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Identification of a novel anti-integrin monoclonal antibody that recognises a ligand-induced binding site epitope on the β1 subunit

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Abstract Integrins are the major family of receptors involved in the adhesive interactions of cells with extracellular matrix macromolecules. Although it is known that integrins can exist in active or inactive states, the molecular mechanisms by which integrin activity is modulated are poorly understood. A novel anti-integrin monoclonal antibody, 12G10, that enhances α5β1-fibronectin interactions has been identified. 12G10 binds to the β1 subunit and appears to recognise a region of the subunit that contains the epitopes of several previously described activating or inhibitory monoclonal antibodies. However, unlike other activating anti-β1 antibodies, the binding of 12G10 to α5β1 is increased in the presence of ligands (fibronectin fragment or RGD peptide). This is the first report for the β1 integrin family of an antibody that recognises a ligand-induced binding site, and further emphasises the functional importance of a specific region of the β1 subunit in regulating integrin–ligand interactions.

Key words: Integrin; Activation; Ligand binding; Monoclonal antibody; Conformational change

1. Introduction

Many cell–cell and cell–matrix interactions are mediated by members of the integrin superfamily of cell-surface receptors. Integrins are α,β heterodimers that have been classified into eight different groups according to the identity of their β subunit. The β1 family is the principal group of cell-matrix receptors [1–3].

A general property of integrins is that they can exist in active or inactive states; ligand recognition requires that the integrin be in the activated state [1]. Ligand recognition has been shown to cause a conformational change in integrins, leading to the exposure of neo-epitopes known as ligand-induced binding sites (LIBS) [4,5]. A number of monoclonal antibodies (mAbs) have been described that can activate integrins directly, a subset of these are also anti-LIBS antibodies [5].

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 recognised by a wide variety of cell types lies close to the centre of the fibronectin subunit and contains the tripeptide RGD as a key active site [6,7]. The integrin α5β1 is the major receptor for this central cell-binding domain (CCBD) and is expressed on many cell types. Here we have identified a novel anti-β1 mAb that stimulates α5β1-fibronectin interactions and also has the characteristics of an anti-LIBS antibody.

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Abbreviations: LIBS, ligand-induced binding site(s); CCBD, central cell binding domain of fibronectin; mAb, monoclonal antibody; kDa, kilodalton; H/120, recombinant fragment of fibronectin that lacks the CCBD; HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); S.D., standard deviation.

2. Materials and methods

2.1. Materials

Rat mAbs 16 and 13 recognising the human α5 and β1 integrin subunits respectively, were produced and purified as previously described [8]. Mouse anti-human β1 integrin mAbs JB1B and K20 were purchased from Serotec (Oxford, UK), and The Binding Site (Birmingham, UK), respectively. Mouse mAbs TS2/16 and 8A2 against the human β1 subunit were gifts from F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain) and N. Kovach (University of Washington, Seattle, USA), respectively. All antibodies were used as purified IgG, with the exception of 8A2 (ascites). An 80-kDa fragment of fibronectin containing the CCBD was purified from a trypsin digest of plasma fibronectin as described [9]. A recombinant fragment of fibronectin (H/120) that lacks the CCBD was produced as previously described [10]. The synthetic peptides GRGDS and GRDGS were synthesised using Fastmoc chemistry on an Applied Biosystems 431A peptide synthesiser and purified as described previously [11,12]

2.2. Production of mAb 12G10

Methods were based on those previously described [13]. Briefly, BALB/c mice were immunised twice subcutaneously with ~200 μg of purified β1 integrins from HT-1080 cells (see below) in the presence of RIBI adjuvant (Universal Biologicals, London, UK). Three days before sacrificing, mice were injected intraperitoneally with HT-1080 integrins without adjuvant. Spleens were removed and fused with X63-Ag8.653 myeloma cells. Hybridomas were screened by ELISA against the immunogen. Positive hybridomas were cloned by limiting dilution, and further characterised by Western blotting under non-reducing conditions. One clone, 12G10, was identified as an anti-β1 mAb because it reacted with the β1 subunit in Western blots. 12G10 IgG was purified from the medium of serum-free culture supernatants using Protein G-Sepharose (Pharmacia).

