The high-affinity interaction of integrin α5β1 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 α5β1, identified by phage display, which blocks α5β1-mediated cell adhesion to fibronectin. A key question is the location of the binding sites for these ligands within the integrin. In this study we have identified residues that form part of the epitopes of three inhibitory anti-α5 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-β1 mAbs, indicating that its epitope is close to the interface between the α and β subunits. Residues in human α5 were replaced with the corresponding residues in mouse α5 by site-directed mutagenesis; wild-type or mutant human α5 was expressed on the surface of α5-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 α5. As expected, disruption of these epitopes had no effect on ligand recognition by α5β1. 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 α5β1 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 α5β1, 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 α5β1 [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 α5β1, 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 α5β1 are overlapping [7].

A subset of anti-α5β1 monoclonal antibodies (mAbs) are able to inhibit the interaction between α5β1 and FN. Most inhibitory anti-α5 mAbs do not compete directly with FN for binding to α5β1 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 sevenfold repeated unit of approx. 200 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 α5β1) 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-propeller secondary structure [13] and to fold cooperatively 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 α3 affected the binding of invasin and laminin [21]. The epitopes for inhibitory anti-α4

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

1 To whom correspondence should be addressed (e-mail mhumphri@fs1.scg.man.ac.uk).

© 1999 Biochemical Society
mAbs have been localized to the second and third N-terminal repeats of z4 [22,23] and swapping predicted loop regions on the upper surface of the z5 subunit p-propeller within the second and third repeats with the corresponding loops from z4 blocked the binding of two inhibitory anti-z5 mAbs [3].

A series of mouse and rat anti-(human z5) mAbs have been described that are informative with regard to ligand binding. P1D6 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 z5p1 [3]. SNAKA52 is a novel anti-z5 mAb that behaves in a manner similar to JBS5 [3,8]. The binding of JBS5 to z5p1 is cation-regulated and is inhibited by anti-z1 mAbs 13 and 12G10; it is therefore likely to bind close to the interface between z and p subunits [8]. mAb 16 has similar properties to those of JBS5 but in addition its binding to z5p1 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 z5 p-propeller with the corresponding residues from mouse z5. The information has been used to map antibody epitopes on the p-propeller model and to predict the spatial topology of ligand-binding sites.

**EXPERIMENTAL**

**Materials**

Rat mAbs 16 and 11 recognizing the human z5 subunit and z5 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 z5 mAbs VCS, P1D6 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 z5 mAbs SAM-1 and SAM-2 were from Serotec and TCS Biologicals Ltd. (Botolph Claydon, Bucks., U.K.) respectively. Mouse anti-human z5 mAb SNAKA52 was prepared as follows: BALB/c mice were immunized with z5p1 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 z4p1 to determine which subunit the antibody was directed against.

All antibodies were used as purified IgG except P1D6, which was used as ascites. Mouse and rat IgG were obtained from Sigma (Poole, Dorset, U.K.). The synthetic peptide GACRR-ETAWAGCA 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-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-Sephasel (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 z5 cDNA in pCDNA3 (Invitrogen, San Diego, CA, U.S.A.) was then subcloned into pUC119. Site-directed mutagenesis was performed with the Mutagen kit (Bio-Rad) [26]. The primers used to generate mutations were: 1, 5’-GCAGAGGAGGAGGTCCACAGGCGACCCGGTGG-3’ for the E116D/L118Q mutation; 2, 5’-CCTGATCAACCCGGTTTCCAGGGGCA-3’ for the L212P mutation; 3, 5’-GTCCTCTAGTGACAGGTCAAGGGG-3’ for the S75Y mutation. Primers were designed similarly for the remaining mutations. The KpnI/XhoI fragment was subcloned into pCDNA3 containing z5 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% (v/v) trypsin/0.02% EDTA in PBS, washed twice in PBS, resuspended to a concentration of 10^6 cells/ml in PBS, and 8 × 10^6 cells were placed into 0.4 cm electroporation cuvettes (Bio-Rad). Wild-type or mutant z5 DNA (20 μg) was added and the cells were left on ice for 10 min. Cells were electroporated at 25 μF 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% (v/v) glucose 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/CO_2 (19:1) atmosphere.

