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A Series of Function Blocking Antibodies against the αvβ3 Integrin Bind Allosteric to the Ligand Binding Site and Induce Ligand Dissociation

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The αvβ3 integrin plays a critical role in bone resorption, angiogenesis, and tumor cell invasion. A blockade of this receptor has therapeutic potential in osteoporosis, vascular restenosis, and cancer. In this report, we characterize the mechanism by which six monoclonal antibodies inhibit the function of αvβ3. All six antibodies interact with a common site that is partially comprised of residues 164–202 within the β3 subunit. This domain is physically separate from the RGD binding site, and appears to regulate ligand binding allosterically. Thus, the blocking antibodies function, in part, by inducing the dissociation of ligand from αvβ3. Although this family of antibodies is able to virtually abolish αvβ3-mediated cell adhesion, they only block about one-half of soluble ligand binding to the integrin. This observation is consistent with the idea that two functionally distinct populations of αvβ3 are present on the cell surface. The unique mechanism of action of these antibodies provides new insight in the structure–function relationships of αvβ3, and also suggest that such antibodies are likely to behave differently than RGD mimetics if used as drugs.

INTRODUCTION

Integrins are cell surface receptors that mediate the interaction between cells and the extracellular matrix (Hynes, 1992; Ruoslahti, 1996). The αvβ3 integrin is expressed on osteoclasts, vascular smooth muscle cells, angiogenic endothelial cells and on many tumors (Brooks et al., 1994; Horton et al., 1993; Liaw et al., 1995). This integrin binds to several proteins containing the arginine-glycine-aspartic acid (RGD)[†] tripeptide motif. αvβ3 can mediate ligand internalization (Pijuan-Thompson and Gladson, 1997; Wickham et al., 1993), cell adhesion and cell migration (Leavesley et al., 1992).

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[†] The abbreviations used are: RGD, arginine-glycine-aspartate; FACS, fluorescence-activated cell sorting; mAb, monoclonal antibody.

The $\alpha v\beta 3$ integrin is involved in several adhesive interactions that have clinical significance. For example, $\alpha v\beta 3$ on the osteoclast mediates cell adhesion to bone, so a blockade of $\alpha v\beta 3$ may significantly reduce the rate of bone resorption (Engleman et al., 1997) and slow osteoporosis. On injured or inflamed vascular smooth muscle cells, $\alpha v\beta 3$ mediates cell migration into the intima (Liaw et al., 1995; 1997); therefore, it has been suggested that antagonizing $\alpha v\beta 3$ may prevent vascular restenosis following coronary angioplasty. $\alpha v\beta 3$ is also expressed on proliferating endothelial cells, where it is required for tumor angiogenesis (Brooks et al., 1994). Consequently, interfering with the function of $\alpha v\beta 3$ may be of benefit in slowing tumor growth.

Many integrins, including $\alpha v\beta 3$, bind to the RGD motif. Considerable effort has been devoted to synthesizing RGD mimetics as integrin antagonists. Several such peptide mimetics are effective in preventing disease in animal models (Choi et al., 1994; Coller et al., 1995; Engleman et al., 1997). However, the use of RGD-based antagonists is not without potential complications. Mimetics that lack specificity for a particular integrin may produce unwanted side effects. In this regard, no small molecule RGD-mimetic has been reported to distinguish the integrins that contain the αv subunit, three of which ($\alpha v\beta 3$, $\alpha v\beta 1$, and $\alpha v\beta 5$) bind to RGD with high affinity. Another disadvantage of using small molecule antagonists as drugs is that they are likely to have a short half-life in vivo.

Humanized monocloal antibodies (mAbs) provide an alternative strategy for inhibiting integrin function. Using conventional hybridoma technology (de St.Groth and Scheidegger, 1980), monoclonal antibodies can be generated which are exquisitely specific for individual integrin heterodimers. Indeed, several such antibodies have been used to sort out integrin expression patterns and to define the role of individual integrins in several biological processes (Cheresh and Spiro, 1987; Wayner et al., 1991). The methods for humanizing murine monoclonal antibodies are now relatively well-established and such humanized antibodies have proven effective as therapeutic agents.

At present, the only clinically approved antagonist of an integrin is the antibody abciximab, which is a humanized Fab fragment of Mab 7E3. This antibody blocks the function of platelet integrin $\alpha IIb\beta 3$ and prevents ischemic complications associated with coronary surgery (Genetta and Mauro, 1996; Tcheng et al., 1993). Now there are function-blocking mAbs against other integrins, including $\alpha v\beta 3$, and the role of such integrins in disease is better understood. Consequently, such mAbs are being considered as potential drugs, and are being evaluated in clinical trials. To properly administer antibody therapy, and to interpret the results obtained from such a blockade, it is important to understand the mechanism of action of these antibodies precisely. It is also important to know how inhibition by an antibody differs from inhibition by small molecule ligand mimetics.

In this report, we provide the first information on the mechanism by which a series of six mAbs inhibit the function of the $\alpha v\beta 3$ integrin. All six antibodies are highly specific for the complex between αv and $\beta 3$. All block $\alpha v\beta 3$-mediated cell attachment. The antibodies appear to bind a common epitope that is partially comprised of residues 164–202 on the $\beta 3$ subunit. The antibodies act allosterically; their binding is not effected by RGD ligand. Moreover, the antibodies can bind to integrin even when ligand is bound, and are able to induce the dissociation of bound ligand. Interestingly, the antibodies have different effects on the binding of soluble ligand vs. cell adhesion, suggesting that there are two populations of $\alpha v\beta 3$ present on the cell surface. The findings of the study provide new insight into the structure–function relationships that govern the function of $\alpha v\beta 3$.

METHODS

Generation of Monoclonal Antibodies against $\alpha v\beta 3$

The P112 and P113 series of mAbs were generated using standard procedures (de St.Groth and Scheidegger, 1980). Monoclonal antibodies were

derived from different mice, either P112 or P113, in two separate fusions. Six-week-old female Balb/c mice (Charles River, Wilmington, MA) were immunized intraperitoneally (i.p.) with 1×10^6 BHK cells transfected with human αvβ3 (BHK/αvβ3) in Freund's Complete Adjuvant on day 0. Mice were injected i.p. with 1×10^6 BHK/αvβ3 cells in Freund's Incomplete Adjuvant three and five weeks following the initial injection. Three weeks later, mouse P112 was injected both i.p and intravenously with 5 μg purified human αvβ3 suspended in saline. Three days later, P112 was sacrificed by CO_2 overdose and the P112 spleen cells were fused with myeloma P3X63 Ag8.653 (American Type Culture Collection; ATCC, Rockville, MD) using polyethylene glycol, MW 1300–1600 (ATCC).

