An Allosteric Ca$^{2+}$ Binding Site on the $\beta3$-Integrins That Regulates the Dissociation Rate for RGD Ligands*

(Received for publication, February 23, 1996, and in revised form, May 1, 1996)

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Here we use a model RGD-containing ligand to study how Ca$^{2+}$ and Mg$^{2+}$ regulate ligand binding to $\beta3$-integrins. Fab-9, an antibody that contains an optimized RGD loop in its antigen binding site (Barbas, C. F., Languino, L., and Smith, J. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10003-10007), was used as the model ligand. Across a physiologic range of Mg$^{2+}$, Fab-9 bound to both $\alpha_v\beta3$ and $\alpha1\beta3$ with a monophasic binding isotherm. Across the same range of Ca$^{2+}$, the binding of Fab-9 to the $\beta3$-integrins was biphasic. Low concentrations of Ca$^{2+}$ ($\mu$M) promoted the binding of Fab-9. Higher concentrations of Ca$^{2+}$ ($\mu$M) and Mg$^{2+}$ ($m$M). The Fab-9 binding data suggest that Ca$^{2+}$ binds to two distinct classes of sites on the $\beta3$-integrins, with the low affinity Ca$^{2+}$ binding site(s) being an inhibitory site. We designate this inhibitory site(s) as the I site. Further biochemical characterization showed that the I site has the following characteristics: (1) it is specific for Ca$^{2+}$; (2) it is allosteric to the ligand binding site; (3) its occupation increases the dissociation rate between integrin and RGD ligand; and (4) occupation of the I site can induce cellular deadhesion.

Integrins are $\alpha\beta$ heterodimers that mediate cell adhesion to the extracellular matrix (1, 2). The subjects of this study are two integrins containing the $\beta3$ subunit, $\alpha_v\beta3$ and $\alpha1\beta3$. These two integrins bind the Arg-Gly-Asp (RGD) motif, and both are associated with several significant tissue remodeling events. The platelet integrin $\alpha1\beta3$ mediates platelet aggregation and also platelet adhesion to the subendothelial matrix (3, 4). Since platelets are a major constituent of thrombi, $\alpha1\beta3$ is thought to play a causal role in myocardial infarction, stroke, and atherogenesis. The $\alpha_v\beta3$ integrin is expressed on osteoclasts, tumor cells, and angiogenic endothelial cells. On osteoclasts, $\alpha_v\beta3$ directs adhesion to the bone matrix and thus initiates bone resorption (5, 6). The expression of $\alpha_v\beta3$ on tumors is associated with their metastatic potential presumably because $\alpha_v\beta3$ can mediate cell migration on numerous extracellular matrix proteins. The $\alpha_v\beta3$ integrin is also apparently required for angiogenesis, and it has been postulated that inhibitors of this integrin could prevent tumor growth by interfering with tumor vascularization (7, 8).

Extracellular divalent cations regulate integrin function by controlling the integrin ligand binding event. The integrins contain three different types of structural motifs that can bind divalent ions. The first type is homologous to the EF-hand Ca$^{2+}$ binding motifs (9). There are three or four EF-handlike sites contained within the integrin $\alpha$ subunits (1, 10). A second metal binding motif, called an A domain is present only in selected integrin $\alpha$ subunits. This domain is about 200 residues in length and has considerable homology to an analogous domain in von Willebrand’s factor (11). The recent crystallization of the A domains of two integrins, Mac-1 and LFA-1, showed that this protein module contains a divalent ion (Mg$^{2+}$ or Mn$^{2+}$) bound at the apex of a dinucleotide binding motif (12, 13). The third type of cation binding motif is present within the amino terminus of the integrin $\beta$ subunits. This site, originally identified by Loftus et al. (14), is essential for ligand recognition and was thought to be a modified EF-hand motif. However, it is now thought that the $\beta$ subunit cation binding site is similar to the metal-binding A domain in both $\alpha$M and $\alphaL$ (12, 13).

