A Molecular Basis for Affinity Modulation of Fab Ligand Binding to Integrin $\alpha_{IIb}\beta_3$*

(Received for publication, April 18, 1996, and in revised form, June 15, 1996)

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The Arg-Gly-Asp (RGD) sequence within the third complementarity-determining region (CDR3) of the heavy chain (H3) is responsible for the binding of the recombinant murine Fab molecules, AP7 and PAC1.1, to the platelet integrin $\alpha_{IIb}\beta_3$. AP7 binding is minimally influenced by the conformational state of this receptor, whereas PAC1.1 binds preferentially to the activated state of the receptor induced by platelet agonists. To study the molecular basis for this functional difference, we replaced the AP7 H3 loop (HPFYRGGDGN) with all or segments of the analogous sequence from PAC1.1 (RSP5YRGGDGAP). AP7Fd (VH domain or segments of the analogous sequence from PAC1.1) segments containing these H3 loop sequences were expressed as active Fab molecules by coinfection of Spodoptera frugiperda cell lines with recombinant baculoviruses containing Fd and AP7 $\kappa$ chain cDNA. Replacement of the entire AP7 H3 loop with that from PAC1.1 generated the mutant $\alpha_{IIb}\beta_3$.AP7 Fab molecule, which bound selectively to either activated, gel-filtered platelets or to purified $\alpha_{IIb}\beta_3$ in a manner identical to that of PAC1.1. Identical results were obtained when solely the sequences flanking the amino side of RGD within the respective H3 loops were exchanged. AP7.3 and PAC1.1 exhibited saturable but submaximal binding to activated gel-filtered platelets. Relative to AP7, the number of AP7.3 or PAC1.3 Fab molecules bound per platelet was 17% in the presence of 1 mM Ca$^{2+}$ + 1 mM Mg$^{2+}$ or 40% in the presence of 10 $\mu$M Mn$^{2+}$. The ratio of Fab molecules bound after versus before activation (mean ± S.D.; n = 3) was: for AP7.3, 9.8 ± 0.6; for PAC1.1, 8.8 ± 0.3; and for AP7, 1.4 ± 0.2. In addition, AP7 bound to the stably expressed integrin mutant $\alpha_{IIb}\beta_3$ (S123A), whereas AP7.3 and PAC1 did not. Because AP7.3 behaves in every respect like PAC1.1, we conclude that the ability of RGD-based ligands to distinguish activated from resting conformations of the integrin $\alpha_{IIb}\beta_3$ can be regulated by limited amino acid sequences immediately adjacent to the RGD tripeptide. Furthermore, those Fab molecules that exhibit increased selectivity for the activated conformation of $\alpha_{IIb}\beta_3$ bind to a subpopulation of this integrin on platelets that is modulated by divalent cations.

Several RGD-containing adhesive proteins, such as fibronectin, fibronectin, or von Willebrand factor, when presented in soluble form, will bind to the platelet integrin $\alpha_{IIb}\beta_3$ only when the platelets are first stimulated by an appropriate agonist (1). The molecular properties responsible for this selective binding of macromolecular ligands to $\alpha_{IIb}\beta_3$ on activated platelets are not fully understood. Ligand size is not a critical factor, because small RGD peptides and peptidomimetics, 5-10-kDa RGD-containing disintegrins, the 150-kDa monodonal antibody OPG2, and the large 600-kDa tetramer of the murine monoclonal antibody 7E3 all bind to $\alpha_{IIb}\beta_3$ whether or not platelet activation has occurred, although platelet activation does result in a severalfold increase in apparent binding affinity for some of these ligands (2–5). Ligand valency is also not a likely explanation for selective binding to activated conformations of $\alpha_{IIb}\beta_3$, because the binding of the monoclonal antibody PAC1 to platelets remains dependent upon the activated state of $\alpha_{IIb}\beta_3$ whether the antibody is presented as a 50-kDa recombinant Fab molecule or a 900-kDa pentameric IgM (6).

To address the molecular basis of conformation-sensitive binding of an RGD ligand, we exploited the binding properties of two well-characterized RGD ligands, AP7 and PAC1.1, which are the RGD-containing analogs of the previously described RGD-containing antibodies, OPG2 and PAC1, respectively (6, 7). The characterization of PAC1.1 was accomplished in this study. AP7 Fab molecules bind with high affinity to either the nonactivated or the activated conformational states of $\alpha_{IIb}\beta_3$. On the other hand, recombinant PAC1.1 Fab molecules bind more avidly to the activated conformation of $\alpha_{IIb}\beta_3$ as reflected by an increase in affinity and number of sites. In this regard, the behavior of PAC1.1 is more similar to that of other macro-molecular RGD ligands specific for $\alpha_{IIb}\beta_3$, e.g. fibronectin or von Willebrand factor (1, 8, 9).

