Molecular Basis of Ligand Recognition by Integrin \(\alpha_5\beta_1\)

I. SPECIFICITY OF LIGAND BINDING IS DETERMINED BY AMINO ACID SEQUENCES IN THE SECOND AND THIRD NH\(_2\)-TERMINAL REPEATS OF THE \(\alpha\) SUBUNIT*

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The NH\(_2\)-terminal portion (putative ligand-binding domain) of \(\alpha\) subunits contains 7 homologous repeats, the last 3 or 4 of which possess divalent cation binding sequences. These repeats are predicted to form a seven-bladed \(\beta\)-propeller structure. To map ligand recognition sites on the \(\alpha\) subunit we have taken the approach of constructing and expressing \(\alpha_\Delta\alpha\) chimeras. Although the NH\(_2\)-terminal repeats of \(\alpha_5\) and \(\alpha_\Delta\) are \(>50\%\) identical at the amino acid level, \(\alpha_5\beta_1\) and \(\alpha_\Delta\beta_1\) show marked differences in their ligand binding specificities. Thus: (i) although both integrins recognize the Arg-Gly-Asp (RGD) sequence in fibronectin, the interaction of \(\alpha_5\beta_1\) but not of \(\alpha_\Delta\beta_1\) with fibronectin is strongly dependent on the “synergy” sequence Pro-His-Ser-Arg-Asn; (ii) \(\alpha_5\beta_1\) binds preferentially to RGD peptides in which RGD is followed by Gly-Trp (GW) whereas \(\alpha_\Delta\beta_1\) has a broader specificity; (iii) only \(\alpha_5\beta_1\) recognizes peptides containing the sequence Arg-Glu-Trp-Ala-Trp-Ala (RRETAWA). Therefore, amino acid residues involved in ligand recognition by \(\alpha_\Delta\beta_1\) can potentially be identified in gain-of-function experiments by their ability to switch the ligand binding properties of \(\alpha_\Delta\beta_1\) to those of \(\alpha_5\beta_1\). By introducing appropriate restriction enzyme sites, or using site-directed mutagenesis, parts of the NH\(_2\)-terminal repeats of \(\alpha_i\) were replaced with the corresponding regions of the \(\alpha_5\) subunit. Chimeric subunits were expressed on the surface of Chinese hamster ovary-B2 cells (which lack endogenous \(\alpha_5\)) as heterodimers with hamster \(\beta_i\). Stable cell lines were generated and tested for their ability to attach to \(\alpha_\Delta\beta_1\)-selective ligands. Our results demonstrate that: (a) the first three NH\(_2\)-terminal repeats contain the amino acid sequences that determine ligand binding specificity and the same repeats include the epitopes of function blocking anti-\(\alpha\)-subunit mAbs; (b) the divalent cation-binding sites (in repeats \(4–7\)) do not confer \(\alpha_\Delta\beta_1\)-or \(\alpha_5\beta_1\)-specific ligand recognition; (c) amino acid residues Ala\(^{174}\)-Tyr\(^{226}\) of \(\alpha_i\) (corresponding approximately to repeats 2 and 3) are sufficient to change all the ligand binding properties of \(\alpha_\Delta\beta_1\) to those of \(\alpha_5\beta_1\); (d) swapping a small part of a predicted loop region of \(\alpha_\Delta\) with the corresponding region of \(\alpha_5\) (Asp\(^{154}\)-Ala\(^{159}\)) is sufficient to confer selectivity for RGDGW and the ability to recognize RRETAWA.

Integrins are \(\alpha\beta\)-heterodimeric transmembrane receptors that play central roles in cell adhesion, migration, differentiation, and survival (1). More than 20 distinct integrin heterodimers have been identified, and each integrin recognizes a different set of extracellular matrix or cell-surface proteins. The major sequences recognized by integrins within their ligands have been shown to be short motifs that contain a critical Asp or Glu residue, such as Arg-Gly-Asp (RGD). Analysis of the three-dimensional structures of integrin ligands has revealed that these recognition motifs are displayed in surface-exposed sites (2, 3).

Ligand-binding sites within integrins are less well characterized. Although ligand recognition is known to involve the NH\(_2\)-terminal regions of both \(\alpha\) and \(\beta\) subunits (4, 5), unresolved questions include (i) what is the precise location of the ligand-binding sites? and (ii) how is the specificity of integrin-ligand binding determined?

The tertiary structure of integrins has not yet been determined; however, high quality structure predictions have been made for the ligand-binding domains of both subunits. These predictions are supported by extensive biochemical analyses (6–9). The NH\(_2\)-terminal portion of integrin \(\alpha\) subunits has been shown to contain seven homologous repeats, each 60–70 amino acid residues in length. Repeats \(4–7\) (or in some integrins repeats \(5–7\)) contain putative divalent cation-binding sites (10). The seven NH\(_2\)-terminal repeats are predicted 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 lower or upper surfaces of the propeller. Loops between the first and second, and between the third and fourth \(\beta\)-strands lie on the lower surface of the propeller, whereas loops between the second and third \(\beta\)-strands, and between the fourth \(\beta\)-strand of one blade and first \(\beta\)-strand of the next blade lie on the upper surface. The divalent cation-binding sites are predicted to lie on the lower surface of the propeller.

An inserted (I or A) domain of about 200 amino acid residues is present in about one-third of integrin \(\alpha\) subunits, lying between repeats 2 and 3. In \(\alpha\) subunits that contain an A-domain, this module contains the major sites involved in ligand binding (2, 9, 12–14). Ligands have been shown to interact with the top face of this domain through a metal ion-dependent adhesion
site (MIDAS) motif (15–18). Although the A-domain is present in only a subset of a subunits, the region of the β subunit that participates in ligand recognition has been predicted to have a tertiary fold similar to that of an A-domain (19–22). This domain may also interact with ligand through a MIDAS site (19–25).

