Identification of Amino Acid Residues That Form Part of the Ligand-binding Pocket of Integrin $\alpha_5\beta_1$*

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Arg-Arg-Glu-Thr-Ala-Trp-Ala (RRETAWA) is a novel ligand peptide for integrin $\alpha_5\beta_1$, which blocks $\alpha_5\beta_1$-mediated cell adhesion to fibronectin (Koivunen, E., Wang, B., and Ruoslahti, E. (1994) J. Cell Biol. 124, 373-380). Here we have localized the binding site for RRETAWA on $\alpha_5\beta_1$ using inhibitory monoclonal antibodies (mAbs) and site-directed mutagenesis. A cyclic peptide containing this sequence (*CRRETAWA*) had little effect on the binding of most anti-$\alpha_5$ and anti-$\beta_1$ mAbs to $\alpha_5\beta_1$ but completely blocked binding of the anti-$\alpha_5$ mAb 16 in a directly competitive manner. Hence, the binding site of RRETAWA appears to closely overlap with the epitope of mAb 16. *CRRETAWA* also acted as a direct competitive inhibitor of the binding of Arg-Gly-Asp (RGD) ligand peptide for integrin $\alpha_5\beta_1$. RRETAWA appears to be specific to human $\alpha_5\beta_1$, as it is not recognized by other RGD-dependent integrins or by murine $\alpha_5\beta_1$. Although the RRETAWA sequence appears unrelated to RGD, the binding sites for RRETAWA and RGD on $\alpha_5\beta_1$ were proposed to be closely overlapping because peptides containing the RRETAWA sequence blocked the recognition of RGD by $\alpha_5\beta_1$ and vice versa (5).

The NH$_2$-terminal half of integrin $\alpha$ subunits consist of a seven-fold repeated unit of about 60 amino acid residues. Repeats 4–7 (or in some integrins repeats 5–7) contain putative divalent cation binding sites (6). About one third of integrin $\alpha$ subunits contain an inserted (I or A) domain of about 200 amino acid residues between the second and third repeats. Where present, the A-domain contains the major sites involved in ligand binding (7, 8). Ligand binding sites in non-A-domain-containing integrins (such as $\alpha_5\beta_1$) have been localized to defined regions in the NH$_2$-terminal portions of both $\alpha$ and $\beta$ subunits (8, 9). The NH$_2$-terminal repeats of $\alpha_5\beta_1$ are predicted to have a mainly $\beta$-strand secondary structure (10), and to fold cooperatively into a seven-bladed $\beta$-propeller (11). Each blade of the propeller contains four $\beta$-strands connected by loops of varying length; these strands are tilted such that the connecting loops are either on the upper or lower surfaces of the propeller. In an important recent advance, it was shown that exchanging several putative loop regions on the upper surface of the $\alpha_4$ subunit $\beta$-propeller with the corresponding loops in $\alpha_5$ perturbed ligand recognition by $\alpha_5\beta_1$ and also attenuated the binding of inhibitory anti-$\alpha_4$ monoclonal antibodies (mAbs) (12). Similarly, swapping predicted loop regions on the upper surface of the $\alpha_5$ subunit $\beta$-propeller with the corresponding loops from $\alpha_5$ blocked binding of inhibitory anti-$\alpha_5$ mAbs (4). The region of the $\beta$ subunit that participates in ligand recognition has been predicted to have a von Willebrand factor A-domain-like fold (13–15); the top face of this domain has been suggested to mediate ligand binding through a metal ion-dependent adhesion site (MIDAS) (13–16). A model for the quaternary arrangement of the $\alpha$ and $\beta$ subunit ligand-binding sites has recently been presented (4, 8, 16). However, the precise amino acid residues that participate in ligand recognition are currently unknown.

We have recently described how inhibitory mAbs can be used to map the binding interface between integrin and ligand (4, enzyme-linked immunosorbent assay; ABTS, 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); MIDAS, metal ion-dependent adhesion site.)