Because the heavy chain CDR3 (H3) loops of either AP7 or PAC1.1 contain an RGD motif but differ otherwise in length and flanking amino acid composition, we asked whether differences in amino acid sequence of these flanking regions could be a major factor contributing to the ability of these Fab molecules to distinguish activated from resting conformations of the integrin. By comparing the binding properties of recombinant Fab molecules in which these flanking H3 sequences have been exchanged, we report here that this is indeed the case. An additional unexpected finding, albeit consistent with previous observations, is that the activation-dependent Fab molecules PAC1.1 and AP7.3 bind to a subpopulation of $\alpha_{IIb}\beta_3$ on activated platelets. This subpopulation is still distinguishable.

*References will be provided in the final version of this manuscript. The abbreviations used are: H3, third complementarity-determining region of the heavy chain; PMA, phorbol myristate; CHO, Chinese hamster ovary.
when the total integrin population is isolated from platelets. The proportion of the integrin that is bound by PAC1.1 or AP7.3 can be modulated by divalent cation composition.

**MATERIALS AND METHODS**

Synthesis of Recombinant OPG2 Fd and κ Chain cDNA—The term Fd denotes an Ig heavy chain segment that includes the V<sub>H</sub> domain, the C<sub>H</sub>1 domain, and the hinge region up to and including the cysteine residue that forms a disulfide bond with the light chain (7). Fab molecules represent disulfide-linked heterodimers composed of Fd + κ.

AP7 and PAC1.1 Fd and κ chain cDNAs were prepared as described previously (6, 7). An additional AP7 Fd construct was generated in which the oligonucleotide sequence (CATCAC)<sub>3</sub> was inserted just upstream of the TGA stop codon. This encodes a carboxy-terminal (His)<sub>6</sub> sequence, which was employed to purify Fab or Fd molecules by nickel affinity matrix chromatography, as described (6).

The remaining constructs containing the H3 sequences depicted in Fig. 1 (AP7.1, AP7.2, and AP7.3) were generated by splice overlap extension polymerase chain reaction (7). AP7 Fd cDNA (7) was used as a template to generate AP7.1, AP7.2, and AP7.3 Fd cDNAs. As detailed below, each construct was generated by first producing two cDNA fragments, a 5′ fragment, generally encompassing the Fd signal sequence up to a point within H3, and a 3′ fragment, beginning in H3 and extending to the TGA stop codon. The 5′ and 3′ fragments were ligated, and a final amplification of each DNA with the primer pair HFOR and HREV was performed (Table I). A BglII/XbaI digest of the cDNA product was then ligated into pVL1392.

The 5′ fragment of AP7.1 was obtained with the primers HFOR and CTRREV. The primer CTRREV adds an AffI site while retaining the correct amino acid sequence. The primer AP7.1FOR adds an AffI site and changes the first three amino acids (HPF) of AP7 H3 to the first five amino acids (RPSYS) of PAC1.1 H3. AP7.1FOR was used in combination with HREV to produce the 3′ fragment of AP7.1. Both fragments were digested with AffI and ligated. Ligated cDNA served as template for subsequent polymerase chain reaction reactions using HFOR and HREV to amplify AP7.1 cDNA.

The 3′ fragment of AP7.2 was generated using the primer AP7.2FOR in combination with HREV. AP7.2FOR changes the last two amino acids (GN) of AP7 H3 to the last three amino acids (AGP) of PAC1.1 H3. The cDNA product and the 5′ fragment of AP7 were then digested with SacI and ligated. Oligonucleotides HFOR and HREV were then used to amplify the AP7.2 cDNA.

AP7.3 was constructed by digesting AP7.1 cDNA and AP7.2 cDNA with SacI and ligating the 5′ fragment of AP7.1 with the 3′ fragment of AP7.2. Ligated cDNA was used as template for polymerase chain reaction employing the HFOR and HREV primer pair.

The recombinant viruses containing AP7 Fd and κ DNA have been previously named pVL Fd and pVLκ, respectively (7). Viruses containing the Fd and κ DNA constructs are herein designated pVL7.1Fd, pVL7.2Fd, and pVL7.3Fd.

