Multiple Activation States of Integrin \(\alpha_4\beta_1\) Detected through Their Different Affinities for a Small Molecule Ligand

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We have used the highly specific \(\alpha_4\beta_1\) inhibitor 4-(\(N\)-2-methylphenyl)ureido)-phenylacetyl-leucine-aspartic acid-valine-proline (BIO1211) as a model LDV-containing ligand to study \(\alpha_4\beta_1\) integrin-ligand interactions on Jurkat cells under diverse conditions that affect the activation state of \(\alpha_4\beta_1\). Observed \(K_D\) values for BIO1211 binding ranged from a value of 20-40 nM in the non-activated state of the integrin that exists in 1 mM Mg\(^{2+}\), 1 mM Ca\(^{2+}\) to 100 pM in the activated state seen in 2 mM Mn\(^{2+}\) to 18 pM when binding was measured after co-activation by 2 mM Mn\(^{2+}\) plus 10 \mug/ml of the integrin-activating monoclonal antibody TS2/16. The large range in \(K_D\) values was governed almost exclusively by differences in the dissociation rates of the integrin-BIO1211 complex, which ranged from 0.17 \times 10^{-4} \text{s}^{-1} to >140 \times 10^{-4} \text{s}^{-1}. Association rate constants varied only slightly under the same conditions, all falling in the narrow range from 0.9 to 2.7 \times 10^{4} \text{s}^{-1}. The further increase in affinity observed upon co-activation by divalent cations and TS2/16 compared with that observed at saturating concentrations of metal ions or TS2/16 alone indicates that the mechanism by which these factors bring about activation are distinct and identified a previously unrecognized high affinity state on \(\alpha_4\beta_1\) that had not been detected by conventional assay methods. Similar changes in affinity were observed when the binding properties of vascular cell adhesion molecule-1 and CS1 to \(\alpha_4\beta_1\) were studied, indicating that the different affinity states detected with BIO1211 are an inherent property of the integrin.

Integrins comprise a large family of cell-surface receptors that mediate cell-cell and cell-matrix interactions in diverse biological settings (see Refs. 1 and 2 for reviews). Each integrin is a two-chain heterodimer containing an \(\alpha\)-chain and a \(\beta\)-chain. The leukocyte integrin \(\alpha_4\beta_1\) regulates cell migration into tissues during inflammatory responses and normal lymphocyte trafficking (3–5) and provides a key co-stimulatory signal supporting cell activation (6–10). In vivo studies using blocking monoclonal antibodies (4) and inhibitor peptides (11–13) have demonstrated a critical role for \(\alpha_4\) integrins in leukocyte-mediated inflammation. \(\alpha_4\beta_1\) mediates cell adhesion by binding to either of two protein ligands, vascular cell adhesion molecule-1 (VCAM-1) or the alternatively spliced CS1-containing fibronectin variant (14–17). Whereas expression of \(\alpha_4\beta_1\) is constitutive, its interaction with ligands is strongly enhanced in an activated state that can be induced by various stimuli including antigen, anti-\(T\) cell receptor mAbs, phorbol esters, the divalent cation Mn\(^{2+}\), and certain \(\beta_1\)-specific antibodies (18–20). These changes in affinity and/or avidity ultimately determine whether the interaction is productive and stabilizes the ligand-integrin complex or is nonproductive.

Although all integrins require divalent cations to bind ligand, the regulation of function by metal binding is complex and is not fully understood (21, 22). Integrin \(\alpha\)-subunits contain multiple EF-hand-like Ca\(^{2+}\) binding loops (1, 23), which are in close proximity to ligand-binding sites (24, 25). This region of the \(\alpha\)-chain is made up of seven sequence repeats of about 60 amino acids each, which are presumed to be organized in a \(\beta\)-propeller fold motif found in various enzymes (24, 25). The \(\beta\)-chain contains a second type of metal binding/ligand binding motif that shares homology with the A-domain of von Willbrand's factor (26, 27). A homologous structure is present in some \(\alpha\)-subunits as well. This 200-residue protein module has proven to be surprisingly tractable for biochemical evaluation, and the corresponding regions from \(\alpha_M\), \(\alpha_L\), and \(\alpha_S\) have been successfully crystallized (28–30). The crystal structures revealed a Mg\(^{2+}\) or Mn\(^{2+}\) bound at the apex of a dinucleotide binding motif (28–30). The observed structure defined by the coordination of the cation with the peptide backbone has been postulated to mimic the ligand-occupied structure in what is commonly referred to as the metal ion-dependent adhesion site or MIDAS (see Refs. 21 and 27 for references). Whereas the role of cation-binding sites in regulating integrin function is well established, how the sites are coordinated is unclear. Regulation of ligand binding by cations is further complicated by the fact that submillimolar concentrations of Ca\(^{2+}\) can non-competitively inhibit ligand binding, indicating that certain of the metal-binding sites can play an inhibitory role (22, 31).

Recently, we developed a series of highly selective \(\alpha_4\beta_1\) inhibitors using the tetrapeptide ILDV ligand binding sequence from the CS1 region of fibronectin as the starting point for inhibitor design (32). This sequence is homologous to the tetrapeptide QIDS, which comprises the \(\alpha_4\beta_1\)-binding site in VCAM-1 (33). Whereas both ILDV and QIDS peptides weakly inhibit ligand binding and cell adhesion (33, 34), a compound that was 104 times more potent, BIO1211, was generated by substituting isoleucine with a 4-(\(N\)-2-methylphenyl)ureido)-phenylacetyl N-terminal cap (35). Here we used a tritiated version of BIO1211 as a probe to assess \(\alpha_4\beta_1\) function under various states of activation. The data provide new information on the effect that different activation conditions have on the affinity of \(\alpha_4\beta_1\) for its ligands and establish that these differences in affinity are regulated by changes in the dissociation rate of the ligand-integrin complex.

