Defining the Topology of Integrin α5β1-Fibronectin Interactions Using Inhibitory Anti-α5 and Anti-β1 Monoclonal Antibodies

EVIDENCE THAT THE SYNERGY SEQUENCE OF FIBRONECTIN IS RECOGNIZED BY THE AMINO-TERMINAL REPEATS OF THE α5 SUBUNIT

The high affinity interaction of integrin α5β1 with the central cell binding domain (CCBD) of fibronectin requires both the Arg-Gly-Asp (RGD) sequence (in the 10th type III repeat) and a second site (in the adjacent 9th type III repeat) which synergizes with RGD. We have attempted to map the fibronectin binding interface on α5β1 using monoclonal antibodies (mAbs) that inhibit ligand recognition. The binding of two anti-α5 mAbs (P1D6 and JBS5) to α5β1 was strongly inhibited by a tryptic CCBD fragment of fibronectin (containing both synergy sequence and RGD) but not by GRGDS peptide. Using recombinant wild type and mutated fragments of the CCBD, we show that the synergy region of the 9th type III repeat is involved in blocking the binding of P1D6 and JBS5 to α5β1. In contrast, binding of the anti-β1 mAb P4C10 to α5β1 was inhibited to a similar extent by GRGDS peptide, the tryptic CCBD fragment, or recombinant proteins lacking the synergy region, indicating that the RGD sequence is involved in blocking P4C10 binding. P1D6 inhibited the interaction of a wild type CCBD fragment with α5β1 but had no effect on the binding of a mutant fragment that lacked the synergy region. The epitopes of P1D6 and JBS5 mapped to the NH2-terminal repeats of the α5 subunit. Our results indicate that the synergy region is recognized primarily by the α5 subunit (in particular by its NH2-terminal repeats) but that the β1 subunit plays the major role in binding of the RGD sequence. These findings provide new insights into the mechanisms, specificity, and topology of integrin-ligand interactions.

Integrins are a large family of αβ heterodimeric receptors, many of which play critical roles in cell migration, differentiation, and survival. Integrins recognize a large variety of extra- and cellular and cell surface proteins, and two widespread recognition motifs have been identified within these ligands: Arg-Gly-Asp (RGD) and Leu-Asp-Val (LDV) (1, 2).

The extracellular matrix glycoprotein fibronectin contains a number of domains that mediate its interaction with integrins; the most studied of these domains lies near the center of the fibronectin subunit and is known as the central cell binding domain (CCBD). This region contains a number of homologous repeating polypeptide modules termed fibronectin type III repeats, each about 90 amino acid residues in length. An RGD sequence, located in the 10th type III repeat, is a key recognition site for several different integrins, including the prototypic fibronectin receptor α5β1. Synthetic peptides containing the RGD sequence completely block cell adhesion to the CCBD (3, 4). However, other data show that additional sequences outside the RGD region are sometimes required for full adhesive activity. In particular, a region of the 9th type III repeat which contains the sequence Pro-His-Ser-Arg-Asn (PHSRN) appears to synergize with RGD for recognition by α5β1 (5). Replacement of this sequence with a corresponding sequence from the 8th type III repeat results in a major loss of adhesive activity (5). Similarly, deletion of this region from the CCBD results in -20-fold loss in activity (6). An overlapping, but distinct, sequence plays a similar role in α1β1β3-fibronectin interactions (7). The mode of action of such synergy sequences is not currently understood.

Recently, we described the effect of GRGDS peptide and a tryptic fragment of fibronectin containing the CCBD on the binding of the inhibitory monoclonal antibody (mAb) 13 to α5β1 (8). Our results showed that both GRGDS peptide and the CCBD fragment act as allosteric inhibitors of mAb 13 binding to α5β1. Our data indicated that mAb 13 recognizes an epitope that is strongly attenuated by ligand occupancy rather than a site directly involved in ligand recognition. We hypothesized that inhibitory mAbs recognize sites attenuated during the conformational adaptation of the integrin to ligand and have termed these ligand-attenuated binding sites (8, 9). Although mAb 13 does not appear to recognize a sequence that interacts directly with ligand, its epitope lies very close to regions of the β1 subunit which have been shown to participate in ligand binding (10, 11).

A region of the α5 subunit which plays an important role in fibronectin recognition has been identified by site-directed mutagenesis (12). The NH2-terminal portion of the α5 subunit...
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comprises seven homologous repeats, each of ~60 amino acid residues. Point mutations in a short hydrophobic sequence in the third NH₂-terminal repeat block fibronectin binding. The epitope recognized by the inhibitory anti-α5 mAb 16 was found to lie near to this hydrophobic sequence (12). By analogy with our results for mAb 13, these findings suggested to us that fibronectin recognition by α5β1 may affect the binding of inhibitory anti-α5 mAbs and furthermore, that it might be possible to use inhibitory mAbs as probes to determine whether the α5 and β1 subunits recognize distinct regions of the CCBD.