Cloning and Analysis of Recombinant Baculoviruses—Recombinant viruses were cloned by infection of Sf9 cells (Invitrogen) (2 × 10<sup>6</sup> in 2 ml of complete Grace’s medium) seeded in T25 culture flasks, as described (6, 7). The sequence of each recombinant clone was confirmed prior to its use, using Sequenase version 2.0 (U.S. Biochemical Corp.). Recombinant viruses were used to inoculate either Spodoptera frugiperda Sf9 or High Five insect cells (Invitrogen, Inc.), and Fab molecules were harvested from the medium, normally after 72-h cultures, as described (6, 7). Recombinant Fd and κ chains were detected by a quantitative Western blot assay using rabbit polyclonal anti-murine Fd + κ antibody, developed in our laboratory (7). Protein concentration was determined by the method of Markwell (10).

**RESULTS**

Immunochemical Characterization of Recombinant Anti-a<sub>1ib</sub>b<sub>3</sub> Fab Molecules—AP7 and PAC1 Fab molecules are completely distinct at the level of primary sequence other than the presence of an RGD sequence in the H3 of AP7 and an RYD in H3 of PAC1 (7) (Fig. 1). Because the number and composition of the flanking H3 amino acid residues are different in AP7 and PAC1, we asked whether the AP7 could be endowed with the heactivating-dependent binding characteristics of PAC1 by replacing the H3 flanking residues in AP7 with those of PAC1. Fig. 1 depicts the recombinant Fab molecules that were constructed and expressed in insect cells to address this question. Note that all AP7 variants contained the AP7 scaffold, whereas PAC1.1 contained the PAC1 scaffold. In AP7.1, the amino-terminal H3 flank of AP7 was replaced with that of PAC1; in AP7.2, the carboxy-terminal flank of AP7 was replaced with that of PAC1; and in AP7.3, both flanks were replaced with those of PAC1.

72 h after coinfection of High Five cells with the AP7 light chain baculovirus (pVLκ) and each of four pVL Fd heavy chain viruses (AP7.7, AP7.1, AP7.2, and AP7.3), the average yields of Fab molecules were essentially equal for all of the constructs: AP7.7, 19.4 ± 1.1 μg/ml (mean ± S.D., n = 5); AP7.1, 20.5 ± 4.2 μg/ml (n = 3); AP7.2, 22.8 ± 1.8 μg/ml (n = 3); and AP7.3, 20.6 ± 2.3 μg/ml (n = 3). Identical results were obtained when platelet and integrin binding studies detailed below were carried out with Fab molecules in protein-free culture supernatant or with hexahistidine-tagged versions of these Fab molecules purified by metal ion chelate chromatography.

Binding of Recombinant Fab Molecules to Platelets—Before exchanging AP7 flanking residues with those of PAC1, we tested whether substitution of a glycine for tyrosine in RYD of PAC1 exerted any effect on Fab function. The binding of recombinant PAC1.1 Fab molecules (containing RGD) to gel-filtered platelets was compared with the binding of PAC1 Fab (RYD) by flow cytometry. Both PAC1 and PAC1.1 bound selectively and equivalently to platelets activated by PMA, but neither antibody bound to unstimulated platelets. In both cases, Fab binding to activated platelets was inhibited by EDTA (Fig. 2). In contrast, as described previously (7), a PAC1 derivative in which RYD was replaced by RYE failed to bind to

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**Table I**

| Primer   | Sequence                        | Amino acid       | Amino acid sequence | Restriction site |
|----------|--------------------------------|------------------|---------------------|------------------|
| HFOR     | CTCAGATCTCAATGGAAGACTGCGGTC    | −20 to 15 Signal sequence | BglII              |
| HREV     | CTACCATGATCAATCCCTGCGGAC       | 225 to 228 VPRD   | XbaI                |
| CTRREV   | GTGACCGTTAGATATACATAGGCGGCG   | 91 to 99 TALTYCTR | AffIII             |
| AP7.1FOR | TATACCGTTAGACCCCTACTAGCGGAC    | 96 to 108 TRSPSYRGD | AffIII/SadI        |
| AP7.2FOR | CTTCCGCCGCGACGCGGCGGACCTTATCTAGTGAC | 102 to 114 YRGDAFGYAMD | SadI          |

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platelets under any conditions. Thus, the R(Y/G)D sequence in PAC1 is required for antibody binding to $a_{11b3}$, but the tripeptide sequence by itself does not determine the activation-dependent behavior of the antibody.

