Regulation of integrin function: evidence that bivalent-cation-induced conformational changes lead to the unmasking of ligand-binding sites within integrin $\alpha 5\beta 1$

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The molecular mechanisms that regulate integrin–ligand binding are unknown; however, bivalent cations are essential for integrin activity. According to recent models of integrin tertiary structure, sites involved in ligand recognition are located on the upper face of the seven-bladed β -propeller formed by the N-terminal repeats of the α subunit and on the von Willebrand factor A-domain-like region of the β subunit. The epitopes of function-altering monoclonal antibodies (mAbs) cluster in these regions of the α and β subunits; hence these mAbs can be used as probes to detect changes in the exposure or shape of the ligand-binding sites. Bivalent cations were found to alter the apparent affinity of binding of the inhibitory anti- $\alpha 5$ mAbs JBS5 and 16, the inhibitory anti- $\beta 1$ mAb 13, and the stimulatory anti- $\beta 1$ mAb

12G10 to $\alpha 5\beta 1$. Analysis of the binding of these mAbs to $\alpha 5\beta 1$ over a range of Mn^{2+} , Mg^{2+} or Ca^{2+} concentrations demonstrated that there was a concordance between the ability of cations to elicit conformational changes and the ligand-binding potential of $\alpha 5\beta 1$. Competitive ELISA experiments provided evidence that the domains of the $\alpha 5$ and $\beta 1$ subunits recognized by mAbs JBS5/16 and 13/12G10 are spatially close, and that the distance between these two domains is increased when $\alpha 5\beta 1$ is occupied by bivalent cations. Taken together, our findings suggest that bivalent cations induce a conformational relaxation in the integrin that results in exposure of ligand-binding sites, and that these sites lie near an interface between the α subunit β -propeller and the β subunit putative A-domain.

INTRODUCTION

Cells make adhesive contacts with their environment through cell-surface receptors, many of which belong to the integrin gene superfamily. Integrins are heterodimers containing α and β subunits; receptors containing the $\beta 1$ subunit constitute the principal group of cell-matrix receptors. A striking feature of integrin–ligand interactions is that they can be dynamically regulated: integrins can exist in inactive or active states, with only the active state competent to bind ligand. Acquisition of the active state requires bivalent cations and is accompanied by conformational changes, involving both α and β subunits [1].

The N-terminal portion of integrin α subunits comprises seven homologous, tandemly repeated domains of about 60 amino acids each. Repeats 4–7 (or in some integrins 5–7) each contain cation-binding sequences, similar to the EF-hand motif found in Ca^{2+} -binding proteins such as calmodulin [2]. Recently, the N-terminal repeats have been proposed to form a seven-bladed β -propeller structure in which a bivalent ion is co-ordinated at the centre of the propeller [3]. An additional cation-binding site is found in a highly conserved region close to the N-terminus of the β subunit [4,5]. This region is predicted to have a von Willebrand factor A domain-like fold [6–9].

Sites involved in ligand binding have been identified in the N-terminal regions of both α and β subunits. The N-terminal repeats of the α subunit and the putative A-domain region of the β subunit appear to contain sequences that participate directly in ligand recognition (for a review see [10]). The epitopes of monoclonal antibodies (mAbs) that inhibit $\beta 1$ integrin function have been mapped to a region containing amino acid residues 207–218 within the putative A-domain; intriguingly, this region also includes the epitopes of several mAbs that stimulate integrin

function [11]. The epitopes of inhibitory mAbs that recognize the $\alpha 4$ or $\alpha 5$ subunits cluster in the second and third of the N-terminal repeats [12–15], and involve putative loop regions on the upper surface of the α subunit β -propeller [15–17]. We have shown that the epitopes of inhibitory anti- $\alpha 5$ and anti- $\beta 1$ mAbs are close to, but not directly overlapping with, sites involved in ligand binding [15,18]. These mAbs do not act as ligand mimetics; however, because of the close proximity of their epitopes to ligand-binding sites, these mAbs can be used as probes to detect changes in the exposure or conformation of the ligand-binding domains.

Although it is clear that bivalent-cation occupancy and ligand binding are intimately linked, the precise role of bivalent cations in integrin–ligand interactions remains uncertain. In a recent study, we found that Mn^{2+} , Mg^{2+} and Ca^{2+} have markedly different effects on ligand recognition by $\alpha 5\beta 1$ [19]. Both Mn^{2+} and Mg^{2+} supported ligand binding, but Mn^{2+} supported higher-affinity ligand binding than Mg^{2+} ; in contrast, Ca^{2+} supported ligand binding very poorly, and was a non-competitive inhibitor of ligand binding supported by Mn^{2+} . Our studies also indicated that several distinct cation-binding sites are involved in regulating the ligand-binding activity of $\alpha 5\beta 1$.