A large number of studies have investigated the location of ligand-binding sites in a subunits that lack an A-domain but do not have consensus has emerged. Cross-linking of a peptide from the γ chain of fibronectin to αIIbβ3 showed that the major site of interaction was in the fifth NH2-terminal repeat (26). An RGD-containing peptide also cross-linked to αIIbβ3 via a site in the divergent cation binding repeats, although some cross-linking was also observed to the second and third repeats (27). In addition, recombinant proteins containing repeats 4–7 of αIIb or αb have been shown to possess ligand binding activity (28, 29). In contrast to these findings, the epitopes of function

18). Mouse anti-human α5 mAbs 1D49.F8 and 17E9 were gifts from Dr. S. Goodman (Merck KgaA, Darmstadt, Germany), rat anti-human α5 mAb 69.6.5 was purchased from Coulter-Immunotech (Marseille, France), mouse anti-human α5 mAb LM142 was a gift from Dr. D. Cheresh (Scripps, La Jolla, CA). All antibodies were used as purified IgG. Rabbit, mouse, and rat IgG were obtained from Sigma (Poole, UK). The synthetic peptides GACRRTAWACGA, GCRGDGWCA, and GCRGDGRCA were obtained from Genosys Biotechnologies Ltd. (Cambridge, UK). Peptides were synthesized using 10% MeSO4 according to published protocols (48), and purified by filtration on Sephadex G-10 (Sigma).

Mutagenesis—Full-length clones of human α5 and α6 were gifts from D. Cheresh (Scripps Research Institute, La Jolla, CA) and K. Yamada (National Institute of Dental Research, NIH, Bethesda, MD), respectively. A 1.3-kilobase BamHI/HindIII fragment of α5 was subcloned into pUC118. The following oligonucleotides (listed 5′ to 3′) were used to introduce restriction enzyme sites into α5 by site-directed mutagenesis using the GeneEditor kit (Promega, Southhampton, UK): TTTTGATGACAGCTTCTCATTTCTGTCGGC (AvrII site), GAGGACCTTGAGG- GCCCAGGGTACATATTTTTG (NcoI site), GGACAGGAGGTTTCAAGAGGATTTT- GCAAGAAGGCTGCACTTGTTTCATTTAC (BglII site). The mutations introduced in the AvrII and BglII sites were silent; however, the mutation introducing the NcoI site causes changes to the amino acid sequence and so an additional base change was made to convert the amino acid sequence at this site to that of α5 (S100KQ to A107HG).

The presence of the mutations was verified by restriction enzyme digestion of miniprep DNA (Qiagen) prepared from individual clones. To reconstitute full-length α5, the mutated 5′ BamHI/HindIII fragment of α5 was ligated with a 3′ HindIII/XbaI fragment of α5 into pCDNA3 cut with BamHI and XbaI. To construct the α5/α5(F1–G232) chimeric, a BamHI/HindIII fragment of α5 was ligated with an AvrII/AvrI fragment of α5 into pCDNA3 cut with BamHI and Apal. To construct the α5/α5(F1–G232) chimeric, a BamHI/HindIII fragment of α5 was ligated with an AvrII/XbaI fragment of α5 into pCDNA3 cut with BamHI and XbaI. To construct the α5/α5(A107–C164) chimeric, a BamHI/HindIII fragment of α5 was ligated with a NcoI/BglII fragment of α5, and a BglII/XbaI fragment of α5 into pCDNA3 cut with BamHI and Apal. The constructs were verified by DNA sequencing. Chimeras were designated according to the position of the restriction site in the corresponding amino acid sequence.

The following oligonucleotides (listed 5′ to 3′) were used to exchange amino acid residues within putative loop regions of α5 with the corresponding amino acid residues in α3: CATTTGGGAAAGCTTGGG- GACGATACGGCCCGCATCTGACATGCTTCTCAG (Met118–Glu123 of α5) with Lys122–Asp130 of α3; GCTCATGATGACATGTAGATCAGATTTTGGGCTGCTGACAGGATTTTTGTTG (Glu145–Asp152 of α3 with Asp154–Ala159 of αb); GGTGGCTGCTGTGACAGTTTTTTTTGCAAGTCTGGAC (Phe177–Tyr181 of α5 with Tyr178 of α3); CATTAGGACATCCTGGAAAGACCGATAG (Glu118–Asp126 of αb) with Gly118–Asp126 of α3; CGGTGCTGACAGGCTTTTCTTGCAGGAC (Asp127–Asp132 of α3 with Gly127–Asp132 of αb); ATACATATACATATAGATCATAGTTTTTTGCAAGTCTGCG (Phe177–Tyr181 of α5 with Tyr178–Tyr181 of α3); CATTAGGACATCCTGGCAAGGACCGATAG (Glu118–Asp126 of αb) with Gly118–Asp126 of α3; CGGTGCTGACAGGCTTTTCTTGCAGGAC (Asp127–Asp132 of α3 with Gly127–Asp132 of αb).

