Activation of the Integrin $\alpha_v\beta_3$ Involves a Discrete Cation-binding Site That Regulates Conformation*

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“Activation” of integrins is involved in the dramatic transition of leukocytes and platelets from suspension to adhesion. The integrin $\alpha_v\beta_3$ is not known to take part in this sort of transition, even though it shares its $\beta$ subunit with $\alpha_{11}\beta_3$, the activating integrin on platelets. In the context of a constitutively adhered cell, changes in activation state may be more subtle in their effects, but nonetheless important in regulating cell behavior. We hypothesized that $\alpha_v\beta_3$ can undergo conformational changes analogous to those associated with $\alpha_{11}\beta_3$ activation. Accordingly, we examined $\alpha_v\beta_3$ on the surface of M21 cells (a human melanoma cell line) and found that, like $\alpha_{11}\beta_3$, it can undergo conformational changes upon binding of a ligand analog and can be activated for ligand binding and migration by a monoclonal antibody directed against $\beta_3$. Modulation of the binding of this activating antibody, AP5, ligand binding, and antibody-directed activation all are associated with a discrete cation-binding site shared in both $\alpha_{11}\beta_3$ and $\alpha_v\beta_3$. Based on a measured $K_a$, this site has an apparent $K_a$ for calcium of approximately 20 $\mu$M. At physiological levels of calcium, about 40% of the total $\alpha_v\beta_3$ on a cell’s surface is in a conformation detected by AP5. The data suggest a model for both $\alpha_v\beta_3$ and $\alpha_{11}\beta_3$ function in which the molecule can exist in either of (at least) two conformational states, one stabilized either by AP5 or ligand binding, refractory to calcium binding, and enhanced for ligand recognition, the other stabilized by calcium binding and refractory to AP5 and ligand binding. Functional analysis suggests that AP5 activates $\alpha_v\beta_3$ by preventing occupation of this calcium site, and that the activated form of $\alpha_v\beta_3$ differs functionally from the basal form. The active form is more conducive to migration and the basal to tight adhesion.

Integrins comprise a large family of heterodimeric cell-surface receptors involved in cell-matrix and cell-cell interactions. These receptors mediate adhesion and modulate the cell’s responses to various adhesive ligands (for review, see Ref. 1). The integrin’s role in mediating information flow currently has become a field of growing interest. Several potentially significant biochemical changes are known now to be regulated by integrins. Forexample, manganese ($Mn^{2+}$) appears to enhance the adhesive properties of many integrins (9–11) although the mechanism of this enhancement is not clear. A model has been proposed by Smith and co-workers (9, 12) that a cation must be displaced from the integrin $\alpha_v\beta_3$ as ligand binds. In this model, a calcium or other divalent cation is bound to the integrin and is necessary for proper function, but must be displaced as ligand binds.

Several integrins are conformationally complex molecules. That is, they can exist in different conformational states, or activation states, that affect ligand recognition. Examples of this included $\alpha_{11}\beta_3$ and some $\beta_3$ and $\beta_1$ integrins on leukocytes (13–16). In the case of $\alpha_{11}\beta_3$, binding of ligand analogs, such as RGD-containing peptides, induces a conformational change that can be detected by a change in the Stokes radius of the receptor (17). This change occurs concomitantly with the presentation of “LIBS,” or ligand-induced binding sites, which are epitopes recognized by monoclonal antibodies known as anti-LIBS antibodies (18, 19). “Activation” of $\alpha_{11}\beta_3$ results in a conformational change that can be detected by changes in fluorescence resonance energy transfer (14) and occurs concomitantly with increased efficiency of ligand binding and changes in ligand specificity (reviewed in Ref. 16). It is important to recognize that the so-called “resting” or “inactive” state of $\alpha_{11}\beta_3$ is a functional integrin (3, 20, 21). Therefore, we refer to it as the “basal” state. The binding of some anti-LIBS antibodies results in changes in $\alpha_{11}\beta_3$ consistent with activation; that is, the antibodies stimulate binding of ligand (Ref. 19 and references therein).