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The abbreviations used are: RGD, Arg-Gly-Asp; RRETAWA, Arg-Arg-Glu-Thr-Ala-Trp-Ala; *CRRETAWA*, cyclo-(Gly-Ala-Cys-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys-Ala-Gly); mAb, monoclonal antibody; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, 

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Ligand-binding Pocket of α\(_5\)β\(_1\)

Experimental Procedures

Materials—Rat mAbs 16 and 11 recognizing the human α\(_5\) subunit, and mAb 13 recognizing the human β\(_1\) subunit were gifts from Dr. K. Yamada (NIADDK, National Institutes of Health, Bethesda, MD). Mouse anti-human α\(_5\) mAbs VC5, P1D6 and JBS5 were purchased from PharMingen (San Diego, CA), Life Technologies, Inc., and Serotec (Oxford, UK), respectively. Mouse anti-human α\(_5\) mAbs SAM-1 and SAM-2 were from Serotec and Bradburke Biologicals (Loughborough, UK), respectively. Mouse anti-human β\(_1\) mAbs P4C10, 4B4, and K20 were purchased from Life Technologies, Inc. (Paisley, Scotland, UK), Coulter Corp. (Miami, FL), and The Binding Site (Birmingham, UK), respectively. All antibodies were used as purified IgG, except P4C10 (as ascites). Rabbit, mouse, and rat IgG were obtained from Sigma (Poole, UK). The synthetic peptides GRGDS and GACRRETAWACGA were synthesized using Fastmoc chemistry on an Applied Biosystems 431A peptide synthesizer and purified as outlined previously (18). GACRRETAWACGA was cyclized using 10% MeSO to acrylated protected peptides (19), and purified by filtration on Sephadex G-10 (Sigma). Oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer, or were purchased from MWG Biotech (Southampton, UK). Poly-l-lysine was obtained from Sigma (Poole, UK).

Proteins—Integrin α\(_5\)β\(_1\) was purified from human placenta as described previously (20). Recombinant fragments of the cell-binding domain of fibronectin were produced as preaggregated and purified using DEAE Sephacel (Amersham Pharmacia Biotech, Milton Keynes, UK) and hydroxylapatite (Bio-Rad, Hemel Hempstead, UK) chromatography, as described previously (4).

Biotinylation of Proteins—mAb 11 (~500 µg/ml in buffer A) was mixed with an equal mass of sulfo-N-hydroxysuccinimido biotin (Pierce, Chester, UK) and rotary mixed for 30–40 min at room temperature. The biotinylated mAbs were dialysed against PBS (pH 7.4) for 5 h at room temperature, blocked as described above, and color was developed using ABTS substrate (Sigma). The absorbance of each well at 405 nm was then measured using a multi-scan ELISA reader (Dynatech, Billinghamurst, UK). Measurements obtained were the mean ± S.D. of four replicate wells. In experiments in which the effect of replacing Mn\(^{2+}\) in the assay buffer with Mg\(^{2+}\), Ca\(^{2+}\), or EDTA was examined, buffer A without Mn\(^{2+}\) was used throughout the assay, except during the incubation of mAb with integrin where Mn\(^{2+}\), Mg\(^{2+}\), Ca\(^{2+}\), or EDTA were included at a concentration of 1 mM.

To test if peptides behaved as direct competitive inhibitors or allosterically modulating the binding of mAb 16, the inhibition of antibody binding at different concentrations of peptide was measured as described above over a 10-fold range of mAb 16 concentrations (0.1, 0.3 and 1 µg/ml). The concentration of peptide required to half-maximally inhibit antibody binding, and the maximal extent of inhibition were estimated by non-linear regression analysis as described previously (20). Linear regression analysis of single-reciprocal plots was performed using Sigmaplot Version 6 (Jandel Scientific, Palo Alto, CA).

Solid Phase Assays—Solid-phase receptor-ligand binding was performed using a modification of previously described assays (22). Here a “reverse” assay was used in which ligand (instead of integrin) was adsorbed to the solid phase support, and integrin (instead of ligand) was allowed to bind from the solution phase. ELISA plate wells were coated with III\(_{1-9}\) (SIPSDDN) (50 µg/ml) of RRETAWACGA conjugates (50 µg/ml) for 1 h at room temperature, blocked as described above, and washed three times with 200 µl of buffer A. 100-µl aliquots of purified α\(_5\)β\(_1\) (approximately 1 µg/ml) in buffer A were added to the wells, with or without mAbs (10 µg/ml) or 1:1000 anti-rat or anti-mouse peroxidase conjugate (Dako A/C, Denmark) in buffer A for 20 min. Wells were then washed four times with buffer A, and color was developed using ABTS substrate (Sigma). The absorbance of each well at 405 nm was then measured using a multi-scan ELISA reader (Dynatech, Billinghamurst, UK). Measurements obtained were the mean ± S.D. of four replicate wells.