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The following oligonucleotides (listed 5′ to 3′) were used to exchange amino acid residues within putative loop regions of α5 with the corresponding amino acid residues in α3:

Coulping of Cyclic Peptides to IgG—Rabbit IgG (3 mg) was dissolved in 1 ml of Dulbecco’s PBS without Ca2+ or Mg2+ (PBS–). To this solution, approximately 0.5 mg of his-bisulfosuccinimidyl carbonate (Pierce, Chester, UK) dissolved in 1 ml of PBS– was added. The mixture was incubated for 5 min at room temperature, and then cyclic peptide (1–1.5 mg dissolved in 1 ml of PBS–) was added. After incubation for 5 min at room temperature, the mixture was centrifuged at 13,000 × g for 15 min, and stored in aliquots at −70°C.
**RESULTS**

The First Three NH$_2$-terminal repeats of the $\alpha_5$ Subunit Determine the Specificity of Ligand Recognition—To identify the regions of the $\alpha_5$ subunit that determine the ligand binding specificity of $\alpha_5\beta_1$, chimeric $\alpha_5/\alpha_5$ subunits were generated in which segments of $\alpha_5$ were removed and replaced with the corresponding homologous region of $\alpha_5$. Previous studies have suggested that sites important for ligand recognition by $\alpha_5$ lie in either NH$_2$-terminal repeats 1–3 (35, 36) or in the divalent cation binding repeats 4–7 (29). Therefore, initially two chimeras were constructed. The first, $\alpha_5/\alpha_5$(F1–G232), contained repeats 1–3 of $\alpha_5$ with the remainder of the subunit being $\alpha_5$. The second chimera, $\alpha_5/\alpha_5$(F1–G223), was complementary to the first in that it contained repeats 1–3 of $\alpha_5$ with the remainder of the subunit having the sequence of $\alpha_5$ (Fig. 1). Note that the $\alpha_5/\alpha_5$(F1–G232) chimera contains the divalent cation binding sites of $\alpha_5$ (in repeats 4–7), while the $\alpha_5/\alpha_5$(F1–G223) chimera contains the divalent cation-binding sites of $\alpha_5$.

Chimeric and wild-type subunits were expressed on the surface of $\alpha_5$-deficient Chinese hamster ovary cells (B2 variant, Ref. 50). Stably transfected cell lines were obtained by G418 selection and dilution cloning. Using immunoprecipitation of cell lysates (not shown) each wild-type or chimeric subunit containing 2% of the total) was subtracted from all measurements. Each experiment shown is representative of at least three separate experiments.

*In the nomenclature for the chimeras, the numbering refers to the sequence of amino acid residues exchanged. For example, $\alpha_5/\alpha_5$(F1–G232) contains amino acid residues Phe$^1$-Gly$^{232}$ of $\alpha_5$ with the remainder of the subunit being $\alpha_5$. Therefore, initially two chimeras were constructed. The first, $\alpha_5/\alpha_5$(F1–G232), contained repeats 1–3 of $\alpha_5$ with the remainder of the subunit being $\alpha_5$. The second chimera, $\alpha_5/\alpha_5$(F1–G223), was complementary to the first in that it contained repeats 1–3 of $\alpha_5$ with the remainder of the subunit having the sequence of $\alpha_5$ (Fig. 1). Note that the $\alpha_5/\alpha_5$(F1–G232) chimera contains the divalent cation binding sites of $\alpha_5$ (in repeats 4–7), while the $\alpha_5/\alpha_5$(F1–G223) chimera contains the divalent cation-binding sites of $\alpha_5$. Chimeric and wild-type subunits were expressed on the surface of $\alpha_5$-deficient Chinese hamster ovary cells (B2 variant, Ref. 50). Stably transfected cell lines were obtained by G418 selection and dilution cloning. Using immunoprecipitation of cell lysates (not shown) each wild-type or chimeric subunit containing 2% of the total) was subtracted from all measurements. Each experiment shown is representative of at least three separate experiments.

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receptor mainly recognizes vitronectin, and untransfected cells showed only very low levels of attachment to the ligands used in the current study (see legends to Figs. 2-4).

Three different tests of ligand recognition specificity were used. (i) The dependence of integrin-fibronectin binding on the presence of the synergy region was examined by comparing the level of cell attachment to a wild-type recombinant fragment of the CCBD (IIId-10) with the level of attachment to the same

FIG. 2. Attachment of cells expressing wild-type or chimeric α5/αv to recombinant fibronectin fragments. Attachment of CHO-B2 cells expressing αv/α5(F1-G232) (A), αv/α5(F1-G223) (B), wild-type α5 (C), or wild-type αv (D) to IIId-10 (○), IIId-10(PSDNS) (○), or IIId-10KGE (■). For cells expressing αv/α5(F1-G223) and wild-type αv, IIId-10 was 3–10 times more potent than IIId-10(PSDNS) for promoting half-maximal cell attachment; for cells expressing αv/α5(F1-G232) and wild-type αv, IIId-10 was >100 times more potent than IIId-10(PSDNS) for promoting half-maximal cell attachment. Untransfected cells showed little or no attachment to these proteins (<10% at the highest coating concentration, data not shown). The attachment of cells expressing αv/α5(F1-G232) or wild-type α5 was inhibited >90% by the anti-α5 mAb 16; the attachment of cells expressing αv/α5(F1-G223) or wild-type αv was inhibited >80% by the anti-αv mAb 17E6 (data not shown). Chimeric or wild-type subunits were expressed at comparable levels (mean fluorescence intensity values using P3G8 or mAb 11: 45.8, 63.6, 130.6, and 61.8 for αv/α5(F1-G232), αv/α5(F1-G223), wild-type α5, and wild-type αv, respectively.
fragment in which the synergy region is replaced by the corresponding inactive region of the eighth type III repeat (III6–10(SPSDN)) (49). A recombinant CCBD fragment in which the RGD sequence is mutated to the inactive Lys-Gly-Glu (III6–10KGE) was used as a negative control (5). (ii) To test for specific recognition of the RGDGW sequence the levels of cell attachment to the cyclic RGD peptides ACRGDGWCG (*CRGDGWC*) and ACRGDGRCG (*CRGDGRC*) were compared. (iii) To assay for recognition of the RRETAWA sequence the ability of cells to attach to the cyclic peptide GACRRETAWACGA (*CRRETAWAC*) was examined. Peptides were coupled to a carrier protein (rabbit IgG) for use in these experiments. In each case, cells expressing similar levels of wild-type α5 or αV were analyzed in parallel. In addition, to demonstrate that cell attachment was integrin-mediated, the ability of function-blocking anti-α5 or anti-αV mAbs to perturb cell attach-

