Fine mapping of inhibitory anti- $\alpha 5$ monoclonal antibody epitopes that differentially affect integrin–ligand binding

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The high-affinity interaction of integrin $\alpha 5\beta 1$ with the central cell-binding domain of fibronectin requires both the Arg-Gly-Asp (RGD) sequence (in the tenth type III repeat) and a second site Pro-His-Ser-Arg-Asn (PHSRN) in the adjacent ninth type III repeat, which synergizes with RGD. Arg-Arg-Glu-Thr-Ala-Trp-Ala (RRETAWA) is a novel peptidic ligand for $\alpha 5\beta 1$, identified by phage display, which blocks $\alpha 5\beta 1$ -mediated cell adhesion to fibronectin. A key question is the location of the binding sites for these ligand sequences within the integrin. In this study we have identified residues that form part of the epitopes of three inhibitory anti- $\alpha 5$ monoclonal antibodies (mAbs): 16, P1D6 and SNAKA52. These mAbs have distinct functional properties. mAb 16 blocks the recognition of RGD and RRETAWA, whereas P1D6 blocks binding to the synergy sequence. The binding of SNAKA52 is inhibited by anti- $\beta 1$

mAbs, indicating that its epitope is close to the interface between the α and β subunits. Residues in human $\alpha 5$ were replaced with the corresponding residues in mouse $\alpha 5$ by site-directed mutagenesis; wild-type or mutant human $\alpha 5$ was expressed on the surface of $\alpha 5$ -deficient Chinese hamster ovary cells. mAb binding was assessed by flow cytometry and by adhesion to the central cell-binding domain of fibronectin or RRETAWA by cell attachment assay. All three epitopes were located to different putative loops in the N-terminal domain of $\alpha 5$. As expected, disruption of these epitopes had no effect on ligand recognition by $\alpha 5\beta 1$. The locations of these epitopes are consistent with the β -propeller model for integrin α -subunit structure and allow us to propose a topological image of the integrin–ligand complex.

Key words: adhesion, mutagenesis, receptor.

INTRODUCTION

Integrin $\alpha 5\beta 1$ is a widely distributed cell-surface receptor for the extracellular matrix glycoprotein fibronectin (FN). The central cell-binding domain (CCBD) of FN, which interacts with $\alpha 5\beta 1$, contains a number of repeated modules termed fibronectin type III repeats. An Arg-Gly-Asp (RGD) sequence in the tenth type III repeat is the major binding site for $\alpha 5\beta 1$ [1,2], although an additional ‘synergy’ sequence in the ninth type III repeat is also required for full binding activity [3–5]. Additionally, a novel ligand sequence for $\alpha 5\beta 1$, Arg-Arg-Glu-Thr-Ala-Trp-Ala (RRETAWA), has been identified by panning of a phage library on purified integrin [6]. Although the RRETAWA sequence seems unrelated to RGD, the binding sites for RRETAWA and RGD on $\alpha 5\beta 1$ are overlapping [7].

A subset of anti- $\alpha 5\beta 1$ monoclonal antibodies (mAbs) are able to inhibit the interaction between $\alpha 5\beta 1$ and FN. Most inhibitory anti- $\alpha 5$ mAbs do not compete directly with FN for binding to $\alpha 5\beta 1$ but instead recognize sites that are linked allosterically to the ligand-binding site. Mapping the epitopes of these mAbs has localized sites attenuated by ligand occupancy rather than sites directly involved in ligand recognition [3,8]. Nevertheless, inhibitory mAbs probably recognize sites proximal to the ligand-binding domains because they lie in the same regions of the subunits identified by other techniques (e.g. cross-linking and site-directed mutagenesis) and they are therefore useful in localizing the ligand-binding interface.

The N-terminal half of integrin α subunits consists of a seven-fold repeated unit of approx. 60 amino acid residues. Repeats 4–7 (or in some integrins repeats 5–7) contain putative bivalent cation-binding sites [9]. About one-third of integrin α subunits contain an inserted (I or A) domain of approx. 200 residues between the second and third repeats. Where present, the I

domain contains the major sites involved in ligand binding [10,11]. Ligand-binding sites in non-I-domain-containing integrins (such as $\alpha 5\beta 1$) have been localized to defined regions in the N-terminal portions of both α and β subunits [10,12].

