The Inhibitory Anti-β1 Integrin Monoclonal Antibody 13 Recognizes an Epitope That Is Attenuated by Ligand Occupancy

EVIDENCE FOR ALLOSTERIC INHIBITION OF INTEGRIN FUNCTION*

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Integrin-ligand binding causes conformational changes in the integrin, as evidenced by the increased expression of epitopes known as ligand-induced binding sites. Some monoclonal antibodies (mAbs) that recognize ligand-induced binding sites stimulate ligand binding, possibly by stabilizing the ligand-occupied conformation of the integrin. Here we have investigated the effect of ligand recognition by α5β1 on the binding of a mAb that inhibits β1 integrin function (mAb 13). Ligand (fibronectin fragment or GRGDS peptide) decreased the binding of mAb 13 to α5β1. Analysis of this inhibition showed that at high ligand concentrations, approximately 50% of the total integrin bound mAb 13 with 50-fold lower affinity than in the absence of ligand. The concentration of ligand required for half-maximal inhibition of antibody binding was independent of antibody concentration, suggesting that ligand acts as an allosteric inhibitor of mAb 13 binding. Hence, ligand and mAb 13 did not appear to compete directly for binding to α5β1. The stimulatory anti-β1 mAb 9EG7 was found to increase the maximum level of ligand binding 2-fold, indicating that up to 50% of the total integrin could not bind ligand without 9EG7 stimulation. Analysis of mAb 13 binding in the presence of 9EG7 and ligand (i.e. maximal ligand occupancy) demonstrated that essentially all of the integrin bound mAb 13 with very low or zero affinity. Our results demonstrate that mAb 13 recognizes an epitope that is dramatically attenuated in the ligand-occupied form of α5β1. Hence, since mAb 13 preferentially recognizes the unoccupied conformation of the integrin, the antibody may inhibit ligand binding by stabilizing the unoccupied state of α5β1. In addition, we present evidence that the binding of mAb 13 to ligand-occupied α5β1 may also induce a conformational change in the integrin, resulting in the displacement of ligand.

The adhesive interactions of cells with extracellular matrix macromolecules are mediated by cell surface receptors, many of which belong to the integrin gene superfamily. Integrins are heterodimers containing α and β subunits; receptors containing the β1 subunit constitute the principal group of cell-matrix receptors (1).

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†‡ The abbreviations used are: mAb, monoclonal antibody; LIBS, ligand-induced binding sites; CCBD, central cell binding domain; PBS, phosphate-buffered saline.
IgG-Sepharose was then removed by centrifugation (5 min at 180 it with 10 ml of rat IgG-Sepharose (2 mg IgG/ml of beads) for 2 h on ice. Passing it through a column of Sepharose 4B (30 ml) and then by mixing 13-Sepharose (2 mg IgG/ml of beads) for 2 h on ice, the suspension was packed into a 1.6-cm diameter column (Pharmacia) and washed using Fastmoc chemistry on an Applied Biosystems 431A peptide synthesizer and purified as outlined previously (22, 23).

Purification of α5β1 from Human Placenta—Term placenta was obtained from Dr. J. Aplin, St. Mary's Hospital, Manchester, United Kingdom. Placenta (~500 g) was cut into small chunks with scissors and homogenized in a blender (Philips) with 400 ml of 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, and 0.005% (w/v) digitonin (buffer A). The homogenate was stored at −70 °C. Homogenate was thawed at room temperature and centrifuged at 5000 × g for 10 min. The pellet 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 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 2% (w/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 2 μg/ml bovine serum albumin. Fractions (1.5 ml) were collected into 0.5 ml of 1M Tris-HCl, pH 8.2. Aliquots of the fractions (25 μl) were analyzed using SDS-polyacrylamide gel electrophoresis using a 6% nonreducing resolving gel and Coomassie Blue staining and found to contain β1 integrins of ~90% purity.