2.3. Purification of α5β1 from HT-1080 cells

HT-1080 fibrosarcoma cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK) and were grown to confluence in 225 cm^2 flasks (Costar). Cells (from 100 flasks) were detached with 3 mM EDTA in HBSS (GIBCO-BRL), washed with PBS and extracted with 40 ml of 2% (w/v) Triton X-100, 5 mg/ml BSA, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 25 mM Tris-HCl, pH 7.4, on ice for 30 min. β1 integrins were purified from these extracts by mAb 13 affinity chromatography, essentially as previously described for the purification of β1 integrins from lymphocytes [14]. Pooled fractions were then mixed with 2 ml mAb 16 Sepharose (5 mg IgG/ml Sepharose) for 2 h at room temperature. The suspension was then packed into a 0.8-cm diameter column and washed with 20 ml of 0.1% (w/v) Triton X-100, 150 mM

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NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.4. Bound material was eluted with 0.1% (w/v) Triton X-100, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM NaOAc, pH 3.5, and 0.5 ml fractions were collected and neutralised with 0.1 ml of 1 M Tris-HCl, pH 8.2. 50-μl aliquots were analysed by SDS-PAGE using a 7.5% non-reducing resolving gel. The only bands detected by Coomassie Blue staining were those corresponding to expected positions of the α5 and β1 subunits, and α5 and β1 were the only integrin subunits detected in the eluted fractions by ELISA.

2.4. Solid phase assay

Solid-phase ligand–receptor binding was performed by a modification of the method of Charo and co-workers [15]. 80-kDa CCBD fragment of fibronectin (500 μg/ml in PBS) 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 dialysed against several changes of 150 mM NaCl, 25 mM Tris-HCl, pH 7.4 to remove excess biotin. Purified α5β1 integrin (at a concentration of ~100 μg/ml) was diluted 1:100 with PBS containing divalent cations, and 100-μl aliquots were added to the wells of a 96-well ELISA plate (Dynatech Immulon 3). 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, 1 mM $MnCl_2$, 25 mM Tris-HCl, pH 7.4, containing 1 mg/ml BSA (buffer A), and 100-μl aliquots of biotinylated CCBD fragment (~0.01 μg/ml) diluted in buffer A were added, with or without mAbs. The plate was then incubated at 30 °C for 3 h. Biotinylated ligand was aspirated and the wells washed three times with buffer A. Bound ligand was quantitated by addition of 1:200 ExtrAvidin-peroxidase conjugate (Sigma) in buffer A for 10 min. Wells were then washed four times with buffer A, and colour was developed using ABTS (Sigma). Measurements obtained were the mean ± S.D. of six replicate wells. The amount of non-specific 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.

2.5. Competitive ELISA experiments

12G10 IgG was biotinylated as described above for the CCBD fibronectin fragment. ELISA plates were coated with purified α5β1 and blocked as described above. Wells were then washed three times with buffer A. Biotinylated 12G10 (0.2 μg/ml in buffer A) was added to the wells in the absence or presence of excess unlabelled mAbs. The concentration of competitor antibodies was 20 μg/ml for purified IgG, ascites was used at 1:500 dilution. The plate was then incubated at room temperature for 1–2 h. Unbound antibodies were aspirated and the wells washed three times with buffer A. Bound biotinylated antibody was quantitated by addition of 1:500 ExtrAvadin-peroxidase in buffer A. Wells were then washed four times with buffer A, and colour was developed using ABTS substrate. Measurements obtained were the mean ± S.D. of six replicate wells. The amount of non-specific binding was measured by determining the level of biotinylated antibody binding to wells coated with BSA alone. These values were subtracted from the corresponding values for receptor-coated wells.