The N-terminal repeats of α subunits are predicted to have a mainly β -strand secondary structure [13] and to fold co-operatively into a seven-bladed β -propeller [14]. Each blade of a β -propeller structure contains four β -strands connected by loops of various lengths; these strands are tilted such that the connecting loops are either on the upper or lower surfaces of the propeller. The upper surface of the β -propeller consists of long, variable loops that make extensive contacts with each other. These loops are believed to contain the sites involved in contacting ligands [10,12].

The region of the β subunit that participates in ligand recognition has been predicted to have a von Willebrand factor A-domain-like fold [15–17]; the top face of this domain has been suggested to mediate ligand binding through a metal-ion-dependent adhesion site (‘MIDAS’) [15,17,18]. A model for the quaternary arrangement of the α and β subunit ligand-binding sites has recently been presented in which the top face of the putative β subunit A domain and the upper surface of the α subunit β -propeller are co-planar in the ligand-occupied state [3,10]. However, the precise amino acid residues that participate in ligand recognition are currently unknown.

A large body of evidence suggests that the N-terminal repeats of the α subunit contain residues involved in ligand binding. Exchanging the N-terminal third of αv with the corresponding residues of αIIb altered the specificity of the chimaeric receptor [19]. In addition, mutations in the third N-terminal repeat of αIIb blocked the binding of fibrinogen [20] and mutations in the second and third N-terminal repeats of $\alpha 3$ affected the binding of invasin and laminin [21]. The epitopes for inhibitory anti- $\alpha 4$

Abbreviations used: CCBD, central cell-binding domain; CHO, Chinese hamster ovary; FN, fibronectin; mAb, monoclonal antibody.

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mAbs have been localized to the second and third N-terminal repeats of $\alpha 4$ [22,23] and swapping predicted loop regions on the upper surface of the $\alpha 5$ subunit β -propeller within the second and third repeats with the corresponding loops from $\alpha 4$ blocked the binding of two inhibitory anti- $\alpha 5$ mAbs [3].

A series of mouse and rat anti-(human $\alpha 5$) mAbs have been described that are informative with regard to ligand binding. PID6 is a mAb that specifically blocks the recognition of the synergy sequence in FN; it is therefore likely to bind close to the synergy recognition sequence in $\alpha 5\beta 1$ [3]. SNAKA52 is a novel anti- $\alpha 5$ mAb that behaves in a manner similar to JBS5 [3,8]. The binding of JBS5 to $\alpha 5\beta 1$ is cation-regulated and is inhibited by anti- $\beta 1$ mAbs 13 and 12G10; it is therefore likely to bind close to the interface between α and β subunits [8]. mAb 16 has similar properties to those of JBS5 but in addition its binding to $\alpha 5\beta 1$ is inhibited by RGD peptides in an allosteric manner and directly by RRETAWA. mAb 16 is therefore likely to bind close to the RGD recognition site and at the RRETAWA-binding site [7].

To localize the epitopes of these antibodies, we have exchanged non-conserved residues in predicted loop regions on the upper surface of the human $\alpha 5$ β -propeller with the corresponding residues from mouse $\alpha 5$. The information has been used to map antibody epitopes on the β -propeller model and to predict the spatial topology of ligand-binding sites.