Pooled fractions were then mixed with 2 ml mAb 16-Sepharose (5 mg mAb/ml of beads) for 2 h on ice. The suspension was then packed and washed with a 0.8-mm diameter column (Pharmacia) and washed overnight (16 h) at 15 ml/h with 150 ml of NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM CaCl2, 1 mM MgCl2, and 0.1% (w/v) Triton X-100 (buffer C). Fractions (1.5 ml) were collected into 0.5 ml of 1 × Tris-HCl, pH 8.2. Aliquots of the fractions (25 μl) were analyzed by SDS-polyacrylamide gel electrophoresis using a 6% nonreducing resolving gel and Coomassie Blue staining and found to contain β1 integrins of ~90% purity.

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Cells were washed three times in buffer D, and biotinylated mAb 13 (1 μg/ml) in buffer D was added to the cells in the absence or presence of CCBD fragment (20 μg/ml). Cells were washed three times in buffer D and then incubated with a 1:200 dilution of avidin-fluorescein isothiocyanate conjugate (Sigma) in buffer D for 20 min. Cells were then washed, fixed, and analyzed as described above. Measurements obtained were the mean ± S.D. of three replicate samples. To estimate the amount of nonspecific fluorescence, the level of biotinylated mAb 13 binding was measured as described above in the presence of a 100-fold excess of unlabeled mAb 13.

**RESULTS**

Ligand Decreases the Binding of mAb 13 to α5β1—We first investigated whether ligand influenced the binding of mAb 13 to α5β1. For ligands, we used both the synthetic peptide GRGDS, which represents the key integrin attachment sequence in the CCBD of fibronectin (26, 27), and a proteolytic fragment of fibronectin that contains synergistic regions in addition to the RGD sequence (28–30). The results (Fig. 1A, 1B) showed that the CCBD fragment or GRGDS peptide decreased the binding of mAb 13 to α5β1 in a dose-dependent manner.

![Graph A](image1.png)

**Fig. 1.** Effect of ligand on the binding of mAb 13 to α5β1. Binding of mAb 13 (1 μg/ml) to α5β1 in a solid phase assay (A and B; bars, S.D.) or on K562 cells (C) was measured for a range of concentrations of CCBD fibronectin fragment (A and C) or GRGDS peptide (B). ●, CCBD fragment in A and C, or GRGDS in B; ○, H/120 recombinant fragment of fibronectin in A and C, or GRGDS peptide in B. In C, a control rat IgG had an mean fluorescence intensity value of approximately 4.

(10 μg/ml) with CCBD fragment (20 μg/ml) for 1 h at room temperature. Cells were washed three times in buffer D, and biotinylated mAb 13 (1 μg/ml) in buffer D was added to the cells in the absence or presence of CCBD fragment (20 μg/ml). Cells were washed three times in buffer D and then incubated with a 1:200 dilution of avidin-fluorescein isothiocyanate conjugate (Sigma) in buffer D for 20 min. Cells were then washed, fixed, and analyzed as described above. Measurements obtained were the mean ± S.D. of three replicate samples. To estimate the amount of nonspecific fluorescence, the level of biotinylated mAb 13 binding was measured as described above in the presence of a 100-fold excess of unlabeled mAb 13.

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(but not the control protein H/120 or the control peptide GRDGS) partially inhibited antibody binding. In both cases, this inhibition reached a maximum of ~50% at high ligand concentrations. However, in agreement with previous comparisons of the relative activities of the CCBD fragment and peptides containing the GRGDS sequence (27), the CCBD fragment was 30–100-fold more active on a molar basis than GRGDS in inhibiting the binding of mAb 13 to α5β1. A similar maximal extent of inhibition of antibody binding was obtained when integrin was preincubated with mAb 13 alone and then incubated with ligand alone, implying that 13 binding is at least partially reversible by ligand (data not shown). We used 1 mM Mn\(^{2+}\) in these experiments because ligand recognition by α5β1 is optimal under these conditions (24). Replacing Mn\(^{2+}\) with Mg\(^{2+}\) (which also supports ligand recognition) also resulted in decreased binding of mAb 13 to α5β1 in the presence of either the CCBD fragment or GRGDS peptide. However, replacing Mn\(^{2+}\) with Ca\(^{2+}\) or EDTA (which do not support ligand recognition) did not lead to reduced binding of mAb 13 (results not shown). These data strongly suggest that the de-
Increased binding of mAb 13 to $\alpha_5\beta_1$ is a consequence of ligand occupancy. As described previously (17), binding of the anti-LiBS mAb 12G10 to $\alpha_5\beta_1$ in this assay was increased by both the CCBD fragment and GRGDS.

