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Affinity modulation of the $\alpha_{IIb}\beta_3$ integrin (platelet GPIIb-IIIa) is an intrinsic property of the receptor*

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To analyze the basis of affinity modulation of integrin function, we studied cloned stable Chinese hamster ovary cell lines expressing recombinant integrins of the β_3 family ($\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$). Antigenic and peptide recognition specificities of the recombinant receptors resembled those of the native receptors found in platelets or endothelial cells. The $\alpha_{IIb}\beta_3$-expressing cell line (A5) bound RGD peptides and immobilized fibrinogen (Fg) but not soluble fibrinogen or the activation-specific monoclonal anti-$\alpha_{IIb}\beta_3$ (PAC1), indicating that it was in the affinity state found on resting platelets. Several platelet agonists failed to alter the affinity state of ("activate") recombinant $\alpha_{IIb}\beta_3$. The binding of soluble Fg and PAC1, however, was stimulated in both platelets and A5 cells by addition of IgG papain-digestion products (Fab) fragments of certain β_3-specific monoclonal antibodies. These antibodies stimulated PAC1 binding to platelets fixed under conditions rendering them unresponsive to other agonists. Addition of these antibodies to detergent-solubilized $\alpha_{IIb}\beta_3$ also stimulated specific Fg binding. These data demonstrate that certain anti-β_3 antibodies activate $\alpha_{IIb}\beta_3$ by acting directly on the receptor, possibly by altering its conformation. Furthermore, they indicate that the activation state of $\alpha_{IIb}\beta_3$ is a property of the receptor itself rather than of the surrounding cell membrane microenvironment.

Introduction

Integrins comprise a family of α, β heterodimeric cell-surface receptors involved in a wide variety of cell-cell and cell-substratum interactions (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Ginsberg *et al.,* 1988). Because of the vital role integrins play in cellular processes, the regulation of integrin function has important biological consequences. One way of modulating integrin function is to regulate its affinity for macromolecules. In particular, some members of the β_2 and β_3 subfamilies require activation to manifest full competence (Bennett and Vilaire, 1979; Marguerie *et al.,* 1979; Wright and Meyer, 1986; Detmers *et al.,* 1987) to bind ligands. The major platelet integrin, $\alpha_{IIb}\beta_3$, plays a central role in platelet aggregation (Bennett and Vilaire, 1979; Peerschke and Zucker, 1981). In resting platelets this receptor avidly binds RGD-containing peptides (Pytela *et al.,* 1986; Lam *et al.,* 1987; Parise *et al.,* 1987; Frelinger *et al.,* 1988) but not soluble macromolecular ligands such as fibrinogen (Fg) (Marguerie *et al.,* 1979) or the PAC1 monoclonal antibody (Shattil *et al.,* 1985). Only when stimulated with agonists such as ADP, thrombin, epinephrine, or phorbol esters will platelets bind Fg or PAC1 from solution with high affinity.

The change in platelets responsible for activation of $\alpha_{IIb}\beta_3$ is not fully understood, but morphologic (Loftus and Albrecht, 1984) and immunochemical analysis (Shattil *et al.,* 1985; Coller, 1986) has suggested that activation results in increased access of macromolecules to a ligand-binding pocket. The limited access of macromolecules to the binding pocket in resting platelets could then be due either to the cellular microenvironment (Loftus and Albrecht, 1984; Coller, 1986) or the conformation of the receptor itself (Shattil and Brass, 1987).

In this study we have examined the ligand-binding function of recombinant platelet $\alpha_{IIb}\beta_3$ expressed on a different cellular background and found that it also must be "activated" to avidly bind fluid-phase macromolecular ligands. Activation was achieved with certain monoclonal antibodies reactive with β_3. These antibodies activated the receptor even in fixed cells, which presumably lack physiologic signal transduction mechanisms. Moreover, these antibodies also activated the solubilized receptor, thus

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Table 1. Reactivity of VNRC3 ($\alpha_V\beta_3$) and A5 ($\alpha_{IIb}\beta_3$) CHO transfectants with monoclonal antibodies

Antibody (specificity)	Cell type		
	A5	VNRC3	CHO
Tab (α_{IIb})	265.3	5.2	4.5
Ab15 (β_3)	280.7	29.9	4.4
10E5 ($\alpha_{IIb}\beta_3$)	269.5	4.1	4.9
LM142 (α_V)	7.0	33.1	4.5
None	6.2	4.3	4.5

Stable cloned CHO cell lines transfected with the β_3 and α_V or α_{IIb} subunits were stained with the indicated antibodies. Results are expressed as mean fluorescence intensity in arbitrary units. The VNRC3 cell line expressed ~1/9 of the β_3 subunit that A5 does.

precluding a critical role for the membrane microenvironment. The data show that the affinity modulation of $\alpha_{IIb}\beta_3$ is not a unique property of platelets, but rather a property of the receptor, and that activation need not involve the cellular microenvironment.

Results

Characterization of human β_3 integrins expressed in Chinese hamster ovary (CHO) cells

Cloned CHO cell lines expressing human β_3 integrins were established as described in Methods. These cell lines expressed surface antigenic determinants appropriate for the transfected integrin as indicated by fluorescence-activated cell sorting (FACS) (Table 1). The A5 line was strongly positive for α_{IIb} (Tab), $\alpha_{IIb}\beta_3$ (4F10), and β_3 (Ab15) but not for α_V (LM142). For comparison the VNRC3 line which expresses $\alpha_V\beta_3$ was strongly positive with LM142, and Ab15, but negative with 4F10 and Tab. α_{IIb} subunits from A5 cells and α_V from VNRC3 cells were immunoprecipitable with an anti human β_3 antibody indicating that they were both complexed with the common transfected β_3 subunit (Figure 1).

We next examined the capacity of A5 cells bearing recombinant $\alpha_{IIb}\beta_3$ to bind to immobilized Fg. As shown in Figure 2, the $\alpha_{IIb}\beta_3$-expressing (A5) cell line adhered to Fg-coated microspheres, and adherence was inhibited with 2 mM GRGDSP peptide. In contrast, the parent CHO cell line failed to adhere. Thus, as in resting platelets (Coller, 1980), recombinant $\alpha_{IIb}\beta_3$ mediates cell binding to immobilized Fg.