In all the assays described above, the amount of nonspecific binding was measured by determining the level of antibody or integrin binding to wells coated with BSA alone; these values were subtracted from the corresponding values for receptor- or ligand-coated wells. Each experiment shown is representative of at least three separate experiments. Mapping of the mAb 16 Epitope—A 1.8-kilobase pair KpnI/XhoI fragment of human α\(_5\) in pcDNA3 was subcloned into pUC119. Site-directed mutagenesis (23) was performed using the primer CGCTCA-NHII-CGACGCGC-TCA to reconstruct the full-length cDNA. The presence of the mutation was verified by DNA sequencing.

Chinese hamster ovary cells B2 variant (24) (a gift from R. L. Juliano, University of North Carolina, Chapel Hill, NC) were detached using 0.02% trypsin in PBS, washed twice in PBS, and resuspended to a concentration of 1 × 10⁶ cells/ml in PBS, and 8 × 10⁶ cells were placed into 0.4-cm electroporation cuvettes (Bio-Rad). 20 µg of wild-type or mutant α\(_5\) DNA was added and the cells were left on ice for 10 min. Cells were electroporated at 25 microfarads and 800 V, and then left on ice for another 10 min. Growth medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% glutamine, and 1% non-essential amino acids) was added, and the cells were plated out and incubated at 37 °C, in a humidified atmosphere containing 5% CO\(_2\). After 48 h, the medium was supplemented with 0.7 mg/ml G418 (Life Technologies, Inc.). 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 α\(_5\) subunit), and then with anti-rat IgG-coated magnetic beads (Dako) to select for cells expressing α\(_5\). The expression of wild-type and mutant α\(_5\) was confirmed by flow cytometric analysis in FACScan (Becton Dickinson) using mAb 11. Cells expressing mutant or wild-type α\(_5\) were then cloned by limited dilution to obtain high level expressers. The percentage of cells reactive to a panel of anti-α\(_5\) mAbs was assessed using flow cytometry, using rat IgG or mouse IgG as controls.

Cell Attachment Assay—Chinese hamster ovary-B2 cells, or cells transfected with mutant or wild-type human α\(_5\) were detached using 0.05% (w/v) trypsin, 0.02% (w/v) 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 resuspended in the same buffer with 1 mM MnCl\(_2\) (buffer B) to a concentration of 2 × 10⁶/ml. Assays were performed in 96-well

17. Here we have investigated the molecular basis of RRETAWA recognition using inhibitory anti-α\(_5\) and anti-β\(_1\) mAbs, in conjunction with site-directed mutagenesis. We show that the binding site for RRETAWA is spatially overlapping with the epitope of the anti-α\(_5\) mAb 16 and also with sequences that participate in recognition of RGD. As mAb 16 does not bind to murine α\(_5\)β\(_1\), we exchanged non-conserved residues in predicted loop regions on the upper surface of the human α\(_5\)β-propeller with the corresponding residues from murine α\(_5\). A double mutation (S156G/W157S) was found to specifically block binding of mAb 16. The same mutation also perturbed binding of α\(_5\)β\(_1\) to RRETAWA. Our results pinpoint specific amino acid residues involved in recognition of RRETAWA and imply that residues that interact with RGD may be located in the same loop, or in a closely adjacent region of the integrin. These findings also support the proposed model of the ligand-binding pocket of α\(_5\)β\(_1\) (4, 8).
microtiter plates (Costar, High Wycombe, UK). Wells were coated for 60 min at room temperature with 100-μl aliquots of III-40, or CRRETAWAC*-IgG diluted with Dulbecco's PBS, 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 washed once with buffer B. 100-μl aliquots of the cells in buffer B were then added to the wells and incubated for 60 min at 37 °C in a humidified atmosphere of 5% (v/v) CO2. For experiments examining the effect of anti-α5 mAbs on cell attachment, cells were preincubated with mAbs (10 μg/ml) 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-1-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 twice with 100 μl of buffer B, 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 (22). The absorbance of each well was measured in the absence of peptide (−), or in the presence of 10 μg/ml CRRETAWAC* (+). Mn2+, Mg2+, Ca2+, or EDTA (1 mm) were included in the assay buffer, as indicated.

