Complementary Roles for Receptor Clustering and Conformational Change in the Adhesive and Signaling Functions of Integrin $\alpha_{IIb}\beta_3$

Takaaki Hato,§ Nisar Pampori,* and Sanford J. Shattil*‡

*Department of Vascular Biology, ‡Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California; and §Ehime University School of Medicine, Ehime, Japan

Abstract. Integrin $\alpha_{IIb}\beta_3$ mediates platelet aggregation and “outside-in” signaling. It is regulated by changes in receptor conformation and affinity and/or by lateral diffusion and receptor clustering. To document the relative contributions of conformation and clustering to $\alpha_{IIb}\beta_3$ function, $\alpha_{IIb}$ was fused at its cytoplasmic tail to one or two FKBPI2 repeats (FKBP). These modified $\alpha_{IIb}$ subunits were expressed with $\beta_3$ in CHO cells, and the heterodimers could be clustered into morphologically detectable oligomers upon addition of AP1510, a membrane-permeable, bivalent FKBP ligand. Integrin clustering by AP1510 caused binding of fibrinogen and a multivalent (but not monovalent) fibrinogen-mimetic antibody. However, ligand binding due to clustering was only 25–50% of that observed when $\alpha_{IIb}\beta_3$ affinity was increased by an activating antibody or an activating mutation. The effects of integrin clustering and affinity modulation were additive, and clustering promoted irreversible ligand binding. Clustering of $\alpha_{IIb}\beta_3$ also promoted cell adhesion to fibrinogen or von Willebrand factor, but not as effectively as affinity modulation. However, clustering was sufficient to trigger fibrinogen-independent tyrosine phosphorylation of pp72$^{Syk}$ and fibrinogen-dependent phosphorylation of pp125$^{FAK}$, even in non-adherent cells. Thus, receptor clustering and affinity modulation play complementary roles in $\alpha_{IIb}\beta_3$ function. Affinity modulation is the predominant regulator of ligand binding and cell adhesion, but clustering increases these responses further and triggers protein tyrosine phosphorylation, even in the absence of affinity modulation. Both affinity modulation and clustering may be needed for optimal function of $\alpha_{IIb}\beta_3$ in platelets.

Integrins are type I transmembrane $\alpha\beta$ heterodimers that mediate cell adhesion and signaling in a highly regulated manner (Clark and Brugge, 1995). Several modes of integrin regulation have been demonstrated or postulated, including control of expression on the cell surface by coordinate subunit biosynthesis and recycling (Bennett, 1990; Bretscher, 1992), modulation of receptor affinity by conformational changes in the heterodimer (Sims et al., 1991; Shattil et al., 1998), and modulation of receptor avidity by lateral diffusion of heterodimers to form higher order multimers or clusters (Detmers et al., 1987; van Kooyk et al., 1994; Kucik et al., 1996; Bazzoni and Hemler, 1998). The latter process may be promoted by interactions of integrins with multivalent, extracellular ligands (Peerschke, 1995b; Simmons et al., 1997), and with components of the dynamic actin cytoskeleton (Sastry and Horwitz, 1993; Fox et al., 1996; Kucik et al., 1996). Integrin function must sometimes be regulated acutely over seconds to minutes to enable the kinds of rapid changes in cell adhesion and migration that are required during immune responses, inflammation, and hemostasis. Several integrins in blood cells are targets of this type of activation or “inside-out” signaling, including $\alpha_\beta_1$, $\alpha_\beta_2$, and $\alpha_\beta_3$ in leukocytes, and $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ in platelets (Bennett et al., 1997; Bazzoni and Hemler, 1998; Shattil et al., 1998). It seems likely that some combination of conformational change and receptor clustering is involved in activating the ligand-binding function of these integrins. However, evidence to support one or the other mechanism has been largely indirect, and it has been difficult to determine the relative contributions of each in intact cells. The distinction between integrin affinity and avidity modulation is not academic because the underlying mechanisms may be different, with implications for therapeutic strategies to block or promote integrin functions in pathophysiological conditions (Coller, 1997; Bazzoni and Hemler, 1998).

One of the best-studied integrins from the standpoint of acute regulation is $\alpha_{IIb}\beta_3$, which interacts with Arg-Gly-Asp-containing ligands, such as fibrinogen and von Wille-
suggesting a functional role for binding of multivalent ligands (Huang et al., 1993), indirectly. Banfic et al., 1998; Shattil et al., 1998), seems to require the fested by activation of specific protein tyrosine kinases, lipid 

prostacyclin and nitric oxide can inhibit and, under some conditions reverse these acute changes (Graber and Ha-

wiger, 1982; Freedman et al., 1997). Coupled with evidence from fluorescence resonance energy transfer studies show-

ing that the $\alpha_{IIb}$ and $\beta_3$ subunits undergo changes in relative orientation during platelet activation (Sims et al., 1991), a current hypothesis is that ligand binding to $\alpha_{IIb}\beta_3$ is con-