Here we have investigated the effect of Mn^{2+} , Mg^{2+} and Ca^{2+} on the binding of inhibitory mAbs to $\alpha 5\beta 1$. We also compare the effect of these ions on the binding of the activating mAb 12G10 [20], which recognizes the same region of the $\beta 1$ subunit as inhibitory anti- $\beta 1$ mAbs. We show that cation-induced conformational changes reported by changes in the binding of these function-perturbing mAbs to $\alpha 5\beta 1$ correlate with the effects of cations on the ligand-binding activity of $\alpha 5\beta 1$. Competitive ELISA experiments provide evidence that the epitopes of some function-altering anti- $\alpha 5$ and anti- $\beta 1$ mAbs are spatially close.

Abbreviation used: mAb, monoclonal antibody.

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Our data suggest a mechanism by which conformational changes lead to the exposure of ligand-binding sites within $\alpha 5\beta 1$; this mechanism is consistent with recently proposed models of integrin activation [21,22].

MATERIALS AND METHODS

Materials

Rat mAbs 16 and 11 recognizing the human $\alpha 5$ subunit, and mAb 13 recognizing the human $\beta 1$ integrin, were gifts from Dr. S. K. Akiyama (National Institute of Environmental Health Sciences, NIH, Chapel Hill, NC, U.S.A.). Mouse anti-human $\alpha 5$ mAbs P1D6 and JBS5 were purchased from Gibco-BRL and Serotec (Oxford, U.K.) respectively. Mouse anti-human $\beta 1$ mAbs 4B4 and K20 were purchased from Coulter (Miami, FL, U.S.A.), and The Binding Site (Birmingham, U.K.) respectively. Mouse anti-human $\beta 1$ integrin mAbs TS2/16 and SG/19 were gifts from F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain) and K. Miyake (Saga Medical School, Saga, Japan) respectively. Mouse anti-human $\beta 1$ integrin mAb 12G10 was produced and purified as described previously [20]. All antibodies were used as purified IgG, except P1D6 (as ascites). Integrin $\alpha 5\beta 1$ was purified from human placenta as previously described [17].

Biotinylation of mAbs

mAbs (0.5 mg/ml in PBS) were mixed with an equal mass of sulpho-*N*-hydroxysuccinimido biotin (Pierce) and rotary mixed for 30–40 min at room temperature. The mixture was then dialysed against several changes of 150 mM NaCl/25 mM Tris/HCl, pH 7.4, to remove excess biotin. The dialysate was centrifuged at 13000 *g* for 15 min, and stored at -70°C .

Effect of bivalent cations on the binding of mAbs to $\alpha 5\beta 1$

Purified $\alpha 5\beta 1$ integrin (at a concentration of $\approx 500\ \mu\text{g/ml}$) was diluted 1:500 with PBS containing bivalent cations, and 100 μl aliquots were added to the wells of a 96-well ELISA plate (Dynatech Immulon 3 or 4). 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 three times with 200 μl of 150 mM NaCl/25 mM Tris/HCl, pH 7.4, containing 1 mg/ml BSA (buffer A). Buffer A was treated with Chelex beads (Bio-Rad) to remove any small contaminating amounts of endogenous Ca^{2+} and Mg^{2+} ions. Aliquots (100 μl each) of biotinylated mAbs (0.03–10 $\mu\text{g/ml}$) in buffer A with 1 mM EDTA or 1 mM MnCl_2 were added to the wells. The plate was then incubated at 37°C for 2 h. Unbound antibody was aspirated, and the wells were washed three times with buffer A. Bound antibody was quantified by addition of 1:500 dilution of ExtrAvidin peroxidase conjugate (Sigma) in buffer A for 20 min at room temperature. Wells were then washed four times with buffer A, and colour was developed using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) substrate. Measurements obtained were the means $\pm$ S.D. of four replicate wells. Apparent dissociation constants (K_D) were calculated using the equation $A = A_{\text{max}} \times \{[L]/(K_D + [L])\}$, where A is the absorbance at 405 nm, A_{max} is the maximum absorbance at saturation and is treated as a variable parameter, and $[L]$ is the molar concentration of free antibody ($\approx [L]_0$). K_D values were derived by non-linear regression analysis of the data using Sigma Plot Version 6 (Jandel Scientific, San Rafael, CA, U.S.A.), assuming monovalent binding of the mAbs. Error values quoted are asymptotic S.E.M. values derived from the non-linear regression analysis. A molecular mass of 150 kDa was assumed for IgG.