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**Fig. 3. Attachment of cells expressing wild-type or chimeric αV/α5 to cyclic RGD peptides.** Attachment of CHO-B2 cells expressing αV/α5(F1–G232) (A), αV/α5(F1–G223) (B), wild-type α5 (C), or wild-type αV (D) to *CRGDGWC*-IgG conjugate (●) or *CRGDGRC*-IgG conjugate (○). Untransfected cells showed little or no attachment to either conjugate (<10% at the highest coating concentration). The attachment of cells expressing αV/α5(F1–G232) or wild-type αV was inhibited >90% by the anti-α5 mAb 16; the attachment of cells expressing αV/α5(F1–G223) or wild-type αV was inhibited >80% by the anti-αV mAb 17E6 (data not shown).
The results (Figs. 2–4) showed that cells expressing the \( \alpha V/\alpha 5(F_1–G_232) \) chimera exhibited a strong dependence on the presence of the synergy site for adhering to fibronectin, similar to that observed for cells expressing wild-type \( \alpha 5 \). Cells expressing \( \alpha V/\alpha 5(F_1–G_232) \) showed much higher levels of attachment to \(*\text{CRGDGWC}^*\) sequence than to \(*\text{CRGDGRC}^*\), and gained the ability to attach to \(*\text{CRRETAWAC}^*\), similar to the results obtained with cells expressing wild-type \( \alpha 5 \). In contrast, cells expressing \( \alpha V/\alpha 5(F_1–G_223) \) showed only a weak dependence on the presence of the synergy region for attaching to fibronectin, comparable to that observed for cells expressing wild-type \( \alpha V \). Cells expressing this chimera showed approximately equal levels of attachment to \(*\text{CRGDGWC}^*\) and \(*\text{CRGDGRC}^*\), and lacked the ability to attach to \(*\text{CRRETAWAC}^*\), similar to the results obtained for cells expressing wild-type \( \alpha V \).

In summary, the results showed that \( \alpha V/\alpha 5(F_1–G_232) \beta_1 \) had the same ligand-binding specificity as \( \alpha 5 \beta_1 \), while \( \alpha V/\alpha 5(F_1–G_223) \beta_1 \) had the same ligand-binding specificity as \( \alpha V \beta_1 \).
CHO-B2 cells stably transfected with the indicated wild-type or chimeric α subunit were analyzed for reactivity with anti-α5 and anti-αv mAbs by flow cytometry.

**TABLE I**

**Summary of mAb reactivity with αv/α5 chimeras**

| Subunit transfected | Anti-α5 | Anti-αv |
|---------------------|---------|---------|
|                      | VC5     | 11      | JBS5 | SAM-1 | SAM-2 | 16 | P1D6 | P3G8 | LM142 | 14D9.F8 | 17E6 | 69.6.5 |
| αv Wild type         | +       | +       | +    | +     | +     | +  | +    | +    | +     | +      | +    | +      |
| αv/α5(F1–G232)      | +       | +       | +    | +     | +     | +  | +    | +    | +     | +      | +    | +      |
| αv/α5(F1–G232)      | +       | +       | +    | +     | +     | +  | +    | +    | +     | +      | +    | +      |
| αv/α5(A107–G232)    | +       | +       | +    | +     | +     | +  | +    | +    | +     | +      | +    | +      |
| αv Wild type         | +       | +       | +    | +     | +     | +  | +    | +    | +     | +      | +    | +      |

* a, staining identical to that of negative control (normal mouse or rat IgG).
* b, positive reactivity (similar to that of wild-type α subunit).
* c, weak reactivity (~25% of that of wild-type α subunit).

G223β1 had the same ligand binding specificity as αβ1. Hence, ligand binding specificity appears to be determined by sequences within the first three NH2-terminal repeats of the α subunit. Since the αv/α5(F1–G232) chimera contains the divergent cation-binding sites of αv (in repeats 4–7), and the αv/α5(F1–G232) chimera contains the divergent cation-binding sites of αv, it can be concluded that the divergent cation-binding sites do not play a role in determining the ligand binding specificity.

Cells expressing chimeric or wild-type subunits were analyzed for expression of the epitopes of anti-α5 and anti-αv mAbs using flow cytometry (Table I). The results showed that the αv/α5(F1–G232) subunit contained the epitopes of the function-blocking anti-α5 mAbs JBS5, SAM-1, SAM-2, 16, and P1D6, lacked the epitopes of function-blocking αv mAbs, but expressed the epitopes of the non-function blocking anti-α5 mAbs P3G8 and LM142. Conversely, the αv/α5(F1–G232) subunit contained the epitopes of the function blocking anti-α5 mAbs 14D9.F8, 17E6, and 69.6.5, lacked the epitopes of function blocking αv mAbs, but expressed the epitopes of the non-function blocking anti-α5 mAbs 11 and VC5. These results show that the epitopes of function blocking mAbs anti-α5 mAbs lie within the first three NH2-terminal repeats of αv; similarly the epitopes of function blocking anti-α5 mAbs lie within repeats 1–3 of αv. In contrast, the epitopes of non-function blocking mAbs lie outside the first three repeats. Because the level of epitope expression was similar to that of wild-type αv or wild-type α subunits, it is likely that the chimeras were folded correctly.