We recently showed for the case of $\alpha_{11}\beta_3$, that the conformational state can affect not only ligand recognition, but “outside-in” signals as well (20). We have hypothesized that integrins in general exist on the cell surface as subpopulations in different conformation, or activation, states with specific ligand-binding and signaling functions. Implicit in this hypothesis is the assumption that integrins on constitutively adhered cells can undergo changes in conformation, or activation state, similar to those seen for $\alpha_{11}\beta_3$. Although the role of integrin activation in the case of integrins that mediate the conversion from suspension to adhesion in blood cells is obvious, the effect of integrin activation in the context of an adherent cell might be less dramatic.

To explore this possibility, we have taken advantage of a conformation-sensitive antibody we have described previously. This antibody, AP5, binds a linear epitope corresponding to the first 6 amino acids of the $\beta_3$ subunit (22). It is an anti-LIBS for $\alpha_{11}\beta_3$, that is its epitope is presented following binding of calcium, and phosphorylation on tyrosine of a number of proteins (1–6) (for review, see Ref. 7). Integrins bind divalent cations, which are required for integrin function (8). In addition to this basic requirement of divalent cations, specific divalent cations have been suggested to regulate integrins. For example, manganese ($Mn^{2+}$) appears to enhance the adhesive properties of many integrins (9–11) although the mechanism of this enhancement is not clear. A model has been proposed by Smith and co-workers (9, 12) that a cation must be displaced from the integrin $\alpha_v\beta_3$ as ligand binds. In this model, a calcium or other divalent cation is bound to the integrin and is necessary for proper function, but must be displaced as ligand binds.

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Activation States of Integrin $\alpha_\beta_3$

the assumption that the binding of the antibody over the time measured constitutes all forward rate and therefore can be considered a measure of “initial velocity” of antibody binding. We independently determined that there was no significant off-rate for the antibody over the time course used (not shown). However, if a substantial off-rate component was present, a linear double-reciprocal plot would not be obtained. In all cases the $R^2$ values of the linear regressions were greater than 0.9 and generally greater than 0.9. Calculation of $K_i$ for calcium was performed by comparing the ratio of the slopes of the lines obtained using the relationship:

$$[I] \frac{(S_2/S_1 - 1)}{K_i} = 1$$

where $S_1$ and $S_2$ are the slopes of the line without and with inhibitor, respectively, and $[I]$ is the concentration of inhibitor present. This was necessary because the units of binding, MFI, could not be related directly to concentration of antibody-antigen complex with our instrument. However, the ratio of the slopes is dimensionless and could be used to calculate $K_i$. “Without inhibitor” is $1 \mu M$ Ca$^{2+}$, since this concentration resulted in maximal binding.

Adhesion Assay—The assay used was that of Calof and Lander (24). Briefly, this assay was performed with cells labeled with $^{35}$S-methionine adhered to polystyrene plates coated with the appropriate ligand or control. Following adhesion, nonadhered cells were removed from the plate by centrifugation in the inverted orientation at the indicated G force. Adhered cells are fixed, the plates are exposed to a phosphorimaging plate, and the remaining $^{35}$S (a measure of adhered cells) was quantitated on a phosphorimaging scanner (Applied Biosystems). For Fig. 9, the amount of adhesion obtained on bovine serum albumin (less than 2% maximal adhesion, see Fig. 2) was subtracted to obtain corrected “integrated volume,” a direct measure of cells adhered. In Fig. 2, integrated volume is expressed as a percentage of cells adhering to poly-l-lysine. Values are averages of 6 wells ± 1 S.D. Vitrinectin was obtained from Telios Pharmaceuticals (San Diego, CA), osteopontin made from a recombinant fusion protein was the kind gift of Drs. J effrey Smith and Dana Hu of the La jolla Cancer Research Foundation, and fibrinogen was the kind gift of Dr. Z. Ruggeri of The Scripps Research Institute. Recombinant poly-l-lysine was from Sigma. Ligands were coated at the indicated concentrations for 4 h. Plates were washed once with PBS and blocked with 2% heat-denatured bovine serum albumin (Sigma) for 2 h. Cells were pretreated with 100 $\mu M$ AP5 in 1 $\mu M$ calcium DPBS for the indicated times on ice. Adhesion was carried out in 100 $\mu M$ calcium DPBS or as indicated. Values for counts bound and background were calculated by the Applied Biosystems software.

