relevant to what actually occurs in human platelets, where regulation of αIIbβ3 binding to ligands is biologically relevant to the process of hemostasis, thrombosis and inflammation, we re-examined αIIbβ3 binding to monovalent and multivalent ligands in thrombin activated human platelets. To probe contributions of affinity and clustering on the increase in αIIbβ3 activity that occurs upon platelet activation, thrombin-activated platelets were compared with resting (non-activated) platelets for their binding to either PAC-1 IgM or PAC-1 Fab at two different ligand concentrations for each. Binding to PAC-1 (IgM or Fab) was followed directly by fixation, and then unbound PAC-1 was removed by washing. Bound PAC-1 was then detected by labeled secondary antibody and flow cytometry. Surprisingly, thrombin activation increased multivalent PAC-1 IgM binding but not monovalent PAC-1 Fab binding (Fig. 5A). Again, Mn²⁺ was able to increase the affinity of the integrin as measured by monovalent PAC-1 binding (Fig. 5B). Thus, our results indicate that in platelets, as in CHO cells, αIIbβ3 regulation is at the level of their ability to bind multivalent but not monovalent ligands.

The binding of integrins to their ligands increases their affinity for ligands, in vitro, and results in changes in conformation as detected by LIBS (ligand-induced binding site) antibodies (29-31). Therefore, we next tested whether the affinity of αIIbβ3 for monovalent PAC-1 could be modulated by prior ligand binding. Platelets were incubated with no ligand, a GRGDSP peptide ligand (RGD is a recognized integrin binding motif), or a GRGESP control peptide, and then briefly fixed with formaldehyde. The platelets were then washed to remove the fixative as well as unbound peptides. Those bound peptides, possessing only one reactive amine group at the N-terminus, are unlikely to be fixed to the integrin and are also removed during washing. The fixed integrins on the surface of the platelets were tested for ligand binding in our standard monovalent-fixation assay. We found that binding of monovalent PAC-1 was increased by the GRGDSP peptide but not by the GRGESP peptide (Fig. 6). Thus, we confirmed that ligand binding increases integrin affinity.

DISCUSSION

Talin has been demonstrated to be a key regulator of integrin adhesive activity both in whole organisms and in cell culture models (5,6,32-35). Regulating integrin activity can occur by either changing the affinity of individual integrins for their ligands and/or by clustering of integrins thereby altering their ability to bind multivalent ligands.

αIIbβ3 affinity versus clustering. Our goal has been to distinguish between changes in integrin-ligand affinity versus avidity (clustering plus affinity) on the surface of living cells. Monovalent ligands, like PAC-1 Fab, in solution with cells, can probe the affinity of individual integrins for individual ligands. Simply clustering the integrins without changing their individual affinities should not affect their ability to bind a monovalent ligand. Multivalent ligands, like PAC-1 IgM are able to detect clustering of integrins even in the absence of affinity changes. Each individual multivalent ligand can make multiple interactions with multiple integrins when these receptors are in close proximity (clustered) and this results in increased binding. When the integrins are not in close proximity (unclustered) multiple interactions are not possible and reduced binding results. The binding of multivalent ligands will also be altered by changes in integrin affinity and initial binding of the multivalent ligand can promote clustering of the integrins. The combination of affinity and clustering is often referred to as avidity. By using both types of assays, monovalent and multivalent, we can distinguish between affinity and avidity regulation of integrins.

Using a newly developed binding assay for monovalent ligands that exposes integrins solely to the ligand in its monovalent state we have reported on affinity increases in the Drosophila PS2 integrin due to the presence of Mn²⁺, integrin cytoplasmic and extracellular point
mutations, and deletion of a βPS integrin plexin-semaphorin-integrin domain (19). Here we show that this same assay reports αIIbβ3 integrin affinity modulation by Mn$^{2+}$, a β3 cytoplasmic domain mutation, and ligand-induced activation. All of these results validate the ability of our assay to detect affinity differences in *Drosophila* and vertebrate integrins.