Although each of these structural motifs is known to bind ions, it is not clear how all of the sites coordinate to regulate the ligand binding event. It must be emphasized that ions can have opposing effects on ligand binding to integrins. Divalent ions are certainly required for ligand binding, but in many cases Ca$^{2+}$ has been shown to inhibit both ligand binding and cell adhesion (15–17). Consequently, a current challenge is to understand the distinction between the cation binding sites that promote ligand binding and those that interfere with ligand binding. Three prior reports have addressed the mechanism by which Ca$^{2+}$ interferes with integrin function. Staatz et al. (16) showed that Ca$^{2+}$ is a noncompetitive inhibitor of Mg$^{2+}$-supported collagen binding to the $\alpha2\beta1$ integrin, providing initial kinetic evidence that Ca$^{2+}$ and Mg$^{2+}$ could bind to different classes of sites on integrin. A second report by Mould et al. (18) also indicated the presence of functionally distinct ion binding sites, one of which promotes ligand binding and another that can suppress ligand binding. A study from this laboratory attempted to address the mechanism of cation regulation by measuring kinetic constants for the binding of natural adhesive proteins to two $\beta3$-integrins (19). Because the binding between natural adhesive proteins and the $\beta3$-integrins rapidly becomes nondissociable (20, 21), we were only able to define the “apparent” association rate constant ($k_{app}$) between ligands and integrin. This rate constant is useful in that it describes the rate at which ligands become stably bound to integrin, but it is really a product of three rate constants, $k_1$, $k_2$, and $k_3$ (21). Therefore, measuring $k_{app}$ did not allow us to discern the fine kinetic detail of how divalent ions regulate ligand association and dissociation rates.

In this report we advance substantially the kinetic model of...
how cations regulate ligand binding to both αvβ3 and αIIbβ3 by using a model antibody ligand called Fab-9. This antibody was derived from a human antibody directed against human immunodeficiency virus gp120 (22). However, Fab-9 was engineered to contain an optimized integrin-binding RGD motif in the antigen binding site. Fab-9 was then optimized by phage display to have high affinity for both αvβ3 and αIIbβ3 (23, 24). Unlike natural adhesive proteins, which bind to integrin non-dissociably, Fab-9 binds to integrin in a reversible manner. This is presumably because Fab-9 is monomeric and also because it presents only a single contact point for integrin, the RGD-containing loop in heavy chain complementarity determining region 3. The unique features of Fab-9 and the application of surface plasmon resonance to measure binding in real time allowed us to measure the effects of divalent cations on the individual kinetic constants between Fab-9 and integrin. A key assumption in this study is that Fab-9 does not bind divalent ions. We believe this to be reasonable because the antibody from which Fab-9 was engineered binds to its ligand (human immunodeficiency virus gp120) in a cation-independent manner (22). In the current study, we measured the effects of both Ca\textsuperscript{2+} and Mg\textsuperscript{2+} on the binding of Fab-9 to both β3-integrins. In summary, this study reveals that there are two classes of cation binding sites on the two β3-containing integrins. The first class of sites promote ligand binding to integrin when occupied by divalent cations. The second class of sites are allosteric to the ligand binding site, are highly specific for Ca\textsuperscript{2+}, and when occupied, increase the dissociation rate between RGD and integrin. To our knowledge this is the first evidence showing that divalent cations regulate the dissociation rate for RGD ligand.

**MATERIALS AND METHODS**

Proteins—Integrin αvβ3 was purified from an octylglucoside extract of human placenta by monoclonal antibody affinity chromatography (25). Integrin αIIbβ3 was purified from human platelets by affinity chromatography on KYGRGDS-Sepharose (26). Fab-9 was generated from a combinatorial phage library as described previously (23, 24). Fab-9 was affinity-purified from bacterial lysate by antibody affinity chromatography using goat anti-human IgG-Sepharose. Fab-9 was radiolabeled with \(^{125}\text{I}\) using IODO-GEN (Pierce). Typical specific activities were between 3 and \(5 \times 10^5\) cpm/μg.

Cell Lines—Human embryonic kidney carcinoma 293 cells were obtained from ATCC. The 293 cells were transfected with the cDNA encoding the integrin β3 subunit in the pDNA3 expression vector using DOTAP transfection reagent (Boehringer Mannheim). Stable transfectants were obtained by fluorescence-activated cell sorting after selection in 500 μg/ml G418 (Sigma). Cells were maintained in Dulbecco’s modified Eagle’s medium (BioWhittaker, Inc.) supplemented with 10% fetal calf serum (Irvine Scientific), 20 μg/ml Hepes (pH 7.4), 1% glutamine, 1% penicillin, and 1% streptomycin (Sigma).