RESULTS

A Peptide Containing the RRETAWA Sequence Selectively Inhibits the Binding of the Anti-α5 mAb 16 to α5β1—We have shown that recognition of fibronectin by α5β1 attenuates the binding of inhibitory anti-α5 and anti-β1 mAbs (4, 20), and that these mAbs can be used to localize ligand contact sites. To investigate if recognition of RETAWA causes changes in the binding of inhibitory anti-α5 or anti-β1 mAbs, we examined the effect of a high concentration of the cyclic peptide GACRE-TAWACGA (*CRRETAWAC*) (5) on the binding of a panel of these mAbs to α5β1, VC5 and K20 were used as control (non-inhibitory) anti-α5 and anti-β1 mAbs, respectively. The results (Fig. 1) showed that binding of the anti-α5 mAb 16 to α5β1 was almost completely blocked by *CRRETAWAC*. The binding of other anti-α5 mAbs was unaffected, or slightly increased. *CRRETAWAC* had a small inhibitory effect on the binding of the anti-β1 mAbs 13, P4C10, and 4B4 (maximal extent of inhibition ≤ 25%).

Inhibition of mAb 16 binding by *CRRETAWAC* was dependent on the presence of divalent cations (Fig. 2). Antibody binding was strongly inhibited only when Mn2+ or Mg2+ was present in the assay buffer. A lower level of inhibition was observed in the presence of Ca2+, and the peptide was inactive in the presence of EDTA. These findings strongly suggest that the inhibition of mAb 16 binding is due to recognition of RETAWA by α5β1, which has been shown to be cation-dependent (5). The divalent cation requirements for the interaction of RETAWA with α5β1 appear similar to those for fibronectin (22).

The Binding Site of RETAWA Is Spatially Overlapping with the Epitope of mAb 16—To examine if the *CRRETAWAC* peptide acted as a direct competitive inhibitor or as an allosteric inhibitor of mAb 16 binding, we tested the inhibitory effect of *CRRETAWAC* on mAb 16 binding over a range of mAb concentrations. The results (Fig. 3A) showed that antibody binding could be completely inhibited at high peptide concentrations, and that the concentration of *CRRETAWAC* required for half-maximal inhibition of mAb 16 binding increased in parallel with the antibody concentration (8-fold increase for a 10-fold increase in antibody concentration), as would be expected for a direct competitive inhibition. Plots of 1/antibody binding) versus *CRRETAWAC* concentration were linear (Fig. 3B), confirming that *CRRETAWAC* competed directly with mAb 16 for binding to α5β1. Hence, it appears that the binding site for RETAWA on α5β1 is overlapping with the epitope of mAb 16.

The Binding Site of RETAWA Is Spatially Overlapping with the Binding Site of RGD—To examine the relationship between the binding sites for RETAWA and RGD on α5β1, we tested the inhibitory effect of *CRRETAWAC* on the binding of a recombinant fibronectin fragment to α5β1. This fragment, III-6-10 (21), contains the RGD site in the 10th type III repeat.
The results (Fig. 4A) showed that *CRRETAWAC* peptide completely blocked the binding of mAb 16 to α₅β₁, as binding of mAb 16 was measured at varying concentrations of *CRRETAWAC*, over a 10-fold range of mAb concentrations: 0.1 (▲), 0.3 (■), or 1.0 (●) μg/ml. By non-linear regression analysis, the concentrations of peptide for half-maximal inhibition of mAb binding are 0.22 ± 0.03, 0.50 ± 0.08, and 1.6 ± 0.4 μg/ml, for 0.1, 0.3, and 1.0 μg/ml mAb concentrations, respectively; in each case estimated maximal extents of inhibition are ≥90%. B, single-reciprocal (Dixon) plot of the data shown in A. Lines show linear regression analysis of the data; r² values are 0.998, 0.994, and 0.976, for 0.1, 0.3, and 1.0 μg/ml mAb concentrations, respectively.