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The abbreviations used are: VCAM-1, vascular cell adhesion molecule-1; BIO1211, 4-(\(N\)-2-methylphenyl)ureido)-phenylacetyl-leucine-aspartic acid-valine-proline; mAb, monoclonal antibody; MIDAS, metal ion-dependent adhesion site; HPLC, high pressure liquid chromatography; FACS, fluorescence-activated cell sorter.
EXPERIMENTAL PROCEDURES

Synthesis of \[^{[3H]}\]BIO1211—\[^{[3H]}\]BIO1211 (50 Ci/mmol) was synthesized by NEN Life Science Products using [4,5-\[^{3H}\]]lucine as a precursor. The radiochemical purity of the compound was >95% as measured by reverse phase HPLC on a C18 column, and the compound yielded the predicted spectrum by \[^{1}H\] NMR. \[^{[3H]}\]BIO1211 was dissolved in Me2SO, diluted with 20 volumes of water, and sodium phosphate, pH 8.8, was added to a final concentration of 200 mM. The solution was then aliquoted and stored at -70 °C. The binding affinity of the labeled compound was indistinguishable from unlabeled BIO1211 in the αβ1-direct binding assay with VCAM-lg-alkaline phosphatase as a reporter (36).

Binding of \[^{[3H]}\]BIO1211 to αβ1—Expressing Cells—Jurkat cells that had been enriched for αβ1 expression by FACS sorting were maintained in RPMI 1640 medium plus 10% fetal bovine serum at 37 °C in a tissue culture incubator. K562 cells that had been transfected with either the human α2, human α2, or human α2 gene and selected for high levels of αβ1, αβ1, and αβ1, respectively, by FACS were grown in the same medium supplemented with 1 mg/ml G418, 10 μg/ml gentamicin sulfate, and 50 μg/ml streptomycin. α1 and α2 K562 cells were a gift of Dr. Martin Hemler. For binding studies, the cells were pelleted by centrifugation, washed twice with TBS (50 mM Tris HCl, 150 mM NaCl, 0.1% bovine serum albumin, 2 mM glucose, 10 mM HEPES, pH 7.4), suspended at approximately 2 × 10^6 cells/ml in TBS, and counted using a Neubauer hemocytometer. The cells were further diluted with TBS to the concentration indicated and treated with \[^{[3H]}\]BIO1211 at room temperature. The cells were then pelleted by centrifugation, re-suspended in 100 μl of TBS plus Mn2+, and transferred to a scintillation vial containing 2.9 ml of ScintiVerse II (Fisher). Cell-associated radioactivity was quantified by scintillation counting. All studies were performed in siliconized 1.5-ml Eppendorf tubes with a standard 1-ml sample volume. Each condition was tested in at least two independent studies. In the studies indicated, a 100–1000-fold excess of unlabeled BIO1211 was added to samples after the incubation with \[^{[3H]}\]BIO1211 to prevent further binding. Binding studies testing the effects of cell number, incubation time, and \[^{[3H]}\]BIO1211 concentration were performed in parallel with MnCl2, and Mn2+ was added. Nonspecific binding of \[^{[3H]}\]BIO1211 to cells was assessed at each cell density and \[^{[3H]}\]BIO1211 concentration in TBS but in the absence of added metal ion. Specific counts bound were calculated by subtracting nonspecific counts from total counts bound. Other studies testing the effects of activation on binding were performed as indicated. In the 1 mM Ca2+, 1 mM Mg2+ state where \(K_\text{D} = 20–40\) nM for binding of BIO1211 to αβ1, a high background at the higher BIO1211 concentrations (3,000 cpm at 10 nM) in the standard assay format limited the concentration of \[^{[3H]}\]BIO1211 that could be tested to <10 nM; however, by diluting the specific activity of the label from 50 to 5 Ci/mmoll, binding could be evaluated at BIO1211 concentrations up to 100 nM, albeit with reduced precision.

For kinetic on rate measurements, Jurkat cells were treated with 2 nM \[^{[3H]}\]BIO1211 at room temperature for the times indicated and then treated with a 500-fold excess of unlabeled BIO1211 to quench further binding by the \[^{[3H]}\]BIO1211. Cells were collected by centrifugation and subjected to scintillation counting. For kinetic off rate measurements, Jurkat cells were treated with 5 nM \[^{[3H]}\]BIO1211 at room temperature for 1 h. A 500-fold excess of unlabeled BIO1211 was added, and the cells were further incubated for the times indicated. Cells were pelleted at each time point, and cell-associated \[^{[3H]}\]BIO1211 was measured by scintillation counting. Binding and dissociation data are represented as a percent of the maximum specific counts bound as a function of time. The data were fitted to an exponential curve by nonlinear regression. For \(k_{\text{on}}\), the exponential rate constant is the off rate. For \(k_{\text{on}}\), the observed rate constant is the true rate constant multiplied by the \[^{[3H]}\]BIO1211 concentration. The effect of added unlabeled BIO1211 on dissociation rates was tested over a wide range of concentrations from 100 nM (20-fold excess) to 50 μM (10,000-fold excess). The dissociation curves were superimposable over this range of concentrations, indicating that the excess unlabeled ligand was exerting no allosteric effect on the rate of dissociation (data not shown).