The fusion products were diluted to 1×10^6 cells/ ml in HAT selection media (DMEM containing 20% CPSR supplement with 1X HAT; Sigma Chemical Co. St. Louis, MO), plated in 48-well culture dishes (0.5 cells/well). Five hundred and seventy wells were plated with cells and 225 of these grew in HAT-supplemented medium. Conditioned media was screened for the presence of antibodies that bound ^{125}I-αvβ3 or ^{125}I-αIIbβ3. Ninety six-well plate (Dynatech, Chantilly, VA) were coated with 0.5 μg/well goat anti-mouse IgG Fc-specific monoclonal antibody (Sigma) in borate-buffered saline, pH 8.2 overnight at 4°C. Unoccupied protein-binding sites were blocked with 1% BSA in PBS containing 0.05% Tween-20. The plates were washed with saline and 0.05% Tween-20 (TS). Test samples (50 μl/well; mouse sera, hybridoma supernatant or purified monoclonal antibody) were added and incubated 90 min at 37°C. The plates were washed with TS and 1×10^5 cpm/well of ^{125}I-labeled αvβ3 or αIIbβ3 diluted in Tris-buffered saline containing 1% BSA, 50 mM octyglucoside, 1 mM Ca^{2+}, Mg^{2+}, Mn^{2+}, pH 7.4 were added to each and incubated for 2 h at 37°C. The plates were washed with Tris-buffered saline and bound integrin was quantified by gamma counting.

Ten wells were identified that contained cells producing antibody that absorbed ^{125}I-αvβ3 but not ^{125}I-αIIbβ3. Cells from each of these ten wells were cloned in soft agar. Colonies were picked from agar and grown in HAT selection medium and the exhausted medium of these cultures screened again using the integrin-capture assay. This process was repeated two additional times. All of the antibodies identified were IgG1,κ.

The final immunization of mouse P113 and the fusion and selection of hybridomas was initiated approximately 3 wk later using the same procedures with similar results. Hybridomas from the P112 and P113 fusions that produced antibodies that bound purified αvβ3 but not αIIbβ3 were grown in Balb/c mice for the production of ascites fluid, the monoclonal antibodies purified via Protein A chromatograpy and then further characterized for the ability to block cell adhesion. The mAb M399 is an isotype-matched control IgG raised in a separate fusion.

Monoclonal antibody LM609, which also blocks the function of αvβ3 (Cheresh and Spiro, 1987), was purchased from Chemicon. Monoclonal antibodies 7H2 and AP3 bind the human β3 subunit, and were kind gifts from Dr. Barry Coller (Mount Sinai Medical Center, New York, NY) and Dr. Peter Newman (Blood Research Institute, Milwaukee, WI). Antibody RUU-PL7F12, which also binds to β3, was purchased from Becton Dickinson.

Ligands and Peptides

Fab-9 is a model RGD-containing ligand which was engineered to bind αvβ3 with high affinity (Barbas et al., 1993). The binding of Fab-9 to the β3 integrins has been well characterized (Hu et al., 1996). The cDNA plasmid for expressing Fab-9 was provided by Dr. Carlos Barbas (The Scripps Research Institute, La Jolla, CA). Fab-9 was expressed in E. coli and purified from bacterial lysates by affinity chromatography on a goat-anti human IgG column. Vitronectin was purified from human serum according to Yatohgo et al. (1988). Synthetic peptides with sequences GRGDSP and SPGDRG were obtained from Coast Scientific.

Cell Lines and Expression of Integrin Constructs

The human breast carcinoma cell line MDA-MB-435 was provided by Dr. Janet Price (Price *et al.*, 1990), and were maintained in Eagle's Minimal Essential Media (Irvine Scientific) supplemented with vitamins and nonessential amino acids (Life Sciences, Inc.), 1 mM sodium pyruvate (Bio Whittaker), 2 mM L-glutamine (Sigma), and 10% fetal calf serum (Irvine Scientific). Human embryonic kidney 293 cells were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium (Irvine Scientific) supplemented with 10% fetal calf serum and L-glutamine. Hamster melanoma CS-1 cells (Thomas *et al.*, 1993) were a gift from Dr. Caroline Damsky, and were maintained in RPMI-1640 (Bio Whittaker) supplemented with L-glutamine and 10% fetal calf serum. The 293 cells and CS-1 cells express the integrin αv subunit, but lack the $\beta 3$ subunit. Both cells lines were transfected with the cDNA encoding the human $\beta 3$ subunit using the approaches we have previously described (Lin *et al.*, 1997b) to obtain cell lines expressing recombinant $\alpha v \beta 3$. Some experiments were performed with 293 cells expressing chimeras between the human $\beta 3$ and $\beta 5$ integrin subunits that have been described previously (Lin *et al.*, 1997a). BHK cells used for immunization of mice, were transfected with the cDNA's encoding both the human $\alpha \beta$ and $\beta 3$ subunits to obtain cell lines expressing the $\alpha v \beta 3$ heterodimer. Cell lines that express the human integrin were obtained by selection in antibiotic.

Flow Cytometry

Fluorescence-activated cell sorting (FACS) was performed using standard protocols. Briefly, cells were incubated with 5 µg/ml of primary mAb for 30 min on ice, washed and then incubated with FITC-conjugated goat anti-mouse IgG. Cells were then washed and resuspended in PBS containing 3 µg/ml propidium iodide to identify viable cells. Cells were analyzed using a Becton Dickinson FACSort.

Cell Adhesion Assays

A series of kidney 293 cell lines expressing either $\alpha v \beta 3$, $\alpha v \beta 5$, or $\alpha v \beta 1$ (Hu *et al.*, 1995; Lin *et al.*, 1997b) were used to test the ability of monoclonal antibodies to selectively block $\alpha v \beta 3$. Cell adhesion assays were performed essentially as described (Hu *et al.*, 1995) with minor modifications. Antibodies were diluted in Hank's Buffered Salt Solution (Ca^{2+}/Mg^{2+}-free; HBSS) containing 0.1% BSA, 200 µM Mn^{2+}, 20 mM HEPES, pH 7.4 (HBSS$^+$). An equal volume of cells (2×10^6 cells/ml in HBSS$^+$) were mixed with diluted antibodies and incubated for 30 min at 37°C. The mixture (100 µl/well) was then added to vitronectin-coated plates and incubated for 30 min at 37°C. The number of viable attached cells was determined using the MTT assay kit according to the manufacturers recommendations (Promega, Madison, WI).