EXPERIMENTAL

Materials

Rat mAbs 16 and 11 recognizing the human $\alpha 5$ subunit and $\alpha 5$ cDNA in pBluescript were gifts from Dr. K. Yamada (National Institute for Dental and Craniofacial Research, NIH, Bethesda, MD, U.S.A.). Mouse anti-human $\alpha 5$ mAbs VC5, PID6 and JBS5 were purchased from PharMingen (San Diego, CA, U.S.A.), Gibco-BRL (Paisley, Renfrewshire, U.K.) and Serotec (Oxford, U.K.) respectively. Mouse anti-human $\alpha 5$ mAbs SAM-1 and SAM-2 were from Serotec and TCS Biologicals Ltd. (Botolph Claydon, Bucks., U.K.) respectively. Mouse anti-(human $\alpha 5$) mAb SNAKA52 was prepared as follows: BALB/c mice were immunized with $\alpha 5\beta 1$ purified from human placenta [24] and spleen cells fused with X63-Ag8.653 myeloma cells. The hybridomas were screened by ELISA against the immunogen and against $\alpha 4\beta 1$ to determine which subunit the antibody was directed against.

All antibodies were used as purified IgG except PID6, which was used as ascites. Mouse and rat IgG were obtained from Sigma (Poole, Dorset, U.K.). The synthetic peptide GACRR-ETAWACGA was synthesized, cyclized and coupled to IgG as described previously [7]. Oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer or were purchased from MWG Biotech (Southampton, Hants., U.K.) or Perkin-Elmer Ltd. (Warrington, Cheshire, U.K.). Poly-(L-lysine) was obtained from Sigma. Fluorescein-conjugated rabbit anti-rat and anti-mouse immunoglobulins were obtained from Serotec (Abingdon, Oxon., U.K.). Recombinant fragments of the cell-binding domain of FN were produced as described previously [25] and purified by chromatography on DEAE-Sephacel (Pharmacia, Milton Keynes, Bucks., U.K.) and hydroxyapatite (Bio-Rad, Hemel Hempstead, Herts., U.K.), as described previously [3].

Mutagenesis and transfection

XhoI linkers were ligated to the *HindIII* site in the multiple cloning site of pUC119. A 1.8 kb *KpnI/XhoI* fragment of human $\alpha 5$ cDNA in pCDNA3 (Invitrogen, San Diego, CA, U.S.A.) was

then subcloned into pUC119. Site-directed mutagenesis was performed with the Mutagenesis kit (Bio-Rad) [26]. The primers used to generate mutations were: 1, 5'-GCACAGAGAAGGA-TCCACAGAGCGACCCCGTGG-3' for the E116D/L118Q mutation; 2, 5'-CCTGATCAACCCGGTTCAGGGGCA-3' for the L212P mutation; 3, 5'-GTCCTCACTGTACAGCTCAG-AGGG-3' for the S75Y mutation. Primers were designed similarly for the remaining mutations. The *KpnI/XhoI* fragment was subcloned into pCDNA3 containing $\alpha 5$ to reconstruct the full-length cDNA. Mutant clones were confirmed by nucleotide sequencing of the mutated region.

Chinese hamster ovary cells, B2 variant (CHO-B2) [27] (a gift from R. L. Juliano, University of North Carolina, Chapel Hill, NC, U.S.A.), were detached with 0.05% (w/v) trypsin/0.02% EDTA in PBS, washed twice in PBS, resuspended to a concentration of 10^7 cells/ml in PBS, and 8×10^6 cells were placed into 0.4 cm electroporation cuvettes (Bio-Rad). Wild-type or mutant $\alpha 5$ DNA (20 μ g) was added and the cells were left on ice for 10 min. Cells were electroporated at 25 μ F and 800 V, then left on ice for a further 10 min. Growth medium [Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS, 1% (w/v) glutamine and 1% (w/v) non-essential amino acids] was added and the cells were plated out and incubated at 37 °C, in a humidified air/CO₂ (19:1) atmosphere.

Stable cell lines

At 48 h after transfection, the medium was supplemented with 0.7 mg/ml G418 (Gibco). G418-resistant colonies were harvested after 10–14 days. The cell population was incubated first with mAb 11 (a mAb that recognizes a non-functional epitope on the $\alpha 5$ subunit) and then with anti-(rat IgG)-coated magnetic beads (Dako) to select for cells expressing $\alpha 5$. The expression of wild-type and mutant $\alpha 5$ was confirmed by flow cytometric analysis in a FACScan (Becton Dickinson) by using mAb 11. For populations with a low proportion of expressing cells, a second enrichment with magnetic beads was performed. Cells expressing wild-type $\alpha 5$, E116D/L118Q and L212P mutations were cloned by limiting dilution to obtain high-level expressors. Populations expressing the S75Y mutation were further enriched by using magnetic beads before use in attachment assays. The percentage of cells reactive to a panel of anti- $\alpha 5$ mAbs was assessed by flow cytometry, with rat IgG or mouse IgG as controls.