To analyse the effect of a range of Mn^{2+} , Mg^{2+} and Ca^{2+} concentrations on the binding of mAbs to $\alpha 5\beta 1$, biotinylated mAbs were used at a constant concentration (0.1 $\mu\text{g/ml}$), and Mn^{2+} , Mg^{2+} or Ca^{2+} was added to buffer A to a final concentration of 0.015–4 mM. To estimate the concentrations of bivalent cations required for half-maximal change in antibody binding (apparent K_D values), non-linear regression analysis of the data was performed as described above.

Competitive ELISA experiments

ELISA plates were coated with $\alpha 5\beta 1$, blocked and washed as described above. Biotinylated mAbs (0.1 $\mu\text{g/ml}$) in buffer A with 1 mM EDTA were added to the wells in the absence or presence of a large excess of unlabelled competitor mAbs (10 $\mu\text{g/ml}$ or 1:500 dilution of ascites). The plate was then incubated at 37°C for 2 h. Unbound antibodies were aspirated, and the wells washed three times with buffer A. Bound biotinylated antibody was detected as described above. Measurements obtained were the means $\pm$ S.D. of at least four replicate wells. Other modifications to this assay are described in the Figure legends.

In all the assays described above, the amount of non-specific binding was measured by determining the level of antibody binding to wells coated with BSA alone; these values were subtracted from the corresponding values for receptor-coated wells. Each experiment shown is representative of at least three separate experiments. Under the conditions used in these assays, the ELISA signal appeared to be directly proportional to the amount of bound antibody because plots of 1/absorbance versus 1/[free antibody] did not deviate from linearity at high concentrations. Note, however, that binding constants cannot be precisely determined in these assays, and that only apparent K_D values are quoted.

Mapping of the 12G10 epitope

Construction of mouse/human and chicken/human chimaeras and site-directed mutagenesis of the human $\beta 1$ subunit were carried out as previously described [11]. Chimaeras and mutated human $\beta 1$ were stably expressed on the surface of Chinese hamster ovary cells (as heterodimers with endogenous hamster $\alpha 5$), and the percentage of cells reactive to the mAbs was assessed by flow cytometry.

RESULTS

Bivalent cations modulate the apparent affinity of binding of function-altering mAbs to $\alpha 5\beta 1$

We tested whether the binding of the anti- $\alpha 5$ mAbs JBS5, 16 and P1D6, and the anti- $\beta 1$ mAbs 13, 4B4, TS2/16 and 12G10, to $\alpha 5\beta 1$ was influenced by bivalent cations. These mAbs have been shown to have epitopes close to the ligand-binding domains of $\alpha 5\beta 1$ and to alter integrin function (Table 1). In preliminary experiments (not shown), we found that Mn^{2+} , Mg^{2+} and Ca^{2+} markedly changed the apparent affinity of mAbs JBS5, 16, 13 and 12G10 binding to $\alpha 5\beta 1$. Significantly, Mn^{2+} , a bivalent ion previously shown to be a potent stimulator of $\alpha 5\beta 1$ function, caused the largest change in the binding of the mAbs to $\alpha 5\beta 1$ (Figure 1). Whereas the apparent affinity of mAbs JBS5, 16 and 12G10 binding to $\alpha 5\beta 1$ was increased by Mn^{2+} , the apparent affinity of mAb 13 binding was decreased. The apparent affinity of P1D6, 4B4 and TS2/16 binding to $\alpha 5\beta 1$ was altered < 1.5 -fold in the presence of cations (results not shown). The binding of the non-inhibitory mAbs 11 (against $\alpha 5$) and K20 (against $\beta 1$) was also unaffected by Mn^{2+} , Mg^{2+} or Ca^{2+} (results not shown).

Table 1 Specificity of the mAbs used in this study

Antibodies are categorized according to their function-altering activity (Inhib., inhibitory; or Stim., stimulatory); mAbs 11 and K20 have no effect on integrin activity (n/l, neutral) and are used as controls. The epitopes of JBS5, 16 and P1D6 lie in the second and third repeats (R2/R3) of the α5 subunit [15], and A. Irie and Y. Takada, unpublished work), whereas that of mAb 11 probably lies outside the N-terminal repeats (C-terminal) [15]. The epitopes of mAbs 13, 4B4 and TS2/16 include amino acid residues 207–218 in the putative A-domain of the β1 subunit [11], whereas the epitope of K20 lies in amino acid residues 426–587 of β1 (outside the putative A-domain) [11]. –, not known.