Saturable binding to nonactivated platelets in the presence of 1 mM Ca$^{2+}$ plus 1 mM Mg$^{2+}$ was observed for AP7 Fab molecules at concentrations $\geq 10 \, \mu$g/ml (Fig. 3A). Following platelet activation with PMA, the number of AP7 Fab molecules bound at saturation increased by approximately 40%, almost certainly due to activation-induced surface expression of internal pools of $a_{11b3}$ (13, 14). In sharp contrast to these results for AP7, the binding of AP7.1, AP7.3, or PAC1.1 Fab molecules to nonactivated platelets was negligible (Fig. 3A) and not significantly greater than the binding of nonspecific murine Fab molecules (not shown). Following platelet activation, however, saturable binding of AP7.1, AP7.3, and PAC1.1 was observed at concentrations $\geq 10 \, \mu$g/ml, although the extent of binding was less than that of AP7. In each case, Fab binding was completely inhibited by RGDW ($\geq 10 \, \mu$M) or EDTA (5 mM) (not shown). AP7.2 Fab molecules failed to bind to platelets regardless of the state of platelet activation or divalent cation composition (Fig. 3A and B). We conclude that the pattern of binding of AP7.1 and AP7.3 to platelets is identical to that of PAC1.1 rather than to that of the parent AP7 Fab molecule.

Divalent cations have a profound effect on the binding of fibrinogen or von Willebrand factor to $a_{11b3}$, and the same was true for the Fab molecules studied in this report. In the presence of 10 $\mu$M Mn$^{2+}$, which is known to augment the ligand binding activity of $a_{11b3}$ (15), there was little effect on the binding of AP7, but PMA-induced increases in binding of AP7.1, AP7.3, and PAC1.1 to platelets were more pronounced than those observed in 1 mM Ca$^{2+}$ plus Mg$^{2+}$. Saturable binding of these Fab molecules occurred at lower input concentrations ($\geq 2 \, \mu$g/ml), and the number of molecules bound at saturation increased (compare results in Fig. 3A with those in Fig. 3B). For example, relative to AP7, the number of AP7.3 or PAC1.1 Fab molecules bound per activated platelet was 17% in the presence of 1 mM Ca$^{2+}$ plus Mg$^{2+}$ and 40% in the presence of 10 $\mu$M Mn$^{2+}$.

Fig. 4 provides a direct comparison of the activation-dependent behavior of these various Fab molecules when studied under identical conditions of divalent cation composition, i.e., in the presence of 1 mM Ca$^{2+}$ plus Mg$^{2+}$.

**Fig. 1.** Recombinant Fab molecules developed in this study. The H3 sequence of each of the Fd constructs used in this study is depicted. H3 is arbitrarily defined as the intervening sequence between the consensus terminal residues of the VH gene product (YCTR or YCAR) and the initial residues of the consensus JH germ line sequence (YXXX). Framework refers to the remainder of the VH domain sequence.

**Fig. 2.** PAC1.1 Fab molecules bind selectively to activated platelets. Wild type PAC1 Fab molecules contain an Arg-Tyr-Asp (RYD) sequence at residues 100A-C of the heavy chain CDR3. The RGD-containing mutant PAC1.1 contains a Giv at residue 100B instead of Tyr. 1 $\mu$M wild type PAC1 Fab (A) or PAC1.1 Fab (B) was incubated for 15 min with either resting platelets (in the presence of 0.1 $\mu$M PGI2), platelets stimulated with 0.2 mM PMA, or PMA-stimulated platelets in the presence of 5 mM EDTA. After the addition of fluorescein isothiocyanate-labeled anti-mouse Ig, bound Fab molecules were measured by flow cytometry. This experiment is representative of five iterations that generated comparable findings.

**Table 1.** Framework and sequence of recombinant Fab molecules developed in this study. The H3 sequence of each of the Fd constructs used in this study is depicted. H3 is arbitrarily defined as the intervening sequence between the consensus terminal residues of the VH gene product (YCTR or YCAR) and the initial residues of the consensus JH germ line sequence (YXXX). Framework refers to the remainder of the VH domain sequence.
immunosorbent assay (Fig. 6). The binding of AP7, AP7.3 or PAC1 Fab molecules to α1bβ3 in the presence of 1 mM Ca2+ plus Mg2+ was completely inhibited by >10 µM RGDF or 5 mM EDTA. As had been observed with intact platelets, AP7.2 failed to bind to purified α1bβ3.