**Cell attachment assay**

CHO-B2 cells, or cells transfected with mutant or wild-type human z5, were detached with 0.05% (v/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 MnCl_2 (buffer A) at a concentration of 2 × 10^6/ml. Assays were performed in 96-well microtitre plates (Costar, High Wycombe, Bucks., U.K.). Cells were washed 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 μl of 10 μg/ml heat-denatured BSA. The BSA was removed by aspiration and the wells were washed once with buffer A. Aliquots (100 μl) of the cells in buffer A were then added to the wells and incubated for 20 min at 37°C in a humidified air/CO_2 (19:1) atmosphere. For experiments examining the effect of anti-z5 mAbs on cell attachment, cells were either preincubated with antibody (10 μg/ml) for 30 min at room temperature before being added to the wells (mAb 16) or mAb was added directly to the wells.

© 1999 Biochemical Society
(50 μl, 20 μg/ml) before the addition of 50 μl of cells at 4 × 10⁵/ml (P1D6, JBS5 and SNAKA52). To estimate the reference value for 100 % attachment, cells in triplicate wells coated with poly-L-lysine (500 μg/ml) were fixed immediately by the 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 twice with 100 μl of buffer A, and the remaining bound cells were fixed as described above for reference wells. The fixative was twice with 100 μl of water, and attached cells were stained with Crystal Violet (Sigma), as described previously [28]. The total) was subtracted from all measurements. Each experiment shown is representative of at least two separate experiments.

RESULTS

Generation of chimaeric receptors

An alignment of α5 and α5 sequences [29] was used as the basis for assigning the loops on the upper surface of the β-propeller model as predicted by Springer [14]. These loops correspond to those sequences connecting β-strands 4 and 1 between blades and strands 2 and 3 within each blade; they are believed to contain the inhibitory mAb epitopes and ligand-binding sites.

The epitopes of monoclonal anti-(human α5) antibodies were localized by using mouse/human interspecies chimaeras because the mAbs under investigation do not recognize mouse α5. The sequences of human and mouse α5 were aligned by using ClustalW (Figure 1) [30]. The two sequences show 90 % identity within the seven N-terminal repeats. Non-conserved residues within the 2–3 and 4–1 loops of blades 2–4 were identified and the human residues were replaced with mouse residues at these points as single or double mutations. In addition, a residue predicted to lie within a loop on the lower surface of the propeller was exchanged as a control (A201S). These mutations are listed in Figure 1. Mouse α5β1 recognizes human FN and therefore these mouse/human chimaeras were predicted to bind human FN unless their conformation had been compromised. Chimaeric cDNA species were stably transfected into CHO-B2 cells and the population of expressing cells was enriched by using magnetic beads. CHO-B2 is a variant of the CHO cell line that has a low level of expression of α5 (2 % of wild-type [27]). Human wild-type and mutant α5 were therefore expressed on the surface of CHO-B2 cells associated with hamster β1. Cell-surface expression was analysed by FACS with the anti-α5 mAb 11, which recognizes an epitope in the C-terminal half of the subunit outside the N-terminal repeats. All except one of the mutants was expressed at the cell surface (L118Q/S119N). Mutation A201S had no effect on the binding of mAbs.

FACS analysis

The reactivity of a panel of anti-α5 mAbs towards the α5 chimaeras was tested by flow cytometry (Table 1). This panel

![Figure 1](alignment_of_human_and_mouse_alpha5_amino_acid_sequences_for_blades_2_and_3_of_the-beta-propeller_model.png)

The boundaries between blades 1 and 2 and blades 3 and 4 are indicated by vertical lines. Predicted β-strands are underlined and non-conserved residues in the loops between the second and third, and between the fourth and first, β-strands are marked with an asterisk. The Ala residue shown in bold was used as a negative control. The assignment of β-strands and loops is based on an alignment of the sequence of α5 with that of α4 by Irie et al. [29].