2.6. Effect of ligands on the binding of mAbs to α5β1

ELISA plates were coated with purified α5β1 and blocked as described above. Wells were then washed three times with buffer A. 100-μl aliquots of mAbs (5 μg/ml or 1:1000 dilution of ascites) in buffer A were added to the wells in the presence or absence of CCBD fragment (1 μg/ml) or GRGDS (5 μg/ml); a recombinant fragment of fibronectin (H/120) lacking the CCBD (1 μg/ml), and GRDGS peptide (5 μg/ml) were used as controls. The plate was then incubated at room temperature for 90 min. Unbound antibody was aspirated and the wells washed three times with buffer A. Bound antibody was quantitated by addition of 1:1000 anti-mouse peroxidase or anti-rat peroxidase conjugate in buffer A. Wells were then washed four times with buffer A, and colour was developed using ABTS substrate. Measurements obtained were the mean ± S.D. of four replicate wells. 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.

To analyse the effect of RGD peptides on the apparent affinity of 12G10 or 8A2 binding to α5β1, the same procedures as described above were used, except that antibody binding was measured for a range of antibody concentrations in the presence of either GRGDS, or the control peptide GRDGS. Non-linear regression analysis to estimate apparent affinity ($1/K_d$) and the maximal level of binding was performed as previously described [14].

3. Results

3.1. 12G10 is a novel activating anti-β1 monoclonal antibody

Hybridomas produced by immunising mice with β1 integrins from HT-1080 fibrosarcoma cells were screened by Western blotting to identify those that reacted with the β1 subunit. Several clones were found to recognise β1, including one designated 12G10 (result not shown). 12G10 and other previously described anti-β1 mAbs were then tested for their effect on ligand binding to α5β1 in a solid phase assay (Fig. 1). The results showed that the antibodies could be grouped into three classes: those that enhanced ligand binding (12G10, TS2/16 and 8A2); those that had no significant effect on ligand binding (JB1B and K20); and those that inhibited ligand binding (13). TS2/16 and 8A2 have been previously shown to enhance cell adhesion by activating β1 integrins [16–18], whereas 13 has been shown to inhibit β1 integrin–ligand interactions [8]. Based on these results, 12G10 appears to be a new member of the subset of anti-β1 mAbs that can activate ligand binding.

3.2. The 12G10 epitope overlaps with that of other activating mAbs

To investigate if the epitope recognised by 12G10 overlaps with that of the previously described activating mAbs, we studied the ability of other anti-β1 mAbs to competitively inhibit the binding of 12G10 to α5β1 (Fig. 2). As expected, the binding of biotinylated 12G10 was strongly inhibited by unlabelled 12G10; in addition, 8A2 and TS2/16 completely inhibited 12G10 binding, suggesting that these antibodies recognise the same, or closely overlapping epitopes on the β1 subunit. 12G10

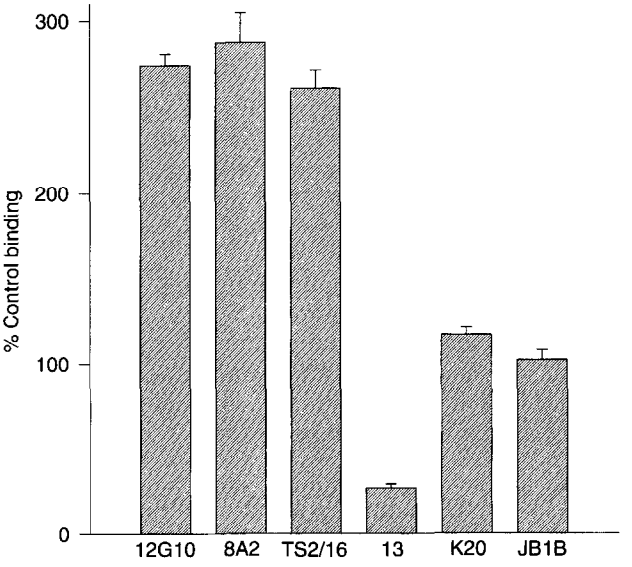

Fig. 1. Effect of anti-β1 mAbs on the binding of CCBD fibronectin fragment to integrin α5β1 in a solid phase assay. Binding of biotinylated CCBD fragment was detected as described in section 2. Results are expressed as % of control binding (in the absence of mAbs).