The solubilized recombinant β_3 integrins bound synthetic RGD peptide ligands as assessed by affinity chromatography (Figure 1). We previously observed that an Fg peptide containing the sequence LGGAKQAGDV (L10) will elute $\alpha_{IIb}\beta_3$ but not $\alpha_V\beta_3$ from an RGD affinity column (Lam *et al.*, 1987), suggesting differential peptide recognition by the β_3 integrins. A quantitative comparison of β_3 integrin binding to L10 and RGD peptides was effected by use of the anti-LIBS1 antibody (Figure 3). This monoclonal binds preferentially the occupied (as opposed to the unoccupied) form of the receptor (Frelinger *et al.*, 1990). The order of peptide

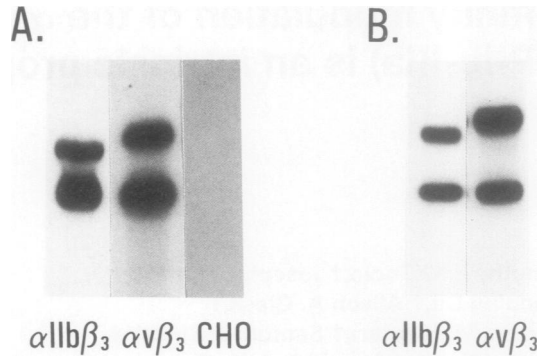

Figure 1. **Analysis of integrin subunits in stable transfectants.** (A) Detergent lysate from surface iodinated transfectants was immunoprecipitated with an anti-β_3 (Mab15), and the resulting products were analyzed by SDS-PAGE (nonreducing 7% gels) followed by autoradiography. The transfectant or cell type is listed below each panel. (B) Detergent lysate from surface iodinated transfectants was applied to GRGDSP sepharose columns. After the unbound proteins were washed with column buffer, bound proteins were eluted with GRGDSP (1.7 mM). Proteins in eluted fractions were analyzed by electrophoresis on 7% acrylamide gels under denaturing conditions and detected by autoradiography. The transfected type is listed below each panel.

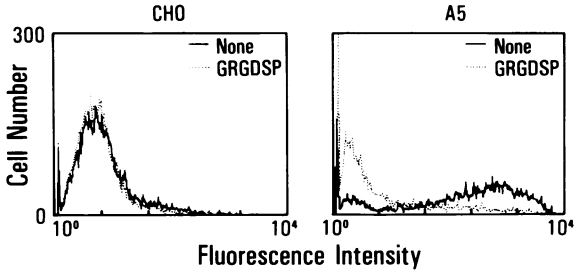

Figure 2. **Recombinant $\alpha_{IIb}\beta_3$ binds insoluble fibrinogen.** Fibrinogen-coated fluorescent beads (25 μl) were allowed to bind to CHO or A5 cells (5×10^5 cells) by incubation at room temperature for 30 min. After centrifugation in a 6% BSA solution, the pelleted cells were resuspended and subjected to FACS analysis. The solid line denotes no added peptide, whereas the dotted line denotes the presence of 2 mM GRGDSP. Although no binding was observed in CHO cells, RGD-inhibitable binding was observed with A5 cells.

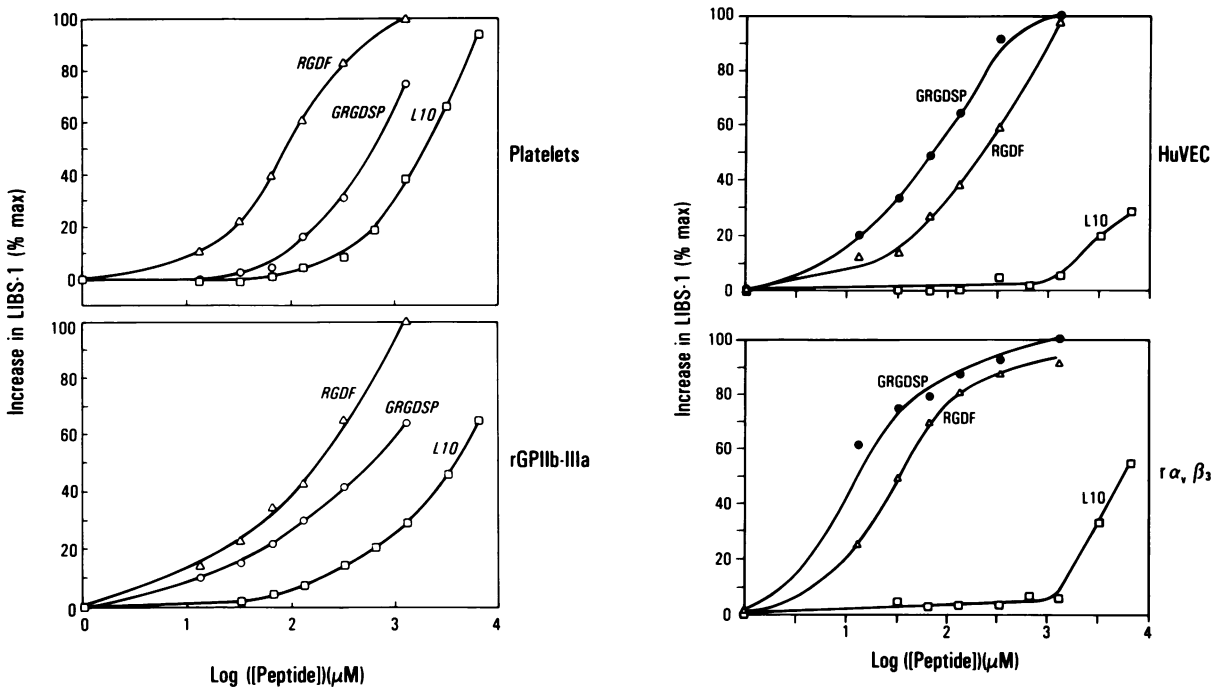

Figure 3. **Analysis of integrin peptide-binding specificities.** The binding of native or recombinant $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ to the peptides GRGDSP, RGDF, and LGGAKQAGDV (L10) was analyzed by the ability of the peptides to up-regulate anti-LIBS1 binding, as described in Methods. The increase in anti-LIBS1 binding is plotted versus increasing doses of the three peptides. The cell or transfectant type is listed to the right of each panel.

potency in stimulating anti-LIBS1 binding to platelets was RGDF > GRGDSP > L10, which is identical to their order of potency in inhibiting Fg binding (Lam *et al.*, 1987; Cheresh *et al.*, 1989). The recombinant $\alpha_{IIb}\beta_3$ manifested a similar order of peptide potency. In endothelial cells, the order of potency was GRGDSP > RGDF > L10. Thus, in platelets, GRGDSP was 5-fold more active than L10, whereas in endothelial cells it was 1000-fold more active. The recombinant $\alpha_v\beta_3$ manifested an identical order of peptide potency to the endothelial cells. Thus, the recombinant $\alpha_{IIb}\beta_3$ is functionally similar to the platelet molecule with respect to both immobilized macromolecular and peptide ligand-recognition specificity.