Ligand Binding Assays—The binding of Fab-9 to integrins was measured using a ligand binding assay that has been previously described (19). To study ligand binding as a function of cations, the type and concentration of divalent cation varied, and \(^{125}\text{I}\)-Fab-9 was used at a fixed concentration, 0.5 nM. To ensure that the immobilized integrin did not present two endogenously bound cations, the plate was washed with EDTA. The plate was then washed six times with cation-free binding buffer (50 mM Tris, 100 mM NaCl, and 1 mM Mg\textsuperscript{2+} bovine serum albumin, pH 7.4). Because the αIIbβ3 integrin cannot withstand exposure to EDTA, this integrin was washed six times with cation-free binding buffer to remove endogenously bound ions. \(^{125}\text{I}\)-Fab-9 was allowed to bind to integrin for 3 h at 37°C, at which time the binding had reached equilibrium. To measure the ability of cations to dissociate ligand from integrin, \(^{125}\text{I}\)-Fab-9 was allowed to bind integrin under the optimal cation conditions (0.05 mM Mn\textsuperscript{2+} for αvβ3 or 0.05 mM Ca\textsuperscript{2+} for αIIbβ3) for 3 h at 37°C. Then free ligand was removed by washing, and a dissociation solution containing the binding buffer and the desired cation(s) was added to the microtiter wells. Ligand dissociation was allowed to proceed for 90 min at 37°C. The amount of ligand remaining bound was determined by γ-counting as described previously (19). In all cases, nonspecific binding was measured in the presence of 20 μM EDTA and was subtracted from the total binding to yield specific binding. The data from these measurements were highly reproducible, with the differences between data points typically below 7%. Results are expressed as the average of triplicate data points, and all experiments were repeated at least three times.

Cell Adhesion Assays—Cell adhesion was measured using previously described methods (17). Purified Fab-9 was coated on to 96-well microtiter plates (Titertek) and incubated overnight at 4°C. The plates were then blocked with 20 mg/ml of bovine serum albumin in binding buffer for 1 h at 37°C. Cells were harvested from tissue culture flasks with phosphate-buffered saline/EDTA, washed and resuspended in adhesion buffer (1 × Hanks’ balanced salt solution, 50 μM Hepes (pH 7.4), 1 mg/ml bovine serum albumin) containing either Ca\textsuperscript{2+} or Mg\textsuperscript{2+} at the specified concentrations. In most experiments 100 μl of cells (1.5 × 10\(^6\) cells/ml) were added to each well. Nonspecific adhesion was measured in the presence of 20 mM EDTA. After a 45-min incubation at 37°C, the nonadherent cells were removed by gentle aspiration. Adherent cells were detached using a colormetric assay for lysosomal acid phosphatase activity with a chromophore that absorbs at 405 nm (27). A standard curve with cells in suspension showed that absorbance values were directly proportional to cell number. All experiments were performed at least three times, yielding identical results.

Surface Plasmon Resonance—The kinetic parameters (association and dissociation rate constants, \(k_1\) and \(k_2\), respectively) and the affinity constant (\(K_d\)) between Fab-9 and integrins were measured by the surface plasmon resonance technique (BIAcore Inc.). The application of surface plasmon resonance to measure kinetic constants for integrins has been described previously (17, 24). Briefly, integrin was coupled to the biosensor chip with the amine coupling kit. Ligand solution was applied to the sensor chip, and the binding was measured as a function of time. Both association and dissociation reactions were performed in Tris-buffered saline (50 mM NaCl, pH 7.4) containing specific cations. These measurements yielded rate constants, \(k_1\) and \(k_2\). The overall affinity constant, \(K_d\), was derived from \(k_1/k_2\). To study ligand dissociation as a function of cations, the initial binding reaction was performed in buffer containing 0.2 mM Mn\textsuperscript{2+}, and then the buffer was changed to Tris-buffered saline containing either Ca\textsuperscript{2+} or Mg\textsuperscript{2+} at the specified concentrations. The Ionic strength of the buffer was maintained at a constant level by adjusting the amount of NaCl.

**RESULTS**

Divalent Cations Differentially Regulate Fab-9 Binding to β3-Integrins—The binding of \(^{125}\text{I}\)-Fab-9 to αvβ3 was measured across a concentration range of Ca\textsuperscript{2+} or Mg\textsuperscript{2+}. Binding was measured at equilibrium. In Mg\textsuperscript{2+} the binding of \(^{125}\text{I}\)-Fab-9 to αvβ3 exhibits a “normal” isotherm, with the binding reaching saturation at 1–2 mM Mg\textsuperscript{2+} (Fig. 1A). A biphasic binding isotherm was observed across the same range of Ca\textsuperscript{2+}. In this cation, the binding of Fab-9 to αvβ3 increased with ion concentration, but at 0.1 mM Ca\textsuperscript{2+} the binding dissipated. At 5 mM Ca\textsuperscript{2+} very little specific binding of Fab-9 to αvβ3 was detected. This result indicates the existence of two classes of Ca\textsuperscript{2+} binding sites on the integrin. Occupation of the first class of sites with low concentrations of Ca\textsuperscript{2+} (less than 0.1 mM) promotes Fab-9 binding to integrin. At higher levels of Ca\textsuperscript{2+} (greater than 0.1 mM), the second class of sites are occupied, and ligand binding is inhibited. Thus, we call the second class of inhibitory or I sites. Identical results were obtained with the αIIbβ3 integrin (not shown). Using the same procedure, we also measured the binding between β3-integrins and several other RGD-containing antibodies obtained from phage libraries (24). In each case, a biphasic binding profile was observed as a function of Ca\textsuperscript{2+} concentration. Thus, the inhibition of ligand binding by Ca\textsuperscript{2+} appears to be a property common to ligands that present only a single RGD contact point.