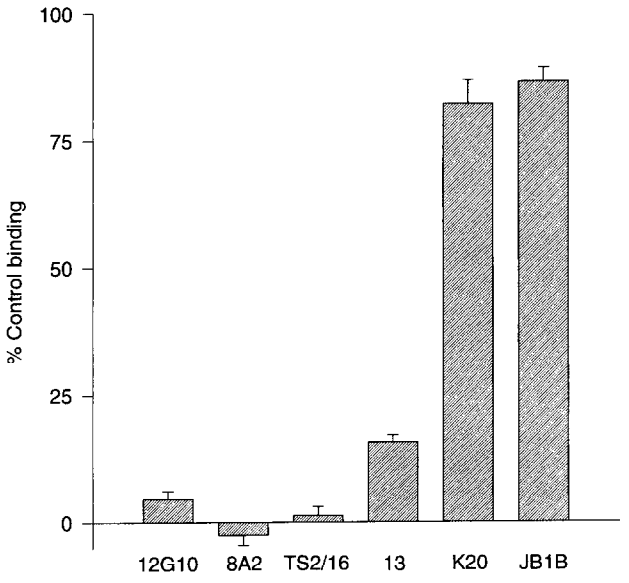

Fig. 2. Effect of anti-β1 mAbs on the binding of biotinylated 12G10 to integrin $\alpha5\beta1$ in a competitive ELISA assay. Binding of biotinylated antibody, in the absence or presence of a large excess of competing unlabelled antibodies, was detected as described in section 2. Results are expressed as the % of control binding (in the absence of unlabelled antibodies).

binding was also inhibited by mAb 13, although to a slightly lesser extent than 8A2 and TS2/16. Other antibodies, such as JB1B and K20, did not block 12G10 binding. It has been shown that 8A2, TS2/16 and 13 all recognise a small sub-region of the β1 subunit (residues 207–218) [19]. Hence it appears that 12G10 binds at, or very close to, this same region of the β1 subunit.

3.3. 12G10 recognises a ligand-induced binding site

To investigate if the binding of any of the anti-β1 mAbs could be modulated by ligands, we measured the amount of antibody binding to $\alpha5\beta1$ in the presence of a CCBD fragment of fibronectin, or GRGDS peptide. A recombinant fragment of fibronectin lacking the CCBD, and GRDGS peptide were used as controls. The results (Table 1) showed that 12G10 binding was increased approximately 1.5-fold in the presence of ligands, whereas binding of the other activating antibodies was unaffected. Binding of the inhibitory mAb 13 was slightly decreased by ligands in this assay.

Comparison of the effects of RGD peptide on 12G10 and 8A2 binding to $\alpha5\beta1$ (Fig. 3A and 3B, respectively) demonstrated that ligand increased both the apparent affinity and the maximal level of 12G10 binding. In contrast, although the apparent affinity of 8A2 binding was increased by ligand, the maximal level of binding was unchanged.

4. Discussion

Our results show that 12G10 is a novel member of a family of anti-integrin mAbs known as anti-LIBS antibodies [4,5]. The epitopes recognised by this group of mAbs are expressed on a greater proportion of receptors in the ligand-occupied state than in the unoccupied state. All of the previously described anti-LIBS antibodies are against either the β3 integrin αIIbβ3 [4,5] or the β2 integrin αMβ2 [20]; this report is the first description of this type of antibody for a β1 integrin.

Interestingly, none of the previously described anti-LIBS antibodies against αIIbβ3 showed increased binding to αIIbβ3 in the presence of GRGDS peptide in solid phase assays [4], whereas this peptide did increase 12G10 binding to $\alpha5\beta1$ in solid phase assays. The $\alpha5\beta1$ solid phase assay also has the useful property that ligand binding can be readily modulated by different mAbs. These features make it a potentially valuable aid for identifying the regions of the $\alpha5$ and β1 subunits that are involved in regulating ligand binding.