In this study we tested whether recognition of the CCBD by α5β1 perturbs the binding of inhibitory anti-α5 mAbs and if this perturbation is different from that observed for inhibitory anti-β1 mAbs. We show that the binding of the α5-α5 mAbs P1D6 and JB55 to α5β1 is inhibited by fibronectin fragments containing both the RGD sequence and the synergy site but is not inhibited by GRGDS peptide or by fibronectin fragments lacking the synergy sequence. In contrast, the binding of the anti-β1 mAb 4P4C10 is inhibited by GRGDS peptide and fibronectin fragments containing the RGD sequence alone. We also present evidence that P1D6 blocks recognition of only the synergy site by α5β1. Taken together, our data suggest that the α5 subunit is largely responsible for recognition of the synergy site, whereas the RGD sequence appears to be recognized mainly by β1 subunit. Based on an x-ray crystal structure for the CCBD (13), these results allow us to predict a spatial topology for α5β1-fibronectin interactions.

EXPERIMENTAL PROCEDURES

Materials—Rat mAbs 11 and 13 recognizing the human α5 and β1 integrin subunits, respectively, were produced and purified as described previously (14). Rat anti-mouse β1 mAb 9EG7 was a gift from D. Verdery, Coopers (Freiburg, Germany); this mAb also cross-reacts with human β1. Mouse anti-human α5 mAbs P4C10, 4B4, and K20 were purchased from Life Technologies, Inc. (Paisley, Scotland, U. K.), Coulter Corp. (Miami, FL), and The Binding Site (Birmingham, U. K.), respectively. Mouse anti-human α5 mAbs VC5, P1D6, and JB55 were purchased from Pharmingen (San Diego, CA), Life Technologies, Inc., and Serotec (Oxford, U. K.), respectively. All antibodies were used as purified antibody (1:500 dilution of ascites. P1D6, 4B4, and P4C10 (as assayed). A recombinant fragment of fibronectin, H/120, which does not contain the CCBD, was produced and purified as described (15). The synthetic peptides GRGDS and GRGDC were synthesized using Fastmoc chemistry on an Applied Biosystems 431A peptide synthesizer and purified as outlined previously (16).

Proteins—Integrin α5β1 was purified from human placenta as described previously (6). An 80-kDa fragment of fibronectin containing the CCBD was purified from a trypsin digest of human plasma fibronectin as described (17). Recombinant fragments of the CCBD were produced as described (5, 18, 19). All recombinant proteins, except proteins comprising the 9th or 10th type III repeats, were purified using DEAE-Sepahex (Pharmacia, Milton Keynes, U. K.) and hydroxyapatylate (Bio-Rad, Hemel Hempsted, U. K.) chromatography, essentially as described previously (19). Recombinant proteins comprising the individual 9th and 10th repeats were purified as described (18). All purified proteins solutions were dialyzed against several changes of 150 mM NaCl, 25 mM Tris-Cl, pH 7.4 (buffer A).

Biotinylation of Proteins—Tryptic CCBD fragment of fibronectin or recombinant CCBD fragments (~500 μg/ml in buffer A) were mixed with an equal mass of sulfo-N-hydroxysuccinimidio biotin (Pierce, Chester, U. K.) and rotary mixed for 30–40 min at room temperature. The mixture was then dialyzed against several changes of buffer A to remove excess biotin. Dialyzed protein solutions were centrifuged at 13,000 × g for 15 min and stored at 4 °C.

Effect of Ligand on the Binding of mAbs P1D6, JB55, and P4C10 to α5β1—Purified α5β1 (at a concentration of ~500 μg/ml) was diluted 1:500 with phosphate-buffered saline containing 1 mM CaCl₂ and 0.5 mM MgCl₂. 100-μl aliquots were added to 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) NaN₃, 25 mM Tris-Cl, pH 7.4. Wells were then washed three times with 100 μl of 150 mM NaCl, 1 mM MnCl₂, 25 mM Tris-Cl, pH 7.4, containing 1 mg/ml BSA (buffer B). 100-μl aliquots of mAbs (0.3 μg/ml or 1.010,000 dilution of ascites in buffer B) were added to the wells in the presence or absence of varying concentrations of tryptic CCBD fragment or GRGDS peptide. The H/120 fragment of fibronectin and the peptide GRGDS were used as controls. The plate was then incubated at 30 °C for 2 h. Unbound antibody was aspirated, and the wells were washed three times with buffer B. Bound antibody was then quantitated by coating wells with 1:1,000 dilution of horseradish peroxidase conjugate (Dako A/C, Denmark) in buffer B for 20 min. Wells were then washed four times with buffer B, and color was developed using ABTS substrate (Sigma, Poole, U. K.). Measurements obtained were the mean ± S.D. of four replicate wells.

