

Regulation of Integrin $\alpha 5\beta 1$ -Fibronectin Interactions by Divalent Cations

EVIDENCE FOR DISTINCT CLASSES OF BINDING SITES FOR Mn^{2+} , Mg^{2+} , AND Ca^{2+} *

(Received for publication, June 19, 1995, and in revised form, August 4, 1995)

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Integrin-ligand interactions are known to be dependent on divalent cations, although the precise role of cations in ligand binding is still unclear. Using the interaction between $\alpha 5\beta 1$ and fibronectin as a model system, we have performed a comprehensive analysis of the effects of Mn^{2+} , Mg^{2+} , and Ca^{2+} on ligand binding. Each cation had distinct effects on the ligand-binding capacity of $\alpha 5\beta 1$: Mn^{2+} promoted high levels of ligand binding, Mg^{2+} promoted low levels of binding, and Ca^{2+} failed to support binding. Studies of the effects of different combinations of cations on ligand binding indicated that the cation-binding sites within $\alpha 5\beta 1$ are not all identical, or of broad specificity, but instead each site shows a distinct preference for one or more cations. Ca^{2+} strongly inhibited Mn^{2+} -supported ligand binding, but this inhibition was noncompetitive, suggesting that Ca^{2+} recognizes different cation-binding sites to Mn^{2+} . In contrast, Ca^{2+} acted as a direct competitive inhibitor of Mg^{2+} -supported ligand binding, implying that Ca^{2+} can displace Mg^{2+} from the integrin. However, low concentrations of Ca^{2+} greatly increased the apparent affinity of Mg^{2+} for its binding site, suggesting the existence of a distinct high affinity Ca^{2+} -binding site. Taken together, our results imply that the ligand-binding capacity of $\alpha 5\beta 1$ can be regulated in a complex manner through separate classes of binding sites for Mn^{2+} , Mg^{2+} , and Ca^{2+} .

Cell adhesion is of fundamental importance to many normal biological processes, including wound healing, embryonic cell migration, and the function of the immune system. Conversely, aberrant cell adhesion contributes to the pathogenesis of a large number of common human disorders such as rheumatoid arthritis, atherosclerosis, and tumor cell metastasis in cancer. Many cell-cell and cell-matrix interactions are mediated by members of the integrin superfamily of cell-surface receptors. Integrins are $\alpha\beta$ heterodimers that have been classified into eight different groups according to the identity of their β subunit. The $\beta 1$ family is the principal group of cell-matrix receptors (Hynes, 1992, 1994; Ruoslahti *et al.*, 1994).

Integrin-ligand interactions are dependent on divalent cations, but the precise role of divalent cations in ligand binding

has not yet been elucidated. The N-terminal portion of the integrin α subunits comprises seven homologous, tandemly-repeated domains of ~50 amino acid residues. Domains 4–7 (or in some subunits, 5–7) contain sequence motifs similar to the Ca^{2+} -binding EF-hands in proteins such as calmodulin. However, the integrin divalent cation-binding motif differs from classical EF-hand sequences in that it lacks an essential oxygenated residue at the $-z$ coordination position. Hence, it has been proposed that integrin ligands, such as RGD, may supply a crucial aspartate residue to complete the coordination geometry of the divalent cation (Corbi *et al.*, 1987; Humphries, 1990). This hypothesis therefore suggests that divalent cations may act as bridge between ligand and receptor.

Chemical cross-linking experiments have provided direct evidence for the role of integrin EF-hand-like sites in ligand binding. Specifically, the binding site of a sequence from the fibrinogen γ chain has been mapped to the fifth repeat of αIIb (D'Souza *et al.*, 1990), and a peptide corresponding to the EF-hand-like sequence in this repeat bound to fibrinogen in a divalent-cation-dependent manner (D'Souza *et al.*, 1991). Similarly, cross-linking of an RGD peptide to $\alpha V\beta 3$ localized the ligand-binding site between the second and sixth domains of the αV subunit (Smith and Cheresch, 1990).

The importance of cation coordination in ligand binding by integrins has been demonstrated directly by the covalent coupling of Co(III) to $\alpha V\beta 3$ (Smith and Cheresch, 1991). In addition, a recombinant fragment of αIIb that spans the EF-hand-like domains has been shown to contain multiple Ca^{2+} binding sites (Gulino *et al.*, 1992), and a modeling study of hybrid integrin-calmodulin EF-hands predicts the integrin loop to support divalent cation chelation (Tuckwell *et al.*, 1992).

A highly conserved region is found toward the N terminus of integrin β subunits suggesting that this sequence may be functionally important. Cross-linking studies have shown that this region in the $\beta 3$ subunit is proximal to the ligand-binding site (D'Souza *et al.*, 1988; Smith and Cheresch, 1988). In addition, mutation of oxygenated residues in this region (Loftus *et al.*, 1990; Bajt and Loftus, 1994) results in a receptor that is deficient in binding both cation and ligand. The sequence containing these residues shows some homology to a cation-binding sequence found in integrin I domains (Michishita *et al.*, 1993; Bajt and Loftus, 1994; Lee *et al.*, 1995). In an important recent advance, a synthetic peptide comprising this region of $\beta 3$ (residues 118–131) was shown to bind both the Ca^{2+} analogue Tb^{3+} and RGD peptides (D'Souza *et al.*, 1994). Ligand binding caused displacement of cation from this peptide, and a similar displacement of cations from $\alpha IIb\beta 3$ by ligands was also observed. Based on these results, a mechanism of integrin-ligand binding has been proposed, termed the "cation displacement hypothesis" (D'Souza *et al.*, 1994). In this mechanism, cation,

* This work was supported by grants from the Wellcome Trust (to M. J. H. and A. P. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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ligand, and receptor initially form a ternary complex in which ligand is bridged to the integrin through the cation, and cation is subsequently displaced from the ligand-binding site.

Cation binding by integrins has also been shown to be associated with conformational changes. For example, expression of the epitope recognized by monoclonal antibody (mAb)¹ 24 on $\alpha L \beta 2$ is dependent on Mg^{2+} (or Mn^{2+}), and mAb 24 epitope expression correlates with the ability to bind ligand (Dransfield and Hogg, 1989; Dransfield *et al.*, 1992a, 1992b). Hence, an alternative hypothesis for the role of divalent ions in integrin function is that cation binding is required to cause a conformational change in the integrin that renders it competent to bind ligand.

A number of important questions concerning the cation-binding sites on integrins are currently unresolved. First, do the cation-binding sites bind only one type of divalent cation, or do they all have a broad specificity? Second, is there only a single cation-binding site involved in ligand recognition or can occupancy of more than one site support ligand binding? Third, why does Mn^{2+} confer a much higher ligand-binding affinity on many integrins than Ca^{2+} or Mg^{2+} (Gailit and Ruoslahti, 1988; Altieri, 1991; Elices *et al.*, 1991; Dransfield *et al.*, 1992b; Kern *et al.*, 1993; Sanchez-Aparicio *et al.*, 1993)?