Important differences in the binding of these Fab molecules to purified α1bβ3 were observed when the effect of divalent cations was analyzed. In the presence of 1 mM Ca2+ plus Mg2+, comparable binding of AP7.1, AP7.3, and PAC1.1 molecules to α1bβ3 was observed (optical density, 0.3-0.5) (Fig. 7A). In contrast, the absolute number of AP7 Fab molecules bound at saturation (optical density, 1.3-1.4) was 2-3-fold higher than that observed for any of the other three Fab molecules. However, in the presence of 10 µM Mn2+, the maximal extent of binding of all four Fab molecules to α1bβ3 was now equivalent (Fig. 7B). Nonetheless, significant differences in apparent affinity persisted, such that the concentrations of each Fab molecule at which half-saturation was achieved was markedly disparate. From the representative experiment shown in Fig. 7B, these approximate values were 0.2 µg/ml for AP7, 2.5 µg/ml for AP7.3, 10 µg/ml for AP7.1, and 20 µg/ml for PAC1.1. The differences in the numbers of α1bβ3 molecules occupied by AP7.3 (or PAC1.1) Fab in the absence versus the presence of Mn2+ must be due to the presence in the former case of stable conformers of α1bβ3 in which the epitope remains inaccessible to the antibody.

**DISCUSSION**

This study is the first to show that the amino acid environment immediately adjacent to the RGD tripeptide in macromolecular ligands can determine whether ligand binding is sensitive to the activation state of α1bβ3. This finding excludes ligand size or valency as requisite factors responsible for affinity modulation of the receptor. The only difference between AP7, which does not bind selectively to activated α1bβ3, and AP7.3, which does, is the length and composition of flanking sequences in H3: AP7 contains HPFYrgdGGN, whereas AP7.3 contains RSPSYrgdGAGP. The fact that AP7.3 behaves identically in parallel binding experiments to the prototype activation-dependent ligand PAC1 argues that a very subtle change in the position and/or orientation of the RGD sequence in this CDR loop is sufficient to modify the dependence of ligand binding on the conformational state of the receptor. These sequence differences would be expected to modify a number of conformational properties of the H3 loop, including the distance of the RGD sequence from the apex of the loop to the plane of the antigen-binding face and the orientations of the Arg and Asp side chains themselves.

X-ray crystallographic studies of OPG2, the parent molecule of AP7, have established that the distance from the apex of the H3 loop to the plane of the antigen-binding face is about 14 Å (16). The insertion of an additional two amino acids to the amino side of the H3 loop, as in the case in AP7.1 and AP7.3, may shift the position of the RGD sequence from the apex of the loop to a position closer to the carboxyl side of the loop. This would decrease the distance between the RGD sequence and the antigen binding face. This interpretation would support the contention of Beer et al. (17), who proposed that the ability of the RGD sequence to extend into a receptor pocket influences its ability to distinguish conformations of α1bβ3. Using a peptide approach, they demonstrated a direct relationship between the platelet activation dependence of peptide binding and the length and composition of the spacer (Gly)ₙ sequence between RGDF and the polyacyrionitrile beads to which the peptide was conjugated. Longer peptides (≥G₉RGDF) bound equally well to both activated and nonactivated platelets, whereas intermediate length peptides (e.g. G₅RGDF) exhibited the greatest sensitivity to the conformation of α1bβ3 (17). This raises the possibility that RGD recognition sites may be recessed from the ligand-binding surface of the α1bβ3 molecule, perhaps situated within a shallow groove. In that case, platelet activation may alter the depth or
size of this groove and thereby modulate accessibility of the relevant integrin contact sequences to RGD ligands.

The decrease in apparent affinity of AP7.3 relative to that of AP7 recapitulates the observed difference in apparent affinity between PAC1 and AP7. In a previous study, we showed that PAC1 Fab molecules exhibit a 60-fold decrease in apparent $K_d$ relative to the parent IgM (6). This difference was reasonably ascribed to loss of multivalency. However, in the present study, we observed that the conversion of AP7 to AP7.3 results in a comparable decrease in affinity. Half-maximal binding of AP7 Fab molecules to activated platelets in the presence of manganese occurred at roughly 1 $\mu$g/ml (20 nM), whereas half-maximal binding of AP7.3 Fab molecules occurred at roughly 30 $\mu$g/ml (600 nM) (Fig. 7B). Thus, a 30-fold decrease in affinity resulted simply by exchanging the H3 of PAC1.1 for the H3 of AP7. Not surprisingly, the affinity of AP7.3 Fab molecules was similar to that of recombinant PAC1.1 Fab molecules, in side by side comparisons. From these results, we conclude that there is a relationship between the affinity of an RGD ligand and its sensitivity to the conformational state of the integrin to which it binds. This is consistent with our hypothesis that increased accessibility of the $\alpha_{IIb}\beta_3$ recognition sites facilitates binding of even lower affinity ligands.