trolled, at least in part, by changes in heterodimer confor-

mation that affect access of the ligand to recognition sites in the receptor (Loftus and Liddington, 1997; Shattil et al., 1998). On the other hand, it is entirely possible that clustering of $\alpha_{IIb}\beta_3$ also occurs during the process of platelet activa-

tion. Indeed, clustering of $\alpha_{IIb}\beta_3$ on the platelet surface has been detected by electron microscopy after ligand binding (Isenberg et al., 1987; Simmons et al., 1997). Were clustering to occur directly in response to platelet agonists, it could en-

hance ligand binding through chelate and rebinding effects. Furthermore, “outside-in” signaling through $\alpha_{IIb}\beta_3$, mani-

fested by activation of specific protein tyrosine kinases, lipid 

kinases, and cytoskeletal reorganization (Fox et al., 1993; Banfic et al., 1998; Shattil et al., 1998), seems to require the binding of multivalent ligands (Huang et al., 1993), indirectly suggesting a functional role for oligomerization of $\alpha_{IIb}\beta_3$.

Since platelets are not amenable to genetic manipula-

tions ex vivo, heterologous expression systems have been used to study the structure and function of $\alpha_{IIb}\beta_3$. (O’Toole et al., 1994; Loh et al., 1996). For example, human $\alpha_{IIb}\beta_3$ expressed in CHO cells binds little or no fibrinogen or PAC1 and is therefore, in a constitutively low affinity/ 

avidity state, just as it is in resting platelets. However, the affinity of $\alpha_{IIb}\beta_3$ can be increased by incubation of the cells with particular “LIBS” mAb Fab fragments that bind to the $\alpha$ or $\beta$ integrin subunit and induce a conformational change in the extracellular portion of the receptor to ex-

pose ligand binding sites (O’Toole et al., 1994). Under these experimental conditions, monovalent LIBS Fab frag-

ments by themselves would not be expected to induce re-

ceptor clustering. In the present study, we have used new modifications of this experimental system to establish the separate contributions of affinity modulation and receptor clustering to the functions of $\alpha_{IIb}\beta_3$. Specifically, single or tandem repeats of the FK506-binding protein, FKBP2 (FKBP), have been fused to the cytoplasmic tail of $\alpha_{IIb}$ to conditionally cluster heterodimers into oligomers from inside the cell using AP1510, a synthetic, bivalent, and mem-

brane-permeable FKBP dimerizer (Amara et al., 1997). The results establish that affinity modulation and receptor clustering can play complementary roles in the adhesive and signaling functions of this prototypic integrin. Whereas affinity modulation is the predominant mechanism for reg-

ulating ligand binding to $\alpha_{IIb}\beta_3$, receptor clustering facil-

itates this process and promotes outside-in signaling, even in the absence of affinity modulation.

1. Abbreviations used in this paper: ECL, enhanced chemiluminescence; FKBP, FK506 binding protein; FKBP12; vWF, von Willebrand factor.

Materials and Methods

Plasmid Constructions and Expression of Recombinant Forms of $\alpha_{IIb}\beta_3$ in CHO Cells

A pCDM8 template containing full-length $\alpha_{IIb}$ (O’Toole et al., 1994) was subjected to PCR with Pfu polymerase (Stratagene, La Jolla, CA) to place XbaI and SpeI restriction sites at the 5’ and 3’ ends of $\alpha_{IIb}$, respectively. The PCR product was cut with XbaI and SpeI and ligated into an XbaI-cut, CMV-based mammalian expression vector, pCF1E (ARIAD Pharmaceutical, Inc., Cambridge, MA). Plasmids with inserts in the correct orienta-

tion were amplified and purified for CHO cell transfections (Maxi-Prep; QIAGEN Inc., Chatsworth, CA). The resulting $\alpha_{IIb}$ (FKBP)/pCF1E plas-

mid encoded $\alpha_{IIb}$ fused in-frame to FKBP, which in turn was fused in frame to a hemagglutinin epitope tag (see Fig. 1). To construct $\alpha_{IIb}$ fused to two tandem FKBP repeats ($\alpha_{IIb}$ (FKBP)), a single FKBP was removed from pCF1E with XbaI/SpeI and ligated into Spe-cut $\alpha_{IIb}$ (FKBP)/pCF1E. The remaining $\alpha_{IIb}$ and $\beta_3$ cDNAs depicted in Fig. 1 were in pCDM8 (O’Toole et al., 1994). cDNA coding full-length human Syk was in EMCV (Gao et al., 1997). Plasmid inserts were analyzed by automated sequenc-

ing to confirm authenticity.

cDNAs were transfected into CHO-K1 cells with lipofectamine accord-

ing to the manufacturer’s instructions (GIBCO BRL, Gaithersburg, MD). Typically, 0.5–2 $\mu$g of each plasmid was used, supplemented when neces-

sary with empty vector DNA (pcDNA3; Invitrogen, San Diego, CA) for a total of 4 $\mu$g per dish. Cells were maintained for 48 h for transient expres-

sion or subjected to antibiotic selection for stable expression. Stable cell lines were selected further for high integrin expression by single cell FACS® 

sorting using an $\alpha_{IIb}\beta_3$-specific murine mAb, D57 (O’Toole et al., 1994).