In contrast to previously reported results we do not find increased binding of monovalent ligand (PAC-1 Fab) to αIIbβ3 integrins in the presence of high levels of talin head domains in CHO cells. The same cells did show increased binding to the same ligand presented in a multivalent state (PAC-1 IgM) in the traditional binding assay. Using our assay we also do not find increased affinity of αIIbβ3 on human platelets activated with thrombin. Despite being able to detect numerous activating conditions we have considered that there might be an artifact of our assay, such as the presence of formaldehyde during the binding phase, that renders it unable to detect integrin activity differences resulting from platelet activation. To test this we used the multivalent PAC-1 IgM in the exact same assay, including the presence of formaldehyde during the binding phase. This ligand did demonstrate activation dependent increase in binding. Thus, our assay does not do away the ability to detect integrin activity increases upon activation-- only changing the valency of the ligand does that. Therefore, we propose that talin increases integrin activity, both in CHO cell culture model assays and in human platelets, by increasing their clustering but not, directly, their affinity.

**Differences between our assay and those previously used.** Our monovalent-fixation assay differs markedly from the ones currently used (monovalent-plus-multivalent-secondary) to detect monovalent PAC-1 ligand binding to αIIbβ3 and we suggest this difference is the cause of our different results in the cases of talin and thrombin activation of αIIbβ3 integrins in CHO cells and platelets. In the commonly used monovalent-plus-multivalent-secondary assay, PAC-1 Fab fragment is incubated with cells for 15-30 minutes at room temperature and then polyclonal secondary antibodies are added and the incubation continues for an additional 20-30 minutes on ice. This is typically followed by a wash step and analysis by flow cytometry of the live cells. We have a number of concerns regarding this procedure. Most importantly, as soon as the secondary antibodies are added to the binding reaction, the PAC-1 Fab ceases to be a monovalent ligand. It is then capable of reporting on clustering as well as affinity changes. Although multivalent, the exact nature of the newly created ligand will depend on the exact concentration of the PAC-1 Fab and the polyclonal secondary antibodies and where the secondary antibodies bind to the PAC-1 Fab. Therefore, it is not expected that this ligand will behave exactly the same as the PAC-1 IgM in all binding assays.

That multivalency is a component of the standard monovalent ligand-secondary assay is illustrated by the fact that in all the reports based on this assay PAC-1 Fab (and secondary antibody) remains bound even following a wash and/or dilution of live cells prior to flow cytometry that removes most or all unbound PAC-1 Fab. If unbound and bound PAC-1 Fab were truly in equilibrium with the integrins, then washes bringing the unbound PAC-1 concentration essentially to 0 should have resulted in dissociation of the bound ligand from the integrins. That it did not, demonstrates that the reactions were not at equilibrium and therefore were not measuring the affinity of the integrins at that time. In preliminary experiments with *Drosophila* cells we have found that washing the cells after binding of a monovalent ligand to PS2 integrins (without adding secondary antibodies) resulted in dissociation of the ligand (data not shown). Therefore, the monovalent ligand must be fixed to the integrins prior to washing.

In the case of activated platelets additional complications suggest that previous binding assays were not a reliable measure of the affinity of integrins prior to ligand binding. On the surface of activated platelets the binding of ligands to αIIbβ3 is a multiphasic, energy dependent, process where ligand binding becomes irreversible over 15-30 minutes (36-38). Time dependent irreversibility has also been seen in CHO cell experiments measuring αIIbβ3 binding to fibrinogen or PAC-1 (18). As the commonly used assays involve binding times of 15-30 minutes at room temperature, followed by a 30 minute incubation with secondary antibodies on ice, time dependent changes make it difficult to determine what the affinity of the integrin is prior to ligand binding at
a time when talin is proposed to increase integrin affinity. We suggest that our binding times of 10 minutes followed directly by fixation gives a more accurate measure of the effects of talin on early integrin-ligand interactions.

The prediction of low affinity interactions between PAC-1 and αIIbβ3 integrins that have not been activated (presumably of low affinity and not clustered) is seen in our monovalent-fixation assay because we fix the bound ligand to the integrins prior to washing (Fig. 1). In the common monovalent-plus-multivalent-secondary or multivalent IgM assays almost no binding is detected. This is exactly what would be expected for assays that involve a wash that removes unbound ligand in the absence of integrin clustering, as in CHO cells expressing only αIIbβ3 integrins or non-activated platelets. Even dimerizing the low affinity integrins resulted in their binding to the PAC-1 IgM and fibrinogen (39). This increase in binding was seen even though the integrins did not show an increased exposure to multiple conformation reporting antibodies, demonstrating that low affinity binding to soluble ligands does occur. This assay did not find binding to PAC-1 Fab under dimerizing conditions suggesting that integrin affinity had not been increased. We would suggest that the PAC-1 Fab-secondary assay used in this study gave different results than PAC-1 IgM due to the sensitivity of clusters of only 2 integrins, linked by a disulfide bond, to the nature of the multivalent ligand formed by the PAC-1 Fab and secondary antibodies.