	Anti-α5 mAb				Anti-β1 mAb				
	mAb ...JBS5	16	P1D6	11	13	4B4	12G10	TS2/16	K20
Type	Inhib.	Inhib.	Inhib. ^a	n/l	Inhib.	Inhib.	Stim.	Stim.	n/l
Epitope (where known)	R2/R3	R2/R3	R2/R3	C-terminal	207–218	207–218	–	207–218	426–587

^a Inhibits recognition of the synergy region of fibronectin only [15].

Figure 1 Effect of bivalent cations on the binding of function-altering mAbs to α5β1

Binding of mAbs JBS5 (A), 16 (B), 13 (C) and 12G10 (D) was measured in the presence of 1 mM Mn²⁺ (●) or in the presence of 1 mM EDTA (○). By non-linear regression analysis, the apparent affinities of binding of the mAbs to α5β1 are estimated to be as follows: JBS5, 18 ± 1 nM (Mn²⁺) and 67 ± 5 nM (EDTA); 16, 2.5 ± 0.1 nM (Mn²⁺) and 15.3 ± 0.5 nM (EDTA); 13, 5.3 ± 0.7 nM (Mn²⁺) and 2.1 ± 0.2 nM (EDTA); 12G10, 0.63 ± 0.05 nM (Mn²⁺) and 2.3 ± 0.3 nM (EDTA). Predicted maximal levels of mAbs 16 and 12G10 binding are also decreased in EDTA, suggesting a lower level of expression of these epitopes in the presence of EDTA compared with Mn²⁺.

Modulation of the binding of function-altering mAbs to α5β1 by Mn²⁺, Mg²⁺ and Ca²⁺ correlates with the occupancy of cation-binding sites that regulate ligand recognition

To investigate further how JBS5, 16, 13 and 12G10 binding is altered by bivalent cations, we tested the effect of a range of

cation concentrations on the binding of these mAbs to α5β1. Using a concentration of each mAb that was well below the apparent K_D, we found that Mn²⁺, Mg²⁺ and Ca²⁺ had distinct effects on the binding of these mAbs to α5β1 (Figure 2). JBS5 binding was increased by Mn²⁺, and to a lesser extent by Mg²⁺ or Ca²⁺. Using non-linear regression analysis, changes in JBS5

Figure 2 Comparison of the effect of Mn^{2+} , Mg^{2+} and Ca^{2+} on the binding of function-altering mAbs to $\alpha 5\beta 1$

Binding of mAbs JBS5 (**A**), 16 (**B**), 13 (**C**) and 12G10 (**D**) ($0.1 \mu\text{g/ml}$) was measured for varying concentrations of Mn^{2+} ($\bullet$), Mg^{2+} ($\blacksquare$) or Ca^{2+} ($\blacktriangle$). By non-linear regression analysis, the concentrations of Mn^{2+} , Mg^{2+} and Ca^{2+} for half-maximal change in JBS5 binding are $34 \pm 5 \mu\text{M}$, $2.3 \pm 0.3 \text{ mM}$ and $74 \pm 7 \mu\text{M}$ respectively; the concentrations of Mn^{2+} , Mg^{2+} and Ca^{2+} for half-maximal change in 12G10 binding are $21 \pm 3 \mu\text{M}$, $2.0 \pm 0.2 \text{ mM}$ and $49 \pm 14 \mu\text{M}$. The data for mAbs 16 and 13 did not fit well to a model that assumes occupancy of a single class of cation-binding site for each bivalent ion.

Table 2 Reactivity of mAb 12G10 to $\beta 1$ chimaeras

Chimaeric $\beta 1$ subunits were constructed by replacing part of the mouse (m) $\beta 1$ or chicken (c) $\beta 1$ sequence with the corresponding sequence from human (h) $\beta 1$. For example, h587/m means the first 587 amino acids of mouse $\beta 1$ are substituted with those from human $\beta 1$; h304/c means the first 304 amino acids of chicken $\beta 1$ are substituted with those from human $\beta 1$. Reactivity of the mAbs to Chinese hamster ovary (CHO) cells stably expressing wild-type (wt) human or chimaeric $\beta 1$ was examined by flow cytometry. The numbers in the Table represent the percentages of positive cells in a typical experiment. /+ represents positive reactivity of the cells to the mAb. mAb SG/19 recognizes the N-terminus of human $\beta 1$ and serves as a positive control.