Recombinant $\alpha_{IIb}\beta_3$ is expressed in an "inactive" state

Platelet $\alpha_{IIb}\beta_3$ requires cellular activation to bind fluid-phase macromolecular ligands, such as Fg, with high affinity (Coller, 1986; Shattil and Brass, 1987). To determine whether the recombinant $\alpha_{IIb}\beta_3$ was activated, we used the monoclonal anti-$\alpha_{IIb}\beta_3$, PAC1, which is reactive with activated but not resting platelets (Shattil *et al.*, 1985). PAC1 did not react with $\alpha_{IIb}\beta_3$ in the A5 cell line or resting platelets, although the receptor was present, as evidenced by staining with the $\alpha_{IIb}\beta_3$ complex–specific antibody 4F10 (Figure 4). In contrast, ADP-activated platelets stained brightly with PAC1. Platelet agonists such as ADP (10–50 μM), thrombin (1–5 U/ml), epinephrine (4–200 μM), phorbol myristate acetate (PMA, 5–500 nM), and ionomycin (5–200 nM) failed to stimulate PAC1 binding to A5 cells (not shown). Three possible explanations for this result are 1) the recombinant $\alpha_{IIb}\beta_3$ lacks the PAC1 epitope, 2) the recombinant $\alpha_{IIb}\beta_3$ cannot be activated, or 3) the CHO cells lack receptors and/or transduction systems required for agonist-mediated activation.

To address these possibilities, we sought a means to directly activate $\alpha_{IIb}\beta_3$ by use of monoclonal antibodies. We screened a panel of monoclonal antibodies raised against intact platelets for the capacity to stimulate PAC1 binding. Of the 46 antibodies tested, 6 stimulated PAC1 binding to platelets. IgG papain-digestion products (Fab) fragments of one of these, P41, stimulated PAC1 binding to both A5 transfectants and platelets but not to wild-type CHO cells (Figure 5). A similar analysis of antibodies against purified $\alpha_{IIb}\beta_3$ from a separate fusion (Frelinger *et al.*, 1990) identified another

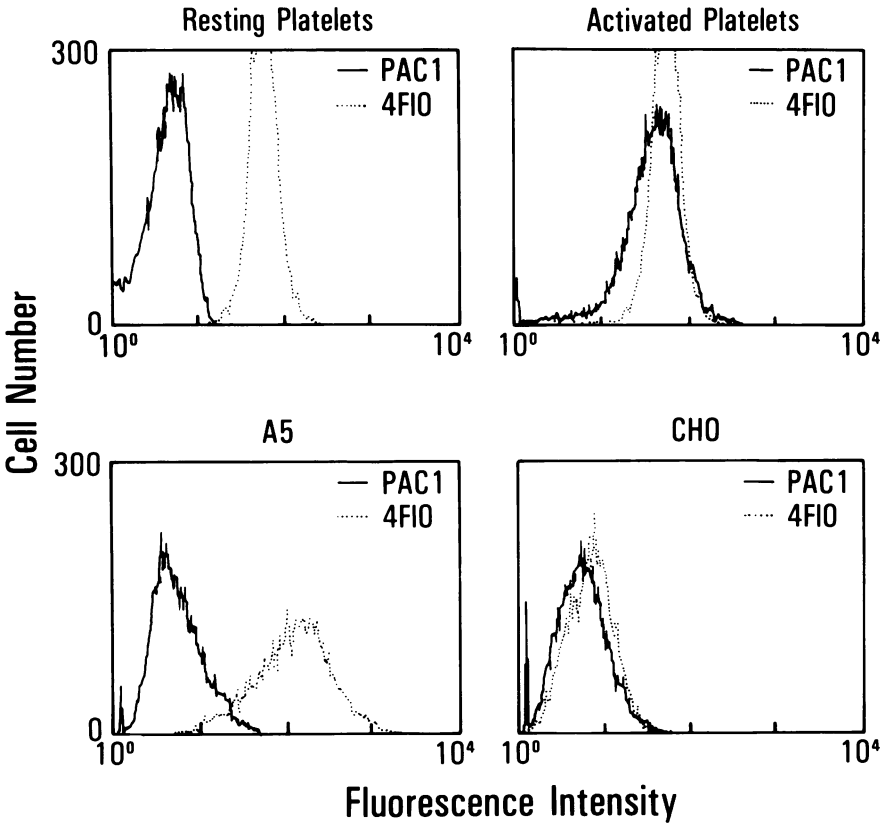

Figure 4. **Recombinant $\alpha_{IIb}\beta_3$ is in a "resting" state.** The binding of the complex-specific antibody 4F10 and the activation-specific antibody PAC1 to resting and activated platelets, A5, or CHO cells was examined by flow cytometry. Fluorescein-labeled antibodies (5 μl) were incubated with 5×10^5 cells in Tyrodes buffer in a final volume of 50 μl. After a 30-min incubation at 37°C, the samples were diluted to 0.5 ml with Tyrodes and analyzed. The solid line denotes binding of PAC1, whereas the dotted line denotes binding of 4F10. The state of the receptor in A5 transfectants resembles that in resting platelets rather than that in platelets activated with 100 nM PMA. Wild-type CHO cells bind neither antibody.

antibody (Ab-62) whose Fab fragments had similar activating properties. Fab fragments of these 2 antibodies also stimulated the binding of soluble Fg to A5 cells (Figure 6). Soluble Fg binding was inhibited with GRGDSP peptide and the anti-$\alpha_{IIb}\beta_3$ monoclonal 2G12, but not with the inactive GRGESP peptide. CHO cell controls did not bind Fg saturably under any condition.

Time course analysis showed that Fg binding reached steady state by 30 min incubation and was initially >75% reversible (not shown). The binding of various concentrations of Fg to activated A5 cells at steady state was analyzed by use of the Ligand computer program (Figure 7). The data were consistent with binding to a single class of sites with $K_a = (9.1 \times 10^6) \pm (1.5 \times 10^6)$ M^{-1} (Kd = 110 nM), receptor concentration = 2.3 ± 0.3 nM (138 000 sites/cell), and nonsaturable binding = $(2.2 \pm 0.4) \times 10^{-3}$. There was day-to-day variation in the quantity of $\alpha_{IIb}\beta_3$ expressed, as measured by the binding of the 2G12 antibody (150 000–440 000 sites per cell) as well as in the number of Fg receptors (138 000–360 000 sites per cell). The ratio of Fg receptors to $\alpha_{IIb}\beta_3$ expressed per cell on a given day was 0.83 ± 0.05; thus, virtually all of the recombinant $\alpha_{IIb}\beta_3$ was functional with respect to Fg binding. These results show that the recombinant $\alpha_{IIb}\beta_3$ in CHO cells lacks the capacity to bind soluble Fg and PAC1 but can be activated to do so. Thus, the lack of high-affinity binding of these ligands to resting platelets is not due to a unique property of the platelet-surface microenvironment.