The results (Fig. 4A) showed that *CRRETAWAC* peptide completely blocked the binding of III₆₋₁₀ to α₅β₁, and the concentration of *CRRETAWAC* required for half-maximal inhibition of binding increased in parallel with the concentration of recombinant fragment (~10-fold increase for a 10-fold increase in III₆₋₁₀ concentration), as would be expected if RRETAWA acted as a direct competitive inhibitor of RGD binding. Plots of 1/III₆₋₁₀ binding versus *CRRETAWAC* concentration (Fig. 4B) were linear, confirming that RRETAWA competes directly with fibronectin for binding to α₅β₁. Although the fibronectin fragment used in these experiments also contains the synergy region of the ninth type III repeat, similar results were obtained with a mutant fragment III₆₋₁₀(SPSDN) (21), in which the synergy sequence is replaced with an inactive sequence from the eighth type III repeat (data not shown). Hence, it appears that the binding site for RRETAWA directly overlaps with that of RGD. This finding is consistent with the previously...
described ability of peptides containing the RRETAWA sequence to block the recognition of RGD by \( \alpha_5 \beta_1 \) and vice versa (5).

The Binding Site of RGD Does Not Overlap with the mAb 16 Epitope—To further study the relationship between RRETAWA and RGD, we examined if recognition of RGD by \( \alpha_5 \beta_1 \) affected the binding of mAb 16, or other anti-\( \alpha_5 \) mAbs. The peptide GRGDS was used as a model RGD-containing ligand, as this peptide has been shown to have similar properties to fibronectin fragments that lack the synergy sequence (4). The results (Fig. 5) showed that mAb 16 binding to \( \alpha_5 \beta_1 \) was partially inhibited by GRGDS. In agreement with our previous study (4), this peptide had no effect on the binding of other anti-\( \alpha_5 \) mAbs but did perturb the binding of inhibitory anti-\( \beta_1 \) mAbs. To examine if RGD peptide acted as a direct competitive inhibitor or as an allosteric inhibitor of mAb 16 binding, we tested the effect of GRGDS on mAb 16 binding over a ten-fold range of antibody concentrations. The results (Fig. 6A) showed that the concentration of RGD peptide required for half-maximal inhibition of antibody binding was approximately the same for each concentration of antibody, and the maximal extent of inhibition decreased with increasing antibody concentration. These data are inconsistent with a direct competitive inhibition and instead suggest that GRGDS is an allosteric inhibitor of mAb 16 binding. Plots of 1/(antibody binding) versus GRGDS concentration were hyperbolic (Fig. 6B), confirming that GRGDS allosterically inhibited mAb 16 binding to \( \alpha_5 \beta_1 \). Hence, GRGDS and *CRRETAWAC* showed distinct modes of inhibition of mAb 16 binding. These distinct modes of inhibition were not simply due to the difference in size between GRGDS and *CRRETAWAC*, or to differences in the affinity of the two peptides for binding to \( \alpha_5 \beta_1 \), because a cyclic RGD peptide of similar size and affinity to *CRRETAWAC* showed the same allosteric pattern of inhibition as GRGDS on mAb 16 binding (data not shown). Thus, it appears that the binding site of RGD does not directly overlap with the mAb 16 epitope, and therefore the binding sites for RRETAWA and RGD, although overlapping, are non-identical.

**RRRETAWA and RGD Binding to \( \alpha_5 \beta_1 \) Show Overlapping but**

![Graph showing effect of GRGDS peptide on binding of anti-\( \alpha_5 \) and anti-\( \beta_1 \) mAbs to \( \alpha_5 \beta_1 \). Binding of mAbs to purified \( \alpha_5 \beta_1 \) was measured in an ELISA-type assay in the presence of 100 \( \mu \)g/ml GRGDS (a concentration that gave a near-maximal effect). Results are expressed as a percentage of mAb binding in the absence of peptide.](image)

![Graph showing analysis of the effect of GRGDS on the binding of mAb 16 to \( \alpha_5 \beta_1 \).](image)
binding of $\alpha_{5}\beta_1$ from the solution phase was detected using a biotinylated non-inhibitory anti-$\alpha_5$ mAb 11 (25). As an RGD-containing ligand, we used the III$_{6-10}$(SPSDN) protein (21), which lacks the synergy region. III$_{6-10}$(SPSDN) and *CRRETAWA*-IgG supported similar levels of $\alpha_5\beta_1$ binding (data not shown). The results (Fig. 7, A and B) showed that the binding of $\alpha_5\beta_1$ to both *CRRETAWA*-IgG and III$_{6-10}$(SPSDN) was blocked by mAb 16. Two other anti-$\alpha_5$ mAbs (JB55 and SAM-2) blocked binding to both ligands. However, one anti-$\alpha_5$ mAb (SAM-1) inhibited recognition of the fibronectin fragment but not *CRRETAWA*-IgG. P1D6, an anti-$\alpha_5$ mAb previously shown to inhibit recognition of the synergy region but not the RGD sequence of fibronectin (4), also did not block binding of $\alpha_5\beta_1$ to *CRRETAWA*-IgG. Most notably, anti-$\beta_1$ mAbs, particularly mAb 13 and P4C10, which strongly perturbed recognition of III$_{6-10}$(SPSDN), were ineffective at blocking binding of $\alpha_5\beta_1$ to *CRRETAWA*-IgG. These data provide further evidence that the binding sites of RRETAWA and RGD on $\alpha_5\beta_1$ are overlapping but non-identical.