<table>
<thead>
<tr>
<th>Mutation</th>
<th>mAb 11</th>
<th>mAb 16</th>
<th>JBS5</th>
<th>P1D6</th>
<th>VS5</th>
<th>SAM-1</th>
<th>SAM-2</th>
<th>SNAKA52</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>80.9</td>
<td>88.3</td>
<td>81.3</td>
<td>75.0</td>
<td>57.9</td>
<td>87.0</td>
<td>93.3</td>
<td>78.4</td>
</tr>
<tr>
<td>L118Q</td>
<td>20.8</td>
<td>22.8</td>
<td>17.7</td>
<td>13.9</td>
<td>6.2</td>
<td>56.9</td>
<td>60.7</td>
<td>71.8</td>
</tr>
<tr>
<td>S119N</td>
<td>35.8</td>
<td>41.9</td>
<td>27.8</td>
<td>30.7</td>
<td>31.5</td>
<td>37.8</td>
<td>35.7</td>
<td>31.8</td>
</tr>
<tr>
<td>A201S</td>
<td>71.4</td>
<td>85.6</td>
<td>64.2</td>
<td>52.6</td>
<td>67.2</td>
<td>56.9</td>
<td>60.7</td>
<td>71.8</td>
</tr>
<tr>
<td>E116D</td>
<td>16.3</td>
<td>11.8</td>
<td>24.7</td>
<td>21.5</td>
<td>26.5</td>
<td>28.8</td>
<td>37.0</td>
<td>30.1</td>
</tr>
<tr>
<td>L212P</td>
<td>50.4</td>
<td>62.3</td>
<td>57.0</td>
<td>47</td>
<td>52.6</td>
<td>67.6</td>
<td>62.2</td>
<td>62.1</td>
</tr>
<tr>
<td>I225V</td>
<td>30.8</td>
<td>36.1</td>
<td>29.1</td>
<td>22.1</td>
<td>24.7</td>
<td>22.5</td>
<td>32.9</td>
<td>17.5</td>
</tr>
<tr>
<td>S75Y</td>
<td>46.1</td>
<td>53.1</td>
<td>29.7</td>
<td>40.5</td>
<td>35.6</td>
<td>46.1</td>
<td>44.9</td>
<td>22.5</td>
</tr>
<tr>
<td>E116D/S119N</td>
<td>30.8</td>
<td>31.7</td>
<td>28.4</td>
<td>27.1</td>
<td>32.2</td>
<td>34.8</td>
<td>36.3</td>
<td>26.6</td>
</tr>
<tr>
<td>S77A/E78K</td>
<td>49.2</td>
<td>50.5</td>
<td>46.1</td>
<td>40.4</td>
<td>43.7</td>
<td>51.0</td>
<td>50.1</td>
<td>46.1</td>
</tr>
<tr>
<td>E116D/L118Q</td>
<td>43.4</td>
<td>7.9</td>
<td>41.6</td>
<td>42.3</td>
<td>35.0</td>
<td>44.0</td>
<td>49.8</td>
<td>45.4</td>
</tr>
<tr>
<td>L69I</td>
<td>43.6</td>
<td>45.7</td>
<td>57.6</td>
<td>36.4</td>
<td>53.5</td>
<td>52.9</td>
<td>59.3</td>
<td>56.7</td>
</tr>
<tr>
<td>E61Q</td>
<td>37.0</td>
<td>40.2</td>
<td>47.0</td>
<td>41.5</td>
<td>42.8</td>
<td>50.1</td>
<td>51.9</td>
<td>45.2</td>
</tr>
</tbody>
</table>
Figure 2  FACS histograms of wild-type (WT) and mutant α5 stably expressed on the surface of CHO-B2 cells

Representative histograms of WT and mutant α5b1 are shown stained with α5-specific mAbs. Cells were harvested after selection with G418 and sorted by using magnetic beads, stained with an α5-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.

The epitope for P1D6 was disrupted by the mutation L212P. Leu115 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 α5 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 α5b1; these chimaeras were therefore deemed functional (Figure 3).

The attachment of cells to RRETAWA was also assessed. Cells expressing α5 with the L212P or S75Y mutations (disrupting the epitopes of P1D6 and SNAKA52 respectively) bound RRETAWA as well as cells expressing wild-type α5 (results not shown). Cells expressing α5 with the E116D/L118Q mutation (disrupting the epitope of mAb 16) also attached to RRETAWA to a similar extent as cells expressing wild-type α5, despite the observation that mAb 16 competes directly for binding to α5b1 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-α5 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 α5b1 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 α5b1 is abrogated by the double mutation E116D/L118Q.