P41 and Ab62 recognize the β_3 subunit of $\alpha_{IIb}\beta_3$

To characterize further the mode of action of these antibodies, we first identified their antigenic targets. Immunoprecipitation of surface-labeled A5 but not CHO cells with either antibody resulted in the isolation of surface proteins with mobilities identical to $\alpha_{IIb}\beta_3$ (Figure 8A). This indicated that these antibodies bind to the recombinant $\alpha_{IIb}\beta_3$ rather than to an endogenous CHO cell constituent. To determine which subunit was recognized, we immunoblotted platelet extracts and purified $\alpha_{IIb}\beta_3$ with these antibodies. In both cases, the β_3 subunit was recognized (Figure 8B). Thus, both monoclonal antibodies activate the receptor by binding to the β_3 subunit.

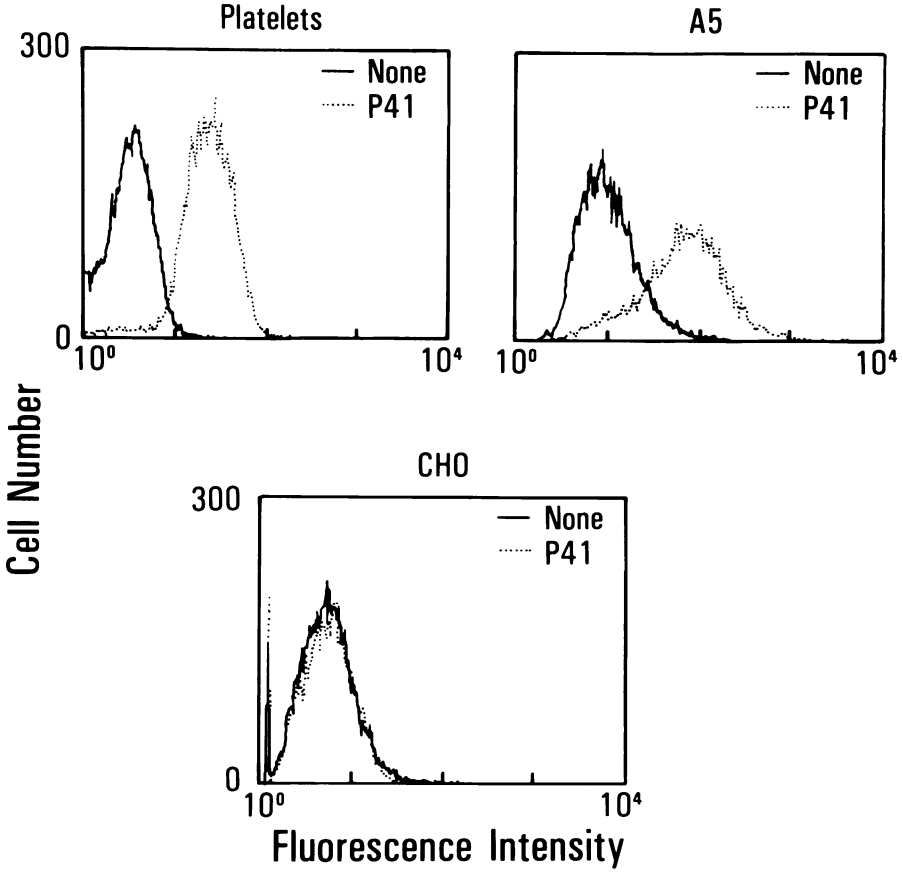

Figure 5. **Recombinant $\alpha_{IIb}\beta_3$ activated with a monoclonal antibody.** The binding of FITC-conjugated PAC1 to various cell types was examined by flow cytometry as described in the legend to Figure 4. The solid line denotes no added agonist, whereas the dotted line denotes the presence of 5 μl of MoAbP41 ascites. Both platelets and A5 transfectants are able to bind PAC1 in the presence of MoAbP41. No binding is observed with wild-type CHO cells.

Antibody activation occurs in the absence of signal transduction or cell membranes

The previous experiments established that Fab fragments of these two antibodies activate A5 cells and platelets to express $\alpha_{IIb}\beta_3$-dependent receptors for fluid-phase Fg and PAC1. To determine whether this activation required intracellular signal transduction events, we examined the capacity of these Fabs to induce PAC1 binding to formaldehyde fixed platelets. Fixation of platelets completely abrogated the PAC1 response to strong agonists such as 50 nM PMA or 1 U/ml thrombin (not shown). In sharp contrast, the response to P41 was not impaired (Figure 9). Thus, platelet viability was not a prerequisite for the activation response to this antibody.

To determine whether these antibodies could activate $\alpha_{IIb}\beta_3$ extracted from the membrane milieu, we examined their capacity to stimulate Fg binding in a solution phase. $\alpha_{IIb}\beta_3$ was solubilized from platelets, incubated in the presence or absence of activating monoclonals, and then bound to microtiter plates coated with a non-activating monoclonal anti-β_3 antibody. Only the captured receptor that was stimulated with either P41 or Ab62 was able to bind soluble Fg (Figure 10). Specificity of Fg binding was verified by inhibition with cold Fg; GRGDSP peptide; and a complex specific monoclonal, 2G12. Activation of $\alpha_{IIb}\beta_3$, therefore, can proceed in the absence of the cell membrane microenvironment.

Discussion

The major findings of this study are as follows: 1) Two different integrin α subunits (α_{IIb} and α_V) form functional complexes with β subunits encoded by the same mRNA, and the α subunit regulates peptide ligand-recognition specificity. 2) Whereas stable $\alpha_{IIb}\beta_3$ transfectants are unable to bind avidly soluble Fg or the activation specific monoclonal PAC1, when stimulated with certain anti-β_3 monoclonals, they do express these binding functions. 3) Activation of $\alpha_{IIb}\beta_3$ with these antibodies can proceed in the absence of signal transduction events or a cell membrane. Thus, $\alpha_{IIb}\beta_3$ activation is not an inherent property of platelets or their microenvironment but is an intrinsic property of the receptor itself.

