The Amino-terminal One-third of α_{IIIb} Defines the Ligand Recognition Specificity of Integrin α_{IIIb}β_3*

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Integrins are heterodimeric adhesion receptors composed of noncovalently associated α and β subunits. The integrin superfam-ily consists of at least 20 members that are composed of differ-ent combinations of nine β and more than 15 α subunits. The different combinations of α and β subunits produce recep-tors that often possess a distinct ligand recognition specificity. With regard to integrin ligands, a number of discrete sites recognized by integrins have been identified and high resolu-tion structures have been obtained for a number of integ-ri

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§The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction; mAb, monoclonal antibody; CHO, Chinese hamster ovary.
against α1β1β2. The ligand mimic property of both mAbs is linked to the tripeptide sequence RYD within the third complementarity-determining region that appears to mimic the RGD recognition sequence (4, 5). The binding of both antibodies to α1β1β2 is blocked by adhesive protein and small competitive peptide ligands (39, 40). Neither antibody binds to ligand binding defective mutants of α1β1β2 (10). However, these two antibodies differ in that the binding of PAC1 is activation-dependent while the binding of OPG2 does not require prior receptor activation. Finally, ligand binding to these receptors can be assessed indirectly by the conformational changes reported by the exposure of LBS epitopes (41). Utilizing this integrin pair, we have defined the region of the α subunit that regulates recognition specificity for both activation-dependent and -independent ligands. We report here that neither the cation binding repeats nor the NH2 terminus alone is sufficient to control the ligand recognition specificity of this integrin pair. Ligand specificity requires both regions. A minimal sequence encompassing the amino-terminal one third of the α subunit was required to transfer ligand recognition specificity.

**Materials and Methods**

Monoclonal Antibodies and Reagents—Murine monoclonal anti-α1β1β2-specific antibodies (mAb) 4F10 and 2G12 were kindly provided by Dr. Virgil Woods (University of California, San Diego, CA). The α1β1β2 complex-specific mAbs AP2 (42) and OPG2 (40) were provided by Dr. Thomas J. Springer (The Scripps Research Institute, La Jolla, CA). The α1β1β2 complex-specific mAb 10E5 was provided by Dr. Barry Coller (State University of New York, Stonybrook, NY). The α1β1β2-specific mAb D57, the anti-β1 mAb 15, the αβ1 anti-LIBS1, and the anti-β1 mAb anti-LIB52 (IgG) have been previously described (30, 41, 43). mAb PAC1 (IgM) binds specifically to activated α1β1β2 (39) and was provided by Dr. Sanford Shattil (The Scripps Research Institute). The α1-specific mAb 3G8 was obtained from Chemicon (Temecula, CA, USA). The peptide mimetic Ro 43-5054 (38) was generously provided by Beat Steiner (Heinmer-LaRoche, Basel, Switzerland). The peptide mimetic SC52012 (37) was provided by Searle (Searle Research and Development, Skokie, IL). The synthetic peptides GRGDSP and KYPGHLG-GADQADGV (K16) were prepared by solid phase synthesis on an Applied Biosystems peptide synthesizer (Applied Biosystems, Foster City, CA) using phenylacetamidomethyl resins and t-butycarbonyl amino acids purchased from Applied Biosystems. Peptide purity was assessed by reverse phase high performance liquid chromatography and amino acid composition verified by fast atom bombardment mass spectrometry.