All of these mutants bound the CCBD of FN and a panel of other anti-α5 mAbs with an affinity comparable to that of wild-type α5, indicating that the gross conformation of the receptor had not been disrupted.
Epitope mapping of the \( \alpha_5 \beta_1 \) integrin

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 9.91). The low levels of attachment of untransfected cells to the III6–10 fragment are probably due to the expression of \( \alpha_v \) integrins by these cells.

Previous studies located the epitopes of JBS5 and P1D6 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 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 P1D6 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 P1D6 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 \( \alpha \) 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. P1D6 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 \( \alpha \) and \( \beta \) subunits. The binding of these three mAbs is also regulated by cations; it has been proposed that cation occupancy leads to a conformational change in the integrin that prevents the mAb from binding at a spatially distant site.
Table 2  Effect of anti-α5 mAbs on the α5β1-mediated cell attachment of mutant α5β1 to III6–10

The attachment of wild-type and mutant α5 transfected CHO-B2 cells to the III6–10 fibronectin fragment was inhibited by mAbs as indicated. Wild-type and mutant α5 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 ± S. D. of three values. Expt. 1: S75Y mutant (wild-type α5: 66.4% positive, mean fluorescence index 14.3; S75Y mutant α5: 83.3% positive, mean fluorescence index 17.68). Attachment in the presence of mouse IgG (MuIgG): S75Y 46%, wild-type 77%. Expt. 2: E116D/L118Q double mutant (wild-type α5: 73.6% positive, mean fluorescence index 10.4; E116D/L118Q mutant α5: 68.2% positive, mean fluorescence index 12.23). Attachment in the presence of μlgG: E116D/L118Q 31%, wild-type 47%. Expt. 3: L212P mutant (wild-type α5: 68.4% positive, mean fluorescence index 13.84; L212P mutant α5: 38.7% positive, mean fluorescence index 18.50). Attachment in the presence of μlgG: 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.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Mutation</th>
<th>P1D6</th>
<th>mAb 16</th>
<th>SNAKA52</th>
<th>JBS5</th>
<th>MuIgG/no mAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S75Y</td>
<td>Wild-type</td>
<td>45.58 ± 12.26</td>
<td>77.57 ± 10.41</td>
<td>74.66 ± 19.61</td>
<td>100 ± 18.50</td>
</tr>
<tr>
<td>2</td>
<td>E116D/L118Q</td>
<td>Wild-type</td>
<td>35.17 ± 15.36</td>
<td>25.84 ± 8.12</td>
<td>36.49 ± 6.88</td>
<td>100 ± 9.21</td>
</tr>
<tr>
<td>3</td>
<td>L212P</td>
<td>Wild-type</td>
<td>22.67 ± 1.49</td>
<td>47.54 ± 1.71</td>
<td>30.11 ± 3.84</td>
<td>100 ± 2.38</td>
</tr>
</tbody>
</table>

Figure 5  Diagrammatic representation of the topological arrangement of mAb epitopes, based on the β-propeller model for the seven N-terminal repeats of α5

The epitopes of P1D6 (within the 4–1 loop connecting blades 2 and 3) and 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 α5β1 is not affected by anti-β1 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 α5β1, RRETAWA, has been shown to compete directly with mAb 16 for binding to α5β1. This implies that the RRETAWA-binding site directly overlaps with the α5β1 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 α5β1 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 to α5β1. 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.

Figure 6  Tentative model for the structure of ligand-occupied form of α5β1

The RGD motif in the type III repeat 10 of FN is recognized by the β I 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. © 1999 Biochemical Society
to α5/β1 [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 α5/β1 might be proximal to the P1D6 and mAb 16 epitopes respectively. Consistent with this suggestion is the prediction of the β-propeller model that the loops containing the synergy sequence and RGD are located on the same face of the α5 subunit.

We thank K. Yamada and S. Aota for the FN III6–10 clone, human α5 cDNA and mAb 16; I. Hesketh for oligonucleotide synthesis; L. Hall and R. Slater for DNA sequencing; and R. Juliano for CHO-B2 cells. This work was supported by grants from the Wellcome Trust.

REFERENCES


Received 26 May 1999/6 September 1999; accepted 24 September 1999