The extracellular matrix glycoprotein fibronectin has served as a prototype substrate for the study of integrin-ligand interactions, and several regions of the molecule have been shown to be responsible for its adhesive activity. One domain that is recognized by a wide variety of cell types lies close to the center of the fibronectin subunit and contains the tripeptide RGD as a key active site (Pierschbacher and Ruoslahti, 1984; Yamada and Kennedy, 1984). The integrin $\alpha 5 \beta 1$ is the major receptor for this central cell-binding domain (CCBD) and is expressed on many cell types. Here we have studied the role of Mn^{2+} , Mg^{2+} , and Ca^{2+} in modulating $\alpha 5 \beta 1$ -fibronectin interactions. We show that either Mn^{2+} or Mg^{2+} , but not Ca^{2+} , can support ligand binding. However, Ca^{2+} strongly modulates ligand binding supported by Mn^{2+} or Mg^{2+} and acts as a direct competitive inhibitor of Mg^{2+} -supported binding but not of Mn^{2+} -supported binding. Our results suggest the existence of distinct classes of cation-binding sites for each divalent ion.

EXPERIMENTAL PROCEDURES

Materials—Rat mAbs 16 and 13 recognizing the human $\alpha 5$ and $\beta 1$ integrin subunits, respectively, were produced and purified as described previously (Akiyama *et al.*, 1989). An 80-kDa fragment of fibronectin containing the CCBD was purified from a trypsin digest of plasma fibronectin as described by Garcia-Pardo *et al.* (1989). The synthetic peptide GRGDS was synthesized using Fmoc (*N*-9-fluorenyl)-methoxycarbonyl chemistry on an Applied Biosystems 431A peptide synthesizer and purified as described previously (Humphries *et al.*, 1986, 1987).

Cell Attachment Assay—K562 erythroleukemia cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK) and were grown in RPMI 1640 medium containing 10% (v/v) fetal calf serum, and 2 mM glutamine (all from Life Technologies, Inc., Paisley, Scotland, UK). Cell attachment assays were performed in 96-well microtiter plates (Costar, High Wycombe, Bucks, UK). Wells were coated for 60 min at room temperature with 100- μ l aliquots of 80-kDa CCBD fragment (1 μ g/ml) diluted with Dulbecco's phosphate-buffered saline, and then sites on the plastic for nonspecific cell adhesion were blocked for 30 min at room temperature with 100 μ l of 10 mg/ml heat-denatured BSA. Cells were resuspended to 2×10^6 /ml in 150 mM NaCl, 25 mM Hepes, 2 mM EDTA, pH 7.4, and incubated at 37 °C for 30 min. Cells were then washed twice in Hepes-buffered saline (HBS; 150 mM NaCl, 25 mM Hepes, pH 7.4) and resuspended in the same buffer. Aliquots of cells (50 μ l) were then added to the microtiter wells and

incubated with 50- μ l aliquots of Hepes-buffered saline containing $2 \times$ the final concentration of divalent cations ($MnCl_2$, $MgCl_2$, or $CaCl_2$) for 20 min at 37 °C in a humidified atmosphere of 6% (v/v) CO_2 . To estimate the reference value for 100% attachment, cells in quadruplicate wells coated with polylysine (500 μ g/ml) were fixed immediately by direct addition of 100 μ l of 5% (w/v) glutaraldehyde for 30 min at room temperature. Unbound and loosely bound cells in experimental wells were removed by shaking, and the remaining cells were then fixed as described above for reference wells. The fixative was aspirated, the wells were washed 3 times with 200 μ l of H_2O , and attached cells were stained with Crystal Violet (Sigma) by a modification of the method of Kueng *et al.* (1989). 100 μ l of 0.1% (w/v) Crystal Violet in 200 mM MES, pH 6.0, was added to each well and incubated at room temperature for 1 h. Excess dye was removed by three washes of 200 μ l of H_2O , and bound dye was solubilized with 100 μ l of 10% (v/v) acetic acid. The absorbance of each well at 570 nm was then measured using a multi-scan ELISA reader (Dynatech, Billingshurst, UK). Each sample was assayed in quadruplicate, and background attachment to BSA was subtracted from all measurements.

Purification of $\alpha 5 \beta 1$ Integrin from Human Placenta—Term placenta was obtained from Dr. J. Aplin, St. Mary's Hospital, Manchester, UK. Placenta (~500 g) was cut into small chunks with scissors and homogenized in a blender (Philips) with 400 ml of buffer A (150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 0.005% digitonin). The homogenate was stored at -70 °C. Homogenate was thawed at room temperature and centrifuged at 5,000 \times g for 10 min. The pelleted material was then mixed with 600 ml of buffer A on ice for 10 min and centrifuged as above. The pellet was extracted on ice for 1.5 h with 400 ml of buffer B (150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 2 mg/ml BSA). The extract was centrifuged at 6,000 \times g for 10 min and then at 40,000 \times g for 30 min. The supernatant was preadsorbed by passing it through a column of Sepharose 4B (30 ml), and then by mixing it with 10 ml of rat IgG-Sepharose (2 mg IgG/ml of beads) for 2 h on ice. IgG-Sepharose was then removed by centrifugation (5 min at 180 \times g) and column filtration, and the eluate was mixed with 8 ml of mAb 13-Sepharose (2 mg of IgG/ml of beads) for 2 h on ice. The suspension was then packed into a 1.6 cm diameter column (Pharmacia Biotech Inc.) and washed overnight (16 h) at 15 ml/h with buffer C (150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% Triton X-100). Bound material was eluted with buffer D (10 mM NaOAc, pH 3.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% Triton X-100) at 45 ml/h. 1.5-ml fractions were collected into 0.5 ml of 1 M Tris-HCl, pH 8.2. Aliquots of the fractions (25 μ l) were analyzed by SDS-polyacrylamide gel electrophoresis using a 6% nonreducing resolving gel and Coomassie Blue staining and found to contain $\beta 1$ integrins of $\geq 90\%$ purity.

Pooled fractions were then mixed with 2 ml of mAb 16-Sepharose (5 mg IgG/ml Sepharose) for 2 h on ice. The suspension was then packed into a 0.8-cm diameter column and washed with 12 ml of buffer C. Bound material was eluted with buffer D, and 0.5-ml fractions were collected and neutralized with 0.1 ml of 1 M Tris-HCl, pH 8.2. Aliquots of the fractions (25 μ l) were analyzed by SDS-polyacrylamide gel electrophoresis using a 6% nonreducing resolving gel. The only bands detected by Coomassie Blue staining were those corresponding to expected positions of the $\alpha 5$ and $\beta 1$ subunits. $\alpha 5$ and $\beta 1$ were the only integrin subunits detected in the eluted fractions by ELISA.