Characterization of Recombinant Integrins in CHO Cells

Cell surface expression of recombinant $\alpha_{IIb}\beta_3$ was assessed by flow cytom-

etry using biotin-D57 and FITC-streptavidin (Leong et al., 1995). $\alpha_{IIb}$ ex-

pression was also evaluated by Western blotting. 48 h after transfection, the cells were lysed in 66 mM Tris-HCl, pH 7.4, containing 2% SDS and 30 $\mu$g of protein were electrophoresed in SDS–7.5% polyacrylamide gels under nonreducing conditions, transferred to nitrocellulose, and then sub-

jected to Western blotting with murine mAb D57 (O’Toole et al., 1994). cDNA coding full-length human Syk was in EMCV (O’Toole et al., 1994). cDNA coding full-length human Syk was in EMCV (Gao et al., 1997). Plasmid inserts were analyzed by automated sequenc-

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Confocal Microscopy

to establish whether API510 could induce clustering of $\alpha_{IIb}$ (FKBP)$\beta_3$ that was detectable morphologically, cells stably expressing $\alpha_{IIb}$ (FKBP)$\beta_3$ were incubated in the presence of 1.000 nM API510 (or 0.5% EtOH as a vehicle control) for 30 min at room temperature. Then, analogous to the method used by Yauch and co-workers to detect antibody-induced integ-

рин clustering (Yauch et al., 1997), the cells were incubated with 10% goat serum for 30 min at room temperature, followed by 10 $\mu$M FITC-

D57 or unlabeled D57 for 30 min on ice. After washing, the sample con-

taining unlabeled D57 was incubated for 30 min with FITC-labeled goat anti-

mouse IgG (Biosource International, Camarillo, CA), blots were developed 

for 0.1–1 min by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

Confluent Cells

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Measurements of Ligand Binding Due to Clustering and Affinity Modulation of $\alpha_{IIb}\beta_3$

Ligand binding to $\alpha_{IIb}\beta_3$ in CHO cells was assessed by flow cytometry us-

ing a saturating amount of the fibrinogen-mimetic, murine monoclonal 

IgM antibody, PAC1 (Kashiwagi et al., 1997). CHO cells were resus-

pended to 10$^5$ cells/ml in Tyrode’s buffer supplemented with 2 mM CaCl$_2$ and MgCl$_2$ (O’Toole et al., 1994). For most experiments, 4 × 10$^5$ cells were added to tubes containing a final concentration of 0.4% PAC1 ascites or 40 nM purified PAC1 in a final vol of 50 $\mu$L, and then incubated for 30 min
at room temperature. In some experiments, nonrecombinant PAC1 Fab produced in insect cells and purified by nickel-agarose chromatography was used instead of PAC1 IgM at a final concentration of 30 nM (Abrams et al., 1994). As indicated for each experiment, cell incubations were also carried out in the presence of one or more of the following reagents: 10–50,000 nM AP1510 (or vehicle buffer) to cluster αIIb(FKBP)β3 or αIIb(αFKBP)β3 (Amara et al., 1997), 150 μg/ml anti-LIBS6 Fab to convert αIIbβ3 into a high affinity form through conformational changes (Du et al., 1993; Kashiwagi et al., 1997), and 10 μM integrin, an αIIbβ3 antagonist to specifically block PAC1 binding (Scarborough et al., 1993). Preliminary experiments with AP1510 and anti-LIBS6 Fab indicated that a 10–30-min incubation of cells with these reagents was sufficient to achieve their maximum effects. Cells were then washed and incubated on ice for 30 min with biotin-D57, followed by phycocyanin-streptavidin and either FITC-labeled goat anti-mouse Ig μ heavy chain antibody (to label PAC1 IgM) or FITC-labeled goat anti-mouse heavy and light chain antibody (to label PAC1 Fab) (both from Biosource International). Samples were diluted with 0.5 ml Tyrode’s buffer containing 2 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO) and analyzed on a FACSCalibur® flow cytometer (Becton Dickinson Co., Mountain View, CA). After electronic compensation, PAC1 binding (FL1 channel) was analyzed on the gated subset of live cells (propidium iodide–negative, FL3) that was positive for αIIbβ3 expression (FL2). To control for variations in integrin expression from transfection to transfection, PAC1 binding, measured as mean fluorescence intensity in arbitrary units, was expressed relative to the levels of αIIbβ3, measured simultaneously with biotin-D57.