Migration Assay—The assay employed was a “Transwell” plate assay (Transwell plates with a pore size of 8 $\mu M$, Costar, Cambridge, MA). The lower side of the membrane was coated with VN or Opi as indicated for 3 h at room temperature. Both sides were blocked with 1% bovine serum albumin for 1 h. Cells were removed from growth plates as above and treated with 100 $\mu M$ AP5 for 20 min on ice in 10 $\mu M$ calcium DPBS, or mock-treated. 5 x 10$^4$ cells were added per well in the indicated buffer and centrifuged briefly to bring them in contact with the membrane. Following a 20-h incubation at 37°C, cells were fixed and stained with “Diff-quick” (Baxter Scientific), and cells were removed from the top surface of the membrane with cotton swabs. Cells that had migrated to the lower side of the membrane were counted; values presented reflect the averages from at least 25 fields ± 1 S.D.

RESULTS

AP5 Is an Activating, Anti-LIBS Antibody for $\alpha_\beta_3$—The human melanoma cell line, M21, which expresses high levels of $\alpha_\beta_3$ (11, 25) but no detectable $\alpha_\beta_5$ (11, 25)$^1$ was used in these studies. AP5, directly labeled with FITC, was incubated with M21 cells at the indicated concentrations either in the presence or absence of a cyclic RGD analog. G4120 at 40 $\mu M$ (23). G4120 binds with high affinity to both $\alpha_\beta_3$ and $\alpha_\beta_5$ (23) and stimulates binding of AP5 to cells expressing $\alpha_\beta_3$ (20). Following the incubation and washes, bound AP5 was quantitated by flow cytometry. Fig. 1A shows histograms of fluorescence distribution for 10,000 cells incubated either with a control antibody or 10 $\mu g/\mu l$ AP5 with or without G4120. M21 cells comprise a single, homogeneous population of cells with respect to AP5 binding, an indication of “error.” Binding to all cells is increased dramatically by the inclusion of G4120. Fig. 1B shows mean fluorescence intensities (MFI) taken from similar histo-

1 A. J. Pelletier, unpublished data.
2 The abbreviations used are DPBS, Dulbecco’s phosphate-buffered saline; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity; Opi, osteopontin; VN, vitrincin.
Activation States of Integrin α₃β₃

Methods). The assay for adhesion we employed was that of Calof and Lander (24), which includes a low speed centrifugation of the inverted plate to remove unbound or loosely adhered cells. The results, expressed as the percent of cells that remain adhered following 5 min at 100 × g, are shown in Fig. 2. AP5 increased the adhesion of cells to both substrata under these conditions. We have performed more conventional plate assays with similar results on fibrinogen as well (data not shown).

Adhesion to fibrinogen, Opn, and Vn in these latter assays was inhibited completely by inclusion of 40 μM G4120 to block α₃β₃, LM609 (150 μg/ml), a blocking, anti-α₃β₃ antibody, inhibited 100% of the adhesion to Opn and about 85% of adhesion to Vn. This residual adhesion probably was due to α₃β₃ on the surface of M21 (11, 25). For this reason, Opn adhesion was used in most subsequent assays.

AP5 Binding Is Inhibited by Calcium—We previously reported that the reactivity of AP5 toward α₃β₃ is inhibited by calcium (22). We next determined if the same is true for α₃β₃. Fig. 3 shows the same graph of MFI versus AP5 concentration in the presence or absence of 40 μM G4120 as is shown in Fig. 1B. That experiment was performed in DPBS, which includes 900 μM CaCl₂. At the same time, a parallel experiment was performed in DPBS lacking CaCl₂ (all other components of DPBS were included). Under these conditions, binding of AP5 is constitutively high and is insensitive to the addition of G4120. Since the effect of G4120 and that of low calcium are not additive, the ligand analog must act directly to overcome the effect of the calcium, rather than through an independent mechanism. Thus, the binding of calcium to α₃β₃ inhibits the presentation of this ligand-induced binding site, and the ligand analog overcomes that inhibition.