Purification of $\beta 1$ Integrins from Peripheral Blood Mononuclear Cells—Mononuclear cells were purified from ~100 leukocyte concentrates (provided by the Blood Transfusion Service, Lancaster, UK) by centrifugation on Histopaque 1077 (Sigma, Poole, Dorset, UK). Cells were then washed with phosphate-buffered saline, centrifuged at 400 \times g for 10 min, and extracted with 100 ml of buffer B on ice for 30 min. The extract was clarified by centrifugation at 40,000 \times g for 30 min, and the resulting supernatant was precleared by rotary mixing with 4 ml of rat IgG-Sepharose beads for 1 h at room temperature. IgG-Sepharose was prepared by coupling at a ratio of 2 mg of IgG to 1 ml of CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions. The IgG-Sepharose was removed by column filtration, and the filtrate was rotary mixed with 5 ml of mAb 13-Sepharose (2 mg IgG/ml Sepharose) for 2 h at room temperature. The suspension was then packed into a 1.6-cm diameter column and washed overnight at 4 °C with buffer C. Bound material was eluted with buffer D, and 1.5-ml fractions were collected and neutralized with 0.5 ml of 1 M Tris-HCl, pH 8.2. Aliquots of the fractions (50 μ l) were analyzed by SDS-polyacrylamide gel electrophoresis using a 7.5% nonreducing resolving gel and Coomassie Blue staining and were shown to contain $\beta 1$ integrins of $\geq 90\%$ purity. $\alpha 5 \beta 1$ was found to be a major component of this mixture by ELISA, although $\alpha 2 \beta 1$, $\alpha 4 \beta 1$, and $\alpha 6 \beta 1$ were also present. Only trace amounts of other $\beta 1$ integrins ($\alpha 3 \beta 1$ and $\alpha V \beta 1$) could be detected by ELISA.

¹ The abbreviations used are: mAb, monoclonal antibody; CCBD, central cell-binding domain of fibronectin; BSA, bovine serum albumin; MES, 4-morpholinoethane-sulfonic acid; ELISA, enzyme-linked immunosorbent assay.

Solid Phase Receptor-Ligand Binding Assay—80-kDa CCBD fragment of fibronectin (500 $\mu\text{g/ml}$ in phosphate-buffered saline) was mixed with an equal mass of sulfo-*N*-hydroxysuccinimido biotin (Pierce, Chester, UK) and rotary mixed for 30–40 min at room temperature. The mixture was then dialyzed against several changes of 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, to remove excess biotin. Solid-phase receptor-ligand binding was performed by a modification of the method of Charo *et al.* (1990). Purified $\alpha 5\beta 1$ from placenta or $\beta 1$ integrins from mononuclear cells (both at a concentration of ~ 0.5 mg/ml) were diluted 1:500 or 1:200, respectively, with phosphate-buffered saline containing divalent cations, and 100- μl aliquots were added to the wells of a 96-well ELISA plate (Immulon 3, Dynatech). Plates were incubated overnight at room temperature, and wells were blocked for 1–3 h with 200 μl of 5% (w/v) BSA, 150 mM NaCl, 0.05% (w/v) NaN_3 , 25 mM Tris-HCl, pH 7.4. Wells were then washed 3 times with 200 μl of 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, with 1 mg/ml BSA (buffer E). In experiments examining the role of cations in ligand binding, 100- μl aliquots of biotinylated CCBD fragment (typically 0.1 $\mu\text{g/ml}$) were diluted in buffer E with varying concentrations of MnCl_2 , MgCl_2 , or CaCl_2 (alone or in combination). For experiments examining the effect of antibodies or RGD peptides on ligand binding, biotinylated ligand diluted in buffer E with 1 mM MnCl_2 was added to the wells with or without antibodies or peptides. The plate was then incubated at 30 $^\circ\text{C}$ for 3 h. Biotinylated ligand was aspirated, and the wells were washed 3 times with buffer A. Bound ligand was quantitated by the addition of 1:200 ExtrAvidin-peroxidase conjugate (Sigma) in buffer E with 1 mM MnCl_2 for 10 min. Wells were then washed 4 times with buffer E, and color was developed using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma). Measurements obtained were the mean \pm S.D. of six replicate wells. The amount of nonspecific binding was measured by determining the level of ligand binding to wells coated with BSA alone; these values were subtracted from the corresponding values for receptor-coated wells. Results shown are representative of at least three separate experiments. Data shown in Figs. 1–3 were obtained using placental $\alpha 5\beta 1$, data shown in Figs. 4–6 were obtained using $\beta 1$ integrins from mononuclear cells, although both integrin preparations gave essentially identical results. Curve fitting (nonlinear regression analysis) to estimate apparent dissociation constants was performed as described previously (Mould *et al.*, 1994). For double-reciprocal plots, data were normalized to zero ligand binding in the absence of the supporting cation (Mn^{2+} or Mg^{2+}). Linear regression analysis of double-reciprocal plots was performed using SigmaPlot version 6; for clarity, not all of the data points are shown in some of these analyses. Under the conditions used in these assays ($\alpha 5\beta 1$ coating concentration ~ 4 nM) the ELISA signal appeared to be directly proportional to the amount of bound ligand because plots of $1/\text{absorbance}$ versus $1/[\text{free ligand}]$ (not shown) did not deviate from linearity at high ligand concentrations.

RESULTS

Mn^{2+} , Mg^{2+} , and Ca^{2+} Have Distinct Effects on $\alpha 5\beta 1$ -Fibronectin Interactions—To examine the role of divalent cations in regulating the activity of $\alpha 5\beta 1$, we compared the ability of Mn^{2+} , Ca^{2+} and Mg^{2+} to modulate integrin function in a solid phase receptor-ligand binding assay and in a K562 cell attachment assay. We chose to use an 80-kDa fragment from the CCBD of fibronectin as the ligand in these experiments because it contains only one integrin recognition domain. Native dimeric fibronectin, which contains an $\alpha 5\beta 1$ recognition site in both subunits, could show cooperative binding to immobilized integrins. For the solid phase assays described in this report, we used either an affinity-purified preparation of $\alpha 5\beta 1$ from human placenta or a partially pure preparation of $\alpha 5\beta 1$ from human peripheral blood mononuclear cells; essentially identical results were obtained from these two preparations. In the initial characterization of this assay, binding of the CCBD fragment was found to be inhibited $>90\%$ by an antifunctional antibody to the $\alpha 5$ subunit and to be completely inhibited by either EDTA or GRGDS peptide (results not shown). K562 cells were chosen for the attachment assays because $\alpha 5\beta 1$ is the only $\beta 1$ integrin expressed by these cells (Hemler *et al.*, 1987). In agreement with previous studies (Wayner *et al.*, 1989; Faulk *et al.*, 1993), we found that the attachment of K562 cells to the CCBD fragment was mediated solely by $\alpha 5\beta 1$ (data not shown).