Assessing αβ1-Ligand Interactions by Competition—\[^{[3H]}\]BIO1211 was also used to study αβ1 function by competition, using the radioactivity for αβ1 occupation. Jurkat cells in this format, Jurkat cells (1 × 10^6/ml) in the buffers indicated were treated with serial dilutions of test compound for 1 h, and then 5 nM \[^{[3H]}\]BIO1211, an amount sufficient to bind all unoccupied receptors, was added for 10 min before measuring the bound counts. The cells were then pelleted by centrifugation and subjected to scintillation counting. Counts bound under these conditions measure integrin that is not occupied by the test compound and is therefore free to bind the \[^{[3H]}\]BIO1211. The competition format was also used for kinetic binding studies. Binding and dissociation constants were calculated from \(k_{\text{on}}\), that after treatment with test compound was free to bind the \[^{[3H]}\]BIO1211.

Analysis of \[^{[3H]}\]BIO1211 by Reverse Phase HPLC—Samples were analyzed by reverse phase HPLC on a C18 column (Vydac, catalog number 218TP54, 0.46 × 25 cm) at 53 °C. The column was developed at 1 ml/min with the following gradient of acetonitrile in 50 mM sodium acetate, pH 4.5: 0–32 min 17.5–21.5%, 32–45 min 21.5–34.5%, 45–51 min 34.5–50%, 51–48 min 50–17.5%, and 48.1–53 min 17.5%, conditions that maximize the resolution of BIO1211 from potential proteolytic and hydrolytic degradation products (data not shown). The column effluent was monitored at 254 nm, and 0.5-ml fractions were collected. The fractions were then scintillation mixture and analyzed by scintillation counting. Immediately prior to injection on the HPLC, test samples were spiked with 2.5 μg each of cold BIO1211 and cold BIO-1588, an analog of BIO1211 with the C-terminal Val-Pro deleted, and a likely proteolysis product. The elution profiles of the cold inhibitors were used to monitor column performance.

RESULTS

Development of a BIO1211-αβ1 Binding Assay Using \[^{[3H]}\]BIO1211—The ability of \[^{[3H]}\]BIO1211 to bind Jurkat (αβ1 positive) cells and α1-transfected K562 (αβ1 negative) cells was examined under diverse conditions known to alter the activation state of αβ1 (Fig. 1). \[^{[3H]}\]BIO1211 binding to Jurkat cells was greatest in the presence of 2 mM Mn2+ alone, 10 mM Mg2+ alone, and 2 mM Mn2+ + 10 μg/ml mAb TS2/16, treatments that activate αβ1. Binding was greatly reduced under non-activating conditions exemplified by treatment with Ca2+, Mg2+, or Mn2+ (1 mM each) (see Fig. 1, A and B). No binding was observed to the α1-transfected K562 (αβ1-negative) control cell line. The -2,000 cpm background seen in Fig. 1A is residual free \[^{[3H]}\]BIO1211, which was removed if samples were washed prior to analysis. In the studies presented below, specific binding was calculated by subtracting background counts from total counts bound. Background binding was determined for each test sample using a control sample that was subject to the same treatment but in the absence of any divalent cation.

Specific counts bound at saturation provided a direct measure of αβ1 expression levels, and based on this number, we estimate that the Jurkat cells used in these studies have approximately 80,000 copies of αβ1 per cell. To rule out the possibility that αβ1 levels changed under the different treatments, αβ1 levels were compared under the 1 mM Ca2+, 1 mM Mg2+ (non-activating), and 2 mM Mn2+ (activating) conditions by FACS, using the non-neutralizing anti-α1 mAb B5G10 as a reporter (Fig. 1C). The αβ1 levels in the activated and non-activated samples were indistinguishable.

A series of studies were performed to assess selected variables that might affect the performance of the assay. First, no impact of temperature on BIO1211 binding was observed under any of the activating conditions tested when binding was compared at room temperature and at 37 °C (data not shown). Second, the serum or buffer in which samples were prepared had no impact on the observed rate constants that might impact our interpretation of binding. As shown in Fig. 2, over 95% of the bound \[^{[3H]}\]BIO1211 was released by the EDTA treatment unchanged, indicating that only minimal degradation had occurred. Previously, we showed that the kinetically determined \(K_D\) values for binding of \[^{[3H]}\]BIO1211 to Mn2+-activated cells of various types (peripheral blood lymphocytes, -10,000 copies of αβ1/cell; Jurkat cells, -80,000 copies/cell; α1-K562 cells ~250,000 copies/cell) were similar, showing that BIO1211 binding was not affected...
by differences in surface expression of \( \alpha_\beta_1 \) (35). Finally, because of the presence of two carboxylic acid groups in the BIO1211 sequence, we tested whether it could function as a chelator. Using fluorescent metal ion indicators that are sensitive to the concentrations of free \( \text{Mg}^{2+}, \text{Mn}^{2+}, \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) as probes for binding, we were unable to detect an association between BIO1211 and these metal ions (data not shown).

Assessing the \( K_D \) of BIO1211 for \( \alpha_\beta_1 \) by Equilibrium Binding—The low level of occupancy seen in Fig. 1B, for binding of \([\text{3H}]\text{BIO1211 to Jurkat cells under non-activating conditions,}\) suggested that the affinity of BIO1211 for \( \alpha_\beta_1 \) was lower for non-activated than for the activated integrin. In order to confirm this possibility and to obtain a more accurate measure of \( K_D \), the analysis was repeated using higher BIO1211 concentrations. The resulting curves showed dose-dependent binding and, at 100 nM [\text{3H}]BIO1211, specific counts bound were comparable for the 1 mM \( \text{Ca}^{2+} \), 1 mM \( \text{Mg}^{2+} \) state and the 2 mM \( \text{Mn}^{2+} \) state (data not shown). Based on these binding data, a \( K_D \) of 20–40 nM was calculated for the 1 mM \( \text{Ca}^{2+} \), 1 mM \( \text{Mg}^{2+} \) (non-activated) state. Attempts to estimate \( K_D \) values for activated states using equilibrium binding methods were unsuccessful because the \( K_D \) values were lower than the concentration of \( \alpha_\beta_1 \) in the assay. Thus, while we observed dose-dependent and saturable binding (see Fig. 1B), the binding curves were in fact simply measuring a titration of the receptor to full occupancy and could not be used to accurately calculate affinity. A more accurate assessment of the affinity of BIO1211 for activated \( \alpha_\beta_1 \) was obtained using kinetic measurements (see below). For unknown reasons, total counts bound were 20% lower in the 1 mM \( \text{Ca}^{2+} \), 1 mM \( \text{Mg}^{2+} \) + TS2/16 state under conditions that should have been saturating for [\text{3H}]BIO1211 binding compared with other activated states (Fig. 1B). It was not possible to perform FACs analysis in the presence of TS2/16 to test if TS2/16 treatment had altered surface levels of integrin, since the analysis would have required co-treatment with two murine anti-\( \alpha_\beta_1 \) mAbs.