Cell attachment assay

CHO-B2 cells, or cells transfected with mutant or wild-type human $\alpha 5$, were detached with 0.05% (w/v) trypsin/0.02% EDTA in PBS, washed with 150 mM NaCl/25 mM Hepes (pH 7.4), incubated at 37 °C for 30 min in the same buffer and then resuspended in the same buffer with 1 mM MnCl₂ (buffer A) at a concentration of 2×10^6 /ml. Assays were performed in 96-well microtitre plates (Costar, High Wycombe, Bucks., U.K.). Wells were coated for 60 min at room temperature with 100 μ l aliquots of III_{6–10} or *CRRETAWAC*-IgG diluted with Dulbecco's PBS (the asterisks indicate that the peptide is cyclized via a disulphide bond); sites on the plastic for non-specific cell adhesion were then blocked for 40–60 min at 37 °C with 100 μ l of 10 mg/ml heat-denatured BSA. The BSA was removed by aspiration and the wells were washed once with buffer A. Aliquots (100 μ l) of the cells in buffer A were then added to the wells and incubated for 20 min at 37 °C in a humidified air/CO₂ (19:1) atmosphere. For experiments examining the effect of anti- $\alpha 5$ mAbs on cell attachment, cells were either preincubated with antibody (10 μ g/ml) for 30 min at room temperature before being added to the wells (mAb 16) or mAb was added directly to the wells

Figure 2 FACS histograms of wild-type (WT) and mutant $\alpha 5\beta 1$ stably expressed on the surface of CHO-B2 cells

Representative histograms of WT and mutant $\alpha 5\beta 1$ are shown stained with $\alpha 5$ -specific mAbs. Cells were harvested after selection with G418 and sorted by using magnetic beads, stained with an $\alpha 5$ -specific mAb followed with a fluorescein-conjugated rabbit F(ab) $_2$ anti-mouse or rat immunoglobulin, and analysed on a FACScan flow cytometer. The logarithm of the fluorescence intensity is shown on the abscissa and the cell number is shown on the ordinate. A total of 10000 events were collected.

included non-inhibitory mAbs 11 and VC5 as controls; these mAbs probably recognize a region of the $\alpha 5$ subunit that lies outside the β -propeller domain. Most mutations did not affect the binding of the mAbs tested; however, those mutations that did affect the binding of a mAb were highly selective. In each case the binding of only one mAb was affected, with the exception of S75Y, which affected the binding of two mAbs (Figure 2). These results indicate that the gross conformation of the receptor was not affected by the mutations.

The epitope for P1D6 was disrupted by the mutation L212P. Leu²¹² is predicted to lie in the 4–1 loop linking blades 3 and 4 of the propeller.

The binding of SNAKA52 was partly inhibited by the mutation S75Y, which is predicted to lie on the 4–1 loop linking blades 1 and 2. The binding of JBS5 was also affected by the S75Y mutation but to a smaller degree than SNAKA52.

The binding of mAb 16 was disrupted by changing Glu¹¹⁶ to Asp and Leu¹¹⁸ to Gln as a double mutation, but not by the mutation of either of these residues individually. These residues are predicted to lie in the 2–3 loop in blade 2.

Cell attachment

In addition to antibody binding experiments, the integrity of the $\alpha 5$ chimaeras was tested by using cell attachment assays. The binding of each of the mutants to the CCBd of FN was found to be comparable to that to wild-type $\alpha 5\beta 1$; these chimaeras were therefore deemed functional (Figure 3).