**Identification of a Minimal Region of the α5 Subunit That Confers Ligand Binding Specificity**—Since cells expressing the αv/α5(F1–G232) chimera had the same ligand binding properties as cells expressing wild-type αv, we attempted to narrow down the region of α5 required to switch ligand binding specificity by constructing αv/α5 chimeras containing smaller segments of α5 (see Fig. 1). A chimera αv/α5(A107–G232), which contains essentially the second and third repeats of αv, was expressed at high levels. FACS analysis with a panel of anti-α5 or anti-αv mAbs showed that the αv/α5(A107–G232) chimera retained the epitopes of all the function blocking anti-α5 mAbs tested (Table I), although the binding of JBS5 was decreased compared with the αv/α5(F1–G232) chimera. Chimeras containing only the second or third repeat of αv (αv/α5(A107–C164) and αv/α5(G184–G232)) were expressed at low levels than αv/α5(A107–G232), and failed to react, or reacted only weakly, with function blocking anti-α5 and αv mAbs (data not shown). Since it could not be demonstrated that these two chimeras were folded correctly, they were not studied further.

Cells expressing the αv/α5(A107–G232) chimera were tested for their ability to attach to αβ1- or α5β1-sective ligands (Fig. 5). The results showed that cells expressing αv/α5(A107–G232) showed a strong dependence on the synergy region for binding to the CCBD of fibronectin, displayed preferential recognition of RGDGW over RGDR, and possessed the ability to attach to RRETAWA. Therefore, amino acid residues 107–232 of αv were found to be sufficient to confer on αv/α5 the ligand binding specificity of αvβ1. In addition, since the amino acid sequence Asp227-Gly232 of αv is identical to the corresponding sequence Asp218-Gly223 in αv5, residues that confer ligand binding specificity must be contained within Ala107-Trp220 of αv.

**Identification of a Putative Loop Region of αv That Determines Specificity for RGDGW and RRETAWA**—To further narrow down the amino acid residues required to confer on αvβ1 the ligand-binding properties αvβ1, we identified differences in sequence between the αv5/Trp220 region of αv and the corresponding region in αv (Fig. 6). Interestingly, many of these sequence differences were observed to occur within putative loop regions, and were confined mainly to the central portions of these loops. Previous data has suggested that loop regions linking β-strands 2 and 3 in each repeat (2-3 loops) and β strands 4 and 1 between repeats (4-1 loops) are important for ligand recognition by αvβ1 (5, 35, 36). Hence, using oligonucleotide-directed mutagenesis, we made αv/α5 chimeras containing the following exchanges: Met118Glu123 of αv with Lys120 and Asp130 of α5 (αv/α5(K125–D130)), Gly145Asp150 of αv with Asp154Ala159 of α5 (αv/α5(D154–A159)), Phe177–Tyr178 of αv with Tyr186–Phe187 of α5 (αv/α5(Y186–F187)), and Ala209Phes217 of αv with Gly218–Tyr226 of α5 (αv/α5(Q218–Y226)). We also made a chimera in which both Phe177–Tyr178 and Ala209–Phes217 of αv were exchanged with Tyr186–Phe187 and Gly218–Tyr226 of α5, respectively (αv/α5(Y186–Y187, Q218–Y226)). All “loop swapping” chimeras expressed successfully, with the exception of αv/α5(Y186–F187). This latter chimera was expressed only at very low levels, and may therefore have been misfolded. The other chimeras were examined for their reactivity with anti-αv mAbs (Table II). All chimeras expressed the epitopes of the non-function blocking mAbs P3G8 and LM142, and the epitopes of the function blocking mAbs 17E6 and 14D9.F8. However, both αv/α5(K125–D130) and αv/α5(D154–A159) chimeras lacked the epitope of the function-blocking mAb 69.6.5.

Cells expressing the loop swapping chimeras were tested for their ability to attach to αvβ1, or αβ1-selective ligands. Cells expressing each chimera showed only a weak dependence on the presence of the synergy region for attaching to fibronectin, similar to that observed for cells expressing wild-type αv (Fig. 7). However, none of the loop swaps significantly affected recognition of the synergy sequence. Cells expressing the αv/α5(K125–D130) and αv/α5(Q218–Y226) chimeras showed approximately equal levels of adhesion to *CRG-DGWC* and *CRGDGRC*, and failed to attach to *CRRETAWAC*, again similar to the results for cells expressing wild-type αv (Figs. 8 and 9). Cells expressing αv/α5(Y186–F187, Q218–Y226) showed slight selectivity for *CRGDGWC* over *CRGDGRC* but...
DISCUSSION

In this report we have sought to identify the regions of the integrin α5 subunit that are involved in ligand recognition using α5/α5 chimeras. Our major findings are as follows: (i) the first three NH2-terminal repeats contain the epitopes of function blocking anti-α subunit mAbs and the amino acid sequences that determine ligand binding specificity; (ii) the divalent cation-binding sites (in repeats 4–7) do not determine the specificity of ligand recognition; (iii) the amino acid sequence Ala107-Trp126 of α5 (corresponding approximately to the second and third repeats) is sufficient to confer on α5/β1 the ligand binding properties of α5/β1; (iv) swapping a 6-amino acid sequence from a predicted loop region of α5 with the corresponding region of α5 (Asp154-Ala159) is sufficient to confer on α5/β1 selectivity for RGDGW and recognition of RRETAWA.