The CCBD fragment also markedly decreased the binding of mAb 13 to $\alpha_5\beta_1$ on K562 cells (Fig. 1C). However, in contrast, GRGDS had only a small effect on mAb 13 binding to K562 cells ($-10\%$ inhibition at $100 \mu$g/ml; result not shown). The inhibition of mAb 13 binding to K562 cells by the CCBD fragment appeared to be specific because: (i) no decrease in mAb 13 binding was observed in the presence of EDTA; and (ii) the fragment increased the binding of mAb 12G10 to K562 cells (data not shown).

Ligand Decreases the Apparent Affinity of mAb 13 Binding to
of antibody binding should increase in parallel with the anti-binding, the concentration of ligand for half-maximal inhibition of binding of the control anti-

Ligand Acts as a Direct Competitive Inhibitor of mAb 13 Binding to a5b1—To examine the mode of inhibition of antibody binding by ligand, we examined the effect of a constant concentration of GRGDS peptide (20 μg/ml) on the binding of varying concentrations of mAb 13 to a5b1 in solid phase assays. The results showed that initially the level of mAb 13 binding appeared to reach a lower maximum level in the presence of GRGDS peptide (Fig. 2B); however, at high concentrations of mAb 13, the level of binding continued to increase, whereas it reached a plateau level in the presence of the control peptide (Fig. 2A). By double-reciprocal analysis (Fig. 2C), two distinct populations of receptors could be distinguished in the presence of GRGDS, each of which represented about 50% of the total integrin. The first population of receptors bound mAb 13 with approximately the same apparent affinity as in the control, whereas the second bound mAb 13 with >50-fold lower apparent affinity than the control (Fig. 2C legend). Similar results were obtained when the CCBD fragment rather than the GRGDS peptide was used as the ligand, but neither GRGDS nor the CCBD fragment had any effect on the apparent affinity or maximum level of binding of the control anti-β1 mAb K20 (data not shown).

Ligand Acts as an Allosteric Inhibitor of mAb 13 Binding to a5b1—The above results demonstrate that in the presence of ligand (CCBD fragment or GRGDS peptide), a proportion of the total integrin binds mAb 13 with a much reduced affinity. To examine if ligand acts as a direct competitive inhibitor or an allosteric inhibitor of mAb 13 binding, we examined the inhibitory effect of ligand at several different mAb 13 concentrations. The results (Fig. 3, A and B) showed that the concentration of ligand required for half-maximal inhibition of mAb 13 binding was not significantly different over a 10-fold range of antibody concentrations. In addition, the maximal extent of inhibition decreased with increasing antibody concentration. If ligand behaved as a direct competitive inhibitor of mAb 13 binding, the concentration of ligand for half maximal inhibition of antibody binding should increase in parallel with the antibody concentration, and the maximal extent of inhibition should be unchanged. Instead, these results are consistent with an allosteric inhibition, in which ligand does not directly compete with mAb 13 for binding to the β1 subunit, but instead binds to a separate site and decreases the affinity of mAb 13 binding by an allosteric effect on the conformation of the integrin. Single reciprocal plots of 1/(antibody binding) versus ligand concentration were hyperbolic (Fig. 3, C and D), which is also diagnostic of an allosteric type of inhibition (31).

The Maximum Level of Ligand Binding Is Increased by the Activating mAb 9EG7—Since in the presence of ligand only about 50% of the integrin bound mAb 13 with a very low affinity and the affinity of the other 50% was approximately the same as that of the control (i.e. not ligand occupied), we hypothesized that this latter population may be in an inactive (incompetent to bind ligand) state. To test this possibility, we investigated the effect of the activating mAb 9EG7 on the binding of CCBD fragment to a5b1. As shown in Fig. 4, the maximum level of ligand binding in the presence of 9EG7 was 2-fold higher than the maximum level of ligand binding in the absence of 9EG7. A similar result was obtained for a second activating mAb 12G10 (data not shown). These findings support the hypothesis that ~50% of the integrin in the solid phase assays is normally in a state that is incompetent to bind ligand; however, this population can be stimulated to bind ligand in the presence of activating mAbs such as 9EG7.