Clustering of integrins in cultured cells and platelets when binding to PAC-1 IgM and other multivalent ligands has been repeatedly observed. In addition to our observations of PAC-1 IgM binding to CHO cells, integrin αIIbβ3 redistribution into macro-clusters as a result of agonist-induced activation of talin has been demonstrated in CHO cells that express high levels of talin and PKCα (15). Additionally, β-galactosidase complementation and bioluminescence resonance energy transfer assays demonstrated that αIIbβ3 is clustered in CHO cells binding to either bivalent antibodies or bivalent fibrinogen (13). In spreading B16F1 cells talin head expression induced integrin αVβ3 activation and macro-clusters (14). Finally, in platelets there is extensive evidence, by both confocal and electron microscopy, for the clustering of αIIbβ3 bound to PAC-1 IgM, fibrinogen, and peptides in thrombin and ADP activated human platelets (37,40,41). Thus in suggesting that the binding assays that rely on multivalent ligands introduce a clustering component we are proposing something that is perhaps underappreciated but not unobserved.

**Models for integrin “activation” by talin.**

The data presented here call into question simple models proposing that talin’s mechanism of action is to directly increase integrin affinity for ligands. Our data are consistent with a model where talin’s direct role is one of regulating the clustering of αIIbβ3 integrins (Fig. 7A). Clustering may also be accompanied by changes in integrin conformation but our data would suggest that the integrin is still in a low affinity state. Clustering increases the integrin αIIbβ3 avidity for multivalent ligands such as the widely used PAC-1 (either IgM or Fab with secondary antibodies) or fibrinogen. Once these ligands bind to the clustered low affinity integrins they are predicted to stabilize the high affinity state of the integrins. As the ligand is retained in the vicinity of the clustered integrins, due to the talin-induced clustering, it binds to many of the integrins in the cluster and stabilizes the high affinity state that triggers sustained inside-out signaling. Once the integrins are clustered and converted to high affinity states, the multivalent ligand is essentially irreversibly bound. In this model, it is the ligand binding which directly induces the high affinity state of the αIIbβ3 integrin. Talin facilitates this indirectly by increasing the clustering of the integrins prior to
ligand binding. Talin does not convert the conformation of the integrin heterodimers to a high affinity state prior to ligand binding. The model would predict that in the absence of integrin clustering, individual integrins do interact with ligands, such as PAC-1 IgM, and become activated to a high affinity state (Fig. 7B). However, dissociation of the ligand can and does occur, since the integrins and ligand are in true equilibrium, and the integrin reverts to a low affinity state. The level of inside-out signaling resulting from these transient interactions is expected to be low and not result in dramatic cellular responses. A slightly more complicated model, takes into consideration the observation that ligand binding to \( \alpha_{IIb}\beta_3 \) integrins has been shown to promote their clustering (13). It may be that it is ligand binding to the low affinity integrin which triggers clustering in CHO cells and platelets. Talin, in this model, would be required for executing the clustering that again would result in large increases in multivalent, but not monovalent, ligand binding.

In addition to being different in valency, PAC-1 IgM (or PAC-1 Fab clustered with secondary antibodies) and PAC-1 Fab are different in size and this could contribute to differences in binding to different integrin conformations. While some models propose ligand binding to integrins in an extended conformation, results consistent with regulated binding to integrin \( \alpha V\beta 3 \) in the bent conformation, without conversion to the extended form, have also been observed (42). The ligand binding head domain of \( \alpha_{IIb}\beta_3 \) in the bent conformation might be accessible the smaller Fab probe but not to the larger IgM or (or PAC-1 Fab clustered with secondary antibodies). If this is the case, our monovalent assay specifically probes the affinity state of the integrin head domain in either the bent or extended conformation. IgM and other large multivalent ligands are sensitive to three factors: the affinity state of the head domain, the bent state of the integrin, and clustering.