Similar results were obtained in the two assay systems (Fig.

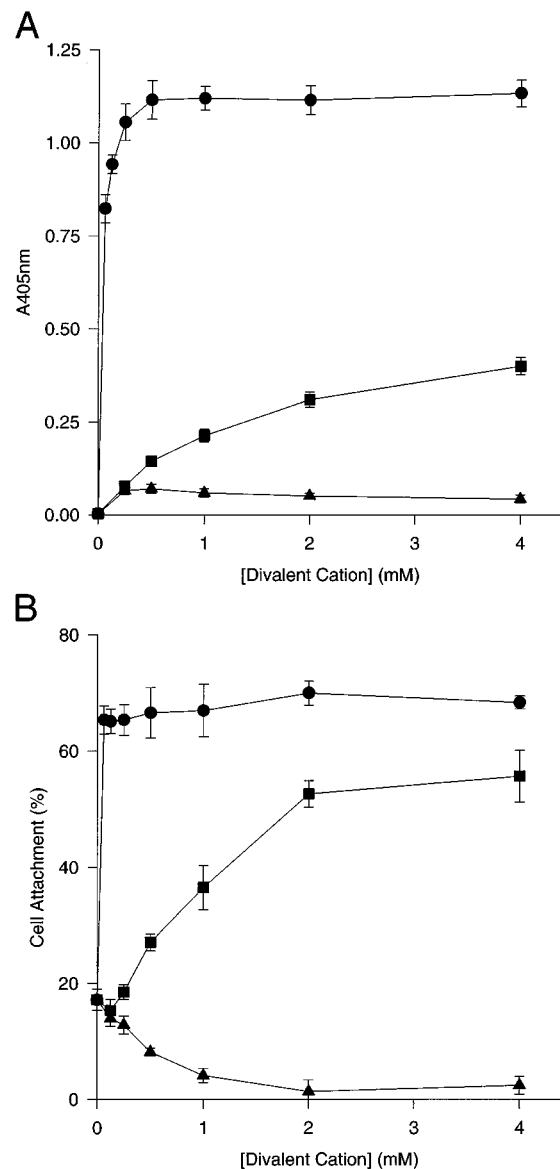


FIG. 1. Effect of Mn^{2+} , Mg^{2+} , and Ca^{2+} on the binding of CCBD fibronectin fragment to $\alpha 5\beta 1$ integrin in a solid phase assay (A) and on the attachment of K562 cells to CCBD fragment (B). Binding of biotinylated CCBD fragment in A or cell attachment in B was measured for a range of concentrations of each individual cation. \bullet , Mn^{2+} ; \blacksquare , Mg^{2+} ; \blacktriangle , Ca^{2+} .

1, A and B). These data suggest that cell-surface $\alpha 5\beta 1$ and $\alpha 5\beta 1$ in solid phase assays behave in a similar manner with respect to the divalent cation dependence of receptor-ligand interactions. Mn^{2+} , Mg^{2+} , and Ca^{2+} had markedly different effects on $\alpha 5\beta 1$ -fibronectin interactions. Both Mn^{2+} and Mg^{2+} promoted ligand binding, although Mn^{2+} supported higher levels of binding than Mg^{2+} . In contrast, Ca^{2+} supported little or no binding. In the cell attachment assay, low levels of attachment were observed in the absence of cations, and this level was decreased with increasing concentrations of Ca^{2+} . Comparison of the concentration of Mn^{2+} and Mg^{2+} to give half-maximal ligand binding in the solid phase assay (Table I) suggested that the affinity of Mn^{2+} for its binding site(s) on $\alpha 5\beta 1$ was ~ 40 -fold higher than that of Mg^{2+} . Scatchard-type analysis of the binding curves (not shown) indicated that there was only a single site (or a single class of sites) for Mn^{2+} and Mg^{2+} on $\alpha 5\beta 1$ for which cation occupancy supports ligand binding. Such sites have been termed "ligand-competent" sites

TABLE I
Affinity of $\alpha 5\beta 1$ for divalent cations

The ability of either Mn^{2+} , Mg^{2+} , or Ca^{2+} to support ligand binding was measured across a wide range of cation concentrations. The concentration of the ion required to support half-maximal ligand binding reflects the apparent dissociation constant (K_D) of the reaction for the ion (Smith *et al.*, 1994).

Divalent cation	K_D
Mn^{2+}	31 ± 4 ($n = 4$) μM
Mg^{2+}	1270 ± 220 ($n = 3$)
Ca^{2+}	ND ^a

^a Not determined. (The levels of ligand binding in Ca^{2+} were too low to obtain a value of K_D for this ion.)

(Smith *et al.*, 1994).

Since the solid phase assay was found to be highly reproducible, and also avoided possible artifacts from cation effects on cellular components other than integrins, we chose to use this assay for a detailed study of the effects of combinations of cations on ligand binding.

Ca²⁺ Is a Noncompetitive Inhibitor of Ligand Binding Supported by Mn²⁺—Since Ca^{2+} failed to support ligand binding, we first investigated whether Ca^{2+} could reduce the high levels of ligand binding supported by Mn^{2+} . Fig. 2 shows the result of an experiment in which the concentration of Mn^{2+} was kept constant at $100 \mu M$ and the concentration of Ca^{2+} was varied. Ca^{2+} strongly inhibited Mn^{2+} -supported ligand binding, although even at a very high Ca^{2+} concentration (8 mM) ligand binding was not abrogated. In contrast, Mg^{2+} did not significantly inhibit Mn^{2+} -supported binding even at 8 mM (results not shown).

To further analyze the effects of Ca^{2+} on Mn^{2+} -supported ligand-binding, we examined the effects of varying the concentration of Mn^{2+} at constant Ca^{2+} . Fig. 3A shows the inhibition of ligand binding by 8 mM Ca^{2+} . Ca^{2+} greatly reduced the maximal level of ligand binding but did not significantly alter the concentration of Mn^{2+} required for half-maximal ligand binding. A double-reciprocal plot of these data (Fig. 3B) indicated that the inhibition observed at high Ca^{2+} concentrations is noncompetitive in nature. A detailed analysis of the effects of lower Ca^{2+} concentrations on Mn^{2+} -supported ligand binding (not shown) suggested that Ca^{2+} binding at multiple sites on the integrin was responsible for its inhibitory effects; however, Ca^{2+} binding to any of these sites did not decrease the apparent affinity of Mn^{2+} for its ligand-competent site. An important inference from these studies is therefore that, Ca^{2+} does not compete with Mn^{2+} for binding to the Mn^{2+} ligand-competent site on $\alpha 5\beta 1$, and therefore appears to bind to different sites.