Adhesion of CHO Cells to Fibrinogen and vWF

Immuno-2 microtiter wells (Dynex Laboratories, Chantilly, VA) were coated with purified fibrinogen (Enzyme Research Laboratories, South Bend, IN) or vWF (Ruggeri et al., 1983) overnight at 4°C. Immobilized fibrinogen or vWF was blocked with 20 mg/ml BSA. CHO cells stably expressing BSA. CHO cells stably expressing

Protein Tyrosine Phosphorylation in CHO Cells

Stable cell lines expressing αIIb(FKBP)β3 were transiently transfected with EMCV-Syk and placed into complete DMEM with 10% FBS. 24 h after transfection, the amount of serum was reduced to 0.5%, and 48 h after transfection, the cells were resuspended to 3 x 10^6/ml in DMEM and slowly rotated at 37°C for 45 min in the presence of 20 μM cycloheximide. Then cells were incubated for 10 min with one or more of the following: 1,000 nM AP1510 to stimulate receptor clustering, 150 μg/ml anti-LIBS6 Fab to increase integrin affinity, or 250 μg/ml fibrinogen to achieve ligand binding. As a positive tyrosine phosphorylation control, one aliquot of cells was allowed to attach for 60 min to a dish coated with αIIbβ3 antibody D57. Cells were lysed in RIPA buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 10 mM Tris, pH 7.4, 1 mM Na3EGTA, 0.5 mM leupeptin, 0.25 mg/ml Pefabloc, 5 μg/ml aprotinin, 20 mM NaF, 3 mM β-glycerophosphate, 10 mM sodium pyrophosphate, and 5 mM sodium vanadate. After clarification, 200 μg of protein were immunoprecipitated with rabbit antisera specific for Syk or FAK (Gao et al., 1997). Immunoprecipitates were subjected to Western blotting with anti-photophosphorylase mAbs, 4G10, and PY20 (Upstate Biotechnology Inc., Lake Placid, NY and Transduction Laboratories, Lexington, KY, respectively), followed by stripping and reprobing with mAb 4D10 to Syk or antisera to FAK (Gao et al., 1997). Immunoreactive bands detected by ECL were quantitated by calibrated densitometry using a flatbed scanner, Power Center Pro 240 computer, and NIH Image software.

Results

Heterologous Expression of αIIbβ3 Containing Dimerization Motifs

The purpose of these studies was to evaluate the possible contributions of integrin clustering and affinity modula-
be detected morphologically, CHO cells stably expressing this integrin were incubated for 30 min at room temperature with 1,000 nM AP1510 (or vehicle buffer as a control), stained with FITC-D57 on ice, fixed, and then examined by confocal microscopy. D57 staining was entirely surface associated, and cells that had been treated with buffer instead of AP1510 exhibited a finely patchy distribution of \( \alpha_{IIb}(FKBP)_{3} \beta_{3} \) (Fig. 3, A–C). In contrast, \( \alpha_{IIb}(FKBP)_{3} \beta_{3} \) in cells treated with AP1510 exhibited a coarse patchiness (Fig. 3, D–G), similar to that observed when the D57 antibody was deliberately cross-linked with a secondary antibody before cell fixation (Fig. 3, H). The same results with AP1510 were obtained with another independent \( \alpha_{IIb}(FKBP)_{3} \beta_{3} \) clone; in contrast, AP1510 caused no discernible clustering of wild-type \( \alpha_{IIb}\beta_{3} \) in CHO cells (not shown). These results are consistent with the conclusion that oligomerization of \( \alpha_{IIb}(FKBP)_{3} \beta_{3} \) can be induced conditionally from within the cell using AP1510, enabling us to conduct a detailed study of the functional consequences of integrin clustering.

**Receptor Clustering in the Regulation of Ligand Binding to \( \alpha_{IIb}\beta_{3} \)**

Activation of \( \alpha_{IIb}\beta_{3} \) is required for the binding of soluble, macromolecular Arg-Gly-Asp–containing ligands, such as fibronogen, vWF, and fibrinogen-mimetic antibodies, such as PAC1. To evaluate the contribution of clustering to \( \alpha_{IIb}\beta_{3} \) activation, flow cytometry was used to quantitate the specific binding of PAC1 to transiently transfected CHO cells. Specific binding was defined as that inhabitable by 10 \( \mu \)M integrilin, an \( \alpha_{IIb}\beta_{3} \)-selective antagonist, and it was expressed relative to the amount of \( \alpha_{IIb}\beta_{3} \) on the cell surface, determined simultaneously with antibody D57. In cells expressing \( \alpha_{IIb}(FKBP)_{3} \beta_{3} \), there was little binding of PAC1, indicating that, like \( \alpha_{IIb}\beta_{3} \), this integrin is in a constitutive low affinity/avidity state. AP1510 caused a dose-dependent increase in PAC1 binding to \( \alpha_{IIb}(FKBP)_{3} \beta_{3} \) cells (Fig. 4, closed circles), without affecting the levels of surface expression of this integrin. However, PAC1 binding due to AP1510 appeared modest compared with binding in response to upregulation of integrin affinity by an activating antibody Fab fragment, anti-LIBS6 Fab (Fig. 4, open circles).