Our work does not address the mechanisms of how ligand binding stabilizes or induces the high affinity state of integrins. One model, with support from structural data, of how this may occur proposes that binding of ligand to the MIDAS domain of \( \beta 3 \) results in rearrangements in MIDAS contacts that are transmitted through changes in interactions between helixes \( \alpha 1 \) and \( \alpha 7 \) in the \( \beta \) integrin I-domain. Helix \( \alpha 7 \) connects directly to the hybrid domain and ligand binding facilitates an outward of the hybrid domain from the I-domain leading to separation of the stalks and cytoplasmic tails of the \( \alpha \) and \( \beta \) subunits. Separation of the \( \alpha \) and \( \beta \) cytoplasmic domains then leads to cytoplasmic signaling and clustering (31).

Though the use of monovalent ligands is important experimentally, it is likely that most extracellular or cell surface integrin ligands in the organism are multivalent. Therefore, regulation of the clustering of low affinity integrins followed by the subsequent multivalent ligand-induced activation of integrin affinity is a parsimonious strategy to convert integrins on the cell surface to a clustered high affinity state.

REFERENCES


FOOTNOTES

This work is dedicated to the memory of my close colleague and friend, Danny Brower, whose untimely death happened just prior to the beginning of the work reported here. I thank him for helpful discussions regarding this project and for securing the funding that made this work possible. I thank Hisashi Kato and Teresa Helsten for cell lines, antibodies, protocols and advice. I thank Paula Campbell and Barb Carolus of the ARL Cytometry Service for much assistance and the University Medical Center Blood Bank for outdated platelets. Greg Rogers and Joyce Schroeder provided useful comments on the manuscript. I thank Candida Morris who assisted with manuscript preparation. This work was supported by a grant from the NIH (R01GM42474).

The abbreviations used are: PBS, phosphate buffered saline; CHO, Chinese hamster ovary; FBS, fetal bovine serum; BSA, bovine serum albumin; FERM, band four-point-one, ezrin, radixin, moesin homology; MFI, mean fluorescence intensity.

FIGURE LEGENDS

Fig. 1. PAC-1 Fab binding to CHO cells expressing αIIbβ3 and αIIbβ3(D723>R). PAC-1 Fab binding levels were determined for cells expressing wild type αIIbβ3 (solid lines) or αIIbβ3(D723>R) (dashed lines) in the absence (solid squares) or presence of Mn2+ (open circles). In this and subsequent figures binding is expressed as a ratio of specific PAC-1 immunofluorescence (PAC-1 MFI) over total integrin detected by the αIIbβ3 antibody HIP8 (HIP8 MFI). “Specific” PAC-1 binding is the total PAC-1 immunofluorescence minus that seen in the presence of EDTA. Values shown are the mean ± S.E. from 3 independent experiments.

Fig. 2. Talin increases αIIbβ3 binding to multivalent PAC-1 IgM but not monovalent PAC-1 Fab. A, binding of PAC-1 IgM (10 µg/ml) to CHO cells expressing αIIbβ3 is increased when the cells also express GFP-talin head F2-F3 domain (TH-GFP) as compared with cells expressing GFP alone (p=0.0001). Binding of PAC-1 Fab (20 µg/ml) was not increased (p=0.79). B, activation of αIIbβ3 by Mn2+ resulted in increased binding of both forms of PAC-1 (p=0.0001 for both). Binding of PAC-1 IgM was followed by washes and analysis of live unfixed cells while binding of PAC-1 Fab was done with a fixation step prior to washing. Values are as in Fig. 1 but are the mean ± S.E. from 6 experiments.

Fig. 3. β3(D723>R) increases αIIbβ3 binding to PAC-1 IgM and Fab. Binding of PAC-1 IgM (10 µg/ml) was followed by washes and analysis of live unfixed cells. Binding of PAC-1 Fab (20 µg/ml) was done
with a fixation step prior to washing. Values are as in Fig. 1 but are the mean ± S.E. for 4 and 5 experiments for the IgM and Fab samples respectively. The difference between PAC-1 Fab binding to the cell lines expressing wild type β3 and β3D->R is significant (p=0.011).