In further experiments (data not shown) binding of $\alpha_5\beta_1$ to *CRRETAWA*-IgG was completely blocked by *CRRETAWA* or GRGDS peptides, but not by control peptides. Conversely, binding of $\alpha_5\beta_1$ to III$_{6-10}$(SPSDN) was blocked by GRGDS or *CRRETAWA* peptides. Binding of $\alpha_5\beta_1$ to both ligands was abolished in the presence of EDTA.

**A Double Mutation in the $\alpha_5$ Subunit Blocks Binding of $\alpha_5\beta_1$ to both mAb 16 and RRETAWA**—Inhibitory anti-$\alpha_5$ mAbs appear to recognize conformation-dependent epitopes in the second and third NH$_2$-terminal repeats of $\alpha_5$; these epitopes involve putative loop regions on the upper surface of the $\beta$-propeller structure formed by the NH$_2$-terminal repeats (4). As mAb 16 does not react with murine $\alpha_5\beta_1$, we took the approach of swapping non-conserved residues in these loop regions with the corresponding residues in the murine $\alpha_5$ subunit to identify amino acid residues that form part of the mAb 16 epitope (Fig. 8). Mutant and wild-type $\alpha_5$ subunits were expressed on the surface of Chinese hamster ovary cells, B2 variant (24). A double mutation, S156G/W157S, was found to block mAb 16 binding (Table I). The same mutation had no effect on the binding of other anti-$\alpha_5$ mAbs, suggesting that only the epitope of mAb 16 involves these residues, and also that the mutation does not cause any gross changes in the tertiary structure of $\alpha_5\beta_1$.

Cells expressing the mutant receptor attached at similar levels to the recombinant fibronectin fragment III$_{6-10}$ as cells expressing wild-type $\alpha_5\beta_1$ (Fig. 9A), demonstrating that the mutation did not significantly affect recognition of the RGD sequence. In contrast, although cells expressing the wild-type receptor attached well to *CRRETAWA*-IgG, cells expressing the mutant integrin failed to attach to this substrate (Fig. 9B). Hence, the mutated residues appear to form part binding site of RRETAWA.

**DISCUSSION**

In this report, we have sought to localize the site of interaction of a novel ligand sequence RRETAWA with $\alpha_5\beta_1$. Our major findings are as follows. (i) The binding site of RRETAWA appears to directly overlap with the epitope of the inhibitory anti-$\alpha_5$ mAb 16. (ii) The binding site of RRETAWA appears to be overlapping with, but is non-identical to, the binding site of RGD. (iii) Two amino acid residues in a putative loop region of the $\alpha_5$ appear to form part of the binding site of RRETAWA and the mAb 16 epitope. These data help to support and refine a model of the ligand-binding pocket of $\alpha_5\beta_1$ (4, 8), and also provide an explanation for the specificity of RRETAWA for human $\alpha_5\beta_1$.

The binding site of RRETAWA appeared to be directly overlapping with the mAb 16 epitope because the *CRRETAWA* peptide acted as a direct competitive inhibitor of mAb 16 binding. For all other inhibitory mAbs studied so far, ligand binding causes an allosteric inhibition of antibody binding, suggesting that the epitopes of these mAbs are close to but not directly overlapping with the ligand binding sites (4, 17, 20). The epitope of mAb 16 involves these residues, and also that the mutation does not cause any gross changes in the tertiary structure of $\alpha_5\beta_1$.