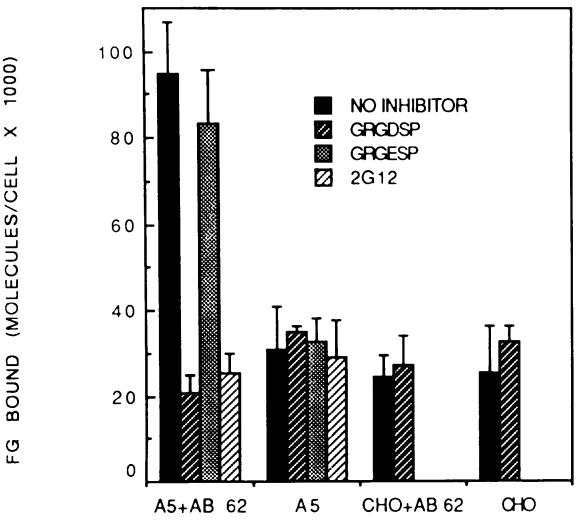

Figure 6. **Fibrinogen binding to recombinant $\alpha_{IIb}\beta_3$.** The binding of ^{125}I-fibrinogen to wild-type CHO or A5 cells was determined by incubating the radiolabeled ligand with cells in the presence or absence of additional antibodies or peptides for 30 min at 37°C. Bound ligand was separated from free by centrifugation in 20% sucrose, and pellet-associated counts were determined by scintillation spectrometry. Results are expressed as total molecules bound per cell. GRGDSP and antibody-inhibitable binding was observed in A5 cells stimulated with AB62 Fabs. Unstimulated A5 recombinants or CHO cells exhibited no binding.

Based on partial sequence identity, immunological cross reactivity, and similar electrophoretic migration (Ginsberg *et al.,* 1987), the β subunits associated with α_{IIb} and α_V have been assumed to be identical or highly similar. Our β_3 clone, isolated from an endothelial cell library and probably the β subunit of $\alpha_V\beta_3$, formed heterodimers with either α_V or α_{IIb}. Each recombinant heterodimer retained its characteristic antigenic specificity and had relative affinities for synthetic peptides similar to those of the native receptor but different from each other. Because they share a β subunit coded by the same mRNA, the different peptide-recognition specificities of the recombinant β_3 integrins are attributable to the α subunit. In particular, the fibrinogen $\gamma_{402-411}$ (L10) peptide was recognized better by $\alpha_{IIb}\beta_3$ than by $\alpha_V\beta_3$. The 21 residues spanning α_{IIb}, 294–314, are proximal to or part of the binding site for a peptide containing $\gamma_{402-411}$ and show significant sequence divergence from the similar region of α_V (D'Souza *et al.,* 1990). Thus, this region of the α subunit may contribute to the difference in peptide ligand-recognition specificity.

Recombinant $\alpha_{IIb}\beta_3$ stably expressed in CHO cells is functionally similar to that in resting platelets with respect to antigenic, peptide, and insoluble macromolecule ligand-binding properties. Recombinant $\alpha_{IIb}\beta_3$ is expressed in an inactive state, because it fails to bind avidly Fg and the activation-specific monoclonal PAC1, whereas it supports cells' adhesion to immobilized Fg. Recombinant $\alpha_{IIb}\beta_3$ does possess the potential capacity to bind fluid phase macromolecules, because stimulation with certain β_3 monoclonals resulted in specific binding of PAC1 and Fg. These activating antibodies are effective on both recombinant and native $\alpha_{IIb}\beta_3$ and exert their effect by acting directly on the receptor, based on three lines of evidence. First, Fab fragments of P41 and Ab62 were effective activators. Thus, these antibodies do not depend on their Fc fragment to mediate this effect. This is in contrast to platelet activation by certain anti-CD9 antibodies that do utilize the Fc region of the activating antibody (Boucheix *et al.,* 1983; Jennings *et al.,* 1990; Worthington *et al.,* 1990). The efficacy of Fab fragments also suggests receptor crosslinking by antibody is not critical in the activation mechanism. Second, these antibodies stimulate PAC1 binding under conditions where strong agonists are unable to activate platelets. Thus, antibody activation does not appear to require signal transduction events or living cells. Indeed, Kouns *et al.* (1989) reported potentially similar antibodies that activate platelet $\alpha_{IIb}\beta_3$ without changes in intracellular Ca^{2+}, phosphorylation, or phospholipid metabolism; and Gulino *et al.* (1990) reported on an anti-α_{IIb} with activating properties. This is in contrast to other agonists, which generally require living, actively metabolizing cells. Finally, P41 and Ab62 are able to stimulate soluble Fg binding to solubilized $\alpha_{IIb}\beta_3$ isolated from platelet membranes. Activation, therefore, is an inherent property of the receptor itself and does not invariably require signal transduction or a cell membrane. The most likely explanation of the antibody effect is alteration of receptor conformation, allowing it to bind fluid-phase macromolecules with higher affinity.

Although platelet $\alpha_{IIb}\beta_3$ may be a prototype of the role activation plays in affinity modulation of integrins, it is clear that other integrin subfamilies also require activation for full functional competence. Stimulation of Mac-I ($\alpha_M\beta_2$) and LFA1 ($\alpha_L\beta_2$) with agents such as phorbol esters, ADP, or *N*-formyl peptides promotes in vitro neutrophil aggregation or lymphocyte adhesion (Wright and Meyer, 1986; Detmers *et al.,* 1987) as well as high-affinity binding of fluid-