To evaluate possible mechanistic differences between integrin clustering and affinity modulation in the control of ligand binding, additional experiments were performed with cells expressing \( \alpha_{IIb}(FKBP)_{3} \beta_{3} \). First, we considered the possibility that AP1510 caused PAC1 binding by increasing integrin affinity rather than (or in addition to) avidity. However, AP1510 failed to stimulate the binding of a monovalent PAC1 Fab fragment to \( \alpha_{IIb}(FKBP)_{3} \beta_{3} \), although this form of PAC1 bound normally in response to anti-LIBS6 Fab (Fig. 5). Since a monovalent ligand might be expected to be sensitive to affinity modulation but less sensitive than a multivalent ligand to avidity modulation, this result suggests that AP1510 was indeed working by clustering the integrin. Second, PAC1 binding in response to AP1510 was completely prevented if the cells were preincubated for 30 min with 4 mg/ml of 2-deoxy-D-glucose and 0.2% sodium azide to deplete metabolic ATP (two separate experiments). Since oligomerization by AP1510 should be energy independent, this suggests that metabolic energy is needed to maintain the receptor in a proper conformation, even when ligand binding is triggered by receptor clustering. Third, the effect of a specific point mutation (\( \beta_{3}(S752P) \)) or a truncation (\( \beta_{3}(A724) \)) of the \( \beta_{3} \) cytoplasmic tail were studied because both have been shown to...
transiently transfected with either high affinity PAC1 binding to wild-type 48 h later. Whereas AP1510 had no effect on mutant [O'Toole et al., 1994], and ligand binding was evaluated (1,000 nM and 150 μg/ml, respectively). CHO cells were transiently transfected with either αIIbβ3, αIIb(FKBP)β3, or αIIbααβ3 (a constitutive, high affinity mutant [O'Toole et al., 1994]), and ligand binding was evaluated 48 h later. Whereas AP1510 had no effect on PAC1 binding to wild-type αIIbβ3, it increased binding to both αIIb(FKBP)β3 and αIIb(FKBP)β3 such that specific PAC1 binding was increased approximately twofold (P < 0.001) (Fig. 7). However, PAC1 binding induced by AP1510 amounted to only 50% of the binding observed with the high affinity αIIb/ααβ3 chimera, and only 25% of the binding induced by anti–LIBS6 Fab (Fig. 7). Nevertheless, the PAC1 binding caused by clustering was statistically significant (P < 0.03) and approximately additive to the binding caused by affinity modulation (Fig. 7).

Fibrinogen and PAC1 binding to activated platelets is initially reversible by the addition of EDTA, but binding becomes progressively irreversible over 15–60 min (Peerschke, 1995a; Fox et al., 1996). In CHO cells that expressed αIIb(FKBP)β3 and were treated with both anti–LIBS6 Fab and AP1510 to achieve maximal PAC1 binding, the added component of ligand binding resulting from AP1510 was fully reversible at 10 min but irreversible at 30 min (Fig. 8). Similar results were obtained when FITC-fibrinogen was used instead of PAC1 to monitor ligand binding (not shown). This series of experiments demonstrates that affinity modulation is the predominant regulator of ligand binding to αIIbβ3. However, receptor clustering plays an additive role in promoting eventual irreversible binding of the ligand.

αIIbβ3 Clustering in the Regulation of Cell Adhesion

Activation of platelets by agonists leads to increased cell adhesion to the αIIbβ3 ligands, fibrinogen, and vWF (Savage et al., 1992). To determine the relative contributions of αIIbβ3 clustering and affinity modulation to cell adhesion, CHO cells that stably expressed αIIb(FKBP)β3 were loaded with BCECF as a fluorescent marker and incubated for 90 min in microtiter wells coated with fibrinogen or vWF. Cell adhesion was dependent on the coating concentration of fibrinogen (Fig. 9, left panel) and vWF (Fig. 9, right panel), as well as on the presence of αIIb(FKBP)β3, since it was blocked by 10 μM integrin. AP1510 (1,000 nM) increased the extent of cell adhesion, but only very modestly and only at the higher coating concentrations of fibrinogen and vWF. On the other hand, increasing integrin affinity with anti–LIBS6 Fab (150 μg/ml) caused a more marked increase in cell adhesion, even at the lower ligand concentrations (Fig. 9, left and right panels). Thus under these assay conditions, receptor clustering is not as effective as affinity modulation in regulating cell adhesion via αIIbβ3.