Kinetics for Binding of BIO1211 to \( \alpha_\beta_1 \) in Different Activation States—Kinetic data for the binding and dissociation of [\text{3H}]BIO1211 to \( \alpha_\beta_1 \) were measured under the following conditions, as shown in Fig. 3: 1 mM \( \text{Ca}^{2+} \), 1 mM \( \text{Mg}^{2+} \); \( \text{Ca}^{2+} \), \( \text{Mg}^{2+} \), \( \text{Mn}^{2+} \) (1 mM each); 1 mM \( \text{Ca}^{2+} \), 1 mM \( \text{Mg}^{2+} \) + 10 \( \mu \)g/ml TS2/16; 2 mM \( \text{Mn}^{2+} \); and 2 mM \( \text{Mn}^{2+} \) + 10 \( \mu \)g/ml TS2/16. Binding...
Cells were pelleted at each time point, and cell-associated [3H]BIO1211 was measured by scintillation counting. The 10 mM Mg^{2+} used in these time courses all fell in the narrow range from 0.9 to 1.2 s\(^{-1}\). Differences in the percent off measurements, 1-ml samples (1.35 × 10^6 cells/ml) were treated with 2 nm [3H]BIO1211 for the times indicated. Cells were pelleted at each time point, and cell-associated [3H]BIO1211 was measured by scintillation counting. The data in A (association) and B (dissociation) were fitted to exponential curves by nonlinear regression, and k_\text{on} and k_\text{off} values were calculated from the curve fits (Table I). For k_\text{on}, 100% refers to maximum [3H]BIO1211 bound in the Mn^{2+}-activated state, whereas for k_\text{off}, 100% bound reflects maximum binding under each test condition (i.e. specific binding at t = 0).

reached a plateau level within 10 min of treatment for all test conditions (Fig. 3A). Association rate constants (k_\text{on}) calculated from these time courses all fell in the narrow range from 0.9 to 2.7 × 10^6 s\(^{-1}\) (Table I). Differences in the percent αβ_1 occupied after 10 min were identical to those seen in the equilibrium experiments shown in Fig. 1B. Time courses for the rates of dissociation of BIO1211 from Jurkat cells are shown in Fig. 3B. Unlike the association rates, which were similar across the different assay conditions, the dissociation rates varied over a wide range and were highly dependent on the activation state of the integrin. Values for k_\text{off} ranged from 0.17 × 10^4 to 1.2 × 10^4 s\(^{-1}\) for the 2 mM Mn^{2+} + TS2/16 state to >140 × 10^4 s\(^{-1}\) for the 1 mM Ca^{2+}, 1 mM Mg^{2+}, Mn^{2+} (1 mM each) state (Table I). The kinetic rate constants from these and other binding studies were used to calculate K_D values, which are summarized in Table I. The close correspondence between the variations in K_D values and in dissociation rate constants indicates that the affinity of BIO1211 for αβ_1 is governed almost exclusively by off rates. K_D values observed under conditions commonly considered to be activating ranged from 470 pm in the Ca^{2+}, Mg^{2+}, Mn^{2+} (1 mM each) state to 18 pm in the 2 mM Mn^{2+} + TS2/16 state. These differences in affinity that resulted from activation were not apparent from the equilibrium binding measurements because the K_D values were lower than the concentration of αβ_1 in the assays and therefore were masked by the format of the assay. Similar problems were encountered when affinities were estimated by measuring the ability of BIO1211 to block cell adhesion to CS1 or VCAM or to block direct binding of VCAM-Ig to Jurkat cells with VCAM-Ig as the reporter (data not shown).

To understand better the role metal ions have on activation, we performed the study shown in Fig. 4, where binding was tested as a function of changing Mg^{2+} concentrations. The dissociation rates were highly dependent on the Mg^{2+} concentration and changed from 8.3 × 10^{-4} s\(^{-1}\) to 4.3 × 10^{-4} s\(^{-1}\) to 3.2 × 10^{-4} s\(^{-1}\) to 2.1 × 10^{-4} s\(^{-1}\) when dissociation rates were measured in the presence of 2, 10, 50, and 300 mM Mg^{2+}. When K_D was plotted as a function of Mg^{2+} concentration, the data fit a hyperbolic curve, suggesting that the measurements were part of a continuum rather than discrete points (Fig. 4B). At high concentrations of added Mg^{2+}, binding appears to be saturating out at an affinity that approximates the value seen at 2 mM Mn^{2+} (Fig. 4B). Suboptimal concentrations of Mn^{2+} produced a similar titration of the dissociation rate to that seen with Mg^{2+}, although at a much lower concentration of the metal ion (data not shown). Variations in the concentration of TS2/16 from 0.1 to 10 μg/ml had no effect on K_D (data not shown), indicating that 10 μg/ml was a saturating concentration.