Generation of α1α1 Chimeric Subunits—Chimeric α1 subunits, which consisted of the backbone of α1 from which various portions were removed and replaced with the homologous regions of α111, were constructed utilizing standard techniques. cDNA clones encoding wild type α111, and α1, have been previously described (9, 45). Oligonucleotide-directed mutagenesis (46) was used to introduce three unique, silent restriction enzyme sites into the cDNA coding for the α1 subunit, resulting in the construct designated α1MNS. Nucleotide sequence numbering for α2 was according to the published sequence (47). The changes were as follows: bp 759–764, ACTCCG was changed to AcGcGct to introduce a MluI site; bp 1098–1103, GCTTCA was changed to GCTagc to introduce a Nhel site; and bp 1469–1474, TGGTGT was changed to aGcGct to introduce a Stul site. Each α1MNS chimeric α1 subunit was constructed utilizing the previously described protocol (49). The MluI fragment of α1MNS was replaced with a MluI/Stul fragment of α111 that contained cation binding repeats 1 through 4 of α111. This fragment was generated by PCR amplification utilizing the wild type α111 cDNA as template and oligonucleosomes that contained the corresponding restriction sites at their 5′ ends. The chimeric α12b(1–4C), which contained cation binding repeats 1 and 2 of α111, was constructed by digesting α1MNS with MluI and Nhel and ligation the corresponding MluI/Nhel α111 fragment generated by PCR. The chimeric α12b(2+3–4C), which contained cation binding repeats 2 and 3 of α111, was constructed by digesting α1MNS with AffIll (bp 911) and Sphl (bp 1232) and ligation the corresponding AffIll/Sphl α111 PCR fragment. The chimeric α12b(3–4C), which contained cation binding repeats 3 and 4 of α111, was constructed by digesting α1MNS with Nhel and Stul and ligation the corresponding Nhel/
only the amino-terminal region of \( \alpha_{IIIb} \). All of these chimeras were expressed on the cell surface as assayed by flow cytometry with the anti-\( \alpha_{v} \) mAb LM142 (Fig. 2). Moreover, the chimeras \( \alpha_{v2b}(L1-Q459) \), \( \alpha_{v2b}(L1-P334) \), and \( \alpha_{v2b}(L1-F223) \) expressed the \( \alpha_{IIIb}\beta_{3} \) complex-specific epitopes recognized by the mAbs AP2 and D57 (Table I). In contrast, the \( \alpha_{v2b}(R140-P334) \) chimera reacted very weakly with both AP2 and D57 mAbs.

Since several of the substitutions resulted in reactivity with \( \alpha_{IIIb}\beta_{3} \)-specific mAbs, the binding of the ligand mimetic mAb PAC1 was examined utilizing flow cytometry. The binding of mAb PAC1 is activation-dependent (39). Therefore, while resting \( \alpha_{IIIb}\beta_{3} \) exhibited low reactivity with mAb PAC1, activation with the mAb anti-LIBS2 significantly increased the binding of mAb PAC1 (Fig. 3). mAb anti-LIBS2 acts directly upon \( \alpha_{IIIb}\beta_{3} \), provoking high affinity ligand binding function (30). The binding of mAb PAC1 was specific since it was completely blocked by GRGDSP peptide. Similarly, cells expressing the chimeras \( \alpha_{v2b}(L1-Q459) \) or \( \alpha_{v2b}(L1-P334) \) specifically bound mAb PAC1 in the presence of activating mAb anti-LIBS2. In contrast, cells expressing wild type \( \alpha_{IIIb}\beta_{3} \) or \( \alpha_{v2b}(L1-F223)\beta_{3} \) or \( \alpha_{v2b}(L1-4C)\beta_{3} \) failed to bind mAb PAC1 after activation with the mAb anti-LIBS2. The lack of mAb PAC1 binding to these chimeras was not due to the failure of anti-LIBS2 to bind to the chimeric receptor as the epitope was present on each of these receptors as assayed by flow cytometry (data not shown). These data suggest that the chimeras \( \alpha_{v2b}(L1-Q459) \) and \( \alpha_{v2b}(L1-P334) \) have a ligand binding pocket very similar to \( \alpha_{IIIb}\beta_{3} \).

Interaction of these \( \alpha_{v/IIIb} \) chimeras with another ligand mimetic mAb, OPG2, was also examined by flow cytometry (Fig. 4). OPG2 inhibits the binding of adhesive proteins to \( \alpha_{IIIb}\beta_{3} \) and its binding is blocked by RGD peptides (40). However, unlike mAb PAC1, the binding of mAb OPG2 to \( \alpha_{IIIb}\beta_{3} \) is activation-independent. Cells expressing wild type \( \alpha_{IIIb}\beta_{3} \) stained brightly with mAb OPG2. Consistent with the results

![Fig. 1. Schematic representation of the \( \alpha_{v/IIIb} \) chimeric \( \alpha \) subunits. Each chimera consists of the backbone of \( \alpha_{v} \) (solid line) from which the indicated portions have been removed and replaced with the homologous region of \( \alpha_{IIIb} \) (shaded boxes). The location of the three silent restriction sites introduced into the wild type \( \alpha \) sequence to facilitate exchanges and the endogenous SphI site are indicated. Solid black rectangles indicate the position of the four divalent cation binding repeats present in each \( \alpha \) subunit. The position of \( \alpha_{IIIb} \) residues that delineate chimeras are indicated.](image)