αIIbβ3 Clustering in the Regulation of Outside-In Signaling

In platelets and CHO cell transfectants, fibrinogen binding to αIIbβ3 leads to tyrosine phosphorylation and activation.
of Syk and FAK. The binding of soluble fibrinogen is sufficient to activate Syk, but additional post–ligand binding events, such as cytoskeletal reorganization, are necessary for activation of FAK (Huang et al., 1993; Clark et al., 1994; Gao et al., 1997). To study the role of integrin clustering in these events, CHO cells stably expressing αIIb(FKBP)2β3 were transiently transfected with human Syk, and tyrosine phosphorylation of Syk and endogenous hamster FAK was examined. Fig. 10 A shows the raw data for a single experiment and Fig. 10 B shows a summary of three separate experiments. Cells maintained in suspension for 10 min in the absence or presence of fibrinogen exhibited a low level of tyrosine phosphorylation of Syk and FAK. However, addition of 1,000 nM AP1510 caused an average 2.8-fold increase in tyrosine phosphorylation of Syk, even in the absence of fibrinogen (P < 0.05), and this response was greater still in the presence of fibrinogen (5.4-fold; P < 0.02). In contrast, in the absence of fibrinogen integrin clustering by AP1510 did not stimulate an increase in FAK tyrosine phosphorylation, but increased FAK phosphorylation was observed in the presence of fibrinogen (3.5-fold; P < 0.001). Thus, integrin clustering can trigger tyrosine phosphorylation of Syk even in the absence of fibrinogen binding, whereas phosphorylation of FAK requires both receptor clustering and fibrinogen binding. Affinity modulation by anti-LIBS6 is not necessary in either case.

**Discussion**

In this study, engraftment of one or two FKBP repeats onto the COOH terminus of the αIIb subunit enabled us to cluster integrin αIIbβ3 in a conditional fashion by treating CHO cells with a synthetic, bivalent FKBP ligand, AP1510. This permitted us for the first time to conduct a detailed comparison of the functional effects of receptor clustering, initiated from within the cell, with the effects of increasing integrin affinity through conformational changes. The major conclusions of this work are: (a) Conformational changes play a predominant role in αIIbβ3 activation in CHO cells, as monitored by ligand binding and cell adhesion assays. (b) Clustering causes a modest increment in reversible and ultimately irreversible binding of multivalent ligands to αIIbβ3, and this binding is additive to that caused by affinity modulation. (c) Ligand binding resulting from receptor clustering is dependent on cellular metabolic energy and is sensitive to some, but not all, of the mutations or deletions in the β3 cytoplasmic tail that block affinity modulation of the receptor. (d) Integrin clustering promotes ligand-independent tyrosine phosphorylation of Syk, and ligand-dependent phosphorylation of FAK, even when cells are maintained in suspension and even in the absence of deliberate affinity modulation. Thus, by being able to manipulate integrin clustering and affinity separately and in a con-
trolled manner, we conclude that these two processes play complementary roles in the function of $\alpha_{IIb}\beta_3$.

**Integrin Clustering and Inside-Out Signaling**

Inside-out signaling is responsible for acute regulation of the ligand binding function of integrins. In the case of integrins that normally engage soluble adhesive ligands in vivo, such as $\alpha_{IIb}\beta_3$, inside-out signaling can be monitored directly using labeled ligands or ligand-mimetic antibodies, such as PAC1. Alternatively, it can be assessed by cell adhesion assays. Although physiologically relevant, cell adhesion is a more indirect measure of integrin activation because it can be influenced by factors, such as actin polymerization, cell spreading, and focal adhesion turnover, that may affect the overall strength of the adhesion process through mechanisms other than regulation of ligand binding (Burridge and Chrzanowska-Wodnicka, 1996; Yamada and Geiger, 1997; Hall, 1998). Thus, when possible, it is preferable to monitor inside-out signaling by ligand binding assays, as in the current study.

Ligand binding to integrins is thought to be regulated by a combination of affinity and avidity modulation (van Kooyk and Figdor, 1993; Diamond and Springer, 1994; Bazzoni and Hemler, 1998). In the case of $\alpha_{IIb}\beta_3$, platelet activation is believed to cause a modification of the conformation or orientation of the integrin cytoplasmic tails that is transmitted across the membrane, leading to increased access of the ligand to binding sites in the receptor (Loftus and Liddington, 1997; Shattil et al., 1998). How-

**Figure 6.** Effects of $\beta_3$ cytoplasmic tail mutations on PAC1 binding caused by receptor clustering and affinity modulation. CHO cells were transiently transfected with the indicated $\alpha_{IIb}$ and $\beta_3$ subunits. 48 h later, they were incubated for 30 min with PAC1 along with AP1510 and/or 150 $\mu$g/ml anti–LIBS6 Fab, and specific PAC1 binding was quantitated by flow cytometry. Note the almost 10-fold difference in scales of the $y$ axes. Data represent the means ± SEM of three experiments.