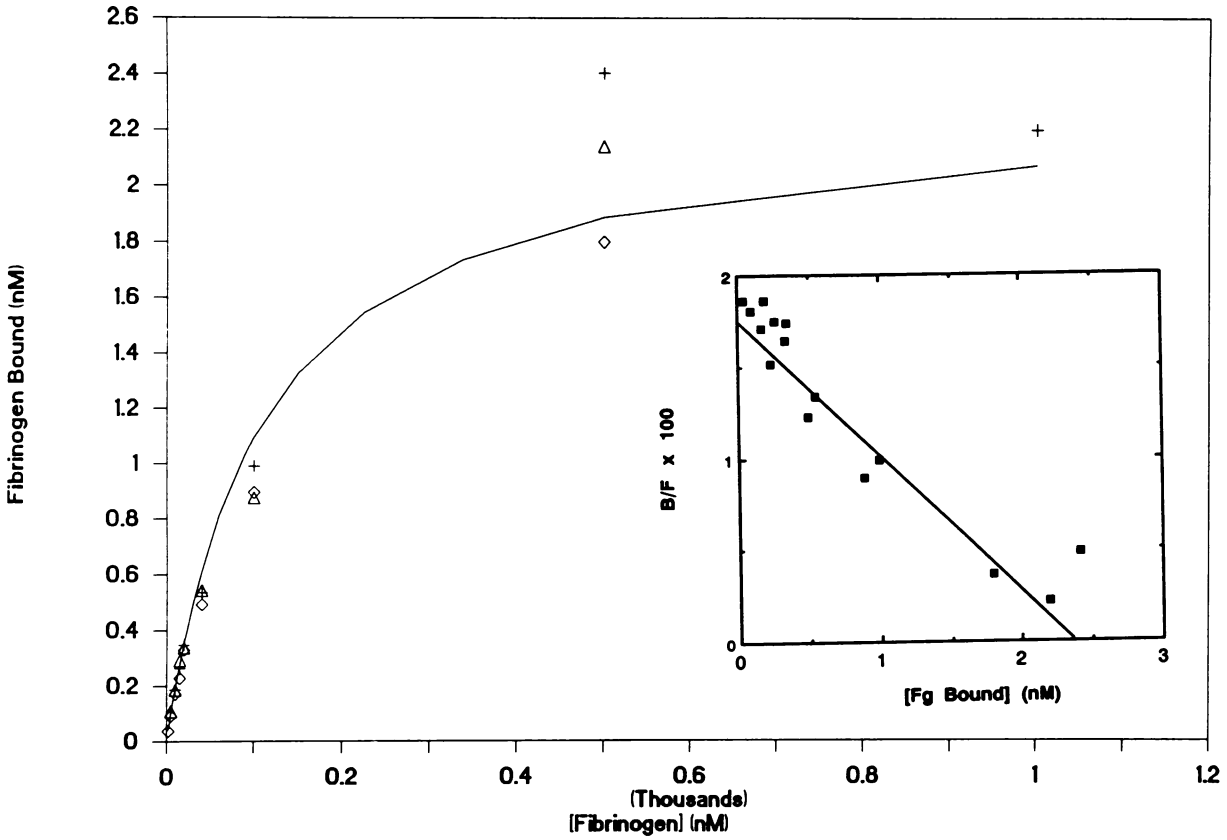

Figure 7. **Equilibrium binding of Fg to recombinant $\alpha_{IIb}\beta_3$.** A5 cells (10^6/ml) were incubated with 6 μM purified Ab 62 for 30 min at 22°C, followed by addition of the indicated concentration of ^{125}I-Fg. After 60 min, bound Fg was measured as described in Methods. Saturable binding from three different experiments is plotted versus the input Fg concentration. A replot of the data according to Scatchard is shown in the inset.

phase Fg (Altieri *et al.*, 1988). Activation of these receptors appears not to require recruitment of new surface receptors, but rather involves a modification of existing receptors (Buyon *et al.*, 1988; Phillips *et al.*, 1988). Recombinant $\alpha_L\beta_2$ expressed in a monkey kidney fibroblastoid cell line (COS cells) (Larson *et al.*, 1990) is constitutively active with respect to adhesion to ICAM-1. This adherence to an immobilized ligand appears similar to the capacity of resting $\alpha_{IIb}\beta_3$ to support platelet (Coller, 1980), 293 cell (Bodary *et al.*, 1989), and CHO cell adhesion to Fg. The failure of agonists to stimulate adhesion to ICAM-1 (Larson *et al.*, 1990) or binding of the L16 activation antibody (Keizer *et al.*, 1988) suggests that the recombinant $\alpha_L\beta_2$ in COS cells was in a similar activation state to recombinant $\alpha_{IIb}\beta_3$ in CHO cells. The possibility that affinity modulation controls the functional activity of integrins and its mechanism, therefore, seems to be an attractive areas for future study. In addition, because the affinity of integrins for specific ligands controls cellular migration, invasiveness, cytoskeletal organization, and adhesion (Hynes, 1987), it seems likely that reagents such as the antibodies described here may modulate these cellular functions in an integrin and adhesive ligand-specific manner.

Methods

Generation and analysis of stable cell lines

CHO cells were cotransfected with equal amounts of β_3 and α_{IIb} or α_V expression constructs (O'Toole *et al.*, 1989) and a CDM8 vector containing the neomycin resistance gene (CDNeo) in a 30:1 ratio. Transfection was by the calcium phosphate method (Graham and Van Der Eb, 1973) followed by a 4-min, 15% glycerol shock. Forty-eight hours after shock, cells were resuspended in selection media containing 700 μg/ml G418, and resistant colonies were isolated after 2 wk in culture. Positive clones were identified by flow cytometry using subunit-specific antibodies (O'Toole *et al.*, 1989). Cloned cell lines were established by single-cell sorting in a FACStar (Becton Dickinson, San Jose, CA) and maintained in media without G418. Cell lines (A5 and VNRC3) exhibiting bright and uniform staining with appropriate antibodies (O'Toole *et al.*, 1989) were maintained for further study.

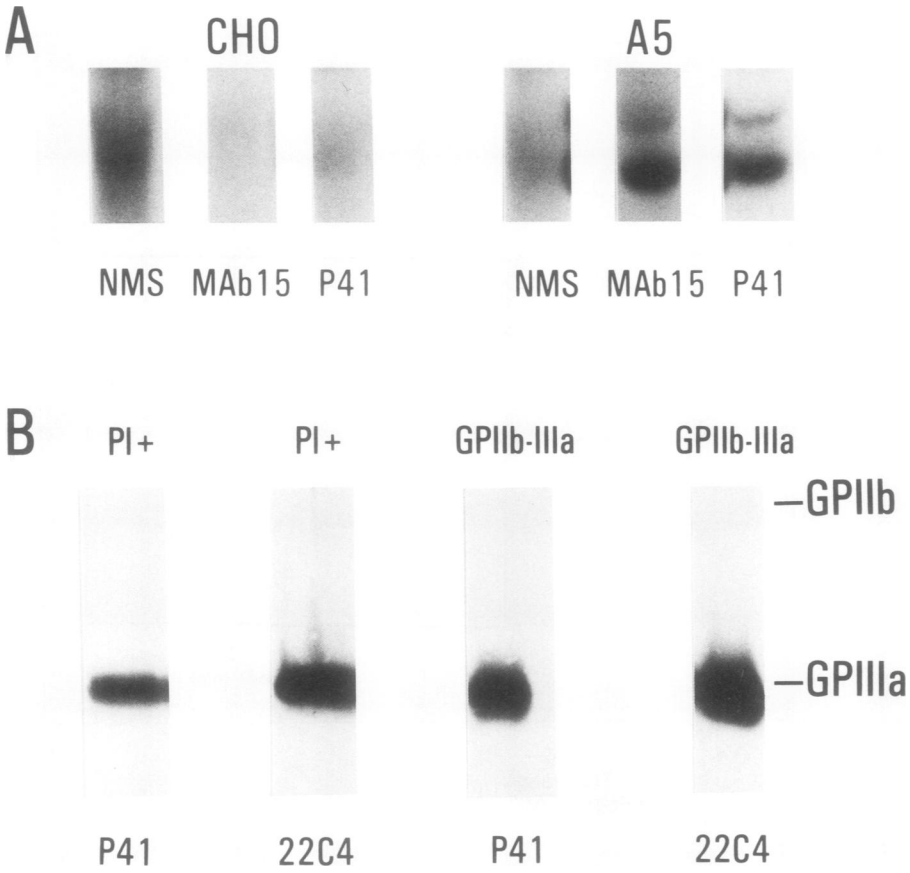

Figure 8. **MoAbP41 recognizes β_3.** (A) Lysate from surface-labeled CHO or A5 cells was immunoprecipitated with MoAbP41, MoAb15 (β_3 specific), or a normal mouse serum (NMS) as indicated. Immunoprecipitates were analyzed on 7% polyacrylamide gels under nonreducing conditions and detected by autoradiography. MoAbP41 immunoprecipitates two bands from A5 cells, which have a migration pattern identical to α_{IIb} and β_3 immunoprecipitated by MoAb15. (B) Platelet lysate or purified $\alpha_{IIb}\beta_3$ was resolved on 7% nonreducing polyacrylamide gels and blotted to nitrocellulose. The blots were incubated with MoAbP41 or 22C4 (β_3 specific) as indicated and developed as described in Methods. MoAbP41 blots a band similar to that identified by 22C4. The location of α_{IIb} and β_3 as determined by Coomassie blue staining is indicated.