The effects of different cation conditions on cell adhesion to immobilized Fab-9 were also measured. Kidney 293 cells expressing the αvβ3 heterodimer were allowed to adhere to Fab-9 across a concentration range of divalent ion (Fig. 1B). Mg\textsuperscript{2+} supported cell adhesion throughout the entire concentration range. In contrast, concentrations of Ca\textsuperscript{2+} above 0.1 mM inhibited cell adhesion to Fab-9. Therefore, divalent ions regulate ligand binding to both purified integrin and integrin in the cell.
first experiment, 125I-Fab-9 was allowed to bind to purified integrin αβ3 was measured as a function of either Ca²⁺ (●) or Mg²⁺ (△). These ligand binding studies were performed under equilibrium conditions as described under the “Materials and Methods.” Each ligand binding assay was repeated at least three times yielding identical results. B, the effect of Ca²⁺ (●) and Mg²⁺ (△) concentration on the adhesion of αβ3-expressing 293 cells to Fab-9 was measured. This adhesion assay was performed three times, each yielding a similar result.

Figure 2. Calcium is a competitive inhibitor of Fab-9 binding to β3-integrins. Kinetic measurements were performed to determine whether Ca²⁺ is a competitive or noncompetitive inhibitor of ligand binding. A, isotherms of 125I-Fab-9 binding to integrin αβ3 were generated at Ca²⁺ concentrations of 0.1 mM (●), 2 mM (△), 10 mM (▲), and 20 mM (★). Note the shift of apparent Kd and that each isotherm approaches the same level of binding at saturation. Several other isotherms were obtained at different concentrations of Ca²⁺ and displayed saturable binding. These isotherms are omitted from this figure for simplicity. B, a Dixon plot was generated from the data in A using the following concentrations of Fab-9: 0.3 nM (●), 1 nM (△), 2 nM(▲), and 5 nM (★). Each set of data were fitted by a linear regression program. The lines of the Dixon plot intersect behind the y axis above the origin, indicating competitive inhibition.

Ca²⁺ was able to promote half-maximal dissociation.

The experiments shown in Fig. 3, A and B, suggest that Ca²⁺ can induce the dissociation of Fab-9 from integrin. Because the readout from these studies was taken following a 90-min incubation with the ion (at steady state), Fab-9 could potentially dissociate and rebind during the second part of the assay. Consequently, the possibility remained that Ca²⁺ acted by preventing re-binding of Fab-9 to integrin. To distinguish between these possibilities and to quantify the effect of cations on the dissociation of Fab-9 from integrin, surface plasmon resonance measurements were made (Fig. 3, C and D). Plasmon resonance monitors a binding reaction in real time and can provide ligand association and dissociation constants separately. We sought to determine the effects of ions on ligand dissociation rate, which is described by the rate constant (k₋₁). After allowing binding between Fab-9 and integrin to reach equilibrium in 0.2 mM Mn²⁺, the buffer was changed to a dissociation buffer containing either 5 mM Ca²⁺ or 5 mM Mg²⁺ (Fig. 3, C and D, double hash mark). Ca²⁺ caused the rapid dissociation of Fab-9 from both αβ3 (k₋₁ = 2.7 × 10⁻³ s⁻¹) and α1β1β3 (k₋₁ = 1.1 × 10⁻¹ s⁻¹). Dissociation was considerably slower in Mg²⁺. For αβ3, the rate constant in Mg²⁺ (5.3 × 10⁻⁴ s⁻¹) was 5-fold lower than in Ca²⁺. The rate constant for α1β1β3 (1.6 × 10⁻⁴ s⁻¹) in Mg²⁺ was 7-fold lower than in Ca²⁺. We also measured the changes in ligand association rate across the range of Ca²⁺ that inhibits ligand binding. Across this range, the association rate constant between Fab-9 and αβ3 was not significantly affected (Table I). However, the dissociation rate constant between Fab-9 and integrin increased 12–15-fold. Ca²⁺ also increased the dissociation rate between Fab-9 and α1β1β3 across the same range of ion (not shown).
**An Inhibitory Ca$^{2+}$ Binding Site on $\beta_3$-Integrins**