Calcium is not exerting its effect on AP5 directly. In equilibrium dialysis experiments, no binding of ⁴⁵Ca to AP5 was detected at concentrations that inhibit binding of AP5 to α₃β₃ (data not shown). Also, since the effect of G4120 clearly is on the receptor, we would expect low calcium and G4120 to have additive effects on the overall binding of the antibody if the effect of the calcium were on the antibody. Furthermore, results from detailed analysis of the inhibition (below) are inconsistent with calcium exerting its effect on AP5.

Fig. 4 shows the effect of calcium concentration over a range from 100 nM to 2 mM on the binding of AP5. Starting with a calcium-minus "DPBS," buffers of the indicated calcium concentration were made. The binding of AP5 is maximal at approximately 1 μM Ca²⁺ and is inhibited significantly at 10 μM.
We performed a similar test of the effect of calcium on AP5 binding over a range of AP5 concentrations from 5–140 μg/ml, which is shown in Fig. 5A. These data show that even at high levels of calcium (900 μM) at which the inhibitory effect of calcium has reached a plateau, AP5 still binds in a dose-dependent manner. The shape of the curves suggests that there are two different effects of calcium, one in the range of 1–100 μM and a second effect at the higher concentrations.

We examined the inhibition of AP5 binding by calcium by expressing the data as a double-reciprocal plot of 1/[AP5] by 1/MFI for the indicated concentrations of calcium between 1 and 100 μM. This can be done because the off reaction of the antibody is not significant over the period of the experiment and thus the binding could be considered to be all forward (not shown, but note that a linear double reciprocal plot could not be obtained if a significant off reaction existed). The results shown in Fig. 5B show that AP5 and calcium binding are mutually exclusive in this range. That is, calcium inhibits AP5 binding and vice versa. This generally is termed “competitive” inhibition. Such an effect does not necessarily imply that the two molecules compete for the same site, but does require that the two sites be linked structurally. From the ratio of the slopes of the lines, we calculated the $K_i$ of calcium. Data from several independent experiments, including that shown in Fig. 5B, resulted in a calculated $K_i$ of 22 ± 8 μM.

At higher concentrations (300 and 900 μM), the character of the inhibition changes; this is the second, high concentration effect mentioned above. The predominant effect at high concentrations of calcium is that the number of sites available for AP5 binding (indicated by the y-intercept) is decreased to approximately 40% of the total. Note that two lines corresponding to 300 and 900 μM calcium are essentially identical, indicating that the inhibitory effect of Ca$^{2+}$ on AP5 binding had reached a plateau, and that this resistant 40% remains accessible to AP5 even at high calcium concentrations.

In true competitive inhibition, the off-rate of AP5 should be insensitive to calcium. The point at which the lines intersect falls slightly to the left of the y axis. This result, consistent in several experiments, could be explained if calcium had a small effect on the off rate of AP5 such that bound AP5 was being lost during the high calcium wash steps. Again, this off-rate would have to be extremely low, or the linear double-reciprocal plots would not be obtained. The left-shift was eliminated if washes...
were carried out in low calcium buffer (data not shown), indicating that the effect was occurring largely during the wash steps.

Our data indicate that calcium and AP5 do not sterically compete for the same physical site on the receptor, yet inhibit each other's binding. This is likely to occur via conformational changes in the receptor. Nonetheless, our data demonstrate that the calcium- and AP5-binding sites are linked structurally.

Effects of Magnesium and Manganese on AP5 Binding—Since AP5 is an activating, anti-LIBS antibody, we reasoned that the conformational changes induced by the binding of Ca\(^{2+}\) that inhibited AP5 binding might be related to calcium's reported inhibition of ligand recognition. We therefore tested whether other divalent cations had effects on AP5 binding to \(\alpha_v\beta_3\) consistent with their effects on ligand binding. Fig. 6 shows that the addition of Mg\(^{2+}\) had no effect on AP5 binding up to a concentration of 5 mM. This is in contrast to the situation with \(\alpha_{IIb}\beta_3\) on platelets, in which high levels of Mg\(^{2+}\) did have an inhibitory effect, albeit a small one, on the binding of AP5 (22). Moreover, in the case of \(\alpha_v\beta_3\), the presence of Mg\(^{2+}\) in the buffer had no effect on the inhibition of AP5 binding caused by Ca\(^{2+}\). This indicates that Mg\(^{2+}\) does not inhibit Ca\(^{2+}\) binding at this site.