The modified behavior of AP7.3, relative to AP7, should not be misconstrued as a coincidence resulting merely from a decrease in affinity. AP7.3 also acquires an altered specificity for the mutant integrin $\alpha_{IIb}\beta_3(S123A)$ that is identical to that of PAC1 but unlike that of AP7. Mutagenesis experiments (12) have clearly shown that alanine substitutions of clustered oxygenated $\beta_3$ residues Asp119, Ser121, or Ser123 do not influence heterodimer formation or surface expression but do alter fibrinogen, PAC1, and OPG2 binding. Although alanine substitution

FIG. 5. Binding of AP7 and AP7.3 to wild type and mutant $\alpha_{IIb}\beta_3$ receptors stably expressed by CHO cells. The binding of AP7 and AP7.3 to CHO cell transfectants was examined by flow cytometry. The results are depicted as histograms with the log of fluorescence intensity on the abscissa and the cell number on the ordinate. Top row, AP7 binding under nonactivated conditions. Middle row, AP7.3 binding under nonactivated conditions. Bottom row, AP7.3 binding in the presence of 10 $\mu$M Mn$^{2+}$ (this treatment is known to convert the receptor from a nonactivated to an activated conformation). Left column, CHO cell transfectants expressing wild type (WT) $\alpha_{IIb}\beta_3$ (A5 cells). Center column, CHO cell transfectants expressing the mutant receptor $\alpha_{IIb}\beta_3(S123A)$. Right column, mock-transfected CHO cells. The results depicted are representative of two independent experiments that gave identical results.

FIG. 6. Binding of AP7, AP7.3, and PAC1.1 Fab molecules to purified $\alpha_{IIb}\beta_3$ versus purified $\alpha_{V}\beta_3$. Binding in the depicted experiment was measured in the presence of 1 $\mu$M Ca$^{2+}$ plus 1 $\mu$M Mg$^{2+}$. The amount of Fab molecules bound in the absence of inhibitors (y axis) is compared with those amounts bound in the presence of 10 $\mu$M RGDW or 5 $\mu$M EDTA (z axis). An amount of each Fab molecule equivalent to 1.5–2× that amount sufficient to saturate all available integrin sites was added in each case. Bars represent means of triplicate determinations. Standard deviations (not shown) did not exceed 10% of the mean, in any case. OD, optical density.
of Asp119 or Ser121 abolished binding of all three ligands, ala-
to purified

"Conformation-sensitive α11b3 Ligands"

Fig. 7. Dose-dependent binding of recombinant Fab molecules
to purified α11b3 in the presence of 1 mm Ca2+ plus 1 mm Mg2+
(A) or 10 μM Mn2+ (B). The concentration of Fab molecules added
is depicted on the z axis, whereas the amount of bound Fab is proportional
to the optical density at 405 nm depicted on the y axis. Fab molecules
tested (x axis) were: AP7, AP7.1, AP7.2, AP7.3, and PAC1.1. Values
represent means of triplicate determinations in a single representative
experiment. Standard deviations (not shown) did not exceed 10% of the
mean, in any case. OD, optical density.

platelet. In the presence of 10 μM manganese, the total number
of sites occupied by AP7.3 increased to 40% of that occupied by
AP7. This difference between AP7 and AP7.3 was also seen
when purified α11b3 adsorbed to plastic microtiter wells was
the antigen target. However, in the case of the purified inte-
grin, the presence of manganese resulted in a comparable sa-
turation of binding by both AP7 and AP7.3. These results pro-
vide direct evidence for the existence of subpopulations of
platelet α11b3 that assume distinctive phenotypes distinguish-
able by ligand and/or antibody binding, predicted by a number
of prior studies (17, 18). Presumably, α11b3 can exist in distinct
stable conformations even after it is isolated from the platelet
membrane. These conformational states of α11b3 remind one of
similar findings by Chan and Hemler (19) with another inte-
grin, α2b1.