While the maximal affinity for binding of BIO1211 to αβ_1 in the presence of divalent cations was achieved with 2 mM Mn^{2+}, a further increase in affinity was observed if samples were co-activated with 2 mM Mn^{2+} + TS2/16, suggesting that the mechanisms by which TS2/16 and divalent cations brought about activation were distinct. Results from these analyses are summarized in Table I. In the presence of 2 mM Mn^{2+} alone and 2 mM Mn^{2+} + TS2/16, K_D values of 100 and 18 pm, respectively, were observed. The K_D value resulting from activation by TS2/16 alone (i.e. 1 mM Mg^{2+}, 1 mM Ca^{2+} + TS2/16) was 220 pm. A similar increase in affinity was seen after treatment of the 10 mM Mg^{2+} alone state with TS2/16 (K_D = 440, 220, and 40 pm for the 10 mM Mg^{2+}, alone state, TS2/16 alone, and 10 mM Mg^{2+} + TS2/16 states, respectively). Interestingly, the final K_D ob-

### Table I

**Binding of [3H]BIO1211 to αβ_1 on Jurkat cells under various states of activation**

| Buffer | k_\text{on} × 10^6 s\(^{-1}\) | k_\text{off} × 10^4 s\(^{-1}\) | K_D pm |
|--------|-----------------------------|-----------------------------|--------|
| 1 mM Ca^{2+}, 1 mM Mg^{2+} | 2.7 | >140 | >5000 |
| 2 mM Mg^{2+} | 1.2 | 8.3 | 700 |
| 1 mM Ca^{2+}, 1 mM Mg^{2+}, Mn^{2+} (1 mM each) | 1.1 | 5.2 | 470 |
| 10 mM Mg^{2+} | 1.2 | 5.3 | 440 |
| 50 mM Mg^{2+} | 1.2 | 4.3 | 360 |
| 100 mM Mn^{2+} | 1.2 | 3.2 | 220 |
| 1 mM Ca^{2+}, 1 mM Mg^{2+} + TS2/16 | 2.3 | 5.1 | 270 |
| 300 mM Mg^{2+} | 1.2 | 2.1 | 170 |
| 2 mM Mn^{2+} | 1.3 | 1.4 | 100 |
| 2 mM Mn^{2+} + HP1/2 | 1.3 | 1.3 | 100 |
| 2 mM Mn^{2+} + TS2/16 | 0.9 | 0.17 | 18 |
| 10 mM Mg^{2+} + TS2/16 | 1.0 | 0.40 | 40 |

* A value of 20–40 nm was determined using equilibrium binding data (see text).

* Data shown were derived from the experiment shown in Fig. 3. k_\text{on} is better defined by a double exponential fit as described in the text.
observed after co-activation by divalent cations plus TS2/16 was strongly influenced by the concentration and type of divalent cation. $K_D$ values varied with metal ion concentrations in a manner analogous to the data observed in the divalent cation alone state, whereas the TS2/16 treatment appeared to produce a quantum step. This distinction in effect is particularly apparent from the dissociation data shown in Fig. 4A. In contrast to the additive effect seen after co-activation by TS2/16 plus divalent cations, the dissociation rate observed in the presence of 2 mM Mn$^{2+}$ plus 10 mM Mg$^{2+}$ was not significantly slower than that seen with 2 mM Mn$^{2+}$ alone, indicating that activation caused by Mn$^{2+}$ and Mg$^{2+}$ was not additive (data not shown).

In the non-activated 1 mM Ca$^{2+}$, 1 mM Mg$^{2+}$ state, the kinetically determined affinity of BIO1211 for $\alpha_\beta_1$ was significantly lower ($>5$ nM) than the affinities determined for the activated states. Whereas the on-rate for the Ca$^{2+}$, Mg$^{2+}$ state was similar to that observed under activating conditions (see Table I), dissociation was too rapid to allow an accurate determination of $k_{off}$ and so the kinetic data gave only a lower limit to $K_D$ (see Fig. 3B). The limit of $K_D$ > 5 nM is consistent with the more accurate estimate of $K_D = 20 - 40$ nM that was obtained from equilibrium measurements described above. Together the data in Table I demonstrate that the affinity of BIO1211 for $\alpha_\beta_1$ is highly sensitive to the activation state of $\alpha_\beta_1$ and can vary by over 1000-fold depending on the assay conditions.

Since $\alpha_\beta_1$ affinity states are dependent on metal ion binding, we investigated the effects of EDTA treatment on dissociation of the BIO1211-$\alpha_\beta_1$ complex under various activating conditions. Results from this analysis are shown in Fig. 5 and Table II. EDTA treatment of the 2 mM Mn$^{2+}$ alone state resulted (Fig. 5A), as expected, in a more rapid release of $[^3H]$BIO1211 from the complex, although even after EDTA treatment the rate of release was not as rapid as from the non-activated state. EDTA treatment also resulted in a more rapid release of BIO1211 from the 2 mM Mn$^{2+}$ + TS2/16 state; however, the rate of release was clearly slower in the presence of TS2/16 than when either the 10 mM Mg$^{2+}$ alone or 2 mM Mn$^{2+}$ alone activated states were treated with EDTA.