Based on their observation that showed that the affinity of 8A2 binding to $\alpha5\beta1$ on K562 cells was ~ 2-fold higher in the presence of GRGDSP peptide than in its absence, Faull and co-workers [21] proposed that 8A2 may be an anti-LIBS antibody. However, in the above study, the maximal amount of 8A2 bound (at saturating antibody concentrations) was not significantly altered by this peptide. In our assays the maximal level of 8A2 binding to $\alpha5\beta1$ was also unaffected by ligands. By definition, for a true anti-LIBS mAb the maximal amount of bound antibody should be increased in the presence of ligands [4,5], suggesting that 8A2 is not a genuine member of this class of antibodies.

None of the anti-LIBS antibodies so far described inhibit the binding of primary ligand, however, several of these antibodies (including 12G10) markedly increase the affinity of ligand binding. It has been proposed that polypeptides may exist in conformational equilibria and that addition of an antibody specific for one conformation may shift the equilibrium in favour of that conformation [22]. Hence, it has been suggested that some anti-LIBS mAbs increase the affinity of binding by shifting the conformational equilibrium between inactivated and activated states of the integrin in favour of the activated state [5]. Activating mAbs that do not recognise a LIBS epitope (such as 8A2) nevertheless show an increased affinity of binding to the ligand-occupied form of the integrin, and may therefore stabilise this conformation.

8A2, TS2/16 (and other previously described activating mAbs) have been shown to recognise a small region of the β1

Table 1
Effect of ligands on the binding of anti-β1 mAbs to integrin $\alpha5\beta1$

Ligand	% Control binding			
	12G10	8A2	TS2/16	13
CCBD fragment	146*	96	103	92**
H/120	98	100	97	99
GRGDS	160*	102	96	91**
GRDGS	99	106	99	96

Antibody binding was detected as described in section 2, in the presence or absence of authentic (CCBD fragment or GRGDS) or control (H/120 or GRDGS) ligands. Antibody concentrations (5 μg/ml or 1:1000 dilution of ascites) were chosen to give near maximal binding. Results are expressed as the % of antibody binding in the absence of ligands. In this assay, the absolute level of 12G10 binding in the presence of ligands ($A_{405} \approx 1.1$) was similar to the level of 8A2 or TS2/16 binding with or without ligands ($A_{405} \approx 1.1$). All standard deviations were < 4% of control binding. Statistical significance of difference from binding in the absence of ligands was evaluated using Student's t-test: *$P < 0.001$; **$P < 0.05$. In other experiments (not shown) the binding of antibodies JB1B and K20 was unaffected by ligands.