In its basal state, α11b3 does not engage soluble ligands and
mediates platelet attachment selectively to surfaces coated
with fibrinogen (20). Once the platelet is activated, however,
the conformation and activity of α11b3 changes, such that it can
engage fibrinogen or other soluble ligands and mediate attach-
ture to surfaces coated with von Willebrand factor, fibronec-
tin, or vitronectin (21, 22). Comparative binding studies of
soluble fibrinogen and fibronectin suggest that conformational
changes in α11b3 are not "all or none" but that intermediate
states in the conformational range of this receptor may further
modulate the selectivity of the receptor for the soluble versus
surface-bound conformation of any one ligand. The ultimate
strength of the adhesive interaction between ligand and recep-
tor in vivo may be influenced by structural determinants in the
ligand other than the RGD sequence (23–26), by post-ligand
binding events, including outside-in signaling (27–29), by mul-
tiple contact points on the receptor (12, 30–33), and by hemo-
dynamic forces (34). Despite this complexity, the similarities
in binding between AP7.3, PAC1, and other natural ligands that
contain RGD sequences and the fact that the structure of OPG2
has been solved by x-ray crystallography make the AP7 deriv-
atives an excellent paradigm for future studies of the molecular
basis of RGD ligand specificity and activation-induced changes
in integrin conformation.

Acknowledgments—We thank Dr. Brunhilde Felding-Habermann
(The Scripps Research Institute) for providing purified human placen-
tal IIb3a.

REFERENCES

1. Du, X., Gu, M., Weise, J. W., Nagaswami, C., Bennett, J. S., Bowditch, R.,
and Ginsberg, M. H. (1993) J. Biol. Chem. 268, 23067–23092
2. Niewiarowski, S., McLane, M. A., Kloczewiak, M., and Stewart, G. J. (1994)
Semin. Hematol. 31, 289–300
3. Plow, E. F., D’Souza, S. E., and Ginsberg, M. H. (1992) Semin. Thromb.
Hemostasis 18, 324–332
4. Tomiyama, Y., Tsubakio, T., Piotrowicz, R. S., Kurata, Y., Lotus, J. C., and
Kunicki, T. J. (1992) Blood 79, 2303–2312
5. Ginsberg, M. H., O'Toole, T. E., Lotus, J. C., and Plow, E. F. (1992) Cdd
Spring Harbor Symp. Quant. Biol. 57, 221–232
6. Abrams, C., Deng, Y.-J., Steiner, B., O’Toole, T., and Shattil, S. J. (1994)
J. Biol. Chem. 269, 18781–18788
7. Kunicki, T. J., Ely, K. R., Kunicki, T. C., Tomiyama, Y., and Annis, D. S. (1995)
J. Biol. Chem. 270, 16660–16665
8. Bennett, J. W., Vilaire, G. (1979) J. Clin. Invest. 64, 1393–1401
9. Ginsberg, M. H., Du, X., and Plow, E. F. (1992) Curr. Opin. Cell Biol. 4,
766–771
10. Markwell, M. K., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978)
Anal. Biochem. 87, 206–210
11. Smith, J. W., Vilaire, D. J., Irwin, S. V., Burke, T. A., and Cheresh, D. A. (1990)
J. Biol. Chem. 265, 11008–11013
12. Bajt, M. L., and Loftus, J. C. (1994)
13. Wencel-Drake, J. D., Plow, E. F., Zimmerman, T. S., Painter, R. G., and
Kunicki, T. J. (1992)
14. Stenberg, P. E., Shuman, M. A., Levine, S. P., and Bainton, D. F. (1984)
J. Clin. Invest. 64, 2268–2273
15. Kirchhofer, D., Gailit, J., Ruoslahti, E., Grzesiak, J., and Pierschbacher, M. D.
J. Biol. Chem. 269, 156–164
16. Kodandapani, R., Veerapandian, B., Kunicki, T. J., and Ely, K. R. (1995)
Curr. Opin. Cell Biol. 7, 2303–2312
17. Steinberg, P. E., Shuman, M. A., Levine, S. P., and Bainont, D. F. (1984) J.
Cell Biol. 98, 748–760
18. Kirchhofer, D., Gailit, J., Ruoslahti, E., Grzesiak, J., and Pierschbacher, M. D.
(Cold Spring Harbor Symp. Quant. Biol. 57, 221–232)
19. Abrams, C., Deng, Y.-J., Steiner, B., O'Toole, T., and Shattil, S. J. (1994)
J. Biol. Chem. 269, 18781–18788
20. Kunicki, T. J., Ely, K. R., Kunicki, T. C., Tomiyama, Y., and Annis, D. S. (1995)
J. Biol. Chem. 270, 16660–16665
21. Bennett, J. W., Vilaire, G. (1979) J. Clin. Invest. 64, 1393–1401
22. Ginsberg, M. H., Du, X., and Plow, E. F. (1992) Curr. Opin. Cell Biol. 4,
766–771
23. Markwell, M. K., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978)
Anal. Biochem. 87, 206–210
24. Smith, J. W., Vilaire, D. J., Irwin, S. V., Burke, T. A., and Cheresh, D. A. (1990)
J. Biol. Chem. 265, 11008–11013
25. Bajt, M. L., and Loftus, J. C. (1994) J. Biol. Chem. 269, 20913–20919
26. Komlos-Drake, J. D., Plow, E. F., Zimmerman, T. S., Painter, R. G., and
Ginsberg, M. H. (1984) Am. J. Pathol. 115, 156–164
27. Steinberg, P. E., Shuman, M. A., Levine, S. P., and Bainont, D. F. (1984) J.
Cell Biol. 98, 748–760
28. Kirchhofer, D., Gailit, J., Ruoslahti, E., Grzesiak, J., and Pierschbacher, M. D.
(Cold Spring Harbor Symp. Quant. Biol. 57, 221–232)
29. Abrams, C., Deng, Y.-J., Steiner, B., O’Toole, T., and Shattil, S. J. (1994)
J. Biol. Chem. 269, 18781–18788
30. Kunicki, T. J., Ely, K. R., Kunicki, T. C., Tomiyama, Y., and Annis, D. S. (1995)
J. Biol. Chem. 270, 16660–16665
31. Bennett, J. W., Vilaire, G. (1979) J. Clin. Invest. 64, 1393–1401
32. Ginsberg, M. H., Du, X., and Plow, E. F. (1992) Curr. Opin. Cell Biol. 4,
766–771
Conformation-sensitive $\alpha_{IIb}\beta_3$ Ligands