The attachment of cells to RRETAWA was also assessed. Cells expressing $\alpha 5$ with the L212P or S75Y mutations (disrupting

the epitopes of P1D6 and SNAKA52 respectively) bound RRETAWA as well as cells expressing wild-type $\alpha 5$ (results not shown). Cells expressing $\alpha 5$ with the E116D/L118Q mutation (disrupting the epitope of mAb 16) also attached to RRETAWA to a similar extent as cells expressing wild-type $\alpha 5$, despite the observation that mAb 16 competes directly for binding to $\alpha 5\beta 1$ with RRETAWA (Figure 4).

In support of the flow cytometric data, for mutants in which the epitope for an inhibitory mAb had been disrupted, this antibody was no longer anti-functional in cell attachment assays (Table 2).

DISCUSSION

We have examined the variable loops on the predicted upper surface of the β -propeller model [14] for the epitopes of inhibitory anti- $\alpha 5$ mAbs that are believed to bind proximally to the ligand-binding sites.

The main findings of this study are as follows.

(1) The binding of P1D6 to $\alpha 5\beta 1$ is abrogated by the mutation L212P.

(2) The binding of SNAKA52 and JBS5 is disrupted by the mutation S75Y.

(3) The binding of mAb 16 to $\alpha 5\beta 1$ is abrogated by the double mutation E116D/L118Q.

All of these mutants bound the CCBd of FN and a panel of other anti- $\alpha 5$ mAbs with an affinity comparable to that of wild-type $\alpha 5$, indicating that the gross conformation of the receptor had not been disrupted.

Figure 3 Effect of mutations on $\alpha 5 \beta 1$ -mediated cell attachment

Attachment of untransfected (▲), $\alpha 5$ wild-type (●) and mutant $\alpha 5$ (■) transfected CHO-B2 cells to the III₆₋₁₀ FN fragment. Wild-type and mutant $\alpha 5$ were expressed at comparable levels, as detected with mAb 11. (A) S75Y mutant (wild-type $\alpha 5$: 66.4% positive, mean fluorescence index 14.3; S75Y mutant $\alpha 5$: 83.3% positive, mean fluorescence index 17.68). (B) E116D/L118Q mutant (wild-type $\alpha 5$: 92.1% positive, mean fluorescence index 13.95; E116D/L118Q mutant $\alpha 5$: 92.5% positive, mean fluorescence index 12.34). (C) L212P mutant (wild-type $\alpha 5$: 92.1% positive, mean fluorescence index 13.95; L212P mutant $\alpha 5$: 67.4% positive, mean fluorescence index 8.91). The low levels of attachment of untransfected cells to the III₆₋₁₀ fragment are probably due to the expression of αv integrins by these cells.

Previous studies located the epitopes of JBS5 and PID6 to the second and third N-terminal repeats by using loop swaps with $\alpha 4$, corresponding to residues 116–134 and 179–191 [3]. Although

Figure 4 Effect of E116D/L118Q double mutation on $\alpha 5 \beta 1$ -mediated cell attachment to *CRRETAWAC*

Attachment of untransfected (▲), $\alpha 5$ wild-type (●) and mutant $\alpha 5$ (■) transfected CHO-B2 cells to the *CRRETAWAC*-IgG conjugate (the asterisks indicate that the peptide is cyclized via a disulphide bond). Wild-type and mutant $\alpha 5$ were expressed at comparable levels, as detected with mAb 11. (wild-type $\alpha 5$: 73.6% positive, mean fluorescence index 10.4; mutant $\alpha 5$: 68.2% positive, mean fluorescence index 12.23).

the residues identified in this study are found in repeats 2 and 3, they do not lie within the loops that were swapped. Therefore either these regions contain additional residues contributing to the epitopes of JBS5 and PID6 that have not been identified in this study, or exchanging such large loop regions indirectly caused the loss of mAb epitopes by inducing changes in tertiary structure.