mAb	Reactivity to cells (%)							
	CHO	wt $\beta 1$	Chimaera ...	h587/m	h425/m	h354/m	h304/c	h189/c
m IgG	2.2	1.9		2.8	1.8	3.8	2.7	1.2
12G10	2.0	75.4/+		94.2/+	79.4/+	95.4/+	37.5/+	0.77
SG/19	1.9	97.5/+		94.7/+	96.6/+	97.8/+	43.0/+	38.4/+

binding fitted well to occupancy of a single (or a single class of) high-affinity Mn^{2+} - or Ca^{2+} -binding site, and by occupancy of a single (or single class of) low-affinity Mg^{2+} -binding site. The

concentrations of Mn^{2+} , Mg^{2+} and Ca^{2+} for half-maximal change in JBS5 binding are estimated to be approx. $30 \mu\text{M}$, 2 mM and $70 \mu\text{M}$ respectively. Hence JBS5 binding appears to be influenced by occupancy of bivalent-cation-binding sites that are of similar affinity to those previously identified from their effects on ligand binding [19]. Qualitatively the results for mAb 16 were similar to those for JBS5, in that occupancy of high-affinity Mn^{2+} or Ca^{2+} sites, or low-affinity Mg^{2+} sites, increased the affinity of antibody binding. However the data for mAb 16 did not fit well to occupancy of a single class of site for each bivalent ion; instead, changes in antibody binding may be due to occupancy of multiple sites with varying affinities. Interestingly, the results for mAb 13 were approximately the converse of those for JBS5 and mAb 16, in that both Mn^{2+} and Ca^{2+} decreased the affinity of mAb 13 binding. 12G10 binding to $\alpha 5\beta 1$ was increased by Mn^{2+} , and to a lesser extent by Mg^{2+} , but was decreased by Ca^{2+} . Changes in 12G10 binding were due to alterations in both the affinity of mAb binding and in expression of the 12G10 epitope ([23]; and results not shown). Non-linear regression analysis of the data indicated that 12G10 binding was increased by binding of Mn^{2+} to a single (or single class of) high-affinity site (apparent $K_D \approx 20 \mu\text{M}$) and by binding of Mg^{2+} to a single (or single class of) low-affinity site (apparent $K_D \approx 2 \text{ mM}$). Occupancy of one or

Table 3 Effects of point mutations in $\beta 1$ on the binding of 12G10

Amino acids Asn-207, Lys-208, Val-211, Lys-218 and Met-287 in human $\beta 1$ were changed to the corresponding residue from mouse $\beta 1$. Reactivity of 12G10 to Chinese hamster ovary (CHO) cells stably expressing wild-type (wt) or mutated human $\beta 1$ was examined by flow cytometry. The numbers in the table represent the percentages of positive cells in a typical experiment. /+ represents positive reactivity of the cells to the mAb. SG/19 recognizes the N-terminus of $\beta 1$ and serves as a positive control.

mAb	Reactivity to cells (%)							
	CHO	wt $\beta 1$	Mutant ...	N207D	K208R	V211F	K218Q	M287V
m IgG	2.0	3.5		1.6	2.0	1.3	3.4	1.5
12G10	1.7	92.4/+		59.4/+	48.2/+	23.9/+	3.0	24.6/+
SG/19	1.8	94.7/+		89.1/+	78.8/+	33.6/+	21.4/+	40.6/+

more high-affinity Ca^{2+} -binding sites (apparent $K_D \approx 50 \mu\text{M}$) appeared to be responsible for decreasing 12G10 binding. In summary, occupancy of $\alpha 5\beta 1$ by Mn^{2+} , Mg^{2+} or Ca^{2+} caused changes in the binding of the function-altering mAbs (particularly JBS5 and 12G10) to $\alpha 5\beta 1$ that appear to be due to occupancy of the same cation-binding sites that modulate ligand recognition; furthermore, these changes mirror alterations in the ligand-binding potential of the integrin [19].

The epitope of 12G10 is overlapping with those of other function-altering anti- $\beta 1$ mAbs

As described above, the epitopes of the function-altering mAbs used in this study have been localized to restricted regions of the $\alpha 5$ and $\beta 1$ subunits. Competitive ELISA experiments had previously suggested that the epitope of 12G10 was very close to the region of the $\beta 1$ subunit that contains the epitopes for inhibitory mAbs such as 13, and stimulatory mAbs such as TS2/16 [20]. To define further the part of the $\beta 1$ subunit that contains the 12G10 epitope, we initially used mouse/human and chicken/human $\beta 1$ chimaeras to localize the epitope to a specific region of $\beta 1$. The results (Table 2) showed that the 12G10 epitope was included in a region containing amino acids 190–304. Since there are only five amino acid changes in this region from mouse to human $\beta 1$ (and 12G10 does not react with mouse $\beta 1$), point mutations in each of these five residues were made. The results (Table 3) showed that mutation of Lys218 abrogated 12G10 binding. Hence the epitope recognized by 12G10 probably includes this residue. Since amino acids 207–218 of $\beta 1$ contain the epitopes of many function-altering anti- $\beta 1$ mAbs [11], the epitope of 12G10 appears to be overlapping with the epitopes of these mAbs.