(1987) J. Clin. Invest. 80, 1624–1630
19. Chan, B. M. C., and Hemler, M. E. (1993) J. Cell Biol. 120, 537–543
20. Savage, B., Shattil, S. J., and Ruggeri, Z. M. (1992) J. Biol. Chem. 267, 11300–11306
21. Haverstick, D. M., Cowan, J. F., Yamada, K. M., and Santoro, S. A. (1985) Blood 66, 946–952
22. Savage, B., and Ruggeri, Z. M. (1991) J. Biol. Chem. 266, 11227–11233
23. Charo, I. F., Nannuzzi, L., Phillips, D. R., Hsu, M. A., and Scarorough, R. M. (1991) J. Biol. Chem. 266, 1415–1421
24. Kloczewiak, M., Timmons, S., Bednarek, M. A., Sakon, M., and Hawiger, J. (1989) Biochemistry 28, 2915–2919
25. Bowditch, R. D., Hariharan, M., Tomlinna, E. F., Smith, J. W., Yamada, K. M., Getzoff, E. D., and Ginsberg, M. H. (1994) J. Biol. Chem. 269, 10856–10863
26. Bowditch, R. D., Halloran, C. E., Aota, S., Obara, M., Plow, E. F., Yamada, K. M., and Ginsberg, M. H. (1991) J. Biol. Chem. 266, 23323–23328
27. Chen, Y.-P., O'Toole, T. E., Shipley, T., Forsyth, J., LaFlamme, S. E., Yamada, K. M., Shattil, S. J., and Ginsberg, M. H. (1994) J. Biol. Chem. 269, 18307–18310
28. Haung, M., Lipfert, L., Cunningham, M., Brugge, J. S., Ginsberg, M. H., and Shattil, S. J. (1993) J. Cell Biol. 120, 537–543
29. O'Toole, T. E., Katagiri, Y., Tamura, R. H., Quaranta, V., Lofts, J. C., Shattil, S. J., and Ginsberg, M. H. (1994) J. Cell Biol. 124, 1047–1059
30. D'Souza, S. E., Ginsberg, M. H., Burke, T. A., Lam, S. C. T., and Plow, E. F. (1988) Science 242, 91–93
31. Lofts, J. C., O'Toole, T. E., Plow, E. F., Glass, A., Frelinger, A. L., III, and Ginsberg, M. H. (1990) Science 249, 915–918
32. D'Souza, S. E., Ginsberg, M. H., Burke, T. A., and Plow, E. F. (1990) J. Biol. Chem. 265, 3440–3446
33. D'Souza, S. E., Ginsberg, M. H., Matsueda, G. R., and Plow, E. F. (1991) Nature 350, 66–68
34. De Nichilo, M. O., Shafrin, D. R., Carter, W. M., Berndt, M. C., Burns, G. F., and Boyd, A. W. (1996) J. Immunol. 156, 284–288