In this study we observed a close correspondence between the regions of the α subunit involved in determining the specificity of ligand recognition and those that contained the epitopes of function blocking mAbs. This finding supports previous evidence that the epitopes of function blocking mAbs are proximal to sites involved in ligand binding (5, 33–36, 54). In addition, these data lend strong support to the β-propeller model, which predicts that the ligand-binding sites lie on the upper face of the β-propeller (11). Recently, we have mapped some of the residues that form part of the JBS5, mAb 16, and P1D6 epitopes by substituting residues in human α5 with the corresponding residues from mouse α5. Ser65 was found to contribute to the JBS5 epitope, Glu126 and Leu126 to the mAb 16 epitope, and Leu212 to the P1D6 epitope (8). All these residues are predicted to lie on the upper face of the β-propeller domain. The results of the present study, including decreased binding of JBS5 to the α5/α5(A107–G232) chimera, are in good agreement with these data.

Two loop swapping mutants, α5/α5(K125–D130) and α5/ α5(D154–A159), failed to react with the function blocking anti-α mAb 69.6.5. However, since they did react with two other function blocking anti-α mAbs it is likely that the structure of the NH2-terminal repeats had not been grossly perturbed. Hence, a probable explanation is that these mutations disrupted the 69.6.5 epitope. Therefore, residues that form part of the 69.6.5 epitope probably lie in the 2-3 loop of repeat 2 and in the 4-1 loop between repeats 2 and 3. These two loops are predicted to lie adjacent to each other on the upper face of the β-propeller. The observation that the α5/α5(D154–A159) chimera bound α5/β1-selective ligands but retained the epitopes of 17E6 and 14D9.F8 is consistent with previous findings that the epitopes of most function blocking mAbs are close to but not directly overlapping with sites involved in ligand recognition (5, 8, 33, 54).

It is intriguing that the α5/α5(Y186–F187) chimera was expressed only at very low levels, whereas the α5/α5(Y186–F187, Q218–Y226) chimera, in which residues from two loops were exchanged, was expressed normally. The reasons for this are unclear. However, it is likely that there is an interaction between these two loops since they are predicted to lie adjacent to each other in the β-propeller model (11). Mutation of Phe177-Tyr178 of α5 could have a destabilizing effect on the tertiary structure, leading to loss of expression at the cell surface. An interaction between the two exchanged loops in the α5/ α5(Y186–F187, Q218–Y226) chimera may compensate for this destabilizing effect, thereby allowing normal levels of expression.
Irie parts of the divalent cation binding repeats of analyzed for reactivity with anti-integrin. Cross-linking of ligand peptides to these recombinant fragments is the same of that of the native binding (28, 29). Importantly, however, it has yet to be demonstrated between the second and third β-strands, and between the fourth and first β-strands (shown boxed) were chosen for swapping with the corresponding residues in \( \alpha_c \). Assignment of β-strands and loops is based on an alignment of the sequence of human \( \alpha_c \) with that of human \( \alpha_c \) by Irie et al. (6).

Although our results show that the divalent cation sites in the \( \alpha \) subunit do not influence the specificity of ligand binding, it is possible that the divalent cation binding repeats of \( \alpha_c \) and \( \alpha_v \) could have an identical function in ligand recognition. For example, these repeats may be involved in interacting with the RGD sequence, which is a property common to both \( \alpha_c \beta_2 \) and \( \alpha_v \beta_1 \). However, it is noteworthy that none of the function blocking mAbs mapped to repeats 4–7. Indeed, it has been shown that a mAb directed against the \( \alpha_c \) L subunit whose NH₂-terminal repeats 2–4 (R2-R4). This observation suggests an attractive general model of receptor-ligand interactions in both A-domain containing and non-A-domain containing integrins (61, 62). Our findings are also consistent with previous mutagenesis data for \( \alpha_c \beta_2 \) and other integrins (33–39).

In a study of \( \alpha_c/\alpha_{1\beta_3} \) chimeras (4), the minimal region of the \( \alpha_{1\beta_3} \) subunit required to switch the ligand binding specificity to that of \( \alpha_c \) was found to be Leu¹-Pro⁶ (corresponding approximately to the first five repeats). A chimera containing only the first 3 repeats of \( \alpha_{1\beta_3} \) (Leu¹-Phe⁶) expressed the epitope of a function blocking anti-\( \alpha_{1\beta_3} \) mAb but, in contrast to the results from the present study, this chimera did not interact with \( \alpha_{1\beta_3} \)-specific ligand mimetic mAbs or a peptidomimetic.

**Table II**

| Subunit transfected | P3G8 | LM142 | 14D9.F8 | 17E6 | 69.65 |
|---------------------|------|-------|---------|------|-------|
| \( \alpha_c/\alpha_5 \) (R125-D130) | + + | + | + | + | + |
| \( \alpha_c/\alpha_5 \) (D154-A159) | + | + | + | + | + |
| \( \alpha_c/\alpha_5 \) (Q218-Y226) | + | + | + | + | + |
| \( \alpha_c/\alpha_5 \) (Y186-F187, Q218-Y226) | + | + | + | + | + |

* +, positive reactivity (similar to that of wild-type \( \alpha_c \) subunit.
  †, ‡, +/−, staining identical to that of negative control (normal rat IgG).
†, ‡, +/−, staining approximately 50% of that of wild-type \( \alpha_c \).**

In two reports, recombinant proteins containing repeats 4–7 of \( \alpha_c \) or \( \alpha_{1\beta_3} \) have been shown to support RGD-dependent ligand binding (28, 29). Importantly, however, it has yet to be demonstrated whether the specificity and affinity of ligand binding of these recombinant fragments is the same of that of the native integrin. Cross-linking of ligand peptides to \( \alpha_{1\beta_3}\beta_2 \) and \( \alpha_c \beta_2 \) also indicated that these peptides interacted (at least in part) with the divalent cation binding repeats (26, 27). Further studies will be required to resolve these inconsistencies, and to show if the findings for \( \alpha_c \beta_2 \) in this report can be generalized to all non-A-domain containing integrins. However, the position of ligand recognition sites in the \( \alpha_c \) subunit deduced here (the second and third repeats) is strikingly similar to the location of the A-domain in \( \alpha_c \) subunits that contain this module (between the second and third repeats). This observation suggests an attractive general model of receptor-ligand interactions in both A-domain containing and non-A-domain containing integrins (61, 62). Our findings are also consistent with previous mutagenesis data for \( \alpha_c \beta_2 \) and other integrins (33–39).