To test if ligand behaved as a direct competitive inhibitor or an allosteric inhibitor, the inhibition of mAb binding at different concentrations of tryptic CCBD fragment was measured as described above for 10-fold different concentrations of mAb (0.3 and 3 μg/ml or 1:10,000 and 1:1,000 dilution of ascites). The concentration of ligand required to inhibit antibody binding half-maximally and the maximal extent of inhibition were estimated by nonlinear regression analysis as described previously (8).

Solid Phase Assays—Solid phase ligand-receptor binding was performed essentially as described previously (20). ELISA plate wells were coated with α5β1, blocked as described above, and washed three times with 200 μl of buffer B. In experiments examining the ability of recombinant proteins and GRGDS peptide to inhibit competitively the binding of the tryptic CCBD fragment to α5β1, 100-μl aliquots of biotinylated tryptic CCBD fragment (0.1 μg/ml) in buffer B were added to the wells, with or without various concentrations of competing peptide or proteins. The plate was then incubated at 30 °C for 3 h. Biotinylated ligand was aspirated, and the wells were washed three times with buffer B. Bound ligand was quantitated by the addition of 1:200 Extravidin-peroxidase conjugate (Sigma) in buffer B for 10 min. Wells were then washed four times with buffer B, and color was developed using ABTS. Measurements obtained were the mean ± S.D. of four replicate wells. To estimate the concentrations of peptide or proteins required for half-maximal inhibition of CCBD fragment binding, nonlinear regression analysis of the data was performed as described previously (15).

In experiments examining the direct binding of biotinylated recombinant CCBD fragments to α5β1, ELISA plate wells were coated with α5β1, blocked, and washed as described above. 100-μl aliquots of biotinylated proteins (0.05–30 μg/ml) diluted in buffer B were added to the wells, and the plate was then incubated at 30 °C for 3 h. Bound ligand was then quantitated as described above. To estimate apparent Kᵦ values, nonlinear regression analysis of the data was performed (15). For experiments examining the effect of anti-α5 and anti-β1 mAbs on the binding of biotinylated recombinant CCBD fragments to α5β1, assays were performed in the presence of 10 μg/ml purified mAbs or 1:50 dilution of ascites.

In all of the assays described above, the amount of nonspecific binding was measured by determining the level of antibody or ligand 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 experiments, the ELISA signal appeared to be directly proportional to the amount of bound antibody or ligand because plots of absorbance versus 1/(free antibody or ligand) did not deviate from linearity at high concentrations. Note, however, that binding constants cannot be determined precisely in these assays and that only apparent Kᵦ values are quoted.

Flow Cytometric Analysis—K562 erythroleukemia cells were grown in RPMI 1640 medium containing 10% (v/v) fetal calf serum, as described previously (24). Cells were washed with 150 mM NaCl, 25 mM HEPES, pH 7.4, incubated at 37 °C for 15 min in 150 mM NaCl, 25 mM HEPES, 2 mM EDTA, pH 7.4, washed twice in 150 mM NaCl, 1 mM MnCl₂, 25 mM HEPES, pH 7.4, containing 1 mg/ml BSA (buffer C), and resuspended in buffer C to a concentration of 1 × 10⁶/ml. Aliquots of buffer C (50 μl) containing 2 × the final concentration of mAbs (1:1,000 dilution of P1D6, 0.6 μg/ml JBS5, and 1:5,000 dilution of P4C10) and 2 × the final concentration of CCBD fragment (50 μg/ml) or GRGDS peptide (200 μg/ml) were added to 50-μl aliquots of cells. Samples were then incubated at room temperature for 1 h. Cells were washed three times in buffer C, and 50 μl of fluorescein isothiocyanate-conjugated F(ab')₂ secondary antibody (Serotec) diluted 1:200 in buffer C with 1% (w/v) normal human serum was added to each sample and the samples were incubated at room temperature for a further 30 min. (The secondary antibody conjugate does not cross-react with rat mAbs.) Cells were then washed twice in buffer C, once in phosphate-buffered saline, and fixed in phosphate-buffered saline containing 0.2% (w/v) formaldehyde. 20,000 cells were analyzed from each sample using a FACScan flow cytometer (Becton Dickinson, Cowley, Oxford, U. K.), and mean
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fluorescence intensity values were calculated. In repeated measurements, standard errors were <5% of the mean fluorescence intensity values.

To estimate the amount of nonspecific fluorescence, the level of binding of an irrelevant mouse mAb 9E10 (anti-myel typh) to K562 cells was measured under the same conditions.