**Other swapping mutations in this region of $\alpha_5$ did not affect mAb 16 binding**. The effects of these mutations will be described elsewhere (L. Burrows, A. P. Mould, and M. J. Humphries, manuscript in preparation).
TAWAC. In further support of this suggestion, mAb 16 was the only anti-α5 mAb tested whose binding was perturbed by RGD peptides, and the S156G/W157S mutation selectively blocked mAb 16 binding. Inhibition of mAb 16 binding by "CRRETAWAC" was specific because it was dependent on the presence of divalent cations. In addition, conservative substitutions in the RRETAWA sequence markedly reduced the ability of the peptide to perturb mAb 16 binding. It is important to note, however, that the binding of mAb 16 to α5β1, although modulated by divalent cations (28), is not cation-dependent. Hence, the mechanisms of binding of mAb 16 and "CRRETAWAC" to α5β1 are not identical, and mAb 16 does not belong to a class of ligand-mimetic antibodies such as PAC-1 (27). Further evidence that the binding sites of RRETAWA and the mAb 16 epitope are directly overlapping comes from the observation that the S156G/W157S mutation perturbed the binding of both mAb 16 and RRETAWA to α5β1, suggesting that these two residues form part of the mAb 16 epitope and the binding site of RRETAWA.

Our results also showed that "CRRETAWAC" acted as a direct competitive inhibitor of the binding of RGD-containing fibronectin fragments to α5β1. Hence, the binding site of RRETAWA appears to be overlapping with that of RGD. In support of this, several anti-α5 mAbs, including mAb 16, blocked the recognition of both RRETAWA and RGD by α5β1. However, several pieces of evidence suggested that the binding sites were not identical. (i) RGD peptides acted as allosteric inhibitors of mAb 16 binding (whereas "CRRETAWAC" acted a direct competitive inhibitor), (ii) recognition of RGD peptides by α5β1 caused a greater attenuation of anti-β1 mAb epitopes than "CRRETAWAC" (Refs. 4 and 20, and this study), (iii) several mAbs that block RGD recognition (particularly anti-β1 mAbs such as JBS5 and P1D6) have little or no effect on recognition of RRETAWA, and (iv) the S156G/W157S mutation perturbed binding of α5β1 to RRETAWA but had no effect on recognition of RGD. A prominent difference between the binding of RRETAWA and RGD to α5β1 is that the β1 subunit appears to contribute relatively little to recognition of RRETAWA, whereas it plays the major role in recognition of RGD (4).

In order to localize the epitopes of anti-α5 mAbs, we previously used a "loop swapping" strategy in which putative loop regions on the upper surface of the α5 subunit–β-propeller were exchanged with the corresponding regions from the α5 subunit. A possible weakness of this approach is that exchanging large loop regions could indirectly cause loss of mAb epitopes, for example by inducing changes in tertiary structure. To minimize possible effects on tertiary structure, we took the approach of exchanging a small number of non-conserved residues in human α5 with the corresponding residues from murine α5. Furthermore, as sites involved in recognition of RGD should be conserved from human to mouse, we predicted that the mutant integrins should retain the ability to recognize fibronectin. Our results showed that the S156G/W157S mutation blocked binding of mAb 16 but not of any other anti-α5 mAb, ruling out the possibility of the mutation causing gross changes in tertiary structure. As predicted, the cells expressing the mutant integrin attached to an RGD-containing fragment of fibronectin to a similar extent as cells expressing the wild-type integrin. Interestingly, however, the S156G/W157S mutation completely blocked the ability of the integrin to recognize RRETAWA. As mouse α5β1 has been reported not to bind to RRETAWA (5), the SW sequence appears to form part of the specific binding site for RRETAWA on human α5β1. Consistent with the specificity of RRETAWA for human α5β1, the SW sequence is not found in this position in any other integrin α subunit.

A previous study suggested that the epitope of mAb 16 may lie in a different region of the α5 subunit, as a mutation Gly190→Ala blocked the binding of this antibody (28). However, this glycine residue is conserved in almost all α subunits, so effects on tertiary structure may explain why this mutation perturbed mAb 16 binding. Nevertheless, it is possible that this residue does contribute to the mAb 16 epitope because it is predicted to lie in the 2–3 loop of the third NH2-terminal repeat, and therefore to be in close proximity to the 4–1 loop that contains the SW sequence.