Some experiments measured adhesion to Fab-9. In these cases Fab-9 (100 nM) was coated in microtiter wells in phosphate buffered saline at 4°C for 18 h. Then, plates were blocked with bovine serum albumin (10 mg/ml). Cells were harvested from tissue culture dishes and resuspended in Binding Buffer (1X Hanks balanced salt solution without cations (Life Technologies, Inc.), containing 50 mM HEPES, pH 7.4, 3 mg/ml BSA, 500 µM $MgCl_2$, 50 µM $MnCl_2$). Cells (2×10^5) and the indicated concentration of competing mAb were added to each well containing immobilized ligand. Cells were allowed to adhere for 30 min at 37°C. Non-adherent cells were removed by gentle washing and the adherent cells were quantified using a colorimetric assay for acid phosphatase (Pratner *et al.*, 1991).

Cell Surface Binding Studies

Fab fragments of the mAb P112-4C1 were generated by papain digestion using the Fab purification kit (Pierce). The Fab fragment of P112-4C1 and Fab-9 were labeled with ^{125}INa (Amersham) using Iodo-gen (Pierce Chemical Co.) to a specific activities ranging from 10,000–40,000 cpm/ng. The affinity of antibodies for $\alpha v \beta 3$ was measured

using cell surface binding studies as described previously (Lin *et al.*, 1997a,b). Briefly, cells were harvested from tissue culture flasks, maintained in a suspended culture, and allowed to bind to radiolabeled ligand in suspension. Bound ligand was separated from free ligand by centrifuging through 20% sucrose cushions. Non-specific binding was determined by competition with excess unlabeled ligand, or with 15 mM EDTA (for Fab-9). The number of binding sites for Fab-9 and the Fab fragment of P112-4C1 was calculated by Scatchard analysis (Scatchard, 1949).

Binding of Ligands and Antibodies to Purified Integrin $\alpha v\beta 3$

Binding studies between purified $\alpha v\beta 3$ and radio-labeled ligands was performed essentially as described (Smith *et al.*, 1994b). To measure the effects of antibodies on ligand dissociation, ^{125}I-Fab-9 was allowed to bind $\alpha v\beta 3$ for 2 h at 37°C. Free Fab-9 was removed by washing. Saturating levels of inhibitors (250 μM GRGDSP peptide, 67 nM mAb P112-4C1, or 67 nM Fab-9) were added in an attempt to induce the dissociation of bound Fab-9. ^{125}I-Fab-9 was allowed dissociate from $\alpha v\beta 3$ for the indicated amount of time at 37°C. Bound ^{125}I-Fab-9 was recovered in boiling 2 N NaOH and quantified by gamma counting.

RESULTS

Generation of Monoclonal Antibodies against the $\alpha v\beta 3$ Integrin

Monoclonal antibodies were raised against the $\alpha v\beta 3$ complex using standard hybridoma technology. Two separate fusions were performed between splenocytes of immunized mice and myeloma cells. Antibodies that were used for this study are named according to the mouse splenocyte donor from which they were generated (P112 or P113). Six mAbs were identified which blocked the function of $\alpha v\beta 3$ using a cell adhesion assay. To obtain relative affinities for each antibody, IgG was

purified from the selected hybridomas and tested for the ability to block cell adhesion. All of the P112 and P113 mAbs, as well as the control mAb LM609, blocked the adhesion of 293 cells expressing recombinant $\alpha v\beta 3$ to immobilized vitronectin. Inhibition of adhesion was dependent on the concentration of the antibody (Fig. 1). The IC_{50}'s ranged from 50 to 200 ng/ml of antibody (0.3–1.3 nM). The irrelevant control antibody, M399, had no effect on cell adhesion. The P112 and P113 antibodies also blocked the adhesion of MDA-MB-435 cells that naturally express $\alpha v\beta 3$ (not shown). The P112 and P113 antibodies had no effect on adhesion of kidney 293 cells expressing recombinant $\alpha v\beta 5$ (data not shown) indicating that the antibodies were specific for the $\alpha v\beta 3$ complex. In support of this conclusion, the P112 and P113
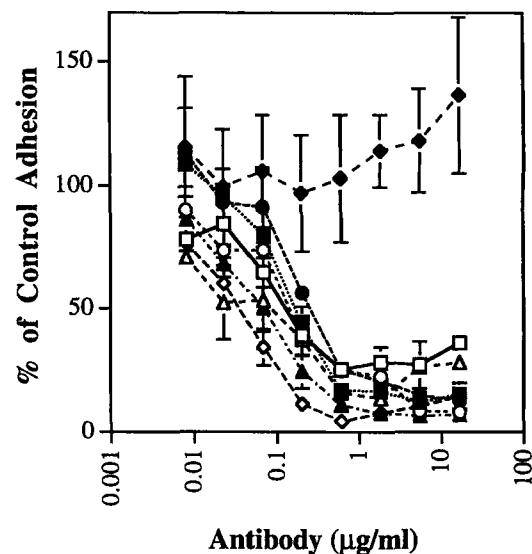