**Fig. 3.** Ca$^{2+}$ increases the dissociation rate between Fab-9 and $\beta_3$-integrins. Measurements were taken to determine the effect of Ca$^{2+}$ on dissociation of Fab-9 from integrin. $^{125}$I-Fab-9 was allowed to bind to purified $\alpha_v\beta_3$ (A) or $\alpha_{IIb}\beta_3$ (B) using the microtiter plate ligand binding assay described under "Materials and Methods." After removing free $^{125}$I-Fab-9 by washing, the $^{125}$I-Fab-9-integrin complex was incubated with a range of Ca$^{2+}$ or Mg$^{2+}$. The dissociation of $^{125}$I-Fab-9 was allowed to proceed for 90 min at 37°C, and the amount of $^{125}$I-Fab-9 remaining bound to integrin was then quantified by $\gamma$-counting. This experiment was performed three times, yielding identical results. The dissociation of Fab-9 from integrin was also measured in real time using BIAcore$^\text{TM}$. Fab-9 was allowed to bind integrin $\alpha_v\beta_3$ (C) or $\alpha_{IIb}\beta_3$ (D) to the sensor chip as described under "Materials and Methods." Following initial binding in Mn$^{2+}$, the mobile phase buffer was changed (double hash mark) to that containing 5 mM of either Ca$^{2+}$ or Mg$^{2+}$. Dissociation of Fab-9 from $\beta_3$-integrins is observed as a decrease in response units (RU). The plasmon resonance studies are representative of at least seven similar experiments.

**TABLE I**

<table>
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<th>[Ca$^{2+}$] M$^{-2}$</th>
<th>$k_a$</th>
<th>$k_b$</th>
<th>$k_d$</th>
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<td>$1.9 \times 10^{-9}$</td>
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<tr>
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</tr>
<tr>
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<td>$1.6 \times 10^{-3}$</td>
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</tr>
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<td>$3.8 \times 10^3$</td>
<td>$3.8 \times 10^{-3}$</td>
<td>$3.8 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

Hence, across this range, Ca$^{2+}$ influences only the ligand dissociation rate. This finding implies that Ca$^{2+}$ is a nonclassical competitive inhibitor of Fab-9 binding to integrin and that Ca$^{2+}$ and Fab-9 bind to separate sites (see "Discussion"). The effects of Ca$^{2+}$ can be distinguished from those of a "pure" competitive inhibitor of ligand binding. For example, the RGD analog SC 52012 (28), which is a competitive inhibitor of ligand binding to integrin $\alpha_{IIb}\beta_3$ (not shown), does not influence the dissociation rate between Fab-9 and the integrin. Even $50 \mu M$ SC 52012, a level of compound that is nearly 1000-fold above its $K_d$ for the integrin, had no effect on the dissociation rate constant between Fab-9 and integrin ($6.1 \times 10^{-4} s^{-1}$ versus $6.9 \times 10^{-4} s^{-1}$). Thus, both Ca$^{2+}$ and the RGD analog competitively inhibit Fab-9 binding to integrin, but they do so by different mechanisms.

The I Site is Specific for Ca$^{2+}$—Fab-9 bound to both $\beta_3$-integrins across a wide range of Mg$^{2+}$ concentration (Fig. 1A). More importantly, Mg$^{2+}$ failed to dissociate Fab-9 from either integrin (Fig. 3, A and B). It is possible that Mg$^{2+}$ does not bind the I site or that Mg$^{2+}$ binds the I site but fails to induce the dissociation of Fab-9. We reasoned that if Mg$^{2+}$ binds the I site without increasing ligand dissociation, then it should compete with Ca$^{2+}$ and protect the ligand-receptor complex from dissociation. This possibility was tested by attempting to block the Ca$^{2+}$-induced dissociation of Fab-9 with Mg$^{2+}$. As shown in Fig. 4, prebound Fab-9 was readily dissociated by Ca$^{2+}$. Neither 1 mM nor 20 mM Mg$^{2+}$ had any effect on the dissociation when included with Ca$^{2+}$. These data indicate that Mg$^{2+}$ is unable to compete with Ca$^{2+}$ for binding to the I site. We conclude that the I site is a calcium-specific inhibitory site.