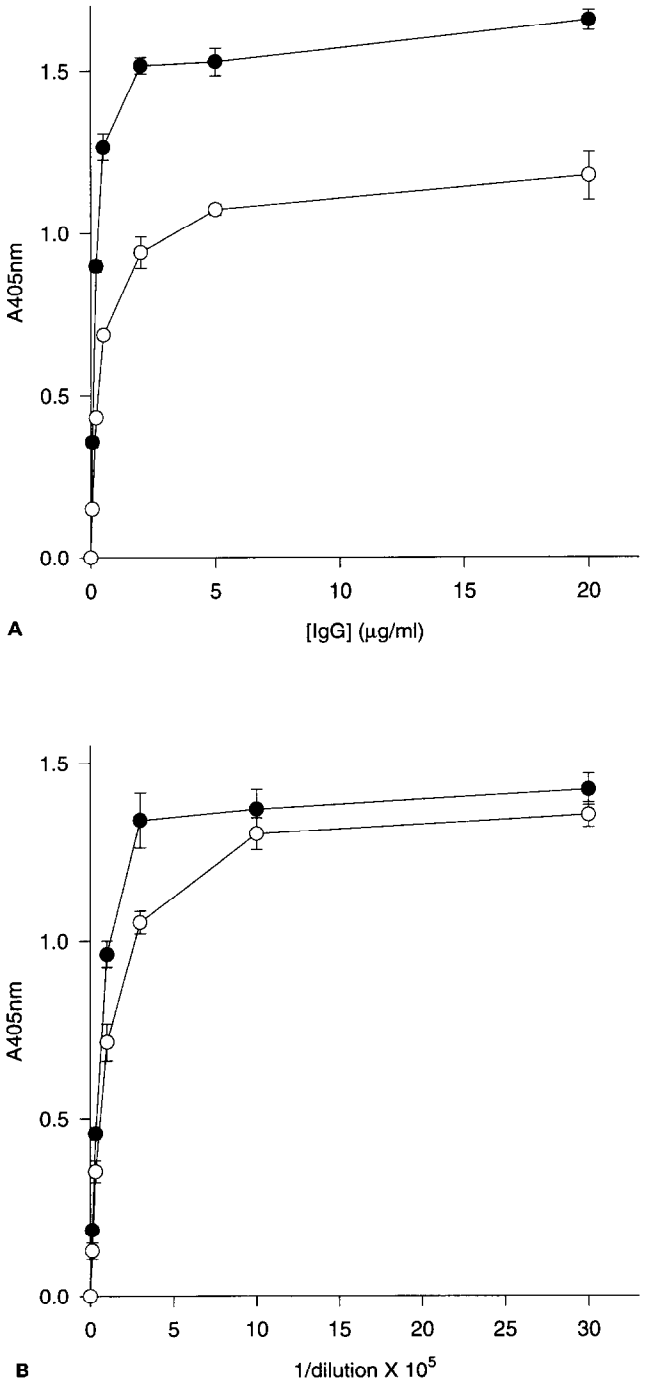

Fig. 3. Effect or RGD peptide ligand on the apparent affinity of binding of mAbs 12G10 (A) and 8A2 (B) to $\alpha 5\beta 1$. Antibody binding was measured in the presence of GRGDS (●) or the control peptide GRDGS (○). By non-linear regression analysis, the apparent affinity of 12G10 binding is increased approx. 2-fold, and the maximal level of binding 1.4-fold, in the presence of ligand. The apparent affinity of 8A2 binding is increased approx. 1.6-fold in the presence of ligand but the maximal level is not significantly changed.

subunit (residues 207–218) that lies between two sequences implicated in ligand recognition [19]. Intriguingly, the same region of the $\beta 1$ subunit also contains the epitopes of inhibitory mAbs such as 13 [19]. Using competitive ELISA experiments, we found that 12G10 binds at, very close to, this region of the $\beta 1$ subunit. While we cannot rule out the possibility that 8A2 and TS2/16 may cause a conformational change that greatly reduces the affinity of 12G10 binding, the essentially complete inhibition of 12G10 binding by these antibodies strongly suggests that the epitopes recognised by 8A2 and TS2/16 are closely overlapping with the 12G10 epitope. However, exposure of the 8A2 and TS2/16 epitopes is unaffected by ligand occupancy. The increase in 12G10 binding to $\beta 1$ caused by ligands nevertheless demonstrates that the conformation of this part of the $\beta 1$ subunit is significantly altered by ligand recognition, leading to increased exposure of certain epitopes. To shed further light on the role of this region of the $\beta 1$ subunit in integrin regulation, it will be important in the future to identify the precise sequence in the $\beta 1$ subunit that contains the 12G10 epitope, and to compare this with the sequences recognised by other activating mAbs.

In summary, we have described a novel activating anti-$\beta 1$ integrin monoclonal antibody that reacts preferentially with the ligand-occupied form of $\alpha 5\beta 1$. The epitope recognised by this antibody is located at, or very near to, a previously defined sub-region of the $\beta 1$ subunit that appears to have a crucial role in regulating integrin activity. If, as predicted, this regulatory region forms part of an A-domain-like structure [23], the epitope recognised by 12G10 may be spatially close to a site involved in both ligand and cation binding.

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