Cell Attachment Assay—K562 cells were washed with 150 mM NaCl, 25 mM HEPES, pH 7.4, incubated at 37 °C for 30 min in the same buffer, and resuspended in buffer C to a concentration of 1 × 10^6/ml. Assays were performed in 96-well microtiter plates (Costar, High Wycombe, U. K.). Wells were coated for 60 min at room temperature with 100-μl aliquots of recombinant proteins (0.2–100 μg/ml) diluted with Dulbecco’s phosphate-buffered saline, and then sites on the plastic for nonspecific cell adhesion were blocked for 40–60 min at 37 °C with 100 μl of 10 mg/ml heat-denatured BSA. The BSA was removed by aspiration, and the wells were then washed once with buffer C. Aliquots of the cells (100 μl) in buffer C were then added to the wells and incubated for 20 min at 37 °C in a humidified atmosphere of 5% (v/v) CO2. For experiments examining the effect of anti-α5 and anti-β1 mAbs on cell attachment, cells were preincubated with mAbs (10 μg/ml or 1:500 dilution of ascites) for 30 min at room temperature before being added to the wells. To estimate the reference value for 100% attachment, cells in quadruplicate wells coated with poly-l-lysine (500 μg/ml) were fixed immediately by direct addition of 100 μl of 5% (w/v) glutaraldehyde for 30 min at room temperature. Loosely adherent or unbound cells from experimental wells were removed by aspiration, the wells washed once with buffer C, and the remaining bound cells were fixed as described above for reference wells. The fixative was aspirated, the wells were washed three times with 200 μl of H2O, and attached cells were stained with Crystal Violet (Sigma) as described previously (20). The absorbance of each well at 570 nm was then measured using a multiscan ELISA reader (Dynatech, Billingshurst, U. K.). Each sample was assayed in quadruplicate, and attachment to BSA (<3% of the total) was subtracted from all measurements.

Epitope Mapping—Wild type human α5 cDNA was subcloned into the pB3-J vector. Putative surface loops in the NH2-terminal repeats of α5 were swapped with the corresponding loops in the human α4 sequence. The positions of the swapping mutations are residues 41–48 in repeat 1 (R1), 116–134 in repeat 2 (R2), 179–191 in repeat 3 (R3), 252–259 in repeat 4 (R4), 308–321 in repeat 5 (R5), and 375–380 in repeat 6 (R6). The expression vector of each mutant was constructed using the overlap extension polymerase chain reaction (21). The presence of the mutation was verified by DNA sequencing. Twenty μg of wild type or mutant α5 cDNA construct was transfected into Chinese hamster ovary-B2 cells (22) (8 × 10^6 cells), together with 1 μg of pFneo of the DNA containing a neomycin resistance gene, by electroporation. Transfected cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37 °C in 6% CO2 for 2 days. Then the cells were transferred to the same medium containing 700 μg/ml G418 (Life Technologies, Inc.). After 10–14 days, G418-resistant colonies were harvested. The expression level of wild type and mutant α5 was confirmed by flow cytometric analysis in FACScan with VCS, a mAb that recognizes a nonfunctional epitope on the α5 subunit. Chinese hamster ovary cells expressing mutant α5 were then cloned to obtain cells expressing high levels of mutant α5 by cell sorting in FACStar (Becton Dickinson). The cloned cells expressed mutant α5 at levels comparable to that of cells expressing wild type α5. Characteristics of these cells will be described in more detail elsewhere.2 To localize the epitopes for P1D6, JBS5, and mAb 11, the reactivity of the wild type and mutant α5-expressing cells with these mAbs was examined by flow cytometry in FACScan.

RESULTS

A Tryptic CCBD Fragment of Fibronectin, but Not GRGDS Peptide, Inhibits the Binding of Anti-α5 mAbs P1D6 and JBS5 to α5β1—We showed previously that the binding of the inhibitory anti-β1 mAb 13 to α5β1 was perturbed by both GRGDS synthetic peptide and a tryptic CCBD fragment (8). We therefore investigated whether the binding of two inhibitory anti-α5 mAbs to α5β1 was affected by these ligands. The results (Fig. 1, A and B) showed that the binding of P1D6 and JBS5 was not inhibited by GRGDS peptide; in fact, we reproducibly observed a slight stimulation (1.2–1.4-fold) of P1D6 binding to α5β1 by GRGDS peptide. However, binding of both P1D6 and JBS5 was strongly attenuated by the CCBD fragment. In correspondence with our previous results for mAb 13 (8), binding of the inhibitory anti-β1 mAb P4C10 to α5β1 was partially blocked by both GRGDS peptide and the CCBD fragment (Fig. 1C).

In control experiments (not shown) the binding of the noninhibitory anti-α5 mAb 11 and the noninhibitory anti-β1 mAb K20 to α5β1 was not affected by either GRGDS peptide or the CCBD fragment. We used 1 mM Mn2+ in all the above experiments because ligand recognition by α5β1 is optimal under these conditions (20). The binding of P1D6, JBS5, and P4C10 to α5β1 was not influenced by GRGDS peptide or CCBD fragment when Mn2+ in the assay buffer was replaced with 1 mM EDTA (data not shown), i.e. conditions under which ligand recognition does not occur (20). These findings strongly suggest that the altered binding of P1D6, JBS5, and P4C10 seen in the above experiments (Fig. 1, A–C) is a consequence of ligand recognition by α5β1.