Ca$^{2+}$ Can Induce Cellular Deadhesion—Studies were conducted to determine whether occupation of the I site by Ca$^{2+}$ could initiate cellular deadhesion. Kidney 293 cells expressing $\alpha_v\beta_3$ were allowed to adhere to immobilized Fab-9 in the presence of 0.2 mM Mn$^{2+}$ (Fig. 5A), an optimal condition for cell adhesion through integrin $\alpha_v\beta_3$. Then the buffer was changed to contain either 2 mM Ca$^{2+}$ or 2 mM Mg$^{2+}$. The morphology of the cells was recorded by photography at 1- and 3-h time points. As shown in Fig. 5, Ca$^{2+}$ caused adherent cells to round up within 1 h (panel D), whereas in Mg$^{2+}$ the cells remained attached and spread for more than 3 h (panels B and C). Thus, it appears that the binding of Ca$^{2+}$ to the I site on the $\alpha_v\beta_3$ integrin can induce the “rounding up” or deadhesion of adherent cells.

**DISCUSSION**

Cytoplasmic factors (29), the physiologic status of the cell (4), and extracellular divalent cations (19) can all influence the affinity between integrins and their ligands. Divalent cations can bind as many as five sites on the integrin (30) and can promote (19) or suppress (15–18) ligand binding. Conversely,
The kinetic details of how divalent cations regulate ligand binding are tightly linked. The objective of this study was to elucidate the binding of cations and ligands to integrins are ligand can also alter the affinity of integrin for divalent cations (31). Thus, the binding of cations and ligands to integrins are competitively inhibited by Mg²⁺ or Ca²⁺ in addition to 2 mM or 20 mM Mg²⁺. This experiment is representative of four repetitions, each yielding identical results.

FIG. 4. The I site is specific for Ca²⁺. The ability of Mg²⁺ to block the Ca²⁺-induced dissociation of Fab-9 from integrin was tested using the purified ligand-receptor binding assay. ¹²⁵I-Fab-9 was allowed to bind to purified integrin αvβ3 in Mn²⁺. The dissociation of bound ¹²⁵I-Fab-9 was induced with a range of Ca²⁺ or Ca²⁺ in addition to 2 mM or 20 mM Mg²⁺. This experiment is representative of four repetitions, each yielding identical results.

FIG. 5. Ca²⁺ induces the rounding up of adherent cells. To test the activity of the I site on integrin expressed on the cell surface, we measured the ability of Ca²⁺ to induce cellular deadhesion. Kidney 293 cells expressing αvβ3 were allowed to adhere to immobilized Fab-9 in adhesion buffer containing 0.2 mM Mn²⁺ (A). After washes, adherent cells were incubated at 37°C with buffer containing 2 mM Mg²⁺ (B, C) or 2 mM Ca²⁺ (D, E). The cells were photographed at 1 h (B, D) and 3 h (C, E) after the buffer change.

The evidence supporting the existence of two classes of cation binding sites is as follows. First, ligand binding across a range of Ca²⁺ is biphasic. At concentrations of ion below 100 μM, Ca²⁺ potentiates ligand binding. Higher concentrations of Ca²⁺ block the binding of Fab-9 to both β3-integrins. Thus, the β3-integrins contain a class of high affinity cation binding sites that promote ligand binding and a class of low affinity cation binding sites that inhibit ligand binding. A similar biphasic isotherm, although somewhat less dramatic, was observed across a range of Mn²⁺ (not shown), indicating that this ion also binds to both classes of cation binding sites. Interestingly, ligand binding as a function of Mg²⁺ was monophasic, with an apparent Kd for Mg²⁺ of 1-2 mM. This ion did not interfere with ligand binding and does not appear to interact with the inhibitory cation binding sites(s). Based on these observations, we conclude that there are two classes of cation binding sites involved in regulating the binding of Fab-9 to integrins. One class of cation binding sites potentiates ligand binding and is functionally identical to the ligand-competent (LC) site we (19) and others have previously proposed (18). The LC site can bind to Ca²⁺, Mg²⁺, or Mn²⁺ and must be occupied for ligand binding. In agreement with this, studies from this laboratory also suggest that Ca²⁺ and Mg²⁺ bind the same LC that promotes ligand binding (data not shown). The other class of sites are specific for Ca²⁺ and interfere with ligand binding. This inhibitory Ca²⁺-binding site is called the I site. The I site is evident in two different types of ligand binding studies. In equilibrium binding experiments, where millimolar concentrations of Ca²⁺ are included in the reaction, ligand binding is suppressed. The presence of the I site can also be inferred from kinetic studies where Ca²⁺ dissociates ligand that is prebound to integrin. The concentration of Ca²⁺ required for both effects is similar (IC₅₀ values are 1-2 mM), strongly suggesting that both effects are the result of Ca²⁺ occupying a single class of binding sites, the I site(s).