Evidence that the epitopes of mAbs JBS5/16 and mAbs 13/12G10 are spatially close

There is evidence to suggest that the ligand-binding pocket on integrins is made up of elements from both α and β subunits that

Figure 3 Competitive ELISA experiments

The binding of biotinylated mAbs JBS5 (A), 16 (B), 13 (C) and 12G10 (D) to $\alpha 5\beta 1$ was measured in the presence of a large excess of the indicated unlabelled mAbs. Results are expressed as a percentage of control binding (in the absence of unlabelled mAbs). Experiments were performed in the presence of 1 mM EDTA. The incomplete inhibition of biotinylated mAb 13 binding by 12G10 in (C) is probably because some of the integrin fails to express the 12G10 epitope in the absence of bivalent cations.

Figure 4 Representation of overlap between the epitopes of function-altering anti- $\alpha 5$ and anti- $\beta 1$ mAbs

Epitopes of anti- $\alpha 5$ mAbs are shown white, epitopes of anti- $\beta 1$ mAbs are shown hatched. Epitopes of mAbs that show altered binding when the integrin is occupied by bivalent cations are marked with an asterisk. The epitope of 4B4 is omitted for clarity, but is closely overlapping with those of mAbs 13 and 12G10. Note that while the Venn diagram cannot be used as a basis for estimating physical distances between clusters of epitopes, it can be used to exclude overlap and to give a qualitative approximation of proximity.

are brought into close proximity through the spatial folding of the polypeptide chains (for a review see [20]). We therefore hypothesized that the epitopes of function-altering mAbs against the $\alpha 5$ subunit may be in spatial proximity to those of function-altering mAbs against the $\beta 1$ subunit, and hence binding of function-altering anti- $\beta 1$ mAbs to $\alpha 5\beta 1$ may sterically hinder the binding of function-altering anti- $\alpha 5$ mAbs and vice versa. To test this hypothesis, we used competitive ELISA experiments to determine whether anti- $\alpha 5$ mAbs and anti- $\beta 1$ mAbs could compete with each other for binding to $\alpha 5\beta 1$ (Figure 3). Binding of biotinylated JBS5 to $\alpha 5\beta 1$ was, as expected, blocked by unlabelled JBS5, and also by mAbs 16 and P1D6, whose epitopes lie in the same region of the $\alpha 5$ subunit. In addition, JBS5 binding was partially inhibited by the function-altering anti- $\beta 1$ mAbs 13, 4B4 and 12G10 (Figure 3A). Interestingly, TS2/16 did not affect JBS5 binding, even though its epitope lies in the same region of the $\beta 1$ subunit as those of 13, 4B4 and 12G10 [11]. Non-function-altering anti- $\alpha 5$ (mAb 11) and anti- $\beta 1$ (K20) mAbs also did not inhibit JBS5 binding. Similar results were obtained for biotinylated mAb 16 (Figure 3B). In the converse experiments, binding of biotinylated mAb 13 to $\alpha 5\beta 1$ was completely blocked by unlabelled mAb 13 and was also strongly inhibited by 4B4, 12G10 and TS2/16; mAbs that have epitopes in the same region of the $\beta 1$ subunit ([11], and the present study). The binding of biotinylated mAb 13 was also partially inhibited by the anti- $\alpha 5$ mAbs JBS5 and 16, but not by P1D6 (Figure 3C). JBS5 and mAb 16 also partially inhibited biotinylated 12G10 binding to $\alpha 5\beta 1$ (Figure 3D). In additional experiments, JBS5 and mAb 16 partially inhibited biotinylated 4B4 but not TS2/16 binding to $\alpha 5\beta 1$ (results not shown). Based on the competitive ELISA results, a Venn diagram was constructed showing overlap between the epitopes of function-altering mAbs (Figure 4). Importantly, this analysis suggests that the epitopes of the anti- $\alpha 5$ mAbs and anti- $\beta 1$ mAbs that are spatially overlapping are mainly those that are affected by bivalent cations. These epitopes must lie close to the interface between α and β subunits.