In the Presence of 9EG7 and Ligand, All of the Integrin Binds mAb 13 with Very Low Affinity—We tested the effect of preincubating a5b1 with 9EG7 and ligand on the binding of mAb 13 to the integrin (Fig. 5A). Since 13 and 9EG7 are both rat monoclonal antibodies, we used biotinylated mAb 13 in these experiments and detected binding using avidin conjugates. As found for unlabelled mAb 13, the CCBD fragment partially (~40%) inhibited the binding of biotinylated mAb 13. Preincubation of integrin with ligand alone did not further reduce mAb 13 binding compared to the level when mAb 13 and CCBD fragment were co-incubated (result not shown); however, preincubation of a5b1 with 9EG7 and CCBD fragment reduced the level of mAb 13 binding to ~15% of control (in the absence of 9EG7 or ligand). Preincubation of a5b1 with 9EG7 alone only slightly reduced mAb 13 binding, confirming previous data that 9EG7 does not directly interfere with mAb 13 binding because it recognizes a distinct part of the β1 subunit (32). Similar results were obtained by examining the binding of biotinylated mAb 13 to a5b1 on K562 cells (Fig. 5B). Although GRGDS alone only slightly reduced mAb 13 binding to K562 cells, in the presence of 9EG7 it was as effective as the CCBD fragment at blocking mAb 13 binding (data not shown). Importantly, although the binding of mAb 13 to a5b1 was strongly attenuated after incubation of the integrin with 9EG7 and ligand, the binding of activating mAbs with epitopes very close to that of mAb 13 was either unchanged (TS2/16) or increased (12G10) (data not shown).

We next examined the effect of preincubating a5b1 with 9EG7 and ligand on the apparent affinity and maximum level of mAb 13 binding, compared to integrin preincubated with 9EG7 alone. The results (Fig. 6) show that in the presence of 9EG7 and CCBD fragment, approximately 50% of the integrin binds mAb 13 with a very low affinity (similar to that of the low affinity population in the absence of 9EG7; compare data in legends to Fig. 6 and Fig. 2), whereas the other 50% appears not to bind mAb 13 at all (had no detectable affinity for the antibody). Similar results were obtained when GRGDS peptide, rather than the CCBD fragment, was used as the ligand (data not shown). It should be noted that in a separate experiment, preincubation with 9EG7 alone did not alter the maximum level of mAb 13 binding but did reduce the apparent affinity of mAb 13 binding compared to a5b1 that was not
preincubated with 9EG7 (results not shown). In conclusion, these studies demonstrate that under conditions where all of the integrin appears to be occupied by ligand, only very low affinity binding sites for mAb 13 can be detected.

Binding of mAb 13 and Ligand Appears to Be Mutually Exclusive—Since ligand appears to act as an allosteric inhibitor of mAb 13 binding and, therefore, to recognize a separate site on the integrin, we examined whether mAb 13 and ligand could bind simultaneously to α5β1. In the experiment shown in Fig. 7, integrin was preincubated with 9EG7 and CCBD fragment (to induce maximal ligand occupancy), and then the amounts of mAb 13 and CCBD binding at different concentrations of mAb 13 were compared. The concentration of CCBD fragment used in these assays gave a maximum level of ligand binding in the absence of mAb 13. The results showed that as the binding of mAb 13 to α5β1 increased, there was a corresponding decrease...
Ligand-attenuated Binding Site on Integrin β1 Subunit