**Figure 7.** Relative effects of receptor clustering and affinity modulation on PAC1 binding to $\alpha_{IIb}\beta_3$. CHO cells were transiently transfected with the indicated integrin constructs. 48 h later, they were incubated for 30 min with PAC1 along with AP1510 and/or 150 $\mu$g/ml anti–LIBS6 Fab, and specific PAC1 binding was quantitated by flow cytometry. Note the difference in scales of the $y$ axes. Data represent the means ± SEM of three to five experiments.
ever, cell activation appears to promote ligand binding to certain \( \alpha_1b \) and \( \alpha_2b \) integrins by also stimulating the lateral diffusion and clustering of these receptors (van Kooyk and Figdor, 1993; Kucik et al., 1996; Bazzoni and Hemler, 1998; Shattil et al., 1998), and the same might be true for \( \alpha_{IIb}b_3 \). Several experimental approaches have been used to cluster integrins, including treatment of cells with multivalent antibodies or chemical cross-linkers, incubation of cells with ligand-coated beads, and promotion of cell spreading (Kornberg et al., 1991; Dorahy et al., 1995; Hotchin and Hall, 1995; Miyamoto et al., 1995). Whereas each of these has provided important information about outside-in signaling, none is entirely suitable for studies of soluble ligand binding to \( \alpha_{IIb}b_3 \). The use here of AP1510 to cluster \( \alpha_{IIb}(FKBP)b_3 \) or \( \alpha_{IIb}(FKBP)b_3 \), while CHO cells were maintained in suspension demonstrates unambiguously that affinity and avidity modulation can complement one another with respect to the control of ligand binding. Given the wide variety of soluble, matrix- and cell-associated ligands that integrins must contend with, it is likely that the relative contributions of affinity and avidity modulation will vary with the integrin and the cell type.

Fortunately, the fusion of single or tandem FKBP repeats to the \( \alpha_{IIb} \) cytoplasmic tail did not interfere with \( \alpha_{IIb}b_3 \) expression or function in CHO cells. Perhaps this means that the very COOH terminus of the \( \alpha \) subunit is dispensable for the integrin functions that were assessed. On the other hand, direct attachment of FKBP to the \( b_3 \) tail interferes with energy-dependent affinity modulation of \( \alpha_{IIb}b_3 \), possibly by disrupting necessary interactions of the \( b_3 \) tail with regulatory proteins (Hato, T., and Shattil, S.J., unpublished observations). We ascribe any functional effects of AP1510 on \( \alpha_{IIb}(FKBP)b_3 \) and \( \alpha_{IIb}(FKBP)b_3 \) to receptor clustering. Although the evidence for this is strong, it is largely indirect. First, AP1510 only affected those forms of \( \alpha_{IIb}b_3 \) that contained FKBP repeats (Figs. 6 and 7). Second, confocal microscopy showed that AP1510 treatment was associated with the appearance of coarse patches of integrin staining in the surface membrane (Fig. 3). Finally, AP1510 caused binding of a multivalent but not monovalent form of PAC1, precisely what might be expected in

Figure 8. Effect of receptor clustering on reversible and irreversible ligand binding to \( \alpha_{IIb}(FKBP)b_3 \). Binding of PAC1 in response to anti–LIBS6 Fab was initiated in CHO cells stably-expressing \( \alpha_{IIb}(FKBP)b_3 \), either in the absence or presence of AP1510. After 10 or 30 min, half of each sample was treated with 5 mM EDTA to displace reversibly bound PAC1 and half was not. Then specific PAC1 binding was determined. Reversible PAC1 binding was defined as specific binding displaced by EDTA, and irreversible binding was defined as specific binding that was not displaced by EDTA. Data represent the means ± SEM of three experiments.

Figure 9. Relative effects of receptor clustering and affinity modulation on CHO cell adhesion to fibrinogen or vWF. As described in Materials and Methods, CHO cells stably expressing \( \alpha_{IIb}(FKBP)b_3 \) were fluorescently labeled with BCECF, and then incubated for 90 min in microtiter wells coated with fibrinogen (left panel) or vWF (right panel) in the presence of 1,000 nM AP1510 and/or 150 \( \mu \)g/ml anti–LIBS6 Fab. After washing, cell adhesion was quantitated by cytofluorimetry. Adhesion was expressed as a percentage of total cells added. This experiment is representative of three so performed. Not shown is the fact that in the absence of AP1510, the adhesion of \( \alpha_{IIb}(FKBP)b_3 \) cells was the same as for cells expressing wild-type \( \alpha_{IIb}b_3 \).
the case of integrin clustering (Fig. 5). Interestingly, the effect of AP1510 on PAC1 binding to αIIbβ3 and αIIbβ3 was nearly equivalent (Fig. 7). Assuming that AP1510 can only dimerize αIIbβ3, this suggests that formation of a dimer of dimers, e.g., (αIIbβ3)2, may be sufficient to initiate some ligand binding to αIIbβ3.