FIGURE 1 The P112 and P113 monoclonal antibodies inhibit $\alpha v\beta 3$-mediated cell adhesion. Kidney 293 cells expressing recombinant $\alpha v\beta 3$ were pre-incubated with the P112 and P113 mAb for 30 min at 37°C prior to plating the cells onto vitronectin-coated wells. Adhesion was allowed to proceed at 37°C for 30 min. Adherent cells were detected using a colorimetric substrate reaction using the MTT dye from Pharmacia. Adhesion is expressed as a percentage of the adhesion obtained in the absence of added antibody. M399 was used as the isotype-matched nonspecific control mAb. Antibodies tested in this experiment include: P113-7D6 (□), P113-1F3 (■), P112-10D4 (○), P113-12A6 (●), P112-4C1 (△), P112-11D2 (▲), LM609 (◇), M399 (◆). This experiment has been repeated twice producing similar results each time.
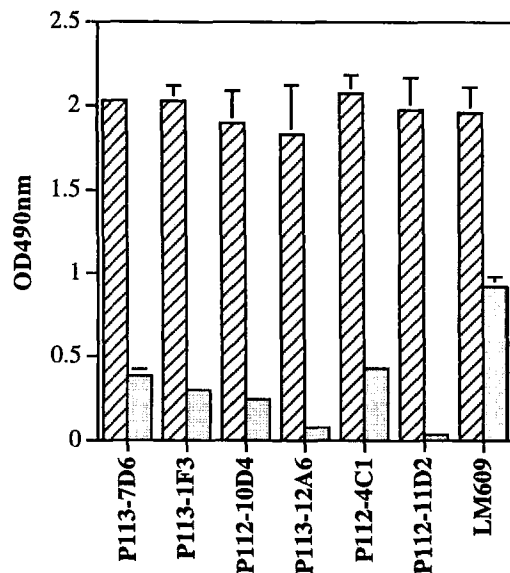
FIGURE 2 The P112 and P113 monoclonal antibodies are specific for $\alpha v \beta 3$. Monoclonal antibodies from the P112 and P113 fusions were tested for the ability to bind to purified $\alpha v \beta 3$ (striped bars) or $\alpha IIb \beta 3$ (filled bars) using and ELISA. Purified integrins were coated in a 96-well plate, and 20 μg/ml of purified IgG from selected hybridomas was allowed to bind to integrin for 1.5 h. The binding of primary mAb was detected with horseradish peroxidase-conjugated goat anti-mouse IgG. The mean optical density derived from triplicate wells is shown. This experiment was repeated twice and yielded identical results.
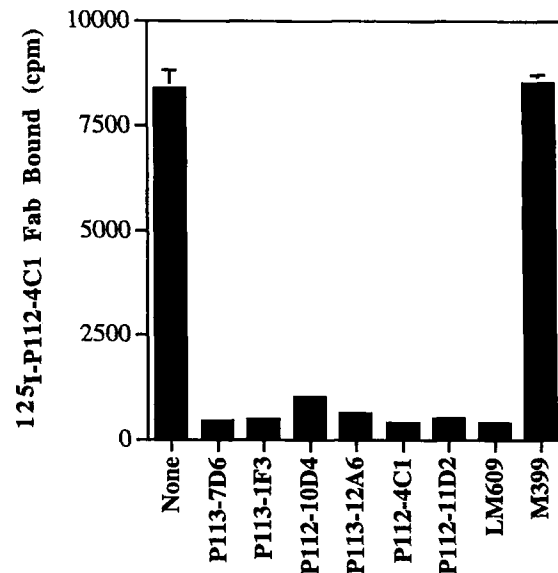
FIGURE 3 The P112 and P113 antibodies bind a common epitope on $\alpha v \beta 3$. Each of the P112 and P113 antibodies and antibody LM 609 (IgG at 20 μg/ml) were tested for the ability to block the binding of ^{125}I-P112-4C1-Fab (2 nM) to CS-1 cells expressing $\alpha v \beta 3$. The irrelevant antibody, M399, was used as the negative control. The binding of ^{125}I-P112-4C1-Fab is expressed as the mean and standard deviation of triplicate data points. This is experiment is an example of four similar experiments which yielded nearly identical results.

antibodies failed to bind to the $\alpha IIb \beta 3$ integrin in ELISA (Fig. 2).

The P112 and P113 Antibodies Bind a Site on $\alpha v \beta 3$ that is Allosteric to the Ligand Binding Pocket

Experiments were conducted to determine if the P112 and P113 antibodies bind a common epitope. We measured the ability of each antibody to competitively inhibit the binding of an Fab fragment of P112-4C1 to $\alpha v \beta 3$. Each of the P112 and P113 mAbs competed with ^{125}I-P112-4C1 Fab for binding to $\alpha v \beta 3$ (Fig. 3). The control mAb M399, did not compete with ^{125}I-P112-4C1 Fab for binding to integrin, nor did antibodies 7H2, AP3 and RUU-PL7F12, all of which bind to the $\beta 3$ subunit (data not shown).

To determine if the P112 and P113 antibodies block cell adhesion by binding to the integrins RGD binding site, we measured the ability of RGD peptide to block binding of ^{125}I-P112-4C1 Fab to $\alpha v \beta 3$ (Fig. 4). The peptide with sequence GRGDSP had no effect on the binding between ^{125}I-P112-4C1 Fab (or any of the other P112 and P113 antibodies) and $\alpha v \beta 3$, even though it can completely block the binding of ^{125}I-Fab-9, ^{125}I-vitronectin and ^{125}I-osteopontin (not shown) to $\alpha v \beta 3$ (Barbas *et al.*, 1993; Hu *et al.*, 1996; Smith *et al.*, 1994a). Collectively, these observations show that the P112 and P113 antibodies bind at a site on the integrin that is separate from the ligand binding site. This conclusion is supported by the observation that the RGD-containing antibody, Fab-9 did not hinder the binding of ^{125}I-P112-4C1 Fab to $\alpha v \beta 3$ (data not shown). Moreover, the binding of the P112 and P113 antibodies to $\alpha v \beta 3$ was not dependent
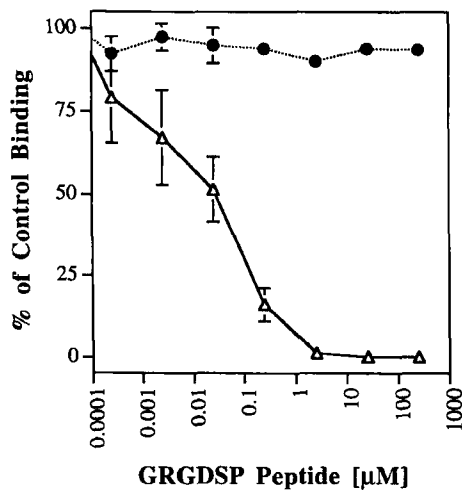
FIGURE 4 Antibody P112-4C1 binds a site distinct from the RGD-binding site. The ability of the peptide GRGDSP to block the binding of ^{125}I-P112-4C1 (●), or the model RGD-ligand, ^{125}I-Fab-9 (△) was measured on purified αvβ3 as described (Lin et al., 1997a). ^{125}I-Fab-9 and ^{125}I-P112-4C1 were used at a concentration of 1 nM. Nonspecific binding of Fab-9 was determined by inclusion of EDTA. Nonspecific binding of ^{125}I-P112-4C1 was measured by including a 100-fold excess of unlabeled P112-4C1 antibody. Specific binding is shown and is the mean of triplicate data points. This experiment was repeated four times yielding identical results.

on divalent ions (not shown), as is the binding of RGD ligand.