FIG. 7. Comparison of the levels of mAb 13 binding (○) and ligand binding (□) to 9EG7-stimulated a5b1 in a solid phase assay. To measure antibody binding, integrin was preincubated with 9EG7 and unlabeled CCBD fragment (20 μg/ml) and then incubated with varying concentrations of biotinylated mAb 13 and unlabeled CCBD fragment (20 μg/ml). The maximum level of antibody binding was estimated by measuring the binding of biotinylated mAb 13 (100 μg/ml) in wells that were preincubated with buffer alone. To measure ligand binding, integrin was preincubated with 9EG7 and biotinylated CCBD fragment (20 μg/ml) and then incubated with varying concentrations of unlabeled mAb 13 and biotinylated CCBD fragment (20 μg/ml). To estimate the maximum level of binding of CCBD fragment, the binding of biotinylated CCBD fragment (20 μg/ml) was measured in the presence of 9EG7 and in the absence of mAb 13. Binding of biotinylated mAb 13 or CCBD fragment was detected as described under "Experimental Procedures." Bars, S.D.

FIG. 8. Model of the modulation of integrin-ligand interactions by mAbs. Three major conformational states of an integrin can be distinguished: I₁, I₂, and I₃, corresponding to the conformations of the inactive, active, and ligand-occupied states, respectively. Only the active state (I₃) is competent to bind ligand (L). Antibodies that recognize epitopes that are preferentially exposed on the ligand-occupied (I₃) state of the integrin (anti-LIBS mAbs) will shift the equilibrium in favor of this state and thereby stimulate ligand binding. Antibodies that recognize epitopes that are preferentially exposed on the unoccupied (I₁) state (anti-ligand-attenuated binding site mAbs (anti-LABS mAbs)) will shift the equilibrium in favor of this state and thereby inhibit ligand binding.

in the binding of the CCBD fragment. For example, at an antibody concentration of 100 μg/ml, ~45% of the total integrin was occupied by mAb 13, but the proportion of the total integrin occupied by ligand decreased to ~55%. Hence, it was found that mAb 13 and the CCBD fragment did not appear to bind simultaneously to α5β1, thus inferring that antibody and ligand binding is mutually exclusive. Although ligand binding could be maximally inhibited only ~50% in this assay by mAb 13, binding of the CCBD fragment to 9EG7-stimulated α5β1 was inhibited >95% by EDTA (data not shown). This failure of mAb 13 to completely inhibit ligand binding is, however, in agreement with the observation (Fig. 6) that only ~50% of the integrin can recognize mAb 13 when it has been preincubated with 9EG7 and ligand.

DISCUSSION

The novel findings of this report are the following. (i) The apparent affinity of binding of the inhibitory mAb 13 to α5b1 is reduced in the presence of ligand, implying that the epitope recognized by this antibody is attenuated by ligand occupancy. (ii) Ligand behaves as an allosteric inhibitor of antibody binding, suggesting that mAb 13 does not perturb integrin function by direct competition for the ligand binding site. (iii) The binding of mAb 13 and ligand appears to be mutually exclusive, suggesting that mAb 13 may induce a conformational change in α5b1 that results in displacement of ligand from the integrin.

Our initial experiments showed that the binding of mAb 13 to α5b1 was reduced by ~50% by both the CCBD fragment and GRGDS peptide. The observation that the short (5-mer) peptide was capable of blocking mAb 13 binding implied that this inhibition was not due to long-range steric hindrance of antibody binding by ligand. Although GRGDS only slightly inhibited binding of mAb 13 to K562 cells, this is probably a consequence of the low activation state of α5b1 on these cells, such that the synergistic regions of the CCBD appear to be required for recognition of the GRGDS sequence by α5b1 on unstimulated K562 cells (33). We found that in the presence of the stimulatory mAb 9EG7, GRGDS was as effective as the CCBD fragment for reducing mAb 13 binding to K562 cells.