Ca²⁺ Can Stimulate or Inhibit Ligand Binding Supported by Mg²⁺—To investigate whether Ca^{2+} could act as an inhibitor of ligand binding promoted by Mg^{2+} , we initially investigated the effect of Ca^{2+} on ligand binding supported by different Mg^{2+} concentrations. At high Mg^{2+} concentrations, only a partial inhibition of ligand binding was observed (result not shown), whereas for low Mg^{2+} concentrations ligand binding was significantly increased at low Ca^{2+} concentrations. Fig. 4 shows an experiment in which the concentration of Mg^{2+} was kept constant at $50 \mu M$ and the concentration of Ca^{2+} varied between 0 and 4 mM. Two phases were apparent. In the first phase there was a marked stimulation in ligand binding, with a concentration of Ca^{2+} for half-maximal increase of $\sim 30 \mu M$ and which reached a maximum at ~ 0.2 – 0.5 mM Ca^{2+} . In the second phase, ligand binding was inhibited at high concentrations of Ca^{2+} ; at very high Ca^{2+} concentrations ligand binding approached the low levels observed in Ca^{2+} alone (not shown). These results suggest that there are at least two Ca^{2+} -binding

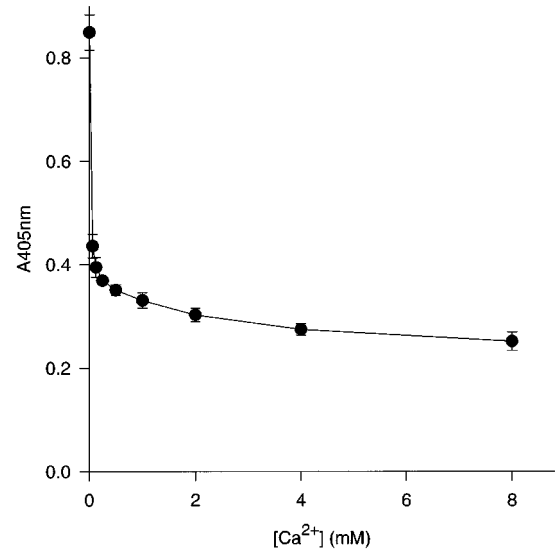


FIG. 2. Effect of Ca^{2+} on Mn^{2+} -supported binding of CCBDFragment to $\alpha 5\beta 1$. The ability of Ca^{2+} to interfere with ligand binding supported by $100 \mu M$ Mn^{2+} was measured for a range of Ca^{2+} concentrations. In the experiment shown above, the level of ligand binding in 8 mM Ca^{2+} alone was 0.032 ± 0.010 .

sites on $\alpha 5\beta 1$ and that these sites have opposing influences on ligand binding supported by low concentrations of Mg^{2+} . Binding of Ca^{2+} to a high affinity site (the first phase) stimulates ligand binding, whereas binding of Ca^{2+} to a low affinity site (the second phase) inhibits ligand binding. To analyze the first of these two phases, we tested the effect of a low concentration of Ca^{2+} (0.25 mM) on ligand binding supported by different Mg^{2+} concentrations (Fig. 5A). The results showed that low Ca^{2+} concentrations have two effects on ligand binding; at low Mg^{2+} concentrations ligand binding is increased, whereas at high Mg^{2+} concentrations the maximal amount of bound ligand is reduced. The concentration of Mg^{2+} required for half-maximal ligand binding in these assays ($\sim 40 \mu M$) was decreased ~ 30 -fold compared with that for Mg^{2+} alone (see Table I). This increase in affinity of Mg^{2+} for its ligand-competent site appears to be the mechanism by which Ca^{2+} stimulates ligand binding at low Mg^{2+} concentrations. A double-reciprocal plot of these data (Fig. 5B) showed that, at low concentrations, Ca^{2+} is a mixed-type inhibitor of ligand binding supported by Mg^{2+} ; it both increases the affinity of Mg^{2+} for its site and reduces the maximal level of ligand binding.

To analyze the inhibition of Mg^{2+} -supported ligand-binding by high Ca^{2+} concentrations (the second phase in Fig. 4) we compared the effect of 0.2 mM Ca^{2+} (a concentration that caused maximal stimulation of binding supported by low Mg^{2+} concentrations) with that of 8 mM (Fig. 6A). High concentrations of Ca^{2+} increased the concentration of Mg^{2+} required for half-maximal ligand binding but did not affect the maximal amount of ligand bound at high Mg^{2+} concentrations. The reciprocal plot (Fig. 6B) shows that the inhibition of ligand binding at high Ca^{2+} concentrations is competitive in nature. By nonlinear regression analysis, the K_I value was calculated as ~ 2 mM. Further analysis of the inhibition of Mg^{2+} -supported ligand binding at different Ca^{2+} concentrations (not shown) indicated that this inhibition is directly competitive, *i.e.* Ca^{2+} is able to compete with Mg^{2+} for binding to the Mg^{2+} ligand-competent site. However, when this site is occupied by Ca^{2+} , the integrin fails to bind ligand.

Taken together, these data suggest: (a) that there is a Ca^{2+} -binding site of high affinity, the occupancy of which converts the Mg^{2+} ligand-competent site into a high-affinity binding site