The P112 and P113 Antibodies Increase the Dissociation Rate for RGD Ligand

Since the P112 and P113 antibodies do not bind at the ligand binding pocket, further studies were conducted to gain insight into the mechanism by which they interfere with cell adhesion. We tested the ability of the mAbs to induce ligand dissociation. This analysis was done using Fab-9 as a representative RGD ligand for αvβ3. The kinetics of binding between Fab-9 and αvβ3 have been measured in detail (Hu et al., 1996); hence it is an excellent model ligand for these types of analyses. In this assay, ^{125}I-Fab-9 was allowed to bind purified αvβ3. Following a 2 h incubation, free ^{125}I-Fab-9 was removed by washing and replaced by either buffer or by the test antibody. At various times, the amount of ^{125}I-Fab-9 remaining bound to the integrin was quantified by gamma counting (Fig. 5A). The irrelevant mAb M399, GRGDSP
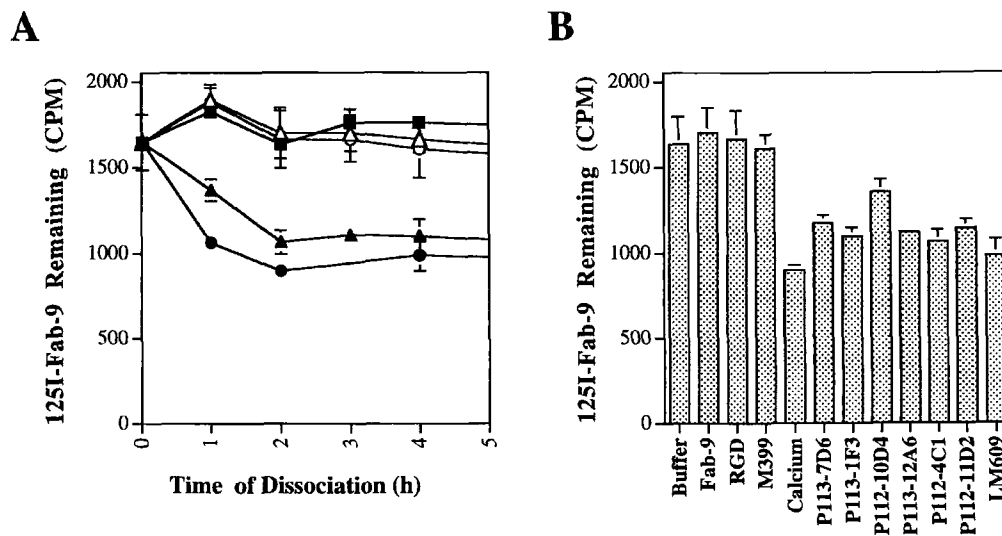

FIGURE 5 The P112 and P113 antibodies dissociate RGD-ligand from αvβ3. (A) ^{125}I-Fab-9 was allowed to bind purified αvβ3 for 2 h at 37°C. Free Fab-9 was removed by washing. Then, ^{125}I-Fab-9 was allowed to dissociate in the presence of buffer (■), Fab-9 (△), RGD peptide (○), mAb P112-4C1 (▲), or 2 mM Ca²⁺ (●). At the indicated times, the ^{125}I-Fab-9 remaining bound to integrin was quantified by gamma counting as described under Methods. Values represent the mean and standard deviation of triplicate data points. (B) In a similar experiment, the ability of level of the other P112 and P113 antibodies and LM 609 to dissociate ^{125}I-Fab-9 within 2 h incubation at 37°C was measured. These experiments have been repeated seven times with similar results.

peptide, and an excess of unlabeled Fab-9 did not effect dissociation when compared to buffer. These controls are key because they show that reductions in the amount of ^{125}I-Fab bound to integrin are not due to the combination of natural dissociation, and then the prevention of rebinding of ligand during the assay. In contrast to these control agents, Ca^{2+}, which causes ligand dissociation by binding to integrin (Hu *et al.*, 1996), induced the dissociation of 30–50% of bound ^{125}I-Fab-9 within 2 h ($n = 7$). P112-4C1 dissociated bound ^{125}I-Fab-9, as effectively as Ca^{2+} (23–46% dissociated after 2 h, $n = 7$). The Fab fragment of P112-4C1 was as effective as the IgG (data not shown). The other P112 and P113 antibodies also dissociated ^{125}I-Fab-9 from $\alpha v\beta 3$ (Fig. 5B).

Residues 164–202 within the β3 Subunit Form Part of the Epitope for the P112 and P113 Antibodies

Although the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins are highly homologous, the P112 and P113 antibodies bind only to $\alpha v\beta 3$. We used this property to map the domain on the β subunit that makes up the epitope for these antibodies. Cells transfected with a series of chimeras between the $\beta 3$ and $\beta 5$ subunits (Lin *et al.*, 1997a) were examined for the ability to bind each antibody using flow cytometry (Fig. 6). The FACS profiles obtained for each antibody in both the P112 and P113 series, and with antibody LM 609, were identical, therefore the profiles obtained with antibody P112-4C1 are shown as an example. P112-4C1 bound to chimeras containing $\beta 3$ exons A–L (not shown), A–I, and C–D. The minimal chimera capable of binding the P112 and P113 antibodies is comprised of $\beta 3$ residues 164–202 swapped into the backbone of $\beta 5$ (Panel G). No binding was observed with cells transfected with vector alone, with cells expressing $\alpha v\beta 5$, or with cells expressing a chimera containing only the first two exons of $\beta 3$ grafted onto $\beta 5$. Three control antibodies (RUU-PL7F12, AP3 and 7H2) which bind the $\beta 3$ subunit, but are not specific for the complex between αv and $\beta 3$, interact with a

separate site, because they bind to the chimera containing $\beta 3$ exons A-I, but not to the $\beta 3(164-202)/\beta 5$ chimera (Fig 6, Panels B, D, F and H).

The P112 and P113 Antibodies Competitively Inhibit Cell Adhesion but not Ligand Binding to Cell Surface αvβ3

Studies were conducted to determine if the prototype antibody, P112-4C1 inhibited cell adhesion and the binding of soluble ligand to a similar extent. Hamster melanoma cells expressing recombinant human $\alpha v\beta 3$ were used for these studies because they lack endogenous $\alpha v\beta 1$, an integrin that is expressed at low levels by the 293 cells and which can complicated the interpretation of cell adhesion assays. To ensure that results from the ligand binding studies and the adhesion experiments were directly comparable, the time and temperature of the binding studies were held constant, and were performed under conditions in which the binding of the Fab fragment to cells had reached equilibrium. In addition, the model RGD-ligand, Fab-9 was used for both studies.