About 50% of the total integrin showed low affinity binding of mAb 13 in the presence of ligand, whereas the affinity of the remainder appeared unaltered. We interpreted the first of these two populations as integrin that is capable of attaining an active conformation (and is, therefore, competent to bind ligand), whereas the second population is locked in an inactive conformation (and incapable of binding ligand). Similar subpopulations of integrin have been noted in other systems (8, 12, 34–36), and as shown previously (34, 37, 38), functionally inactive integrin can sometimes be "rescued" from this state by the addition of activating mAbs. We found that the activating mAb 9EG7 (9), which is a member of the anti-LIBS family of anti-integrin mAbs, increased the maximum level of ligand binding ~2-fold in solid phase assays. Interestingly, 9EG7 only had a small effect on the apparent affinity of ligand binding; in contrast, other stimulatory anti-β1 mAbs such as 8A2 appear to increase the apparent affinity of ligand binding but have little effect on the maximal level of ligand binding (39). In the presence of 9EG7 and ligand, only low affinity binding sites for mAb 13 were observed. These observations strongly support the hypothesis that the population of integrin that binds mAb 13 with only a very low affinity represents integrin that is occupied by ligand. Our results also showed that in the presence of 9EG7 and ligand, about one-half of the total integrin appeared not to bind mAb 13 at all (i.e. had no measurable affinity for the antibody). It is possible that the population of ligand-occupied integrin that fails to bind mAb 13 corresponds to an additional conformational state of α5b1, perhaps with ligand irreversibly bound to the integrin (40).

Since ligand behaved as an allosteric inhibitor of antibody binding, i.e. mAb 13 and ligand appeared to recognize non-overlapping sites on the β1 subunit, it seemed possible that ligand and mAb 13 binding could occur simultaneously. We found, however, that antibody and ligand binding were inversely correlated, suggesting that when mAb 13 binds to the ligand-occupied state of the integrin, it induces a conformational change that results in expulsion of the ligand from the
Ligand-attenuated Binding Site on Integrin β1 Subunit

An important corollary of the above model is that antibodies whose epitopes are preferentially expressed on the unoccupied state may be able to inhibit integrin function by shifting a conformational equilibrium in favor of the unoccupied state (provided that ligand binding is reversible). Here we show that the epitope recognized by the inhibitory anti-β1 mAb 13 is attenuated by ligand occupancy, i.e. the antibody binds with a much lower affinity to the ligand-occupied state than to the unoccupied state of the integrin. It has been suggested that the converse of L1BS epitopes should be termed ligand-induced cryptic site epitopes (44). However, since the receptors in the ligand-occupied state may still weakly express these epitopes, we prefer the term ligand-attenuated binding site epitopes. We propose that mAb 13 be designated an anti-ligand-attenuated binding site mAb, and that antibodies of this type perturb ligand binding by acting in the opposing manner to anti-L1BS mAbs (Fig. 8). Our data suggest that mAb 13 inhibits ligand binding by stabilizing the conformation of the unoccupied state, rather than by sterically blocking a ligand binding site. Indeed, it is difficult to envisage how mAb 13 could act as a direct competitive inhibitor of ligand binding (i.e. block a site directly involved in ligand binding), whereas antibodies with epitopes very close to that of mAb 13 (such as 12G10, TS2/16, and B8A2) strongly stimulate integrin function (17, 45, 46).

Since all known inhibitory mAbs recognize the same region of the β1 subunit (18), other inhibitory mAbs may perturb ligand binding by the same mechanism as mAb 13; in agreement with this suggestion, we have recently found that ligand also appears to act as an allosteric inhibitor of P4C10 binding.2 One question that arises here is: What is the mechanism by which inhibitory anti-β1 subunit mAbs perturb ligand binding? It will clearly be important to determine if these antibodies inhibit ligand binding directly, or like mAb 13, recognize epitopes that are attenuated by ligand occupancy. Our data for mAb 13 clearly highlight the danger of attempting to localize ligand binding sites on integrins by epitope mapping of inhibitory mAbs, since some of these antibodies may recognize sequences that regulate integrin activity, rather than sites that are directly involved in ligand recognition.

Finally, it remains to be determined if activating and inhibitory anti-β1 mAbs mimic the function of biological activators or inhibitors of integrin function. Because the region of the β1 subunit that contains the epitopes for these mAbs is crucially involved in the regulation of integrin-ligand interactions, our current studies are focused on the fine mapping of epitopes within this region.

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