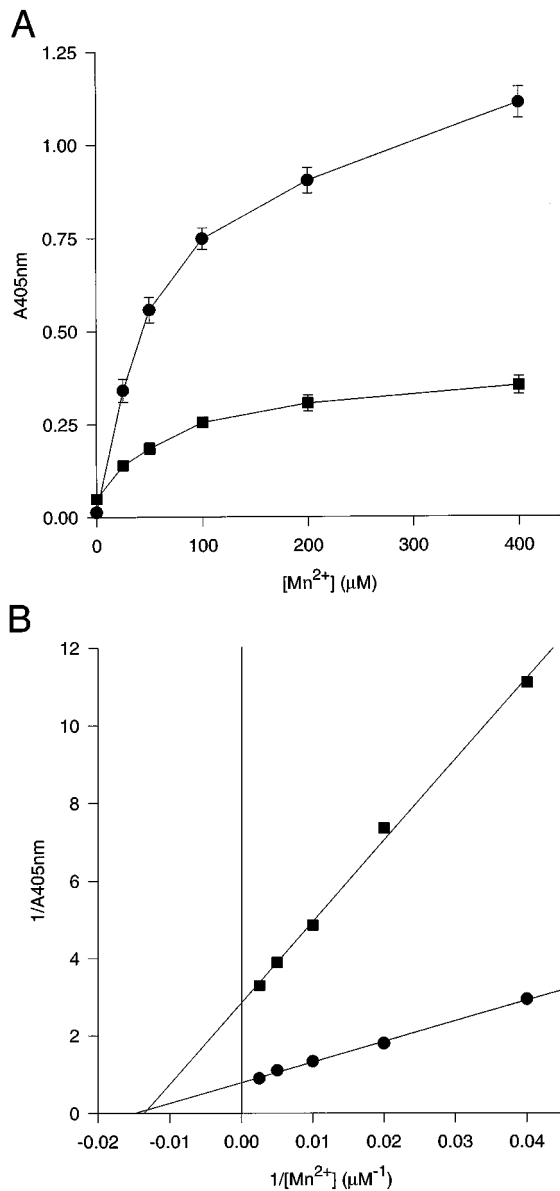


FIG. 3. *A*, effect of 8 mM Ca²⁺ on the binding of CCBDF fragment to $\alpha 5 \beta 1$ at varying Mn²⁺ concentrations. ●, Mn²⁺ alone; ■, Mn²⁺ with 8 mM Ca²⁺. *B*, double-reciprocal plot of the data shown in *A*. By linear regression analysis, the two lines intersect approximately on the x axis, indicative of noncompetitive inhibition. *r*² values are 0.997 (Mn²⁺ alone) and 0.997 (Mn²⁺ with 8 mM Ca²⁺).

for Mg²⁺ and (b) that Ca²⁺ is, however, also able to competitively inhibit the binding of Mg²⁺ to its ligand-competent site. However, since Ca²⁺ binds this latter site with only low affinity, high concentrations of Ca²⁺ are required to oppose the high affinity binding of Mg²⁺. Importantly, the observation that Ca²⁺ can act as a direct competitive inhibitor of Mg²⁺-supported binding but not of Mn²⁺-supported binding also suggests that the ligand-competent sites for these two ions may be distinct.

DISCUSSION

In this report, we have performed a comprehensive analysis of the effects of Mn²⁺, Mg²⁺, and Ca²⁺ ions on the ligand-binding capacity of the integrin $\alpha 5 \beta 1$. Our data show the following. (a) Only Mn²⁺ and Mg²⁺ support ligand binding. Although Ca²⁺ does not support ligand binding, it strongly modulates ligand binding supported by Mn²⁺ or Mg²⁺. (b) Ca²⁺

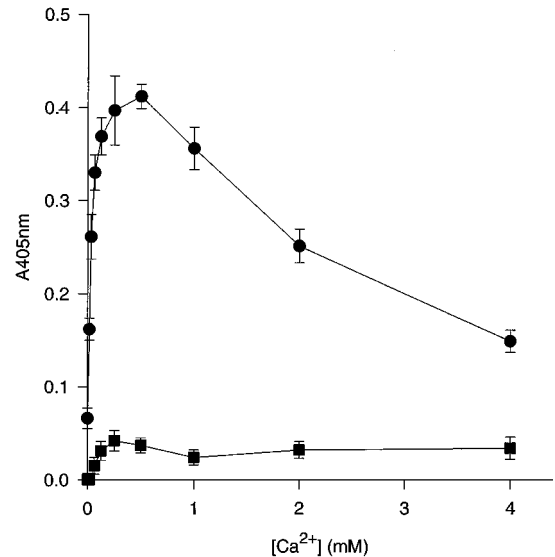


FIG. 4. Effect of Ca²⁺ on the binding of CCBDF fragment to $\alpha 5 \beta 1$ supported by a low concentration of Mg²⁺. The ability of Ca²⁺ to modulate ligand binding was examined for a range of Ca²⁺ concentrations. ●, 50 μM Mg²⁺ with Ca²⁺; ■, Ca²⁺ alone.

is a noncompetitive inhibitor of Mn²⁺-supported ligand binding, suggesting that it does not compete with Mn²⁺ for binding to the Mn²⁺ ligand-competent site. (c) Ca²⁺ can either enhance or inhibit Mg²⁺-supported binding, depending on the concentrations of each ion. The results suggest that Ca²⁺ can compete directly with Mg²⁺ for binding to the Mg²⁺ ligand-competent site, but Ca²⁺ binding to a separate high affinity site also greatly increases the affinity of Mg²⁺ for its ligand-competent site. Taken together, these findings indicate that $\alpha 5 \beta 1$ possesses several distinct cation-binding sites, each of which has a different specificity for Mn²⁺, Mg²⁺, and Ca²⁺.

Our studies of the effects of Mn²⁺, Mg²⁺, and Ca²⁺ on fibronectin binding to $\alpha 5 \beta 1$ showed that this interaction was strongly promoted by Mn²⁺, and to a lesser extent by Mg²⁺. This pattern has been observed for many other integrins including $\alpha 1 \beta 1$ (Luque *et al.*, 1994), $\alpha 2 \beta 1$ (Staatz *et al.*, 1989; Kern *et al.*, 1993), $\alpha 3 \beta 1$ (Weitzman *et al.*, 1993), $\alpha 6 \beta 1$ (Sonnensberg *et al.*, 1988), $\alpha V \beta 1$ (Kirchhofer *et al.*, 1991), and $\alpha L \beta 2$ (Dransfield *et al.*, 1992b). Ca²⁺ does, however, support ligand binding by a small number of integrins, including those of the $\beta 3$ family (Kirchhofer *et al.*, 1991; Smith *et al.*, 1994). In a previous study (Gailit and Ruoslahti, 1988), Mn²⁺ and Mg²⁺ were found to support ligand binding by purified $\alpha 5 \beta 1$ in liposomes, with similar values for the cation concentrations required for half-maximal ligand binding as reported here. However, Ca²⁺ was also found to support ligand binding in the above study. Based on our findings that Ca²⁺ can synergize with low concentrations of Mg²⁺, this result may have been due to contamination of the Ca²⁺ samples with low concentrations of Mg²⁺. Similarly, an explanation for the low levels of K562 cell attachment we observed in the absence of exogenous cations or in the presence of low Ca²⁺ concentrations is probably that small amounts of Mg²⁺ are released from the cells during the time course of the experiment, high concentrations of Ca²⁺ were observed to inhibit this effect. A recent study of myeloid cell adhesion to fibronectin confirms our finding that Ca²⁺ alone does not support $\alpha 5 \beta 1$ -ligand interactions and that Ca²⁺ also inhibits Mn²⁺- and Mg²⁺-supported adhesion (Davis and Camarillo, 1993).