Saturating levels of the Fab fragment of P112-4C1 blocked between 40% and 60% of the binding of soluble Fab-9 to the cell surface (Fig. 7A). This result was highly reproducible and independent of the cell line that was examined. In contrast, P112-4C1 was able to completely inhibit cell adhesion to immobilized Fab-9 (Fig. 7B). Similar results were obtained with the IgG of all other P112 and P113 antibodies. In support of this observation, P112-4C1 was only able to block about one-half of the binding of Fab-9 to purified $\alpha v\beta 3$ (not shown).

Two scenarios can be proposed to explain the different effects of P112-4C1 on cell adhesion vs. the binding of soluble ligand, i.e. Fab-9. In both instances two functionally distinct populations of $\alpha v\beta 3$ are present on the cell surface. In one scenario, the two populations have entirely separate functions, with the first population mediating adhesion and the other the binding of soluble ligand. In this case, P112-4C1 would bind only to both populations of $\alpha v\beta 3$, but only inhibit the
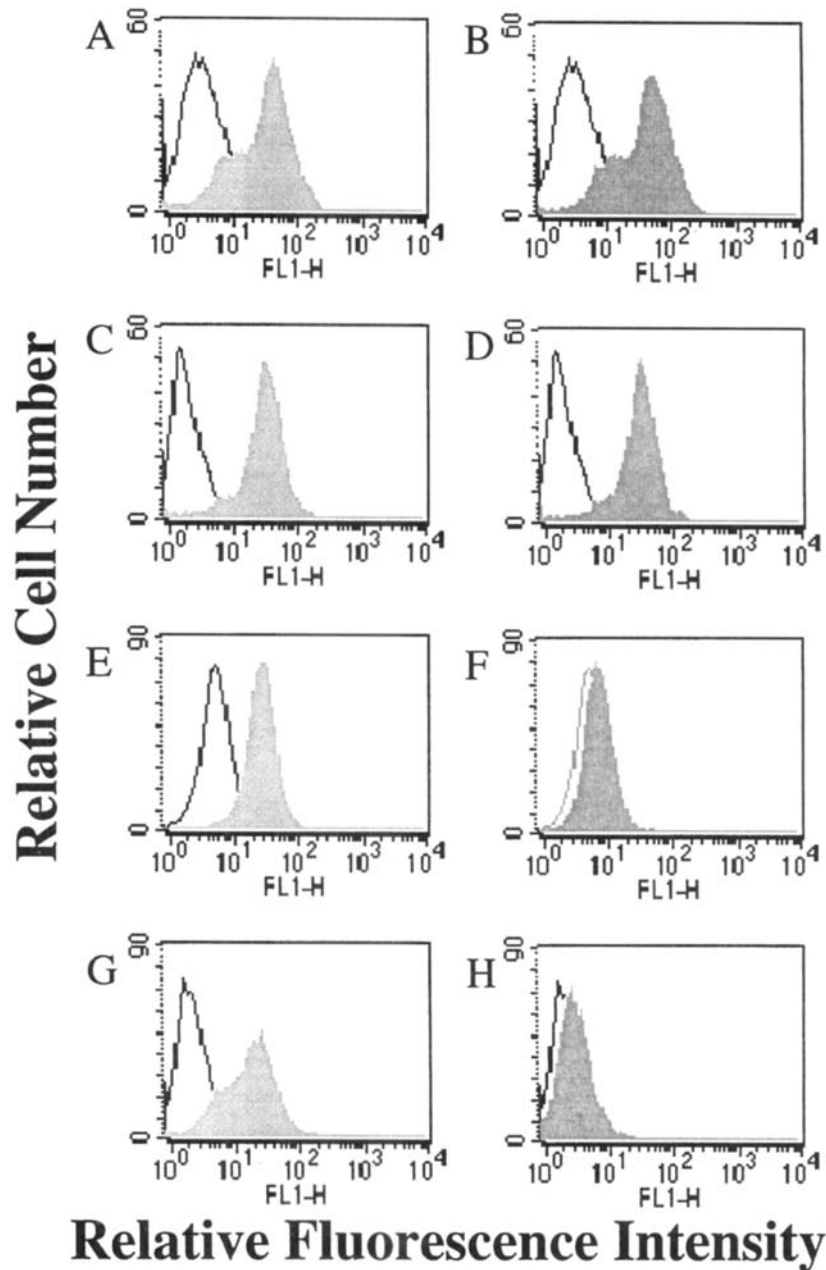
FIGURE 6   The P112 and P113 antibodies bind a site encompassing residues 164–202 on the β3 subunit. The epitope for the P112 and P113 antibodies was mapped by measuring their ability to bind a series of chimeras between the β3 and β5 subunits. Human 293 expressing the chimeras of the integrin β3 and β5 subunits, in association with αv, were used for this analysis (Lin *et al.*, 1997a). The chimeras are comprised of exons of the β3 grafted into the backbone structure of β5. The chimeras used in the analyses include: αvβ3 (Panels A and B); β3(A–I)/β5 (Panels C,D); β3(C–D)/β5 (Panels E,F) and β3(164–202)/β5 (Panels G,H). The binding of mAb P112-4C1 (Panels A, C, E, and G) and the anti-β3 antibody, RUU-PL7F12, (Panels B, D, F, and H) to cells expressing each chimera was measured with flow cytometry and is shown in dark. All antibodies from the P112 and P113 series bound to the chimeras in a manner identical to P112-4C1, as did antibody LM 609 (not shown). The binding of the primary mAb was detected with a secondary FITC-conjugated goat anti-mouse IgG. The level of non-specific binding was measured with an irrelevant primary IgG and is shown as the light histogram.
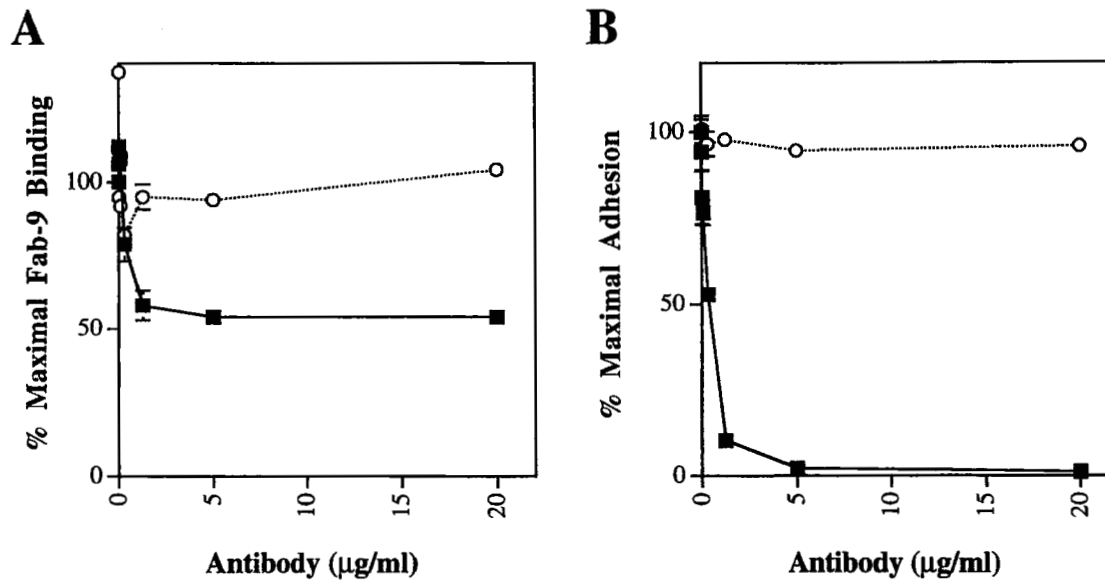
A