We do not currently understand why some anti-α5 mAbs (such as JBS5) block recognition of RGD although their binding is not inhibited by RGD peptides. However, we have shown that the epitopes of these antibodies lie close to the interface between the α5 and β1 subunits (26). As the RGD-binding sites have been suggested to lie at or near this interface (4, 8), these antibodies may perturb the interaction of large RGD-contain-

### Table 1

| Cells      | 11  | 16  | JBS5 | mAb       |
|------------|-----|-----|------|-----------|
| α5 wt      | 58.3/+ | 99.3/+ | 68.3/+ |            |
| SW-GS      | 94.1/+ | 6.2  | 83.3/+ |            |

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* A. P. Mould and J. Humphries, unpublished results.
FIG. 9. Effect of S156G/W157S mutation on α5β1-mediated cell attachment. Attachment of untransfected (●), α5 wild-type (○), and α5 S156G/W157S (■) transfected Chinese hamster ovary-B2 cells to the III156G/W157S fibronectin fragment (A) and *CRRETAWAC*-IgG conjugate (B). Wild-type or mutant α5 were expressed at comparable levels, as detected using mAb 11 (wild-type α5: 94.2% positive, mean fluorescence index 16.1; mutant α5: 87.0% positive, mean fluorescence index 19.9). The attachment of cells expressing wild-type α5 to III156G/W157S or *CRRETAWAC*-IgG could be inhibited by mAb 16 down to the levels of untransfected cells (data not shown), demonstrating that this interaction is mediated by α5β1. The low levels of attachment of untransfected cells to the III156G/W157S fragment are probably due to the expression of αv integrins by these cells.

Implications for the Mechanism of Ligand Binding by α5β1—Based on our studies of fibronectin binding to α5β1, and the crystal structure of the cell-binding domain of fibronectin (30), we proposed that sites involved in ligand recognition by α5β1 were located in loops on the top surface of the α5 subunit β-propeller structure and on the top face of the A-domain-like region of the β subunit, and that these two modules are arranged side-by-side in the ligand-occupied state of α5β1 (4, 8). Our current findings help to confirm and refine the above model. Ser156-Trp157 is predicted to lie near the apex of the 4–1 loop of the third NH2-terminal repeat, on the upper surface of the α5 subunit β-propeller. Our data pinpoint this region of α5 as forming part of the ligand-binding pocket. Additionally, our localization of the mAb 16 epitope, in conjunction with evidence that it lies close to the interface between the α5 subunit β-propeller and the β-subunit putative A-domain (26), gives further support to the side-by-side arrangement previously proposed. The extended loop that contains Ser156-Trp157 could project radially from the α subunit β-propeller, bringing it in close proximity to the A-domain-like region of the β subunit. This loop also appears to play an important role in integrin activation (26). Intriguingly, the region of the α5 subunit identified here as forming part of the ligand-binding pocket of α5β1 is located at precisely the same position as where the A-domain is inserted in integrin subunits that possess this module. Hence there may be parallels between the mechanisms of ligand binding in A-domain containing and non-A-domain containing integrins.

An early study suggested that the cation binding sites in integrin α subunits directly interact with ligand peptides (31). However, the cation binding sites in the α5 subunit are unlikely to participate directly in ligand recognition because they are predicted to lie on the lower surface of the β-propeller, a considerable distance away from the loops on the upper surface of the propeller in the second and third repeats. Recent mutagenesis data also favor an indirect (allosteric) role for these sites (32).

Although the RGD sequence of fibronectin is recognized mainly by the β1 subunit (4), it is likely that the α5 subunit also plays a role in recognition of RGD because only a subset of β1 integrins recognize this sequence. As RRETAWA competes directly with RGD for binding to α5β1 residues on the α5 subunit involved in recognition of RGD are predicted to lie close to those involved in RRETAWA binding, i.e., in the 4–1 loop of the third NH2-terminal repeat or in a closely adjacent loop. A previous study indicated that Y186, F187 and W188 of α5 are involved in α5β1-fibronectin interactions (28). These residues are predicted to be located in the 2–3 loop of the third NH2-terminal repeat, which lies proximal to the 4–1 loop. An alternative possibility is that RRETAWA competes with RGD for binding to the A-domain-like region of the β subunit. A MIDAS motif is present in this domain, and it has been proposed that an acidic residue in the ligand (Asp or Glu) coordinates to the divalent ion at the MIDAS site (13, 16). In support of this possibility, we find that the glutamate residue of RRETAWA is essential for activity.3 This paradigm may also explain the similar divalent cation-dependence of RGD and RRETAWA for binding to α5β1. In the future, cross-linking of *CRRETA WAC* to α5β1 and further mutagenesis studies should allow us to more precisely delineate the RGD and RRETAWA binding sites.

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