We found that the affinity of $\alpha 5 \beta 1$ for Mn²⁺ was ~40-fold greater than that for Mg²⁺. It is a common feature of integrin-ligand interactions that typically >10-fold lower concentra-

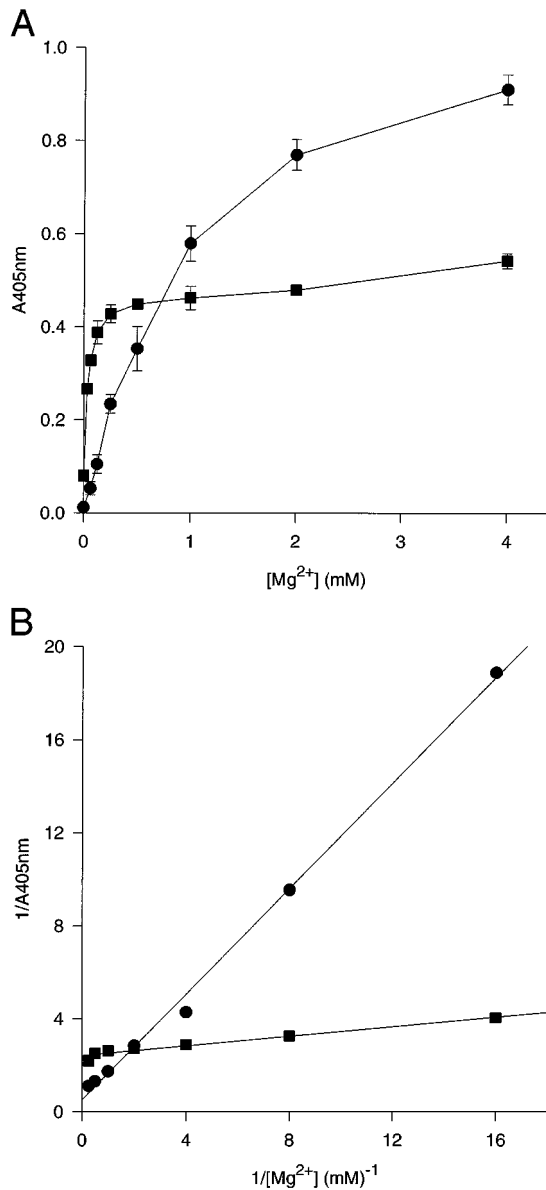


FIG. 5. A, effect of 0.25 mM Ca²⁺ on the binding of CCBD fragment to $\alpha 5\beta 1$ in the presence of varying concentrations of Mg²⁺. ○, Mg²⁺ alone; ■, Mg²⁺ with 0.25 mM Ca²⁺. B, double-reciprocal plot of the data shown in A. By linear regression analysis, the two lines intersect above and to the right of the origin, indicative of a mixed-type inhibition. r^2 values are 0.997 (Mg²⁺ alone) and 0.955 (Mg²⁺ with 0.25 mM Ca²⁺).

tions of Mn²⁺ are required to support ligand binding than Mg²⁺ (Altieri, 1991; Dransfield *et al.*, 1992b; Kern *et al.*, 1993; Michishita *et al.*, 1993; Luque *et al.*, 1994; Smith *et al.*, 1994), indicating that many other integrins also contain one or more high affinity Mn²⁺-binding sites. An important implication from our observation that Ca²⁺ could competitively inhibit Mg²⁺-supported ligand binding but not Mn²⁺-supported binding is that there may be separate ligand-competent sites on $\alpha 5\beta 1$ for Mn²⁺ and Mg²⁺. This may shed light on why Mn²⁺-supported ligand binding is of much higher affinity than that supported by Mg²⁺. We have also found that Mn²⁺ causes a larger increase than Mg²⁺ in the expression of an activation epitope on $\alpha 5\beta 1$ recognized by the mAb 12G10 (Mould *et al.*, 1995),² suggesting that Mn²⁺ is better than Mg²⁺ at stabilizing

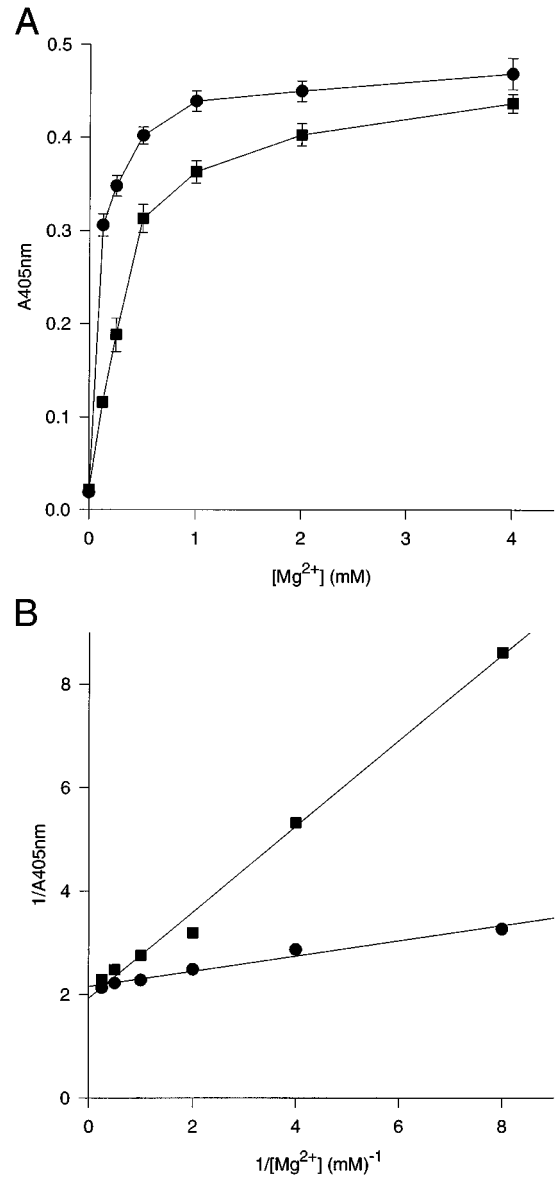


FIG. 6. A, comparison of the effects of 0.2 mM and 8 mM Ca²⁺ on the binding of CCBD fragment to $\alpha 5\beta 1$ in the presence of varying concentrations of Mg²⁺. ●, Mg²⁺ with 0.2 mM Ca²⁺; ■, Mg²⁺ with 8 mM Ca²⁺. B, double-reciprocal plot of the data shown in A. By linear regression analysis, the two lines intersect approximately on the y axis, indicative of a competitive inhibition. r^2 values are 0.973 (Mg²⁺ with 0.2 mM Ca²⁺) and 0.999 (Mg²⁺ with 8 mM Ca²⁺).

a conformational change in the integrin required for ligand recognition.