GRGDS peptide was also ineffective at blocking the binding of P1D6 and JBS5 to α5β1 on K562 cells (Fig. 1D), whereas the CCBD fragment was strongly inhibitory. In contrast, P4C10 binding was attenuated by both GRGDS peptide and the CCBD fragment. (These experiments were performed in the presence of the activating anti-β1 mAb 9EG7 because GRGDS peptide recognition by α5β1 on K562 cells does not occur unless the integrin is stimulated using activating mAbs (8).)

The CCBD Fragment Acts Predominantly as an Allosteric Inhibitor of mAb Binding to α5β1—To examine if the tryptic CCBD fragment acts as a direct competitive inhibitor or as an allosteric inhibitor of P1D6, JBS5, and P4C10 binding to α5β1, we examined the inhibitory effect of the CCBD fragment at 10-fold different mAb concentrations. The results showed that the concentration of CCBD fragment required for half-maximal inhibition of P1D6 (Fig. 2A) and P4C10 (Fig. 2C) binding was not significantly different over this range of antibody concentrations. However, the concentration of CCBD fragment required for half-maximal inhibition of JBS5 (Fig. 2B) binding to α5β1 was increased approximately 4-fold. For all the mAbs, the maximal extent of inhibition decreased with increasing antibody concentration. If ligand behaved as a direct competitive inhibitor of mAb 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. For P1D6 and P4C10, the results are instead consistent with an allosteric inhibition, in which ligand does not compete directly with mAb for binding to α5β1 but instead binds to a separate site and decreases the affinity of mAb binding by an allosteric effect on the conformation of the integrin. However, for JBS5 the inhibition is not purely allosteric in nature; the increased concentration of ligand for half-maximal inhibition of antibody binding with increased mAb concentration indicates that some direct competitive inhibition may also be involved. For each mAb, plots of 1/(antibody binding) versus ligand concentration were hyperbolic (not shown), which is diagnostic of a mainly allosteric type of inhibition (23). Hence, as observed for mAb 13 (8), P1D6, JBS5, and P4C10 appear to recognize sites on α5β1 which are attenuated as a consequence of ligand occupancy.

The Region of the CCBD Responsible for Perturbing the Binding of the Anti-α5 mAbs to α5β1 Lies Outside the 10th Type III Repeat—The above results show that the binding of P1D6 and JBS5 to α5β1 is decreased markedly when the CCBD of fibronectin is recognized by α5β1. However, recognition of the RGD sequence alone does not decrease the affinity of P1D6 and JBS5 binding. To narrow down which region of the CCBD is involved in blocking P1D6 and JBS5 binding, recombinant wild

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type and mutant fragments from the CCBD were produced (Fig. 3). Initially, the activity of these fragments was assessed by testing their ability to inhibit competitively the binding of the tryptic CCBD fragment to \( \alpha_5 \beta_1 \) in a solid phase assay (Fig. 3). In close agreement with a previous study using intact cells (5), mutation of the synergy region led to a 50–200-fold loss of activity in this assay. However, when used at sufficiently high concentrations, such fragments did fully inhibit tryptic CCBD fragment binding. In contrast, fragments lacking the RGD sequence had little or no activity. Since the synergy region and the RGD sequence lie in the 9th and 10th type III repeats, respectively, we first tested whether these repeats were involved in blocking P1D6 and JBS5 binding to \( \alpha_5 \beta_1 \). The results (Fig. 4, A and B) showed that a recombinant protein comprising only the 10th type III repeat (III10) did not perturb P1D6 and JBS5 binding; however, a recombinant protein containing the

**Fig. 1. Effect of GRGDS peptide and tryptic CCBD fragment of fibronectin on the binding of mAbs P1D6, JBS5, and P4C10 to \( \alpha_5 \beta_1 \).** Results shown are for purified \( \alpha_5 \beta_1 \) (panels A–C) or for \( \alpha_5 \beta_1 \) on K562 cells (panel D). Binding of P1D6 (panel A), JBS5 (panel B), or P4C10 (panel C) to purified \( \alpha_5 \beta_1 \) was measured for a range of concentrations of CCBD fragment (●) or GRGDS peptide (○). In a separate experiment the binding of these antibodies was unaffected by the control peptide GRDGS or the control fibronectin fragment H/120 (15) (data not shown). Binding of the antibodies to K562 cells (panel D) was measured by flow cytometry in the presence of the activating rat anti-human \( \beta_1 \) mAb 9EG7, without ligand (open bars) or with 100 \( \mu \)g/ml GRGDS peptide (hatched bars) or with 25 \( \mu \)g/ml tryptic CCBD fragment (filled bars). P1D6, JBS5, and P4C10 binding was detected using a mouse-specific fluorescein isothiocyanate conjugate. The dashed line indicates the level of fluorescence in the presence of an irrelevant mouse mAb. Results are of one experiment, representative of three separate experiments.
9th and 10th type III repeats (III_{9-10}) strongly inhibited the binding of these mAbs. In contrast, binding of P4C10 to a_5b_1 was inhibited by both III_{10} and III_{9-10} (Fig. 4C). A recombinant protein comprising only the 9th type III repeat (III_9) had no effect on the binding of any of the mAbs to a_5b_1. A mixture of III_9 and III_{10} also failed to perturb P1D6 and JBS5 binding (data not shown). These results indicate that the region of the CCBD responsible for blocking P1D6 and JBS5 binding lies in the 9th type III repeat; however, the activity of this region is only observed when the 10th type III repeat is also present in the same protein. In contrast, consistent with the effect of GRGDS peptide on P4C10 binding to a_5b_1, the region of the CCBD responsible for blocking the binding of this mAb appears to lie mainly within the RGD-containing 10th type III repeat.