**Fig. 4.** PAC-1 IgM binding to CHO cells is clustered. PAC-1 IgM bound to CHO cells expressing αIIbβ3 and GFP-talin head F2-F3 domain was fixed, labeled with fluorescent secondary antibodies and visualized by epifluorescence microscopy. *A,* maximum-intensity projection image of a cell showing clustered PAC-1 IgM on the cell surface. PAC-1 IgM fixed to a glass slide and detected with secondary antibodies show significantly less clustering (*B*).

**Fig. 5.** Thrombin activation of human platelets increases the binding of multivalent PAC-1 IgM but not monovalent PAC-1 Fab. *A,* binding of the ligands PAC-1 IgM and PAC-1 Fab, at two different concentrations each, was stopped by the addition of fixative after 10 minutes and then detected by secondary antibodies. *B,* activation of αIIbβ3 by Mn²⁺ increases binding of both forms of PAC-1. PAC-1 IgM 1X and 2X concentrations were 10 and 20 μg/ml. PAC-1 Fab 1X and 2X concentrations were 37.5 and 75 μg/ml. Values are as in Fig. 1 but are the mean ± S.E. from 3 experiments.

**Fig. 6.** RGD peptide binding increases αIIbβ3 binding to PAC-1 Fab. Platelets were incubated without peptide or with 1mM GRGDSP (RGD) or GRGESP (RGE) peptide, fixed, and then used in a standard binding assay for PAC-1 Fab. Pre-incubation with the RGD peptide resulted in increased PAC-1 Fab binding. Binding assays were done using 50 μg/ml PAC-1 Fab and the values given are the mean ± S.E. from 4 experiments. Differences between RGD and no peptide or RGD and RGE are significant (p=0.013 and p=0.0004 respectively).

**Fig. 7.** Model for αIIbβ3 activity regulation by talin. *A* (upper diagram), integrins (αIIb, green; β3 blue) are present on the surface of CHO cells or platelets in a non-clustered, bent, low affinity state. Talin (fuchsia) in the cytoplasm is inhibited from binding to integrins, due to intra- or inter-molecular interactions between its head and tail domains. Unbound multivalent ligands are shown as black lines with triangles for binding motifs. (Middle diagrams) Upon activation of platelets, talin’s inhibition is released and it binds to the cytoplasmic domains of the β3 subunit resulting in clustered integrins. This clustering may leave the integrins in the bent state or a new conformation but in either case the integrin remains in a low affinity state (represented by vertical blue rectangles). The increase in clustering results in multiple low affinity interactions between ligand and integrins. *A* (lower diagram), binding of ligand to the integrins triggers a change in integrin head domains so that they are ultimately in a high affinity state (represented by diagonal blue rectangles). *B,* in the absence of clustering, integrins interact with ligands and promote a change in affinity of individual integrins. Upon dissociation, they return to their low affinity state.
Figure 1

PAC-1 Fab Binding Curves

PAC-1 Binding (PAC-1 MFI/HPB MFI)

[50 100 150]

[PAC-1 Fab] (μg/ml)
Figure 2

A. Talin Increases Integrin αIIbβ3 Avidity Not Affinity

B. Mn²⁺ Increases Integrin αIIbβ3 Affinity

Figure 3

β3D723>R Activation of Integrin αIIbβ3
Figure 4

Figure 5

A. Platelet Activation Increases Integrin \( \alpha_{IIb}\beta_3 \) Avidity Not Affinity

B. Platelet \( \alpha_{IIb}\beta_3 \) Activation by Mn\(^{2+}\)
Figure 6

RGD Peptide Activation of Platelet αIIbβ3

<table>
<thead>
<tr>
<th>Peptide</th>
<th>PAC-1 Binding (PAC-1HIP8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
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</tr>
<tr>
<td>RGD</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>RGE</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>
Figure 7

A.

Ligand
Integrin (low affinity)
Talin (autoinhibited)

Active talin clusters integrins.
Integrins (low affinity) bind multivalent ligand.

Ligand binding activates integrins
Clustered Integrins (high affinity)

B.

Integrin (low affinity)
no ligand bound

Integrin (low affinity) ligand bound

Integrin (high affinity) ligand bound
Integrin αIIbβ3 activation in CHO cells and platelets increases clustering not affinity
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