The degree of cross competition between the binding of the function-altering anti- $\alpha 5$ and the anti- $\beta 1$ mAbs to $\alpha 5\beta 1$ was also influenced by bivalent cations. For each pair of anti- $\alpha 5$ and anti- $\beta 1$ mAbs, there was less cross-competition between these mAbs in the presence of Mn^{2+} than in the absence of cations; an example is shown in Figure 5. Mg^{2+} or Ca^{2+} also reduced the extent of cross-competition (results not shown). Taken together, the results indicate that the regions of the $\alpha 5$ and $\beta 1$ subunits that contain the epitopes of JBS5/mAb 16 and mAb 13/12G10 are in

Figure 5 Effect of bivalent cations on competition between anti- $\alpha 5$ and anti- $\beta 1$ function-altering mAbs for binding to $\alpha 5\beta 1$

The effect of unlabelled mAb 13 on the binding of biotinylated mAb 16 (top), and the effect of unlabelled mAb 16 on the binding of biotinylated mAb 13 (bottom) to $\alpha 5\beta 1$ was examined in the presence of 1 mM Mn^{2+} (●) or in the presence of 1 mM EDTA (○). Results are expressed as a percentage of control binding (in the absence of unlabelled mAbs). Concentrations of biotinylated mAbs were adjusted to give approximately the same levels of control binding in Mn^{2+} and EDTA [0.1 and 0.7 $\mu g/ml$ respectively (top); 0.1 and 0.05 $\mu g/ml$ respectively (bottom)].

spatial proximity to each other, but these two regions move further apart when the integrin is occupied by bivalent ions.

DISCUSSION

The novel findings of this report are the following. (i) Binding of the function-altering mAbs JBS5, 16, 13 and 12G10 to $\alpha 5\beta 1$ is affected by occupancy of cation-binding sites on $\alpha 5\beta 1$; some of these sites are of similar affinity to those identified from their effects on ligand recognition [19]. (ii) The effect of Mn^{2+} , Mg^{2+} and Ca^{2+} on the binding of these mAbs can be correlated with the effects of each bivalent ion on the ligand-binding capacity of $\alpha 5\beta 1$. (iii) Competitive ELISA experiments suggest that the regions of the $\alpha 5$ and $\beta 1$ subunits recognized by JBS5/mAb 16 and mAb 13/12G10 (i.e. the second and third repeats of the $\alpha 5$ subunit β -propeller and amino acids 207–218 in the $\beta 1$ subunit putative A-domain) are spatially close, and that these regions move further apart when the integrin is occupied by bivalent

cations. Taken together, our data indicate that cation occupancy leads to a conformational change in the integrin that unmasks sites involved in ligand recognition, and that these sites lie near the interface between α and β subunits.

Our results suggest an explanation for the differences in the affinity of ligand binding by $\alpha 5\beta 1$ in Mn^{2+} as compared with Mg^{2+} or Ca^{2+} [19], in that each bivalent ion has distinct effects on the conformation of the integrin. Mn^{2+} appears to be highly effective at inducing the conformational changes associated with integrin activation, i.e. exposure of ligand-binding sites, as reported, for example, by enhanced exposure of the mAb JBS5, 16 and 12G10 epitopes that lie near to these sites. Mg^{2+} can also induce these changes, but to a lesser extent than Mn^{2+} . In contrast, although Ca^{2+} appears to cause conformational changes to the $\alpha 5$ subunit that are similar to those caused by Mn^{2+} or Mg^{2+} , it suppresses the exposure of the 12G10 (activation) epitope on the $\beta 1$ subunit. Hence, although occupancy of the integrin by Ca^{2+} may allow the α subunit to achieve an active conformation, it appears to maintain the β subunit in an inactive conformation.