Our results show that Ca²⁺ reduces the overall affinity (Kd) but not Bmax of integrin for Fab-9 (Fig. 2). Therefore, Ca²⁺ is a competitive inhibitor of ligand binding. More importantly, real time binding studies show that Ca²⁺ increases only the ligand dissociation rate. The overall affinity for ligand (Kd) is a product of the ligand dissociation rate (kₙ) and ligand association rate (kₐ). Calcium ion decreases the overall affinity by increasing kₙ, suggesting that Ca²⁺ is a nonclassical inhibitor. In “pure competitive inhibition” the two competing ligands bind to a common binding site (Fig. 6A). In this case one would expect the competitor to change the affinity for ligand by influencing the ligand association rate. However, the behavior of Ca²⁺ is more consistent with the model shown in Fig. 6B, where the ligand binding site and the I site are distinct. The fact that Ca²⁺ can induce dissociation of preformed complexes between Fab-9 and integrin, in which the RGD binding site is occupied, also support the idea that the ion and ligand bind to separate
sites. Consequently, the simplest interpretation of these data is that the I site and the RGD binding site are distinct and that the I site regulates the ligand off-rate by allosteric. We cannot exclude the possibility that the I site and the RGD binding site may share some contact points; however, the data indicate that the I site remains capable of binding to Ca\(^{2+}\) even when ligand is bound to the integrin.

The apparent \(K_d\) of the I site for Ca\(^{2+}\) is between 1 and 2 mM (Fig. 2A, Table I), so under physiologic conditions this site is probably occupied. How then, does occupation of the I site influence the binding of natural ligands to integrins? To address this issue, consideration must be given to the differences in the way that natural ligands and Fab-9 bind to integrins. The natural ligands have multiple contact points for integrin that presumably stabilize the integrin-ligand complex so that it is essentially nondissociable (20). Current thinking is that the RGD motif mediates initial contact and that ancillary contacts create a nondissociable integrin-ligand complex (32). This binding reaction has been previously described kinetically by Reaction 1 (21), a two-step reaction leading to a stabilized integrin-ligand complex,

\[
\begin{align*}
L + R & \rightleftharpoons [LR] \\
\text{REACTION 1} \\
L + R & \rightleftharpoons LR_{\text{stabilized}} \\
\end{align*}
\]

where \(L\) represents ligand and \(R\) represents integrin. The ligand and integrin initially form a reversible ligand-integrin complex (LR). The second step in this reaction, described by the constant \(k_2\), leads to an irreversible complex between ligand and integrin. It is important to emphasize that \(k_2 \gg k_1\) (19, 21); therefore, the rate of the forward reaction predominates, and the stabilized complex is the only species that can be detected using conventional binding studies.

In contrast to the natural ligands, Fab-9 binds to integrins in a simple and reversible manner that can be described by Reaction 2.

\[
\begin{align*}
L + R & \rightleftharpoons LR \\
\text{REACTION 2} \\
\end{align*}
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Thus, the binding of Fab-9 is not stabilized by a secondary binding step, and there is not a kinetic constant \(k_2\) to complicate the kinetic analysis.

These differences may explain why ions do not always regulate the binding of natural ligands in this same way that they regulate the binding of Fab-9. In fact, there are even substantial differences in the way that cations appear to regulate the binding of natural ligands. Ligands like vitronectin and fibronectin bind to \(\alpha_{v}\beta_{3}\) even when Ca\(^{2+}\) is present at millimolar concentrations (19), indicating that the Ca\(^{2+}\)-binding site (the I site) does not prohibit their binding to integrin. One explanation for the ability of these two proteins to bind integrin \(\alpha_{v}\beta_{3}\) even in the presence of Ca\(^{2+}\) is that ligand and Ca\(^{2+}\) bind in a mutually exclusive manner. Thus, vitronectin and fibronectin may exclude Ca\(^{2+}\) from the I site. Other ligands like fibrinogen and osteopontin cannot bind to \(\alpha_{v}\beta_{3}\) when Ca\(^{2+}\) is present (17, 19), suggesting that the binding of these proteins is blocked by Ca\(^{2+}\) binding to the I site. We suggest that Ca\(^{2+}\) occupation of the I site in these equilibrium reactions increases the dissociation rate to the point where it is much faster than the association rate. Therefore, the binding of these proteins to \(\alpha_{v}\beta_{3}\) cannot be measured using the current approaches. Other methods, like stopped-flow technology, may be required to measure these kinetic parameters. We also measured the ability of Ca\(^{2+}\) to induce the dissociation of these ligands when they were prebound to \(\alpha_{v}\beta_{3}\). We did not observe the induced dissociation of any natural ligands by Ca\(^{2+}\) (not shown). Therefore, the stabilization of ligand binding, described by the constant \(k_2\) (Reaction 1), appears to prevent the action of Ca\(^{2+}\) at the I site. In fact, it seems reasonable to speculate that the function of the I site could be lost as a consequence of the stabilized, or nondissociable ligand binding.