In contrast, manganese does reverse the inhibitory effect of calcium. Fig. 7 shows that, at low calcium, manganese does not increase binding of AP5 to M21 cells (in fact, it has a small, but reproducible, inhibitory effect). However, the inhibitory effect of calcium is abrogated partially by 2 mM Mn\(^{2+}\). These data are consistent with manganese inhibiting the binding of calcium to its site, though the mechanism cannot be determined. Mn\(^{2+}\) may bind to a separate site and inhibit calcium binding non-competitively, or it may compete directly with calcium.

AP5 Stimulates Migration by Antagonizing the Inhibitory Effects of Calcium—Now that we have shown that an integrin on a constitutively adhered cell can be activated, the question remains, what is the function of the active integrin in this context? To address this, we looked at migration. Migration was assessed by a Transwell assay in which the bottom of the membrane was coated with 2 \(\mu\)g/ml Opn. We examined migration in either 1 mM or 2 mM calcium, since calcium has been shown previously to inhibit adhesion and migration on Opn mediated by \(\alpha_v\beta_3\) (26). In 1 mM calcium, migration is enhanced by activation of \(\alpha_v\beta_3\) by AP5 (Fig. 8). Similar results have been obtained on Vn (data not shown). Consistent with previous results, higher calcium inhibited migration of nonactivated cells. However, those cells on which \(\alpha_v\beta_3\) had been activated were not affected by the high calcium. These data provide evidence that AP5 activates \(\alpha_v\beta_3\) by antagonizing the binding of calcium to an inhibitory site.

An advantage of the Calof and Lander (24) adhesion assay is that the “wash” step is centrifugation of the plate in the inverted position, which can be varied in a precise manner. This allows us to measure both the efficiency and strength of adhesion of an intact cell. We compared the ability of the basal and active receptors to bind to Opn under increasing G force. The results, shown in Fig. 9 demonstrate the somewhat surprising result that the active receptor is less able to resist force and therefore mediates weaker binding to Opn. As G force is increased, the added adhesion of AP5-treated cells is lost. Thus, adhesion via the active receptor is efficient but weak.

**DISCUSSION**

In this work, we report that the integrin \(\alpha_v\beta_3\) like \(\alpha_{IIb}\beta_3\) undergoes conformational changes related to LIBS presentation and activation. AP5, an antibody that activates \(\alpha_{IIb}\beta_3\), also can activate \(\alpha_v\beta_3\) as measured by increased \(\alpha_v\beta_3\)-mediated adhesion of cells to various ligands and by increased migration. These changes in conformation associated with LIBS presentation and activation can be regulated by a discrete cation-
Activation States of Integrin $\alpha_\beta_3$

binding site, the $K_v$ of which is expected to be approximately 20–25 $\mu$M for calcium, based on our $K_v$ data. Functional analysis suggests that AP5 activates $\alpha_\beta_3$ by preventing the inhibitory effects of calcium. Finally, our data indicate that activation of integrins can be more than a simple on/off switch.

We have reported previously that RGD analogs increase both the apparent affinity and total number of sites bound by AP5 (20, 22). Our data here suggest that low calcium recapitulates this effect. At low levels of calcium, below 100 $\mu$M, AP5 and calcium appear to compete for binding of $\alpha_\beta_3$. However, at calcium concentrations between 300 and 900 $\mu$M, levels still below physiological calcium, the inhibition no longer appears competitive; increasing levels of AP5 no longer can overcome the inhibition. This could be due to two separate cation-binding sites, both affecting AP5 binding. Rather than propose that AP5 is sensitive to calcium binding at two sites, we think the simpler explanation for this is that at millimolar calcium concentrations, the 20 $\mu$M calcium-binding site is occupied effectively 100% of the time, thereby not allowing AP5 access to 60–70% of the receptor at all.