**Fig. 2.** Effect of mAb concentration on the inhibition of P1D6, JBS5, and P4C10 binding to a_5b_1 by the tryptic CCBD fragment. Binding of P1D6 (panel A), JBS5 (panel B), or P4C10 (panel C) to purified a_5b_1 was measured for a range of concentrations of CCBD fragment at 10-fold different mAb concentrations: ●, 1:1,000 dilution and ■, 1:10,000 dilution in panels A and C; ●, 3 \mu g/ml and ■, 0.3 \mu g/ml in panel B. By nonlinear regression analysis, the concentrations of CCBD fragment required for half-maximal inhibition of P1D6 binding are 2.1 \pm 0.6 and 1.2 \pm 0.3 \mu g/ml for 1:1,000 and 1:10,000 dilutions, respectively; the maximal extents of inhibition are estimated to be 57 and 76%, respectively. The concentrations of CCBD fragment for half-maximal inhibition of JBS5 binding are estimated to be 5.5 \pm 0.8 and 1.5 \pm 0.4 \mu g/ml for 3 and 0.3 \mu g/ml, respectively; the maximal extents of inhibition are estimated to be 62 and 77%, respectively. The concentrations of CCBD fragment required for half-maximal inhibition of P4C10 binding are estimated to be 1.7 \pm 0.5 and 1.8 \pm 0.5 \mu g/ml for 1:1,000 and 1:10,000 dilutions, respectively; the maximal extents of inhibition are estimated to be 39 and 48%, respectively.
The PHSRN Site in the 9th Type III Repeat Is Involved in Perturbing the Binding of the Anti-α5 mAbs to α5β1—To define further the region of the CCBD involved in blocking P1D6 and JBS5 binding, we examined the effect of mutations in the 9th type III repeat on the capacity of the III9–10 protein to block JBS5 binding, we examined the effect of mutations in the 9th type III repeat on the capacity of the III9–10 protein to block JBS5 binding, we examined the effect of mutations in the 9th type III repeat on the capacity of the III9–10 protein to block JBS5 binding. The results shown in Fig. 3, a mutant protein in which 16 amino acid residues in the synergy region of the 9th type III repeat were replaced with corresponding sequence from the 8th type III repeat (III9–10SPSDN) also bound to α5β1, but with >100-fold lower apparent affinity than the wild type protein. A second mutant protein in which the RGD sequence in the 10th type III repeat was mutated to KGE (III9–10KGE) bound very poorly to α5β1 (Fig. 6A). None of the proteins bound to α5β1 in the presence of EDTA (data not shown). Parallel results were obtained in K562 cell attachment assays (Fig. 6B).

The effects of inhibitory anti-α5 and anti-β1 mAbs on III9–10 and III9–10SPSDN binding to α5β1 were then tested (Fig. 7A), using concentrations of these proteins which gave similar levels of binding in the absence of mAbs. The results showed that, as predicted, P1D6 inhibited binding of III9–10 but not II9–10SPSDN to α5β1. However, JBS5 inhibited both II9–10 and II9–10SPSDN binding. All of the inhibitory anti-β1 mAbs tested (P4C10, 13, and 4B4) blocked both II9–10 and II9–10SPSDN binding. There was no inhibition of either II9–10 or II9–10SPSDN binding by control anti-α5 or anti-β1 mAbs. Again, parallel results were obtained in cell attachment assays (Fig. 7B). Taken together, these data show that P1D6 inhibits recognition of the synergy region but not the RGD sequence by α5β1. However, it appears that JBS5 does affect binding of the RGD sequence to α5β1.