Extracellular Ca\(^{2+}\) regulates the adhesion and morphology of osteoclasts. This observation has been taken to indicate that osteoclasts contain a Ca\(^{2+}\) receptor that regulates adhesion (33, 34). We have previously suggested that the \(\alpha_{v}\beta_{3}\) integrin may be the osteoclast Ca\(^{2+}\) receptor (17). One potential function of the I site may be to regulate cellular deadhesion. As shown in Fig. 5, Ca\(^{2+}\) can induce the rounding up of adherent cells. This may be relevant in situations like bone resorption, where the osteoclast rounds up from the bone surface after resorption is completed. The identification of the I site on \(\alpha_{v}\beta_{3}\) provides a mechanism by which Ca\(^{2+}\) could induce cellular deadhesion by acting directly on the integrin. One of the limitations in providing an absolute proof of this hypothesis is that natural ligands bind to purified integrin in a nondissociable manner (see above). Therefore, it is difficult to extrapolate directly from binding studies with purified protein to the conditions that exist on the cell surface. Despite the above difference, we have observed instances on whole cells, where the I site may still regulate integrin function. For example, on cells adherent to vitronectin, integrin \(\alpha_{v}\beta_{3}\) clusters into focal contacts when Mg\(^{2+}\) is present but fails to assemble in focal contacts when Ca\(^{2+}\) is present. In fact, Ca\(^{2+}\) can induce the dissociation of the integrin from organized focal contacts (35). In conjunction with the data presented here, it is tempting to speculate that the allosteric cation binding site regulates cell surface organization or clustering of integrins. This hypothesis is currently under investigation.

We recently put forth a “displacement hypothesis” that may explain much of the regulation of ligand binding to integrins by divalent cations (31). We found that just as divalent ions can regulate ligand binding affinity, ligand can also regulate divalent cation binding affinity. Specifically, we found that the addition of RGD ligand to the \(\alpha_{v}\beta_{3}\) integrin could “displace” the binding of two of three bound Mn\(^{2+}\) ions. We interpreted this result to imply that ligand and divalent cation may actually compete for the same binding site on integrin. The findings in the current study provide support for the displacement hypothesis but also suggest some important modifications. The data presented here show that the binding of ligand to its site and the binding of ion to the I site are mutually exclusive. Yet, the inhibition of ligand binding by ion occupation of the I site is not “purely” competitive. In fact, a major finding of this report is that the I site is allosteric or is physically distinct from the ligand binding site. Presumably, Ca\(^{2+}\) binding to the I site induces a conformational change in the ligand binding site that favors ligand dissociation. Thus, the results from this study indicate that ligand and the displaced divalent cation do not necessarily bind to the same site on integrin as was implied (31). Consequently, we conclude that the ligand binding site and the inhibitory Ca\(^{2+}\)-binding I site are physically separate sites.

Finally, the observations presented here have important implications for drug development. Both integrins, \(\alpha_{v}\beta_{3}\) and \(\alpha_{v}\beta_{3}\), are important pharmaceutical targets. Antagonists of \(\alpha_{v}\beta_{3}\) could be used to prevent osteoporosis and perhaps tumor spread. Antagonists of \(\alpha_{v}\beta_{3}\) have already proven efficacious in preventing thrombus formation in clinical trials. All of the
current inhibitors are either monoclonal antibodies or RGD analogs that bind to the integrin’s ligand binding site. The data presented here establish the inhibitory Ca$^{2+}$-binding site as a rational target for inhibitors of integrin function. Antagonists that bind to this site and increase the dissociation rate for ligand may be expected to have pharmacological properties different from compounds that interact with the ligand binding pocket.

Acknowledgments—We acknowledge Rosalie Gonzalez for technical assistance and Crystal Herndon for preparing this manuscript. We thank Larry Feigen at Searle for providing SC 52012.

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