It is possible that the binding of SNAKA52, mAb 16, JBS5 and PID6 has been abrogated by an allosteric effect rather than by disrupting the epitopes directly, i.e. that the mutation of one or two residues caused a conformational change in the integrin that prevented the mAb from binding at a spatially distant site. Although this explanation cannot be ruled out entirely, it is unlikely. In each case we have disrupted the binding of a single mAb. It is very unlikely that an induced conformational change would be specific enough to affect the binding of only one mAb but not that of a series of mAbs that are known to bind within the same region. Furthermore, each of these mutants retained ligand-binding activity. Additionally, the region of the α subunit identified in this study has already been implicated in mAb and ligand binding by other approaches [3,19–23].

The mAbs investigated in this study are useful for probing the nature of the $\alpha 5 \beta 1$ -FN interaction because they each have distinct functional properties.

(1) PID6 is known to block the recognition by $\alpha 5 \beta 1$ of the PHSRN synergy sequence in FN but does not block the recognition of RGD [3]. Its epitope might therefore be close to the synergy recognition site in the $\alpha 5$ subunit.

(2) The binding of mAb 16 to $\alpha 5 \beta 1$ is inhibited allosterically by RGD peptides; it has therefore been proposed that mAb 16 lies close to the RGD recognition site [7].

(3) The binding of SNAKA52, mAb 16 and JBS5 has been shown to be inhibited by the anti- $\beta 1$ mAbs 13 and 12G10 by competitive ELISA ([8], and K. Clark, unpublished work). SNAKA52, mAb 16 and JBS5 are therefore believed to bind within regions close to the interface of the α and β subunits. The binding of these three mAbs is also regulated by cations; it has been proposed that cation occupancy leads to a conformational

Table 2 Effect of anti- $\alpha 5$ mAbs on the $\alpha 5\beta 1$ -mediated cell attachment of mutant $\alpha 5\beta 1$ to III₆₋₁₀

The attachment of $\alpha 5$ wild-type and mutant $\alpha 5$ transfected CHO-B2 cells to the III₆₋₁₀ fibronectin fragment was inhibited by mAbs as indicated. Wild-type and mutant $\alpha 5$ were expressed at comparable levels, as detected with mAb 11. Levels of attachment were normalized with regard to the attachment level of negative-control cells. Results are means $\pm$ S. D. of three values. Expt. 1: S75Y mutant (wild-type $\alpha 5$: 66.4% positive, mean fluorescence index 14.3; S75Y mutant $\alpha 5$: 83.3% positive, mean fluorescence index 17.68). Attachment in the presence of mouse IgG (MulG): S75Y 46%, wild-type 77%. Expt. 2: E116D/L118Q double mutant (wild-type $\alpha 5$: 73.6% positive, mean fluorescence index 10.4; E116D/L118Q mutant $\alpha 5$: 68.2% positive, mean fluorescence index 12.23). Attachment in the presence of mulG: E116D/L118Q 31%, wild-type 47%. Expt. 3: L212P mutant (wild-type $\alpha 5$: 68.4% positive, mean fluorescence index 13.84; L212P mutant $\alpha 5$: 38.7% positive, mean fluorescence index 13.4). Attachment in the presence of mulG: L212P 56%, wild-type 71%. The results in **bold** indicate where the mutant protein was found to be unable to block the binding of a particular monoclonal antibody because the mutated residue forms part of the antibody epitope.

Expt.	Mutation	P1D6	mAb 16	SNAKA52	JBS5	MulG/no mAb
1	S75Y		45.58 $\pm$ 12.26	77.57 $\pm$ 10.41	74.66 $\pm$ 19.61	100 $\pm$ 18.50
	Wild-type		35.17 $\pm$ 15.36	25.64 $\pm$ 8.12	36.49 $\pm$ 6.88	100 $\pm$ 9.21
2	E116D/L118Q	7.89 $\pm$ 5.16	83.68 $\pm$ 10.73			100 $\pm$ 9.09
	Wild-type	14.02 $\pm$ 2.79	8.23 $\pm$ 0.30			100 $\pm$ 19.60
3	L212P	72.26 $\pm$ 2.09		47.54 $\pm$ 1.71		100 $\pm$ 10.54
	Wild-type	22.67 $\pm$ 1.49		30.11 $\pm$ 3.84		100 $\pm$ 2.38