What are the biological implications of αIIbβ3 clustering during inside-out signaling? Ligand binding resulting from clustering of αIIbβ3 required a normal β3 cytoplasmic tail since a tail point mutation (S752P) disrupted this process (Fig. 6). This same mutation disrupts energy-dependent affinity modulation of αIIbβ3 in CHO cells and in human platelets, where it is responsible for a bleeding diathesis (Chen et al., 1992; Fox and Simons, 1992). In contrast, another β3 cytoplasmic modification, a truncation starting at residue Arg 724, did not disrupt ligand binding induced by clustering of αIIbβ3, although it does abolish affinity modulation of αIIbβ3 in CHO cells and platelets (O’Toole et al., 1994; Wang et al., 1997). This suggests that there are differences in the structural elements of the β3 tail that are needed for affinity and avidity modulation. A growing number of proteins have been shown to interact directly with the cytoplasmic tails of α or β integrin subunits, at least in vitro, and overexpression of some of them, including calreticulin (α tails) (Coppolino et al., 1997), cytohesin-1 (β3 tail) (Kolanus et al., 1996), and β3-endonexin (β3 tail) (Kashiwagi et al., 1997) can affect ligand binding and cell adhesion. When overexpressed in CHO cells, several other signaling proteins, including H-ras (Hughes et al., 1996), R-ras (Zhang et al., 1996), and CD98 (Fenczik et al., 1997) have been shown to modulate the ligand binding properties of αIIbβ3; however, it is not known if any of these proteins interact directly with the integrin. The relative effects of potential regulatory molecules such as these on receptor clustering and receptor affinity remain to be determined.

The extent of PAC1 binding observed in response to integrin clustering was only a fraction of that observed with affinity modulation. Yet ligand binding resulting from clustering and affinity modulation was additive (Fig. 7). Clustering also facilitated CHO cell adhesion to immobilized fibrinogen and vWF, but once again, this effect was relatively minor compared with the effect of affinity modulation and it was apparent only at the higher coating concentrations of the ligands (Fig. 9). On the other hand, if clustering of αIIbβ3 were to cause increased ligand binding in platelets as it does in CHO cells, it could affect the ultimate size of a platelet aggregate and hence the delicate balance between adequate and inadequate hemostasis or the difference between partial and total arterial occlusion by a platelet-rich thrombus. Conceivably, the effects of integrin clustering on ligand binding may be even more pronounced in platelets than in the CHO cell model system because receptor density may be higher in platelets and clustering may be stimulated by agonists that trigger integrin interactions with multivalent, polymerizing ligands on both sides of the plasma membrane (Hartwig, 1992; Fox et al., 1996; Simmons and Albrecht, 1996). Furthermore,
Integrin Clustering and Outside-In Signaling

A potential limitation of the chemical dimerization approach used here is that it may not reflect or trigger the types of interactions between αIIbβ3, cytoskeletal proteins, and signaling molecules that take place normally during outside-in signaling. For example, in platelets, the binding of fibrinogen to αIIbβ3 (Kaminski et al., 1997), these results suggest that the binding of multivalent fibrinogen to αIIbβ3 is sufficient to trigger tyrosine phosphorylation and activation of Syk, whereas tyrosine phosphorylation of FAK requires additional post–ligand binding events that occur during platelet aggregation or spreading (Haimovich et al., 1995; Huang et al., 1995). In this regard, clustering of αIIbβ3(FKBP)2 in CHO cells by AP1510 caused significant tyrosine phosphorylation of Syk, even when the cells were maintained in suspension without fibrinogen (Fig. 10). Since integrin-dependent tyrosine phosphorylation of Syk correlates with induction of Syk kinase activity in both platelets and CHO cells (Clark et al., 1994; Gao et al., 1997), these results suggest that the binding of multivalent fibrinogen to αIIbβ3 triggers Syk activation, at least in part, by inducing integrin clustering.

In contrast to the results for Syk, clustering of αIIbβ3(FKBP)2 by AP1510 was not sufficient to cause tyrosine phosphorylation of FAK in cells maintained in suspension. However, fibrinogen binding together with receptor clustering were sufficient to induce the response (Fig. 10). These results highlight the apparent differences in coupling mechanisms between αIIbβ3 and Syk and αIIbβ3 and FAK (Gao et al., 1997). At the same time, they demonstrate unambiguously that conditional clustering of αIIbβ3 in CHO cells can recapitulate a pattern of outside-in signaling that is characteristic of platelets. In nucleated cells, integrin and growth factor signaling pathways collaborate to regulate gene expression, cell adhesion, and motility (Schwartz et al., 1995; Juliano, 1996; Sastry and Horwitz, 1996; Yamada and Geiger, 1997). One hallmark of integrated signaling networks is tight control of enzyme activity and protein subcellular localization though regulated protein–protein interactions (Pawson and Scott, 1997). Chemical inducers of dimerization can be used to promote controlled homodimerization and heterodimerization of proteins in vivo as well as ex vivo (Rivera et al., 1996; Spencer et al., 1996; Clackson, 1997; Yang et al., 1998). Consequently, they should prove useful in evaluating diverse aspects of integrin signaling.

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