An unexpected feature of the dissociation curve for the release of BIO1211 from the divalent cation plus TS2/16-activated state was that it required a double exponential fit to account for the data. A similar biphasic curve for the release of BIO1211 was observed for $\alpha_\beta_1$-BIO1211 complexes formed in the presence of 1 mM Ca$^{2+}$, 1 mM Mg$^{2+}$ + TS2/16 and treated with EDTA (see Fig. 5B). Unlike the large effect of EDTA treatment on the release of BIO1211 from the divalent cation alone and divalent cation plus TS2/16-activated states, the effect of EDTA on the 1 mM Ca$^{2+}$, 1 mM Mg$^{2+}$ + TS2/16 state was modest, causing only a 2-3-fold increase in the rate of the fast phase of release. Although we originally had assumed that the dissociation of BIO1211 from $\alpha_\beta_1$-BIO1211 complexes would follow a single exponential based on the release data that had been generated for the Mn$^{2+}$-activated integrin, a reevaluation of the data summarized in Table I indicated that this was not necessarily true for all methods of activation.
Additional experiments with more data points added along the relevant regions of the dissociation curves supported our original finding that BIO1211 release from divalent cation alone activated \( \alpha \beta_1 \) follows a single exponential and reconfirmed the observation that the dissociation of BIO1211 from the 1 mM Ca\(^{2+} \), 1 mM Mg\(^{2+} \) + TS2/16 state was biphasic. By extrapolating the line defined by the slowly dissociating component back to the y intercept, we can estimate that about 50% of the integrin-BIO1211 complex for the 1 mM Ca\(^{2+} \), 1 mM Mg\(^{2+} \) + TS2/16 state was in the EDTA-resistant form. As shown in Table I, there were two phases of dissociation (see Fig. 5B), one that had been immobilized on plastic and is therefore free to bind the \([^{3}H]BIO1211\) and another that is specific to BIO1211 or whether it reflects a property of \( \alpha \beta_1 \) integrins in their responsiveness to calcium. A more thorough study is needed to understand better the interplay between Ca\(^{2+} \) and other metal ion binding and how these effects are connected to ligand binding.

### Table II: Rate constants for the dissociation of BIO1211-integrin complexes under various conditions

| Treatment | \( k_{\text{off (-EDTA)}} \times 10^{-4/s} \) | \( k_{\text{off (+EDTA)}} \times 10^{-4/s} \) |
|-----------|---------------------------------|---------------------------------|
| a. (+/-) EDTA treatment | | |
| 2 mM Mn\(^{2+} \) | 1.3 | 15 |
| 2 mM Mn\(^{2+} \) + TS2/16 | 0.17 | 12.5 |
| 2 mM Mg\(^{2+} \) + TS2/16 | 0.76 | 17.3 |
| 1 mM Ca\(^{2+} \) + TS2/16 | 10.5 | 32.8 |
| 1 mM Ca\(^{2+} \) + TS2/16 | 14 | 1.2 |
| b. Ca\(^{2+} \) treatment | | |
| Mn\(^{2+} \) + TS2/16, 1 mM Ca\(^{2+} \) | | |

\( ^* \) Kinetic data could not establish the presence or absence of a slow phase because of the small percentage of BIO1211 released over the 120-min incubation.

The effects of Ca\(^{2+} \) on \( \alpha \beta_1 \) activation are complex. For \( \alpha \beta_1 \) BIO1211 complexes, formed in the presence of 2 mM Mn\(^{2+} \) + TS2/16, Ca\(^{2+} \) treatment was as effective as EDTA at promoting release of BIO1211 and, like EDTA treatment, required a double exponential fit to account for the data (Fig. 5A). Ca\(^{2+} \) had less of an effect on the divalent cation alone or TS2/16 states (see Table I). In particular for activation by Mn\(^{2+} \), 1 mM Ca\(^{2+} \) treatment resulted in only a modest 3-fold effect on \( k_{\text{off}} \) (\( k_{\text{off}} = 5.3 \times 1.4 	imes 10^{-4} \text{s}^{-1} \) for the Ca\(^{2+} \), Mg\(^{2+} \), Mn\(^{2+} \) (1 mM each) and 2 mM Mn\(^{2+} \) states, respectively). In the studies shown in Fig. 1A, we observed that increased Ca\(^{2+} \) concentration from 1 to 10 mM slightly stimulated BIO1211 binding. Although the ability of Ca\(^{2+} \) to induce the release of bound BIO1211 from the 2 mM Mn\(^{2+} \) + TS2/16 state is consistent with the published model based on studies for RGD-binding integrins in which high concentrations of Ca\(^{2+} \) can function as a non-competitive inhibitor of ligand binding (22, 31), the results observed for Ca\(^{2+} \) on other states show that Ca\(^{2+} \) can have a wide range of effects on activation. Recent findings (37) have further highlighted differences between \( \alpha \beta_1 \) and other \( \beta_1 \) integrins in their responsiveness to calcium. A more thorough study is needed to understand better the interplay between Ca\(^{2+} \) and other metal ion binding and how these effects are connected to ligand binding.

### CS1 and VCAM-Ig Detect the Mn\(^{2+} \) + TS2/16 High Affinity State

As a result of its high affinity and slow rate of dissociation, the \([^{3}H]BIO1211\) can also be used as a probe for ligand-integrin interactions through competition measurements in which \([^{3}H]BIO1211\) binding is used as a reporter for receptor occupancy. In this format, Jurkat cells are first incubated with test compound and then subjected to a brief treatment with \([^{3}H]BIO1211\) and counted. Counts bound under these conditions measure integrin that is not occupied by the test compound and is therefore free to bind the \([^{3}H]BIO1211\). Typical results from this type of analysis are shown in Fig. 6. Competition for \([^{3}H]BIO1211\) binding was seen with BIO1211, VCAM-Ig, and CS1 at concentrations that are consistent with their known binding constants for \( \alpha \beta_1 \) (35, 36). An interesting aspect of the binding data was that the VCAM-Ig titration required a non-hyperbolic curve fit to account for the data, as had been previously seen in FACS binding experiments using VCAM-Ig (38). The binding equation used to fit the data for VCAM-Ig describes the data expected for a bivalent ligand (39) and differs from the simple hyperbolic competition curve seen for the monovalent CS1. VCAM-Ig binding was also evaluated by competition using a kinetic readout. Surprisingly, the \( t_{1/2} \) for dissociation of the VCAM-Ig-\( \alpha \beta_1 \) complex was only 8 min for the Mn\(^{2+} \) state, which identified a limitation in using VCAM-Ig as a reporter of integrin function (\( t_{1/2} = 70 \text{ min} \) for BIO1211 under these conditions). \([^{3}H]BIO1211\) as a reporter type readout should have broad applications as a probe for \( \alpha \beta_1 \) structure-function.