Figure 5 Diagrammatic representation of the topological arrangement of mAb epitopes, based on the β -propeller model for the seven N-terminal repeats of $\alpha 5$

The epitopes of P1D6 (within the 4–1 loop connecting blades 3 and 4), mAb 16 (within the 2–3 loop in blade 2 and the 4–1 loop connecting blades 2 and 3) and SNAKA52/JBS5 (within the 4–1 loop connecting blades 1 and 2) are indicated.

change in integrin that unmasks sites involved in ligand recognition and that these sites lie near to the interface between the α and β subunits [8]. The loops containing the epitopes of SNAKA52/JBS5 (4–1 loop between blades 1 and 2) and mAb 16 (4–1 loop between blades 2 and 3 and 2–3 loop in blade 2) are therefore predicted to lie close to the interface between the α and β subunits. In contrast, the binding of P1D6 to $\alpha 5\beta 1$ is not affected by anti- $\beta 1$ mAbs or bivalent cations, suggesting that the epitope of P1D6 (4–1 loop between blades 2 and 3) does not lie close to the α/β interface [8].

The novel ligand peptide for $\alpha 5\beta 1$, RRETAWA, has been shown to compete directly with mAb 16 for binding to $\alpha 5\beta 1$. This implies that the RRETAWA-binding site directly overlaps with the mAb 16 epitope. Indeed, the double mutant S156G/W157S (4–1 loop between blades 2 and 3) binds neither mAb 16 nor RRETAWA [7]. We have reported in this study a second mutation E116D/L118Q on an adjacent loop within the same blade of the propeller (2–3 loop of blade 2) that prevents the binding of mAb 16 but does not affect the binding of RRETAWA. The binding site of RRETAWA therefore overlaps only partly with the epitope for mAb 16, whereas mAb 16 binds to $\alpha 5\beta 1$ via at least two loops that are predicted to lie adjacent to each other on the upper surface of the β -propeller.

The results of this study provide support for the β -propeller. SNAKA52, JBS5, mAb 16 and P1D6 inhibit each other's binding

Figure 6 Tentative model for the structure of ligand-occupied form of $\alpha 5\beta 1$

The RGD motif in the type III repeat 10 of FN is recognized by the $\beta 1$ domain; the synergy sequence in type III repeat 9 engages a site in the α subunit β -propeller [10]. The 4–1 loop connecting blade 1 and 2 is found close to the interface of the α and β subunits.

to $\alpha 5 \beta 1$ [8]; their epitopes must therefore be spatially close. The residues involved are 137 residues apart in the amino acid sequence and are in different repeats. It is difficult to reconcile these results with a model in which the FG-GAP repeats represent tandem independent domains; however, they are highly consistent with a β -propeller model in which all FG-GAP repeats contribute to a common surface [14]. Additionally, the epitope of mAb 16 consists of residues from at least two loops: the 4–1 loop between blades 2 and 3, and the 2–3 loop within blade 2 [7]. These two loops are predicted to be adjacent to each other in the β -propeller fold. A summary of the epitope mapping data is presented in Figure 5, based on this model.

The X-ray crystal structure of a recombinant protein spanning type III repeats 7–10 of the CCBD of FN revealed that the synergy sequence and RGD are located on the same face of the FN molecule and that the distance separating them is 3–4 nm [31]. Similarly, a solution structure of the ninth and tenth type III repeats obtained by NMR suggested that these motifs were 3.3 nm apart [32]. The recognition sites for the synergy region and RGD on $\alpha 5 \beta 1$ might be proximal to the PID6 and mAb 16 epitopes respectively. Consistent with this suggestion is the prediction of the β -propeller model that the loops containing the PID6 and mAb 16 epitopes would be approx. 3 nm apart and would both lie on the upper face of the propeller (Figure 6). Further studies are currently in progress to localize more accurately the ligand-recognition sites in the $\alpha 5$ subunit.

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