We used competitive ELISA experiments to test whether the domains of the $\alpha 5$ and $\beta 1$ subunits that contain the epitopes of function-perturbing anti- $\alpha 5$ and anti- $\beta 1$ mAbs are spatially overlapping. A similar approach has been used, for example, to map spatially overlapping biologically active domains in vitronectin [24]. Our results indicated that the ligand-binding domains on the α and β subunits are in close proximity to each other. However, an alternative explanation for the cross-inhibition we observed between the binding of anti- $\alpha 5$ and anti- $\beta 1$ mAbs to $\alpha 5\beta 1$ could be that these effects are due to allostery, i.e. the binding of one antibody causes a conformational change in the integrin that causes a second mAb to bind with lower affinity at a spatially distant site. For example, it might be expected that inhibitory mAbs could perturb the binding of stimulatory mAbs through allosteric effects on integrin conformation, and vice versa. Although we cannot rule out this explanation, we deem it unlikely for following reasons. (i) Inhibitory anti- $\alpha 5$ mAbs JBS5 and 16 perturbed the binding of both stimulatory (12G10) and inhibitory (mAb 13 and 4B4) anti- $\beta 1$ mAbs (and vice versa). (ii) Only a subset of the function-altering mAbs showed the capacity to cross-inhibit (P1D6 and TS2/16 failed to cross-inhibit). (iii) The binding of mAbs JBS5 and 16 is perturbed by 12G10 but not by other stimulatory mAbs, such as N29, 9EG7 and 15/7, which recognize other regions of the $\beta 1$ subunit [25–27] (A. P. Mould and A. N. Garratt, unpublished work). (iv) The degree of cross-inhibition was greatest in the inactive (i.e. conformationally constrained) state of the integrin. All these observations cannot readily be explained if the cross inhibition is due to allosteric effects, but they can be accounted for by a spatial overlap between the JBS5/mAb 16 and mAb 13/12G10 clusters of epitopes. Spatial proximity between the second and third N-terminal repeats of the α subunit and the putative A-domain region of the β subunit is also implied by the cross-linking of an RGD-containing peptide to both these regions in $\alpha V\beta 3$ [28]. More recently, evidence has been obtained that folding of the β -propeller domain formed by the N-terminal repeats of the αL subunit and the putative A-domain region of the $\beta 2$ subunit is dependent on an association between these two modules [29,30]. Point mutations in the putative A-domain region of the $\beta 2$ and $\beta 3$ subunits have also been shown to affect subunit–subunit interactions [31,32]. Our findings for $\alpha 5\beta 1$ suggest that loop regions on the upper surface of the α subunit β -propeller (in the second and third N-terminal repeats) and residues 207–218 of the β subunit putative A-domain may form part of an interface between these two modules.

Interestingly, we found that the epitope of P1D6, an mAb that blocks recognition of the synergy site but not the RGD site in fibronectin by $\alpha 5\beta 1$, appears not to lie close to the epitopes of the function-altering anti- $\beta 1$ mAbs, whereas the epitopes of JBS5 and 16, mAbs that do block recognition of the RGD sequence in fibronectin ([15], and A. P. Mould, unpublished work), are predicted to be close to those of function-altering anti- $\beta 1$ mAbs. Hence, the epitopes of mAbs JBS5 and 16 may be close to the RGD-binding site (mainly on the β subunit), whereas the epitope of P1D6 appears to be relatively distant from this site but close to a site involved in recognition of the synergy sequence (mainly on the α subunit).

Our previous studies on the binding of fibronectin fragments to $\alpha 5\beta 1$ [15] allowed us to propose that the β -propeller domain of the $\alpha 5$ subunit and the putative A-domain of the $\beta 1$ subunit are arranged side by side in the ligand-occupied state of $\alpha 5\beta 1$. Our current findings suggest that these two domains are partly overlapping in the inactive state. A model of integrin quaternary structure has been proposed recently [22], based on the crystal structure of heterotrimeric G-proteins. In this model, the β subunit putative A-domain is proposed to lie on the top surface of the α subunit β -propeller, and to mask ligand-binding sites in the second and third repeats. In the active conformation of the integrin, the β subunit putative A-domain is released from its contacts with the propeller and exposes the ligand-binding sites in the second and third repeats. Our findings are consistent with this model, and we further speculate that amino acids 207–218 of the putative $\beta 1$ A-domain may form part of a conformational switch, analogous to the switch II region of heterotrimeric G-protein α subunits [33,34].

Finally, it should be pointed out that our findings do not exclude a possible direct role of cations in integrin–ligand recognition [5,6,35]. It is conceivable that bivalent ions could be required both for a proposed bridging interaction between integrin and ligand, and to induce conformational changes that expose ligand-binding sites. In the future it will be important to elucidate more fully the nature of these conformational changes, how subunit–subunit interactions are altered by bivalent-cation occupancy, and the precise location of the cation-binding sites involved in this regulation.

We thank J. Aplin for providing human placenta, S. E. Craig for purifying 12G10, and S. K. Akiyama and F. Sánchez Madrid for supplying mAbs. We are grateful to D. S. Tuckwell and A. G. Lowe for helpful discussions. These studies were supported by grants from the Wellcome Trust (to M.J.H.) and by grants GM47157 and GM49899 from the National Institutes of Health (to Y.T.).

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Received 28 November 1997/3 February 1998; accepted 13 February 1998