To understand better whether the difference in affinities between the 2 mM Mn\(^{2+} \) alone and 2 mM Mn\(^{2+} \) + TS2/16 states is specific to BIO1211 or whether it reflects a property of \( \alpha \beta_1 \) common to its interaction with other ligands, we tested VCAM-Ig and CS1 binding to Jurkat cells under the same conditions described for BIO1211. As shown in Table III, VCAM-Ig and CS1 were also sensitive to differences between the 2 mM Mn\(^{2+} \) alone and 2 mM Mn\(^{2+} \) + TS2/16 states. These observations support the notion that BIO1211 mimics many of the properties of \( \alpha \beta_1 \) ligands and that the different activation
The Mn$^{2+}$ + TS2/16 high affinity state is a property of a subpopulation of integrin αβ₁ that affects binding of VCAM-Ig and CS-1. One possible explanation for this selectivity is that the TS2/16 high affinity state is a property of a subpopulation of integrin αβ₁ that affects binding of VCAM-Ig and CS-1. This suggests that the activation by divalent cations and by TS2/16 on the dissociation rate of the integrin. This notion was further supported by studies in which dissociation rates were measured in the presence of suboptimal concentrations of Mn$^{2+}$ and Mg$^{2+}$ plus TS2/16. However, the observed differences in the affinities and dissociation rates between 2 mM Mn$^{2+}$ + TS2/16 and 10 mM Mg$^{2+}$ + TS2/16 suggested that the higher affinity state can be distinguished from the more physiological ligands.

### Table III

| Test compound | Apparent $K_D$ values |
|---------------|-----------------------|
|               | 2 mM Mn$^{2+}$ + 2 mM Mg$^{2+}$ + TS2/16 | 2 mM Mn$^{2+}$ + 2 mM Mg$^{2+}$ + 1 mM Ca$^{2+}$ |
| BIO1211 (μM)  | 15                     | 100                      |
| VCAM-Ig (μM)  | 0.2                    | 16                       |
| CS1 (μM)      | 2.0                    | 38                       |

states of αβ₁, it distinguishes in most physiological ligands.

### DISCUSSION

We have used [3H]BIO1211 as a model LDV-containing ligand to study αβ₁ integrin function. When binding was tested under various conditions that affect the activation state of αβ₁, $K_D$ values were observed that ranged from a value of 20–40 nM in the 1 mM Mg$^{2+}$, 1 mM Ca$^{2+}$ (non-activated) state to 100 pM in the 2 mM Mn$^{2+}$ (activated) state to 18 pM in a newly identified state detected when binding was measured in the presence of 2 mM Mn$^{2+}$ + 10 μg/ml mAb TS2/16. The differences in affinity were regulated almost exclusively by changes in the dissociation rate of the complex. Although we had expected to see affinity differences between non-activated and activated αβ₁, the large variation in affinity among the various activated states was surprising. Over a 25-fold difference in affinity for BIO1211 was detected between the Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$ (1 mM each) state and the 2 mM Mn$^{2+}$ + TS2/16 state. To verify that the variations in the integrin affinity state are relevant to ligands other than BIO1211, we tested VCAM-1 and CS-1 binding under selected conditions and showed that similar trends were also seen for these more physiologically relevant ligands.

A striking feature of the activated aβ₁ was that activation was not defined by a single high affinity state but rather by a continuum of affinities that was easily manipulated by changes in the assay conditions. This result is particularly apparent from the data shown in Fig. 4B where the affinity changed from 700 to 440 to 360 to 220 to 170 pM by simply changing Mg$^{2+}$ concentrations. At high concentrations of added Mg$^{2+}$, BIO1211 affinity and dissociation rate converge to the values seen at 2 mM Mn$^{2+}$ (Fig. 4B). This observation suggests that the activating effects seen with Mg$^{2+}$ and Mn$^{2+}$ at saturation involve formation of the same affinity state of the integrin. This conclusion was supported by the observation that no further increase in affinity was observed when samples that had been treated with 2 mM Mn$^{2+}$ were also treated with 10 mM Mg$^{2+}$. Suboptimal concentrations of Mn$^{2+}$ produced a similar titration of the dissociation rate to that seen with Mg$^{2+}$, with the most dramatic effects seen at concentrations of less than 50 μM Mn$^{2+}$ (data not shown). Presumably, this continuum represents the effects of metal ion-dependent interconversion of a finite number of discrete states of the integrin. If the interconversion between the states is fast as few as two states could account for the data shown in Fig. 4B. Further studies are needed to define the exact number of affinity states involved.

In the presence of 10 mM Mg$^{2+}$ + TS2/16 or 2 mM Mn$^{2+}$ + TS2/16, a further increase in affinity was seen. The additive effect of activation by divalent cations and by TS2/16 on the $K_D$ for [3H]BIO1211 binding indicates that the mechanisms of activation by divalent metals and by antibody are distinct. The kinetic data are less informative about the transition between the “2 mM Mn$^{2+}$” state and the higher affinity state observed with Mg$^{2+}$ or Mn$^{2+}$ plus TS2/16. However, the observed differences in the affinities and dissociation rates between 2 mM Mn$^{2+}$ + TS2/16 and 10 mM Mg$^{2+}$ + TS2/16 suggested that the higher affinity state can be distinguished from the more physiological ligands.