B

FIGURE 7   Antibody P112-4C1 has distinct effects on cell adhesion and the binding of soluble ligand. P112-4C1 (■) was tested for the ability to block the binding of soluble ^{125}I-Fab-9 (Panel A), or the ability to prevent cell adhesion to immobilized Fab-9 (Panel B). The irrelevant mAb M399 (○) was included as the negative control. Both experiments were conducted under identical conditions using CS-1 cells expressing recombinant $\alpha v \beta 3$. The binding of ^{125}I-Fab-9 to cells was quantified by gamma counting as described under Methods. The adhesion of CS-1 cells to Fab-9 was quantified with a colorimetric substrate as described (Pratner *et al.*, 1991). Results are presented as a percentage of specific binding, or adhesion, in the absence of competing P112-4C1. Both experiments were repeated three times in parallel yielding similar results.

function of the population mediating adhesion. Alternatively, two populations of integrin may be present, with only one able to bind to the P112 and P113 antibodies. In this case, P112 would bind only to the population of receptor that mediates adhesion.

To distinguish between these two possibilities, we compared the number of binding sites for the Fab fragment of P112-4C1 to the number of binding sites for Fab-9 by generating binding isotherms for each ligand (Fig 8). Scatchard plots of these isotherms revealed that the number of P112-4C1 and Fab-9 binding sites were virtually identical. A summation of these binding studies indicated $416,107 \pm 120,078$ binding sites for Fab-9, and $411,845 \pm 63,477$ sites for the Fab fragment of P112-4C1. Since the number of P112-4C1 binding sites is equivalent to the number of ligand binding sites, we conclude that the antibody binds the full complement of integrin on the cell, but is only able

to block the ligand-binding function of a subpopulation of the integrin.

DISCUSSION

The purpose of the present investigation was to characterize six monoclonal antibodies (mAbs) that bind the $\alpha v \beta 3$ integrin and inhibit its function. The major findings of the study are as follows: (1) the P112 and P113 antibodies block $\alpha v \beta 3$-mediated cell adhesion with IC_{50}'s ranging from 50–200 ng/ml; (2) the P112 and P113 antibodies all appear to bind to a common epitope partially comprised of amino acid residues 164–202 within the integrin $\beta 3$ subunit; (3) this antibody binding site is allosteric to the ligand binding site, so antibodies are able to bind to integrin even when it is occupied by ligand; (4) the antibodies are able to induce the dissociation of ligand from the integrin; (5) the antibodies
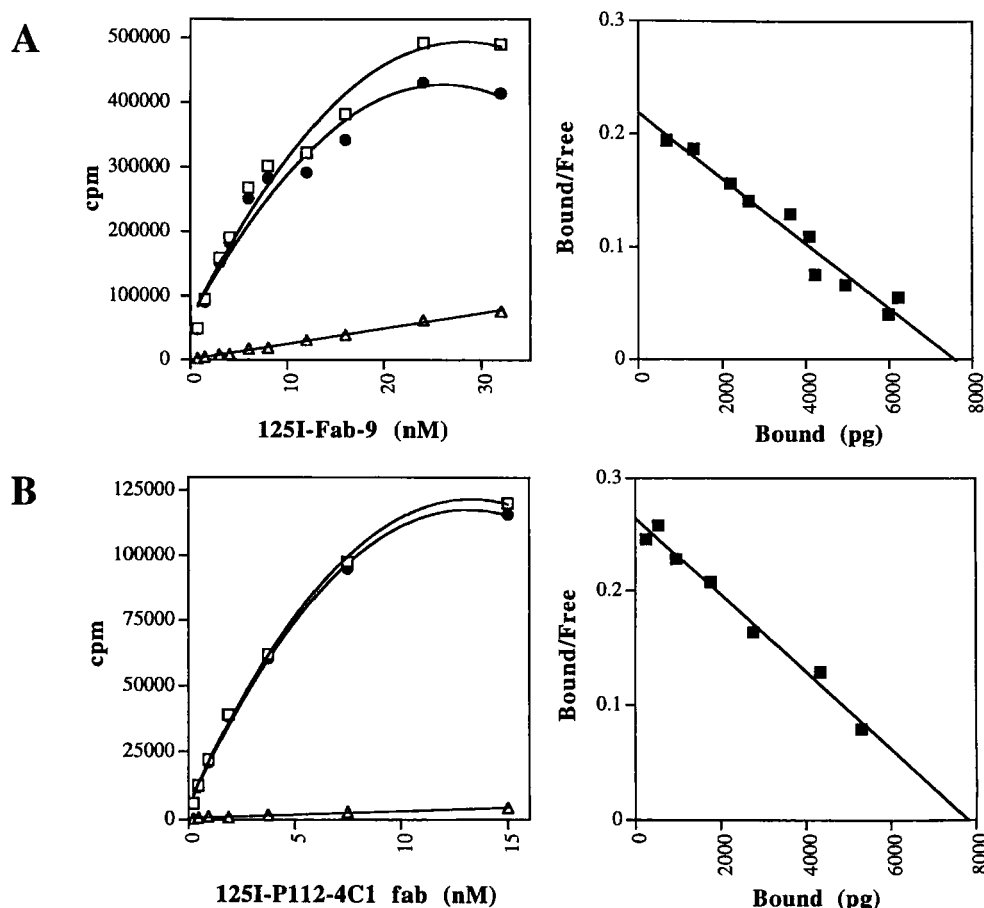
FIGURE 8   P112-4C1 and Fab-9 bind an equivalent number of binding sites on the cell surface. The binding of ^{125}I-Fab-9 (Panel A) and ^{125}I-P112-4C1-Fab (Panel B) to CS-1 cells expressing αvβ3 was measured on cells held in suspension. Binding was measured across a concentration range of each ligand as described under Methods. Binding was measured at 14°C to prevent internalization of ligand, and was allowed to proceed to equilibrium (70 min). A binding isotherm (Left Panel) and Scatchard plot (Right panel) from representative experiments are shown. Specific binding (●) was calculated by subtracting nonspecific binding (△) from total binding (□). This analysis was repeated three times, with each yielding a similar value for the number of P112-4C1 and Fab-9 binding sites.

block virtually all of the cell adhesion mediated by αvβ3, but block only about one-half of the binding of soluble ligand, suggesting the existence of two functionally separate populations of αvβ3 on the cell surface.