The existence of a separate high affinity Ca²⁺-binding site, distinct from the Mg²⁺ ligand-competent site, was suggested by the observation that low concentrations of Ca²⁺ greatly increased the apparent affinity of Mg²⁺ for its ligand-competent site. In summary, our studies suggest that at least three distinct cation-binding sites on $\alpha 5\beta 1$ are involved in the regulation of integrin activity; a tentative model of these sites is shown in Fig. 7. Site 1 binds Mn²⁺ with high affinity; Ca²⁺ does not appear to compete with Mn²⁺ for binding to this site. Site 2 binds Mg²⁺ with low affinity. Both sites 1 and 2 are ligand-competent sites (Smith *et al.*, 1994). Since, at high concentrations, Ca²⁺ acts as a direct competitive inhibitor of Mg²⁺ binding, Ca²⁺ can also bind to site 2, although with low affinity. Site 3 is a Ca²⁺-binding site of high affinity with characteristics of the "effector" site proposed by Smith *et al.*, (1994). Ca²⁺ binding

² A. P. Mould, A. N. Garrat, J. A. Askari, S. K. Akiyama, and M. J. Humphries, manuscript in preparation.

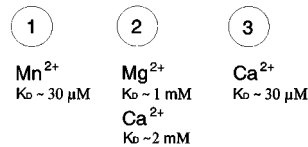


FIG. 7. Model of the type and specificity of cation-binding sites in $\alpha 5\beta 1$. Occupancy of site 1 by Mn^{2+} , or occupancy of site 2 by Mg^{2+} , renders the integrin competent to bind ligand. Although Ca^{2+} can compete with Mg^{2+} for binding to site 2, Ca^{2+} occupancy of this site does not permit ligand binding. Site 3 is a Ca^{2+} -binding site of high affinity; occupancy of this site by Ca^{2+} increases the affinity of Mg^{2+} for site 2 ($K_D \sim 40 \mu M$). The term "site" in the above context could refer either to individual cation-binding sites or to classes of site; our data do not allow us to distinguish between these possibilities.

to this site dramatically increases the affinity of Mg^{2+} for site 2. Ca^{2+} binding at sites 2 and 3 (or possibly at additional sites) may be responsible for its ability to noncompetitively inhibit ligand binding supported by Mn^{2+} . Our results clearly indicate that the cation-binding sites on $\alpha 5\beta 1$ are not all equivalent, neither are they of broad specificity, but instead each site shows a distinct selectivity for one or more cations. Binding of one cation to its site(s) can also affect the affinity of a second cation for its site(s); a similar cooperativity in cation binding has been observed for proteins such as calmodulin that contain multiple EF-hands (Strynadka and James, 1989). How the proposed cation-binding sites in Fig. 7 correspond to the putative divalent cation-binding sites in the $\alpha 5$ and $\beta 1$ subunits and the molecular basis of their specificity will be the subject of future investigations. Such studies have the prospect of mapping key sites involved in modulating integrin function.

The model shown in Fig. 7 is similar in several respects to that proposed to explain the effects of Ca^{2+} and Mn^{2+} on ligand binding to $\alpha V\beta 3$ (Smith *et al.*, 1994). In this study, Ca^{2+} was found to be a mixed-type inhibitor of Mn^{2+} -supported fibrinogen binding to $\alpha V\beta 3$: low concentrations of Ca^{2+} slightly increased the affinity of Mn^{2+} binding to $\alpha V\beta 3$ but decreased the amount of ligand bound at saturating Mn^{2+} concentrations. Since high concentrations of Ca^{2+} completely inhibited ligand binding, it was suggested that Ca^{2+} could also competitively inhibit the binding of Mn^{2+} to its site. Based on these results, a two-site model for $\alpha V\beta 3$ was proposed in which Ca^{2+} binds to an effector site and Mn^{2+} (or Ca^{2+}) to a ligand-competent site (Smith *et al.*, 1994). However, in a recent study of osteopontin binding to $\alpha V\beta 3$ (Hu *et al.*, 1995), it was found that Ca^{2+} only decreased the association-rate of Mn^{2+} -supported ligand binding but had no effect on the dissociation-rate, suggesting that Mn^{2+} and Ca^{2+} must bind to different sites on the integrin, with one cation influencing the on-rate and the other the off-rate of ligand binding. Hence this latter report endorses the concept that there are cation-binding sites on integrins that selectively bind only one type of divalent ion.

Our model of the cation-binding sites in $\alpha 5\beta 1$ may be broadly applicable to the other integrins that show similar divalent-cation requirements for ligand binding. For example, it has been shown for the $\alpha L\beta 2$ -ICAM-1 interaction that high concentrations of Ca^{2+} can compete with Mg^{2+} , but not with Mn^{2+} , for binding to the integrin (Jackson *et al.*, 1994). Low concentrations of Ca^{2+} can, however, synergize with low concentrations of Mg^{2+} to increase ligand binding (Marlin and Springer, 1987). Hence $\alpha L\beta 2$ may have a similar arrangement of cation-binding sites to $\alpha 5\beta 1$.

One intriguing implication from our results is that there may be one cation-binding site on the integrin that selectively binds Mn^{2+} . Whether or not Mn^{2+} (or other transition metals) have a role in the regulation of integrin activity *in vivo* has been a matter of some speculation. It has been estimated that the concentration of Mn^{2+} in tissues is in the range 1–14 μM (Smith

et al., 1994). Significant binding of fibronectin to $\alpha 5\beta 1$ was induced by these concentrations of Mn^{2+} *in vitro*, hence Mn^{2+} could potentially act as a physiological effector of $\alpha 5\beta 1$. Since the majority of Mn^{2+} in the body is sequestered in bone, Mn^{2+} might also be an important regulator of integrins during bone resorption. Alternatively, the effects of Mn^{2+} observed *in vitro* could be fortuitous and not relevant *in vivo*. If it is possible to identify the Mn^{2+} -binding site in $\alpha 5\beta 1$ (e.g. through site-directed mutagenesis), then several approaches could be adopted to resolve this question. This will be the subject of future work.

In conclusion, we have shown that Mn^{2+} , Mg^{2+} , and Ca^{2+} appear to recognize different sites on $\alpha 5\beta 1$ and that each ion has distinct effects on the capacity of $\alpha 5\beta 1$ to bind ligand. Since these cations have the ability to differentially regulate $\alpha 5\beta 1$ function, it will be important in the future to examine the *in vivo* consequences of fluctuations in divalent cation concentrations on $\alpha 5\beta 1$ -mediated cell adhesion and migration, for example, during wound healing (Banai *et al.*, 1990; Sank *et al.*, 1989).

Acknowledgments—We thank J. Aplin for providing human placenta, and Lancaster Blood Transfusion Service for supplying leukocyte concentrates. We also thank W. S. Argraves, D. Tuckwell, and P. Newham for advice and helpful discussions.

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