Flow cytometry

Antibody binding to cells was analyzed by flow cytometry. Cells were harvested with 3.5 mM EDTA; and an aliquot of 5×10^{-5} cells was pelleted, resuspended in media or Tyrodes buffer (Frelinger *et al.*, 1990) containing a specific first antibody, and incubated for 20 min on ice with or without peptide ligands. Cell were pelleted, washed once, and incubated for 20 min with the second antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Tago, Burlingame, CA). Cells were pelleted, resuspended in 0.5 ml media or Tyrodes, and analyzed on a FACS IV analyzer (Becton Dickinson).

The activated form of $\alpha_{IIb}\beta_3$ was identified by its ability to bind the monoclonal antibody PAC1. This antibody was fluoresceinated (Taub *et al.*, 1989) and used directly in flow cytometry. Briefly, 5×10^5 cells were harvested and resuspended to 50 μl in Tyrodes buffer (Frelinger *et al.*, 1990) containing 10 μg/ml of FITC-PAC1 in the presence or absence of additional agonists. After a 30-min incubation at 37°C, the samples were diluted to 0.5 ml with Tyrodes and analyzed by flow cytometry as described (O'Toole *et al.*, 1989).

Immobilized Fg was prepared by coating 0.8 μM fluorescent latex beads (Fluoricon Fluorescent Particles, Baxter, McGraw Park, IL). These beads were prepared by incubating 1 mg of purified Fg with 650 μl of Fluoricon beads for 1 h at room temperature. The beads were pelleted; resuspended in 1 ml of 30 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.4, 170 mM NaCl, and 1% bovine serum albumin (BSA); and stored at 4°C until ready for use. The binding of these particles to transfected cells was assessed by incubating 5×10^5 cells with 25 μl of beads in the presence or absence of inhibitors for 30 min at room temperature. This solution was layered over a 6% BSA cushion and spun 5 min in a microfuge; the pelleted cells were resuspended in 500 μl of Tyrodes and subjected to FACS analysis as usual.

In FACS experiments using fixed platelets, blood from a normal donor was centrifuged at 1000 rpm for 20 min. The resulting platelet-rich plasma was incubated with 2 μg/ml PGE1 and 1 mM theophylline for 15 min at room temperature, followed by a 15-min room-temperature incubation with 0.1% paraformaldehyde. Fixed or normal platelets were analyzed for FITC-PAC1 binding as described above.

Affinity chromatography and immunoprecipitation

Stable $\alpha_{IIb}\beta_3$ transfectants and wild-type CHO cells were surface labeled by the lactoperoxidase-glucose oxidase method, and octylglucoside extracts were prepared. Immunoprecipitations with specific antibody were performed as described (Plow *et al.*, 1986) and analyzed by nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Additional lysate was applied to an affinity matrix of Gly-Arg-Gly-Asp-Ser-Pro-Lys and specifically eluted with 1 mM free peptide (Lam *et al.*, 1987). Fractions of 2.5 ml were collected, and proteins in each fraction were analyzed by non-reducing SDS-PAGE followed by autoradiography.

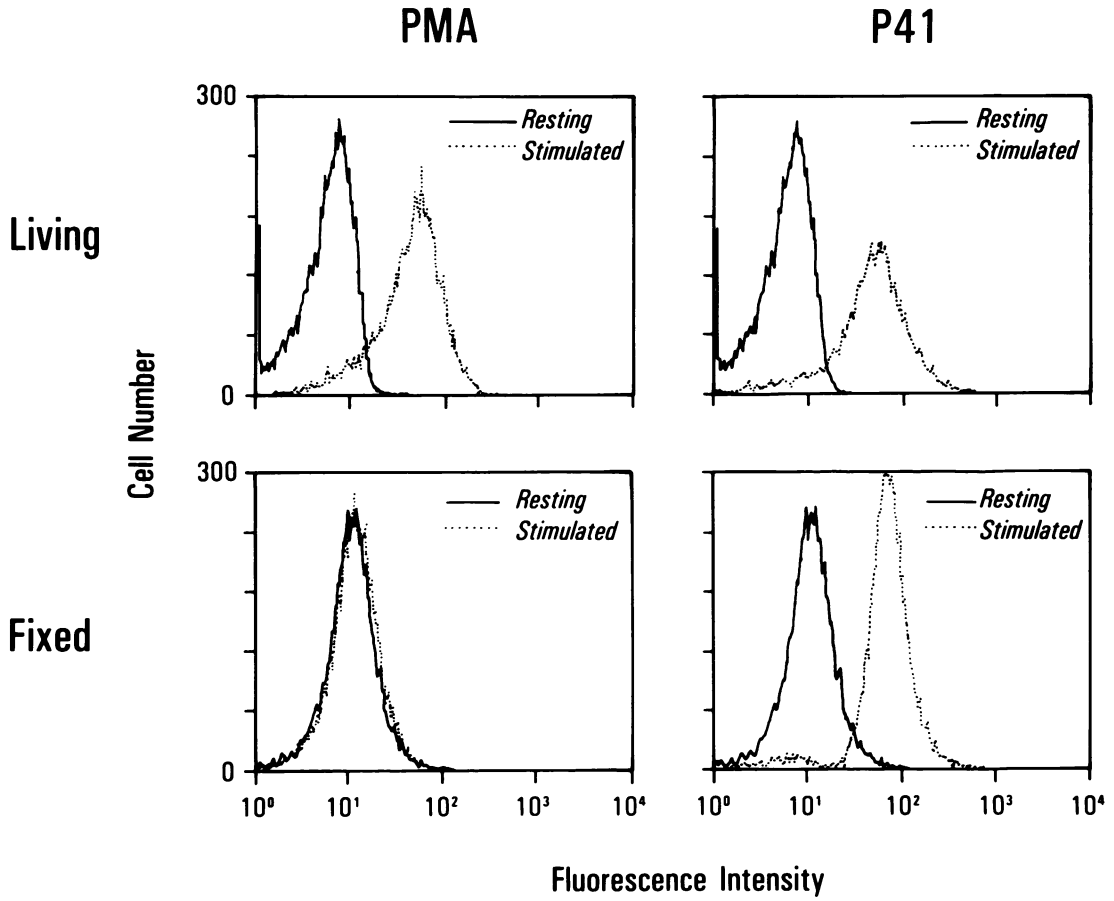

Figure 9. **Antibody activation in the absence of signal transduction.** Living platelets or platelets fixed with 0.1% paraformaldehyde were stimulated with PMA or MoAbP41 Fabs. The binding of FITC-PAC1 to these cell types was analyzed by flow cytometry. The solid line denotes unstimulated cells, whereas the dotted line denotes stimulation with the above-noted agonist. MoAbP41 is able to activate PAC1 binding on both living and fixed platelets, whereas PMA only activates such binding to living platelets.