Epitopes of P1D6 and JBS5 Lie within the NH2-terminal Repeats of the α5 Subunit—For the integrin α5β1 it has been shown that the epitopes of inhibitory anti-α4 mAbs map to sites mainly within the third NH2-terminal repeat (24, 25) and are probably close to, or within, predicted loop structures (12, 26, 27). To localize the epitopes of P1D6 and JBS5, putative loop regions within the NH2-terminal repeats of the α5 subunit were swapped with the corresponding loops from the α4 subunit. Mutant α5 subunits (which formed heterodimers with endogenous hamster β1) were expressed on the surface of Chinese hamster ovary-B2 cells and tested for their ability to bind P1D6 and JBS5 using flow cytometric analysis. The results (Table I) showed that swapping of loops in the NH2-terminal repeats, particularly in repeats 2 and 3, perturbs P1D6 and JBS5 binding. Hence it appears that the epitopes of these mAbs comprise several noncontiguous residues in these repeats. Binding of the noninhibitory anti-α5 mAb 11 to α5β1 was not affected by any of the loop swaps, suggesting that its epitope lies outside the NH2-terminal repeats. The epitopes of the inhibitory anti-β1 mAbs used in this study (P4C10, 13, and 4B4) have been shown previously to map to a region of the β1 subunit which contains amino acid residues 207–218 (10).

**DISCUSSION**

The NH2-terminal portions of both integrin α and β subunits appear to cooperate in ligand recognition; however, it is not yet understood how this cooperativity arises, how specificity is generated, and whether the α and β subunits recognize the same or distinct sequences in the ligand. The prototypical interaction of α5β1 with the CCBD of fibronectin studied here allows us to begin to address these questions.

The main findings of this report are as follows. (i) Binding of the anti-α5 mAbs P1D6 and JBS5 to α5β1 is strongly inhibited by CCBD fragments containing both synergy and RGD sequences, but not by GRGDS peptide or CCBD fragments containing only the RGD site. In contrast, binding of the anti-β1 mAb P4C10 to α5β1 is inhibited to a similar extent by GRGDS
peptide, CCBD fragments containing both synergy and RGD sequences, or CCBD fragments containing only the RGD site. (ii) P1D6 inhibits the interaction of a wild type recombinant CCBD fragment with α5β1 but has no effect on the binding of a mutant CCBD fragment that lacks the synergy sequence. In contrast, the binding of both wild type and mutant fragments to α5β1 is inhibited by P4C10 (and by other anti-β1 mAbs). (iii) The epitopes of P1D6 and JBS5 lie in the NH2-terminal repeats of the α5 subunit. Taken together, our results indicate that the synergy region is recognized primarily by the α5 subunit (in particular by its NH2-terminal repeats) but that the β1 subunit plays the major role in binding of the RGD sequence.

Although our data suggested that ligand acted mainly as an allosteric inhibitor of antibody binding, we believe that this should not be taken as evidence that these antibodies recognize epitopes that are spatially distant from the ligand binding sites. Inhibitory mAbs probably recognize sites proximal to the ligand binding domains because they lie in the same regions of the subunits implicated by other techniques (such as cross-linking and site-directed mutagenesis) as containing ligand binding sequences (9). Our epitope mapping results indicate that the epitopes of JBS5 and P1D6 include a putative β-turn sequence in the third NH2-terminal repeat of α5, a region that has previously been shown to pay a role in fibronectin recogni-
tion (12). These findings are consistent with the concept that inhibitory mAb epitopes are at sites that are attenuated as a result of the conformational changes that take place in the integrin upon ligand recognition and that these changes take place close to the ligand binding sites. Furthermore, our results suggested that the inhibition of JBS5 binding to $\alpha_5\beta_1$ by ligand was not purely allosteric but also involved a direct competition; hence the epitope recognized by this mAb appears to be partly overlapping with the ligand binding pocket.

P1D6 only inhibited binding of the wild type CCBD fragment that contained both synergy sequence and RGD to $\alpha_5\beta_1$ but had no effect on the binding of a mutant fragment lacking the synergy site but retaining the RGD sequence. These results imply that P1D6 only blocks recognition of the synergy sequence by $\alpha_5\beta_1$. A similar conclusion was reached in a study examining the effect of P1D6 on epithelial cell adhesion to mutant fibronectin fragments (28). In contrast, however, we found that the other anti-$\alpha_5$ mAb used in this study (JBS5) inhibited binding of both wild type and mutant CCBD fragments to $\alpha_5\beta_1$. Since not all $\beta_1$ integrins recognize RGD sequences, it is likely that the $\alpha_5$ subunit plays some role in RGD binding, and thus it is possible that the JBS5 epitope may be spatially close to a region of the $\alpha_5$ subunit involved in recognition of RGD. In agreement with this suggestion, mutations in the third NH$_2$-terminal repeat of the $\alpha$IIb subunit blocked binding of mAbs containing an RGD-type motif to $\alpha$IIb$\beta_3$ (29).
Alternatively, as seen with the anti-β1 mAb 13 (8), JBS5 may induce a conformational change in α5β1 which precludes binding of the RGD sequence. All of the inhibitory anti-β1 mAbs tested blocked the binding of both wild type and mutant fragments, as would be predicted if they allosterically affect an RGD-binding region of the β1 subunit. Our proposal that the β1 subunit contains the main site involved in RGD recognition is consistent with studies examining the cross-linking of RGD-containing peptides to αIIbβ3 and αVβ3, which showed that the β3 subunit contained the major site of cross-linking (30, 31). A possible RGD binding motif in integrin β subunits has recently been identified from a phage display library (32).