In a study of \( \alpha_c/\alpha_{1\beta_3} \) chimeras (4), the minimal region of the \( \alpha_{1\beta_3} \) subunit required to switch the ligand binding specificity to that of \( \alpha_c \) was found to be Leu¹-Pro⁶ (corresponding approximately to the first five repeats). A chimera containing only the first 3 repeats of \( \alpha_{1\beta_3} \) (Leu¹-Phe⁶) expressed the epitope of a function blocking anti-\( \alpha_{1\beta_3} \) mAb but, in contrast to the results from the present study, this chimera did not interact with \( \alpha_{1\beta_3} \)-specific ligand mimetic mAbs or a peptidomimetic.

The reason for this discrepancy is unclear. However, a possible explanation is that the \( \alpha_c/\alpha_5 \) (F1–G232) and \( \alpha_c/\alpha_5 \) (F1–G223) chimeras used here contain all of the putative loop between repeats 3 and 4, whereas the \( \alpha_c/\alpha_{1\beta_3} \) (L1–F223) chimera lacks most of this loop. Mutation of Asp²²⁴ in this loop of \( \alpha_{1\beta_3} \) to Val has been shown to lead to loss of ligand-binding function by \( \alpha_c/\alpha_{1\beta_3} \) (39). Importantly, in the \( \alpha_c/\alpha_{1\beta_3} \) (L1–F223) chimera \( \alpha_c/\alpha_{1\beta_3} \) is replaced by Thr²¹⁰ of \( \alpha_c \). Hence, the \( \alpha_c/\alpha_{1\beta_3} \) (L1–F223) chimera may lack one or more additional residues necessary for ligand binding specificity, whereas these residues are contained in the \( \alpha_c/\alpha_{1\beta_3} \) (F1–G232) and \( \alpha_c/\alpha_5 \) (F1–G223) chimeras.

In the same study of \( \alpha_c/\alpha_{1\beta_3} \) chimeras (4), it was shown that the \( \alpha_c/\alpha_{1\beta_3} \) (R140–P334)β3 receptor did not interact with \( \alpha_c/\beta_3 \)-specific ligands. However, this chimera is missing much of the second repeat of \( \alpha_{1\beta_3} \) (39). In contrast, the \( \alpha_c/\alpha_{1\beta_3} \) (L1–F223) chimera lacks second and third NH₂-terminal repeats determine the specificity of ligand recognition. In further support of our finding that the second and third NH₂-terminal repeats determine the specificity of ligand recognition, these repeats show the greatest degree of sequence divergence between \( \alpha_c \) subunits. In contrast, the divalent cation-binding repeats are more similar in sequence (7, 63).

None of loop swapping mutations led to a “gain” of synergy sequence binding (i.e. a change from the weak dependence on the presence of the synergy sequence for binding to fibronectin seen for \( \alpha_c \beta_1 \) to the strong dependence seen for \( \alpha_c \beta_2 \)). It is possible that the residues involved in high affinity recognition of the synergy sequence lie outside the regions exchanged. Alternatively, however, the overall fold of second and third repeats may determine the shape of the fibronectin-binding pocket. It is noteworthy that in addition to \( \alpha_c \beta_2 \) recognizing the synergy sequence of fibronectin more strongly than \( \alpha_c \beta_1 \), it also recognizes the RGD site more weakly. Both these characteristics were evident in the \( \alpha_c/\alpha_{1\beta_3} \) chimera. Interestingly, this chimera retained all the epitopes of the function blocking anti-\( \alpha_c \) mAbs; thus the second and third NH₂-terminal repeats appear to have the same conformation as those of wild-type \( \alpha_c \). We therefore propose that the overall fold of the
second and third repeats determines the shape of the fibronectin-binding pocket, and slight differences in the shape of this pocket between αVβ1 and α5β1 may lead to the differential recognition of the synergy and RGD sequences by these two integrins. Support for the suggestion that the overall fold of repeats 2–3 is important comes from our finding that αV/α5 chimeras containing only the second repeat, or only the third repeat, of α5 appeared to be misfolded.

Based on the structure of the central cell-binding domain of fibronectin (64, 65), the recognition site of synergy sequence on the α5 subunit is predicted to lie a distance of ~35 Å away from the RGD-binding site. As we show in an accompanying paper (66), the loop between the second and third repeats of α5 lies within ~7 Å of the RGD-binding site; this places constraints on possible locations of the RGD and synergy sequence recognition sites on αβ1. Based on these constraints, the divalent cation-
binding sites are too far away from this loop to participate in RGD binding. However, an interaction of the RGD sequence with the MIDAS site of the A-domain-like region of the β subunit is possible if this domain lies adjacent to repeats 2 and 3 of the α subunit, as previously proposed (5). Two potential positions for the synergy sequence recognition site on the α5 subunit are close to the center of the β-propeller, or near the junction of repeats 3 and 4. In support of this suggestion, the epitope of P1D6, an anti-α5 mAb that blocks recognition of the synergy region but not the RGD sequence (5), has been shown to contain Leu<sup>212</sup> in repeat 3 (8).