The findings presented here indicate that the P112 and P113 antibodies bind an epitope that is partially comprised of residues 164–202 within β3. This domain is similar to a domain within the β1 integrin that makes up the epitope for antibodies that modulate the function of β1 integrins (Takada

and Puzon, 1993). During the course of the present study, we also obtained circumstantial evidence that the homologous domain on the integrin β5 subunit is an epitope for function blocking antibodies. Antibodies P3G2 and P1F6 bind only to the αvβ5 integrin and block its function (Leavesley et al., 1992; Wayner et al., 1991). Although not shown in this report, we found that the substituting residues 164–202 from β3 into the backbone of β5 eliminates the binding of P3G2 and P1F6, even though the chimeric integrin retains ligand binding

function. Collectively, these data indicate this domain within the integrin β subunits is a regulatory domain that is a binding site for many of the antibodies that have been raised to modulate integrin function.

One of our prior reports showed that residues 164–202 of β3 play a role in determining ligand binding specificity (Lin et al., 1997a). This finding is supported by that of Takagi et al. who identified a similar same domain as key in determining ligand binding specificity (Takagi et al., 1997). However, the results of the present study show that neither RGD peptide, Fab-9, nor any natural ligand we tested, interfered with the binding of the P112 and P113 antibodies to $\alpha v\beta 3$. Consequently, residues 164–202 appear to be part of the antibody epitope that lies outside of the ligand binding pocket.

All ligand binding reactions can be broken into the steps of ligand association and ligand dissociation, and we have found that in the case of integrins, these two steps can be regulated independently. Two distinct classes of ion binding sites on the integrin control ligand association and dissociation (Hu et al., 1996; Smith et al., 1994b). One site, called the I site, is specific for Ca^{2+} and when occupied increases the off-rate for RGD-ligand. It is interesting that the P112 and P113 antibodies have a similar effect, and are the only proteins to our knowledge that induce dissociation of ligand from integrin. It is conceivable that the P112 and P113 mAbs function as mimics of calcium, and bind the allosteric calcium binding site, although we found no effect of Ca^{2+} on the binding of the antibodies. However, the large difference in the affinity of $\alpha v\beta 3$ for the mAbs (nanomolar) versus calcium ion (millimolar) makes it impossible to make conclusions about whether Ca^{2+} and the P112 and P113 antibodies interact with a common site. We cannot exclude the possibility that the P112 and P113 antibodies also block the association of ligand with integrin, as no convenient methods are available for rapid measurement of this parameter.

Several proteins have been identified recently that associate with integrins and modify their ligand binding function. These include proteins like IAP

(Lindberg et al., 1993; Schwartz et al., 1993) and the TMSF proteins (Berditchevski et al., 1997; Hemler et al., 1996), both of which can co-purify with integrin and which regulate integrin function. Several aspects of the domain encompassing β3 residues 164–202 suggest it could be a docking point for such accessory proteins. First, the results presented here show that the binding of proteins to this domain can modulate integrin function. Second, the domain is allosteric to the ligand binding site, and is available for binding even when ligand is pre-bound at the integrins RGD binding site. Third, we previously demonstrated that this site is involved in determining the integrins ligand binding specificity (Lin et al., 1997a), and others have shown that an adjacent site in the β1 integrins can control the activation of the integrin (Takada and Puzon, 1993). Finally, this region is highly divergent among the integrin β subunits, providing each integrin with a highly specific docking point for potential accessory proteins.

Because the P112 and P113 antibodies block the function of $\alpha v\beta 3$ by a mechanism that is inherently different than that of ligand mimic peptides, the antibodies may have different pharmacologic activities. Antibody LM 609 (Cheresh and Spiro, 1987) has been humanized and is now being tested in clinical trials as an inhibitor of tumor growth and spread. Interestingly, the findings presented here show that LM 609 binds to the same site as the P112 and P113 antibodies, and also has essentially the same mechanism of action. Several efforts are underway to obtain orally bioavailable small molecule antagonists that bind the ligand binding pocket of $\alpha v\beta 3$. Given the success of similar efforts targeting the $\alpha IIb\beta 3$ integrin (Coller, 1997), it is likely that such compounds will be obtained and will also enter clinical trials. It is difficult to predict how the biochemical distinctions between the two classes of antagonist of $\alpha v\beta 3$ will influence their efficacy in vivo. However, it is important to know that they do function differently, as this understanding may greatly assist in interpreting the information gained from clinical trials with each type of antagonist.

One of the more intriguing findings of the study is that the P112 and P113 antibodies virtually abolish αvβ3-mediated cell adhesion, yet, under carefully controlled and identical experimental circumstances, the antibodies only block a portion of the binding of soluble ligand. We have observed such partial inhibition of soluble ligand binding regardless of the source of αvβ3. It occurs in cells expressing recombinant αvβ3, cells expressing endogenous αvβ3, or even with αvβ3 purified from human placenta (unpublished observations, E.C.K. Lin). We take these observations to indicate that two populations of αvβ3 are present on the cell surface. Although both populations bind to the P112 and P113 antibodies, the antibodies only block the ligand binding function of one population. Our results are consistent with the idea that the antibody-sensitive population mediates about one-half of the soluble ligand binding, but virtually all of cell adhesion. Even though the antibodies are able to bind the remaining population of αvβ3, it is not evident why they fail to inhibit its ligand binding function. We suspect that there is some, as yet, unexplained structural differences between the two populations of the integrin.

In summary, we report that a series of mono-clonal antibodies specific for the αvβ3 integrin block ligand binding by interacting with an allosteric regulatory site that is physically distinct from the RGD ligand binding site. The antibodies function, in part, by inducing ligand dissociation. These antibodies could provide a basis for an alternative class of inhibitors that regulate the function of integrins that are already engaged with ligand.

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