At concentrations of calcium above 300 $\mu$M, only about 30–40% of the total $\alpha_\beta_3$ is accessible to AP5 at all, and this 30–40% remains accessible even up to 10 $\mu$M calcium (not shown, but note that there is essentially no change in the binding of AP5 between 300 and 900 $\mu$M calcium). Thus, AP5 reveals two distinct populations of $\alpha_\beta_3$ on the cell surface under physiological calcium conditions. The AP5-positive population of receptor must be maintained in that conformation by some mechanism, perhaps regulated by the cell. The significance of the AP5-accessible population is not yet known, although it should be noted here that it is not strictly speaking an “activated” population.

The inhibition of AP5 binding by calcium at low concentrations shows the hallmarks of competitive inhibition. However, we have shown that AP5 binds to a linear epitope comprising the first 6 amino acids of $\beta_3$ GPNICT (22). This sequence is extremely unlikely to bind a divalent cation directly, since it lacks any acidic residues and is near a basic residue, Arg-8. Furthermore, the data in Fig. 5, when examined in detail, suggest that AP5 and calcium do not compete directly for the same site. Residue Cys-5 is predicted to take part in a long-range disulfide bond (27) which suggests that this epitope is part of a complex tertiary structure. A likely explanation for the mutually exclusive binding of calcium and AP5 is that each binds only one of two conformations of the receptor and, when bound, stabilizes that conformation. In stabilizing its preferred conformation of the receptor, each molecule inhibits the binding of the other.

Since this calcium site is present in both $\alpha_\text{IIb} \beta_3$ and $\alpha_\beta_3$, it must be a structure common to both heterodimers. It seems likely that this site resides at least in part within the $\beta_3$ molecule itself, although the $\alpha_\alpha$ and $\alpha_\text{IIb}$ chains may be directly involved.

A specific cation-binding domain has been proposed for the $\beta_3$ subunit (28, 29). This site was originally proposed to be an “E-F hand” type of cation-binding domain, but mutational analysis was not consistent with this hypothesis (28, 29). This same region of $\beta_3$ shares considerable secondary structure similarity with another divalent cation-binding domain, the “A” domain of the integrin $\alpha$ subunit MAC-1. The crystal structure of this domain recently has been solved (30). Coordination of the metal in this domain is incomplete, with the last coordination site provided by the adjacent molecule in the crystal lattice. This is a symmetry-related artifact, and the authors suggest that in the normal case this last coordination site is provided by ligand. The authors name this motif a MIDAS domain, for metal ion-dependent adhesion site.

A second possible E-F hand metal coordination site has been proposed to comprise residues Ser-211–Gln-227 in $\beta_3$ (28). Interestingly, this sequence resides within the overall structure containing the putative “MIDAS” domain in $\beta_3$ (30) in a proposed hydrophilic loop between $\alpha$-helix 4 and $\beta$-strand D in the secondary structure prediction. Cierniewski et al. (28) reported that a short peptide corresponding to this sequence did not bind terbium, a trivalent surrogate for calcium. However, the failure of a short peptide to bind a trivalent cation such as terbium does not exclude the possibility that the same sequence in the context of the whole protein coordinates divalent cations.

As an assay, AP5 binding has the advantage of permitting assessment of calcium binding within the intact heterodimer. Therefore, analysis of mutants in the $\beta_3$ subunit, or even in the $\alpha_\alpha$ and $\alpha_\text{IIb}$ subunits, should be useful in defining the location of the inhibitory calcium-binding site and in testing which model (the MIDAS or the E-F hand) best fits the cation-binding site in $\alpha_\beta_3$.

Smith and co-workers (9) have reported that manganese stimulates binding of ligand to $\alpha_\beta_3$ and that calcium is a mixed-type inhibitor of the manganese-mediated stimulation. They also report (26) that calcium directly inhibits $\alpha_\beta_3$ binding to osteopontin. Our data show that manganese antagonizes the ability of calcium to inhibit AP5 binding. It seems likely that our observation and those of Smith and co-workers (9) are related and that manganese and calcium antagonize each other’s binding to $\alpha_\beta_3$. Whether this is by direct competition for the same site or not, we currently cannot say.