In summary, we have defined a minimal domain of the α5 subunit (Ala<sup>107</sup>-Trp<sup>226</sup>) that determines the specificity of ligand binding, and identified a sequence that plays a critical role (Asp<sup>154</sup>-Ala<sup>159</sup>). Our findings are consistent with the β-propel-

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FIG. 8. Effect of αV loop swapping mutations on cell attachment to cyclic RGD peptides. Attachment of Chinese hamster ovary-B2 cells expressing αV/α5(K125-D130) (A), αV/α5(D154-A159) (B), αV/α5(Q218-Y226) (C), or αV/α5(Y186-F187, Q218-Y226) (D) to *CRGDGWC*-IgG conjugate (●) or *CRGDGRC*-IgG conjugate (○). In each case, cell attachment was inhibited >70% by the anti-αV mAb 17E6 (data not shown).

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<sup>3</sup>In support of this finding, the chimera containing only the third repeat of α5 (αV/α5(C164-G232)) was weakly reactive with P1D6 but not with other function-blocking anti-α5 mAbs (A. P. Mould, unpublished results).
ler model and appear to exclude a function for the divalent cation binding repeats in ligand recognition. In the accompanying paper (66) we determine further the molecular basis of the interaction of $\alpha_5\beta_1$ with RGDGW and RRETAWA.

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Addendum—While this manuscript was under review, a publication from Banerres and co-workers (Banerres J. L., Roquet, F., Martin, A., and Parello, J. (2000) J. Biol. Chem. 275, 5888–5903) presented new data showing that a recombinant fragment of $\alpha_5$ spanning the divalent cation-binding repeats can interact with fibronectin. The fragment lacks the second and part of the third repeat of $\alpha_5$, which we have identified here as playing an essential role in ligand recognition. Further analysis of the specificity and affinity of ligand binding, and of the extent to which such “minimized” integrins reproduce the functions of the native receptor, is clearly required to resolve this apparent discrepancy. In a separate publication (Puzon-McLaughlin, W., Kamata, T., and Takada, Y. (2000) J. Biol. Chem. 275, 7795–7802) the epitopes of

**FIG. 9. Effect of $\alpha_5$ loop swapping mutations on cell attachment to *CRRETAWAC**. Attachment of Chinese hamster ovary-B2 cells expressing $\alpha_\text{V}/\alpha_5$(K125–D130) (A), $\alpha_\text{V}/\alpha_5$(D154–A159) (B), $\alpha_\text{V}/\alpha_5$(Q218–Y226) (C), or $\alpha_\text{V}/\alpha_5$(Y186–F187, Q218–Y226) (D) to *CRRETAWAC*-IgG conjugate. The attachment of cells expressing $\alpha_\text{V}/\alpha_5$(D154–A159) was inhibited >90% by the anti-$\alpha_\text{V}$ mAb 17E6 (data not shown).
ligand-mimetic mAbs against α_{IIb}β_{3} have been shown to be contained within the second and third repeats of α_{IIb} and the A-domain-like region of β_{3}. Our data are fully consistent with this latter study.

REFERENCES

1. Hynes, R. O. (1992) Cell 99, 11–25
2. Humphries, M. J., and Newham, P. (1998) Trends Cell Biol. 8, 78–83
3. Tan, K., Casasnovas, J. M., Liu, J., Briskin, M. J., Springer, T. A., and Wang, J. (1998) Structure 6, 793–801
4. Loftus, J. C., Halloran, C., Ginsberg, M. H., Feigen, L. P., Zahlocki, J. A., and Smith, J. W. (1996) J. Biol. Chem. 271, 2353–2397
5. Meuld, A. P., Askari, J. A., Asta, S., Yamada, K. M., Irie, A., Takada, Y., Fardon, H. J., and Humphries, M. J. (1997) J. Biol. Chem. 272, 17283–17292
6. Irie, A., Yamada, K. M., and Takada, Y. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7188–7203
7. Oszvig, C., and Springer, T. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4870–4875
8. Burrows, L., Clark, K., Mould, A. P., and Humphries, M. J. (1999) Biochem. J. 344, 527–533
9. Takada, Y., Kamata, T., Irie, A., Puzon-McLaughlin, W., and Zhang, X.-P. (1997) Metab. Biol. 16, 145–151
10. Tuckwell, D. S., Brass, A. M., and Humphries, M. J. (1992) Biochem. J. 285, 325–331
11. Springer, T. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 65–72
12. Diamond, M. S., Garcia-Aguilar, J., Bickford, J. K., Corbi, A. L., and Springer, T. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1657–1662
13. Randi, A. M., and Hogg, N. (1994) J. Biol. Chem. 269, 12995–12998
14. Dickerson, S. K., and Santoro, S. A. (1998) Cell. Mol. Life Sci. 54, 556–566
15. Huang, C., and Springer, T. A. (1995) J. Biol. Chem. 270, 19008–19016
16. Zhang, L., and Plow, E. F. (1996) J. Biol. Chem. 271, 17283–17292
17. Edwards, C. P., Fisher, K. L., Presta, L. G., and Bodary, S. C. (1998) J. Biol. Chem. 273, 28937–28944
18. Kamata, T., Liddington, R. C., and Takada, Y. (1999) J. Biol. Chem. 274, 32108–32111
19. Irie, A., Askari, J. A., Asta, S., Yamada, K. M., and Takada, Y. (1997) Biochemistry 36, 14424–14431