FIG. 6. Analysis of CS1 and VCAM-Ig binding through competition. The apparent affinities of CS1 and VCAM-Ig for αβ₁ were assessed on Jurkat cells through competition with [3H]BIO1211. Jurkat cells (2 × 10⁶ cells/ml) in TBS plus 2 mM Mn$^{2+}$ buffer were incubated for 1 h at room temperature with serial dilutions of CS1, VCAM-Ig, or unlabeled BIO1211 at the concentrations indicated. At the end of the incubation, 5 mM [3H]BIO1211 was added, and the cells were further incubated for 10 min. The cells were then pelleted, and the bound [3H]BIO1211 was quantified by scintillation counting. The titration data were plotted as a percent of maximum counts bound with no added competitor. BIO1211, VCAM-Ig, CS1.
slowly dissociating population was similar to that for the divalent cation plus TS2/16 state in the absence of EDTA. Similar biphasic release data were obtained regardless of whether samples were activated by TS2/16 alone or by TS2/16 plus divalent cation, indicating that the complex dissociation curves were not unique to the high affinity state. Thus, it appears either that the divalent cations are not accessible to EDTA to the same degree in the different activated states of $\alpha_2\beta_1$ or that a different step governs the release rate. Further studies are needed to distinguish between these alternatives. It is not clear whether activation with TS2/16 induces heterogeneity in the integrin or allows preexisting heterogeneity to become visible. Nevertheless, the fact that dissociation data after EDTA treatment can distinguish between divalent cation and TS2/16-activated states provides additional support for the idea that the two stimuli bring about activation of $\alpha_2\beta_1$ in distinct ways.

Although the precise mechanism by which the affinity states of integrins are regulated is unknown, a large body of data implicates the cation-binding sites as a key component (21, 22, 31). Since ligand-binding sites map to the same regions on the $\alpha$- and $\beta$-chains as the cation-binding sites, various models have been proposed in which the cations coordinate with ligands or compete with ligand for binding (28–30, 32, 40), or in which cation binding regulates the opening of the integrin dimer to expose the ligand-binding site (21). Our data argue against the latter model since only the affinities and not the association rate constants for ligand binding are altered by changes in the activation state, indicating that the ligand-binding site is equally accessible for binding under all states of activation. In one published study where a peptide spanning the MIDAS site from the $\beta_2$ subunit of $\alpha_{\text{int}}\beta_2$ was used as a model for the ligand-binding site, the authors suggested ligand binding could directly compete for $\text{Mn}^{2+}$ binding (40). We see no evidence for this competition using $[^3\text{H}]\text{BIO1211}$ as a reporter for $\alpha_2\beta_1$ function.

Although many models have been proposed for how integrin activation is regulated, the simplest explanation for the independent and additive effects of divalent cations and TS2/16 on $\alpha_2\beta_1$ activation is that the metal-binding sites on the $\alpha_2$-chain and the MIDAS site on $\beta_1$-chain can be independently regulated. Thus, the dependence of $\alpha_2\beta_1$ affinity on divalent cations could reflect regulation on the $\alpha$-chain elements, whereas the dependence of the affinity on TS2/16 binding could reflect regulation on the $\beta$-chain. Because of the multiple putative metal-binding sites on the $\alpha_2$-chain, variations in the type and concentration of metal ions could affect occupancy of these sites and thereby change the affinity of $\alpha_2\beta_1$ for ligand. In contrast, since TS2/16 would either be bound or not bound, one would expect a quantum effect of the antibody on affinity through a conformational change induced by antibody binding that can either directly induce ligand binding or, alternatively, that affects metal binding at the MIDAS which would indirectly affect ligand binding. Although the current studies do not allow us to evaluate BIO1211 binding at a molecular level, the availability of $\alpha$-chain mutants (41) that are targeted at these key regulatory sites should allow us to define more precisely the effects of these distinct elements on ligand binding.

Ligand binding and integrin activation induce a cascade of conformational changes within the integrin that ultimately lead to the activation of intracellular signaling pathways. These changes have been studied in detail using mAbs whose epitopes are either exposed (termed LIBS) or lost following ligand binding (21, 42). BIO1211 is a LIBS inducer and therefore might be expected to exhibit the same profile of effects that natural ligands exhibit (35). The small size of BIO1211 makes it particularly well suited for this type of analysis, since it minimizes the chance of steric inhibition of antibody binding. This notion is particularly apparent from the data for HP1/2 shown in Table I. HP1/2 is a B1 class anti-$\alpha_4$-antibody, which is defined as a potent inhibitor of $\alpha_2\beta_1$-ligand interactions that does not induce leukocyte homotypic aggregation (42). Although HP1/2 is a potent inhibitor of VCAM-Ig binding (36), our data clearly demonstrate that HP1/2 has no effect on BIO1211 binding and therefore that it blocks ligand binding through steric effects rather than through direct binding at the ligand-binding pocket. The exquisite sensitivity of the $[^3\text{H}]\text{BIO1211}$ to differences in $\alpha_2\beta_1$ activation suggests that the labeled probe will prove to be an extremely valuable readout for this type of analysis.

Although many assays have been used to study $\alpha_4\beta_1$ function (36, 43, 44), the data we generated with $[^3\text{H}]\text{BIO1211}$ revealed various features about ligand binding that were not evident from these conventional assay methods. Most significant were the observations that activation is not defined by a single state but rather by several distinct states that give rise to a range of affinities and that the affinity differences are tightly coupled to dissociation rates of the integrin-ligand complex. As soluble, monovalent probes for $\alpha_2\beta_1$ function, BIO1211, and related inhibitors represent novel tools that should aid in further unraveling the complexities associated with integrin activation.

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Activation States of Integrin α\(\beta_1\)

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