Production and characterization of monoclonal antibodies

Monoclonal antibodies prepared against intact platelets (Lombardo *et al.*, 1985) or purified $\alpha_{IIb}\beta_3$ (Frelinger *et al.*, 1990) were generated and isolated as described elsewhere. IgG was purified from ascitic fluid on protein A-sepharose (Bio Rad, Richmond, CA). Fab fragments from purified IgG were prepared by digestion with papain (200:1 wt/wt of Ig to papain) for 6 h at 37°C. Undigested Ig and Fc fragments were removed by chromatography on protein A sepharose, and the resulting Fab fragments were analyzed by SDS-PAGE. Less than 8% undigested heavy chain was detected.

For immunoblotting experiments, platelet lysate or purified $\alpha_{IIb}\beta_3$ were resolved on 7.5% polyacrylamide gels under nonreducing conditions and transferred to nitrocellulose. Blots were probed with specific first antibody and developed using the Vectastain ABC method (Vector Laboratories, Burlingame, CA). Immunoprecipitation of surface-labeled cells was accomplished as described (Plow *et al.*, 1986).

Fibrinogen binding

The binding of ^{125}I-Fg to transfected cells was assayed as described previously for platelets (Marguerie *et al.*, 1980). Binding was initiated by the addition of cells to radiolabeled Fg and incubation at 37°C for 30 min. Bound ligand was separated from free ligand by centrifugation through 0.3 ml of 20% sucrose, and the amount of radiolabeled ligand associated with the cell pellet was determined by scintillation spectrometry.

Fibrinogen binding to solubilized $\alpha_{IIb}\beta_3$

Fresh platelets were obtained as described (Ginsberg *et al.*, 1980) and washed twice in Tyrodes pH 6.5 without calcium or magnesium. The platelet pellet was resuspended and solubilized in ice-cold 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) containing 150 mM NaCl, 50 mM octyl-B-D-glucypyranoside (Calbiochem, La Jolla, CA), 1 mM PMSF, 50 ng/ml PGE, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 μM leupeptin, and 1 mg/ml *N*-ethylmaleimide (Sigma, St. Louis, MO) and centrifuged at 100 000 g for 30 min. Platelet lysate was incubated for 15 min at 4°C with activating monoclonals and then added to microtiter wells (Immunlon 2 removawell strips, Dynatech Labs, Chantilly, VA) precoated with 15 μg/ml of a different β_3 monoclonal (MAb15). The microtiter wells were incubated at 4°C overnight, washed twice with a modified Tyrodes-HEPES buffer (2.5 mM

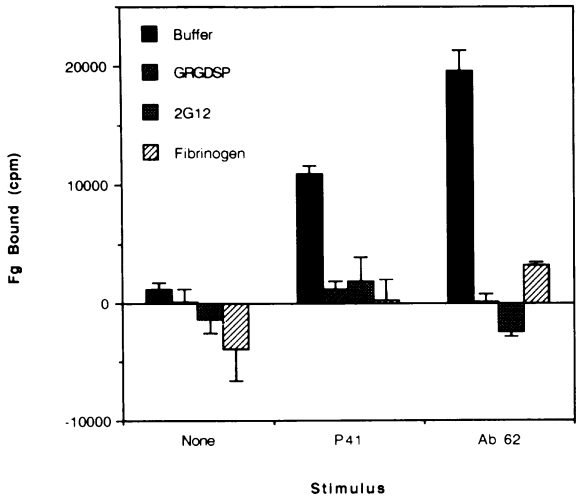

Figure 10. **Activation of purified $\alpha_{IIb}\beta_3$.** The binding of ^{125}I-fibrinogen to purified $\alpha_{IIb}\beta_3$ captured in a microtiter well is described in Methods and depicted above as ^{125}I-Fg cpm bound. Stimulated but not unstimulated receptor exhibits increased binding. Fibrinogen binding is inhibited with excess unlabeled ligand, the GRGDSP peptide, and the anti-$\alpha_{IIb}\beta_3$ antibody 2G12.

HEPES, 150 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 5.5 mM D-glucose, and 1 mg/ml BSA, pH 7.4) containing 50 mM octyl-B-D-glucopyranoside and 0.1 μM activating monoclonal antibody, and then washed twice in Tyrodes-HEPES buffer only. For binding studies, the $\alpha_{IIb}\beta_3$-bound microtiter wells were incubated with 50 μl of 50 nM ^{125}I-labeled Fg in the presence or absence of peptide and antibody inhibitors at room temperature for 4 h. Bound radioactivity was counted after two washes with Tyrodes-HEPES buffer. In control experiments, platelet lysate was incubated in microtiter wells coated with an irrelevant antibody (TIB115), or microtiter wells coated with MAb15 were incubated with Tyrodes-HEPES lacking platelet lysate.

Other reagents

$\alpha_{IIb}\beta_3$ complex-specific monoclonals 10E5 and 2G12 were generous gifts from Barry Coller (State University of New York, Stony Brook) and Virgil Woods (University of California, San Diego), respectively. An α_{IIb}-specific monoclonal (Tab) (McEver *et al.*, 1983) was kindly provided by Rodger McEver (University of Texas, San Antonio). LM142, a monoclonal reactive with α_V, was graciously supplied by David Cheresh (Research Institute of Scripps Clinic, La Jolla, CA). Other monoclonals were produced or obtained as described (Frelinger *et al.*, 1990). Anti-VnR1 was produced against purified platelet $\alpha_V\beta_3$. The peptides RGDF, GRGDSP, and L10 were prepared by solid-phase synthesis on a peptide synthesizer (model 430, Applied Biosystems, Foster City, CA) using phenylacetamidomethyl resins and t-butoxycarbonyl amino acids purchased from Applied Biosystems. Fg was prepared and labeled (Marguerie *et al.*, 1980) by standard methods. All other reagents or chemicals obtained were of the highest available quality.

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