Our observation that the 9th type III repeat appears unable to bind to α5β1 in the absence of the 10th type III repeat is consistent with a previous report showing that the 9th repeat alone cannot support α5β1-mediated cell attachment (18). It has also been shown previously that a peptide containing the

**FIG. 6.** Panel A, binding of III<sub>e-10</sub> proteins to α5β1 in a solid phase assay. Binding of biotinylated wild type III<sub>e-10</sub> (●), III<sub>e-10</sub>(SPSDN) (■), or III<sub>e-10</sub>KGE (▲) was measured for a range of concentrations of each recombinant protein. By nonlinear regression analysis, the apparent affinity of wild type III<sub>e-10</sub> binding to α5β1 is 1.1 ± 0.2 nM; the apparent affinity of III<sub>e-10</sub>(SPSDN) binding is 180 ± 20 nM. Panel B, attachment to K562 cells in wild type III<sub>e-10</sub> proteins. Cell attachment to wild type III<sub>e-10</sub> (●), III<sub>e-10</sub>(SPSDN) (■), or III<sub>e-10</sub>KGE (▲) was measured for a range of coating concentrations of each recombinant protein. The maximal levels of binding to wild type III<sub>e-10</sub> and III<sub>e-10</sub>(SPSDN) correspond to approximately 98 and 50% cell attachment, respectively.

**FIG. 7.** Panel A, effect of anti-α5 and -β1 mAbs on the binding of wild type III<sub>e-10</sub> (0.05 µg/ml) (open bars) and III<sub>e-10</sub>(SPSDN) (10 µg/ml) (filled bars) to purified α5β1. Results are expressed as a percentage of recombinant protein binding to α5β1 in the absence of mAbs. Panel B, effect of anti-α5 and -β1 mAbs on the attachment of K562 cells to wild type III<sub>e-10</sub> (0.2 µg/ml) (open bars) or III<sub>e-10</sub>(SPSDN) (50 µg/ml) (filled bars). Results are expressed as a percentage of cell attachment to the recombinant proteins in the absence of mAbs. The noninhibitory anti-β1 mAb K20 did not affect binding of wild type III<sub>e-10</sub> or III<sub>e-10</sub>(SPSDN) to purified α5β1 (108 ± 12% and 96 ± 1% of control values, respectively), or attachment of K562 cells to wild type III<sub>e-10</sub> or III<sub>e-10</sub>(SPSDN) (94 ± 4% and 106 ± 4% of control values, respectively).
P95SN sequence is unable to inhibit α5β1-mediated cell adhesion to fibronectin; however, when the same peptide was positioned via a covalent bond in the corresponding site of the normally inactive 8th type III repeat in a recombinant fragment containing the 8th and 10th repeats, adhesive activity was partially restored (5). Hence it was proposed that the PHSRN sequence may need to be located a specific distance from the RGD sequence to be recognized by α5β1. Our data suggest an explanation for this observation, i.e., the recognition site for the PHSRN sequence on the α5 subunit is positioned a specific distance from the RGD recognition site on the β1 subunit. The synergistic activity of the PHSRN site can be readily explained if it provides a second (weaker) site for integrin recognition. α5β1 recognizes fibronectin with high affinity but binds only poorly, or not at all, to other extracellular matrix proteins containing an RGD sequence. The interaction of α5β1 with the synergy region provides a mechanism for achieving higher affinity and higher fidelity binding that could be conferred by the RGD site alone. By analogy, the binding of an RGD/LDV site in an integrin ligand to the β subunit and the binding of a second (synergistic) sequence to the α subunit may constitute a widespread paradigm for generating specificity of integrin-ligand interactions.

The X-ray crystal structure of a recombinant protein spanning type III repeats 7–10 of the CCBD revealed that the synergy sequence and the RGD site are located on the same face of the fibronectin molecule, separated by 3–4 nm (13). Since the large globular head of an integrin (about 12 nm across) can easily span this distance, it is possible that an integrin could interact simultaneously with both sites. Based on the structure of the CCBD (13), the recognition site for the PHSRN sequence on the α5 subunit is probably positioned 3–4 nm away from the RGD binding site on the β1 subunit. The NH2-terminal repeats of integrin α subunits have recently been proposed to form a β-propeller structure in which the sequences implicated in ligand recognition are within loops on the upper surface of the propeller (27). The NH2-terminal portion of integrin β subunits has been proposed to contain a region with an α-domain-like fold (11, 33–35), with the ligand binding site on the top face of this domain. Since the synergy site and the RGD sequence lie on the same side of the fibronectin molecule, our results imply that the top surface of the α subunit β-propeller and the top face of the β subunit A-domain must be approximately coplanar in the ligand-occupied state of α5β1. This arrangement would also place sites involved in ligand binding and the epitopes of inhibitory mAbs near the α subunit/β subunit interface, as proposed in models of integrin activation (9, 36, 37).