Calcium Regulation of $\alpha_\gamma$ and $\alpha_\text{IIb} \beta_3$ Conformation and Ligand Binding: A Structural Link between a Discrete Calcium-Binding Site, the AP5-LIBS, and Receptor Activation—Our data, in conjunction with prior published work, suggest that the inhibitory calcium site proposed by Smith and co-workers (9) and the AP5 LIBS are structurally linked. Furthermore, they suggest a specific mechanism whereby AP5 might activate $\beta_3$ integrins: AP5 binding to $\alpha_\beta_3$ or $\alpha_\text{IIb} \beta_3$ prevents occupation of this regulatory calcium site and increases binding to the ligand.

Available data on integrin-ligand interactions on cells indicates that the effect of activation is on the kinetics of the interaction, not the thermodynamics. For example, Cai and Wright (31) showed that antibody binding could not contribute the energy necessary to account for the increase in ligand
binding observed for activated $\beta_3$ if the increase was due to an increase in affinity (that is, a change in the thermodynamics or a $\Delta G$). To explain some of the functional distinctions between the active and basal forms of $\alpha_\beta_3$, we offer the following formalism:

$$k_1 \frac{[I][L]}{[IL]} \xrightarrow{k_{-1}} IL^*$$  
(Eq. 2)

where $I$ and $L$ are integrin and ligand, $[IL]$ is an intermediate bound form, and $IL^*$ is a functionally irreversible bound form. $k_2$ is a zero order rate constant with an unknown $T_{\beta_3}$. The reaction that would be given by a $k_{-2}$ is negligible and may require input chemical energy. Note that the process to irreversible binding may go through more than one intermediate state, with associated rate constants.

The less efficient but strong adhesion via the basal receptor could be explained if $k_2 \gg k_{-1}$ and $k_1$ is relatively low and rate-limiting for formation of $IL^*$. The initial interaction is slow, and effectively all receptors that reach $[IL]$ proceed to $IL^*$ or irreversible binding. The efficient but weak adhesion via the active receptor could be explained if the active conformation resulted in increased $k_1$ and $k_{-1}$, such that $k_{-2}$ is significant compared to $k_2$. Additionally, $k_1$ could be increased and $k_2$ significantly decreased with the same net result. In either case, $k_2$ becomes rate-limiting for formation of the $IL^*$. Thus, with no change in affinity and therefore no requirement that the activating antibody contributes energy, the observed properties of the active receptor could be achieved.

What Is the Role of “Activation States” in Adherent Cells?—
Activation of integrins is involved in the dramatic transition from suspension to adhesion exhibited by platelets (reviewed in Ref. 6), leukocytes (13, 32), and macrophages (33). In contrast, $\alpha_\beta_3$ is not known to take part in this sort of transition. We view the dramatic examples of activation as specialized adaptations of a property shared with other integrins.

A commonly held view is that integrins that mediate adhesion of constitutively adhered cells must be activated, since they function. We find, however, that both the active and basal forms of $\alpha_\beta_3$ are functional, but are functionally distinct. In the case of M21 cells, activation appears to stimulate adhesion that is more efficient, but weaker, than adhesion via the basal integrin and to facilitate migration. Therefore, activation is not a simple on/off switch.

Implicit in our model is the prediction that a given integrin exists on the surface of the cell in different populations, which generally have been known as “activation states.” In fact, sub-sets of integrins can be distinguished antigenically, for example, by AP5, as well as biochemically and functionally. For example, $\alpha_1\beta_3$ is phosphorylated in response to various stimuli (34), and ADP-ribosylation has been reported on another integrin, $\alpha_\beta_3$ (35). In these cases, the biochemical modification has been detected on a subset only, between 5 and 40% of that integrin. Substoichiometric modification of an integrin makes sense if the modified integrin has a specific function not requiring all the receptors; for example, initiating a specific signaling cascade. In fact, our recent work on $\alpha_{11}\beta_3$ suggests that integrins in different activation states are associated with different signaling pathways (20).

The conclusion we draw from all of these data is that integrins are structurally complex, existing in discrete forms, or activation states, that differ functionally. We hypothesize that these states contribute to cell regulation both by altering ligand recognition and by initiating specific signals that lead to changes in cell behavior.

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