![Fig. 2. Flow cytometric analysis of stably transfected cell lines expressing \( \alpha_{v/IIIb} \) chimeric \( \alpha \) subunits. CHO cells co-transfected with wild type \( \beta_{3} \) and the indicated \( \alpha \) subunit and were examined for receptor expression by flow cytometry. Cells transfected with wild type \( \alpha_{v} \) or the indicated \( \alpha_{v/IIIb} \) chimeric \( \alpha \) subunit were stained by indirect immunofluorescence with the anti-\( \alpha_{v} \) mAb LM142. Cells transfected with \( \alpha_{IIIb} \) were stained with the \( \alpha_{IIIb}\beta_{3} \)-specific mAb D57. Results are depicted as histograms with the log of the fluorescence intensity on the ordinate and the cell number on the abscissa.](image)

| Table I | Summary of monoclonal antibody reactivity with recombinant \( \beta_{3} \) integrins |
|---------|--------------------------------------------------|
| Receptor | 142 | 15 | 10E5 | D57 | AP2 | 4F10 | 2G12 | OPG2 | PAC1 | LIBS2 |
| \( \alpha_{IIIb}\beta_{3} \) | - | + | + | + | + | + | + | + | + | + |
| \( \alpha_{v}\beta_{3} \) | - | + | - | - | - | - | - | - | - | - |
| \( \alpha_{v2b}(1-4C)\beta_{3} \) | + | + | - | - | - | - | - | - | - | + |
| \( \alpha_{v2b}(1-2C)\beta_{3} \) | + | + | - | - | - | - | - | - | - | + |
| \( \alpha_{v2b}(2-3C)\beta_{3} \) | + | + | - | - | - | - | - | - | - | + |
| \( \alpha_{v2b}(3-4C)\beta_{3} \) | + | + | - | - | - | - | - | - | - | + |
| \( \alpha_{v2b}(1-4Q59)\beta_{3} \) | + | + | - | - | - | - | - | - | - | + |
| \( \alpha_{v2b}(1-4G59)\beta_{3} \) | + | + | ND | + | + | + | + | + | + | + |
| \( \alpha_{v2b}(1-3P34)\beta_{3} \) | + | + | ND | - | - | - | - | - | - | - |
| \( \alpha_{v2b}(R140-P334)\beta_{3} \) | + | + | ND | - | - | - | - | - | - | - |

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obtained with mAb PAC1, mAb OPG2 bound to cells expressing αv2b(L1-Q459)β3 or αv2b(L1-P334)β3. No specific mAb OPG2 staining was observed with cells expressing wild type αvβ3, αv2b(L1-F223)β3 or αv2b(1–4C)β3. The fact that neither mAb bound to the chimera αv2b(L1-F223) indicates that the NH₂-terminal region alone does not control ligand binding specificity.

The Divalent Cation Binding Repeats Alone Do Not Determine Ligand Binding Phenotype—Integrin α subunits contain seven homologous repeats, of which the last three or four ap-
binding repeats (2b1–4C, and 2b3–14C) were substituted together (2b1–4C) or consisting of two adjacent cation binding repeats of the divalent cation repeats to bind small activation-dependent ligands. While the chimeras v2b(1–2C), v2b(1–4C), and v2b(2–3C) all exhibited strong staining with the anti-LIBS1 mAb, none of these chimeras reacted with the activation-dependent ligand mimetic mAb. An exception was the complex-specific mAb OPG2 (Table I). MAb LM142, none of these chimeras reacted with the anti-LIBS1 mAb LM142. The immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis on 7% nonreducing acrylamide gels and detected by autoradiography. Lanes 1, immunoprecipitate of K16-eluted material; lanes 2, immunoprecipitate of subsequent EDTA-eluted material.

To determine the capacity of the chimeras containing substitutions of the divalent cation repeats to bind small activation-independent ligands specific for α1β3, we examined the capacity of the α1β3-selective peptidomimetic Ro 43-5054 (38) to increase the binding of mAb anti-LIBS1 by flow cytometry. Since the mAb anti-LIBS1 binds preferentially to the occupied conformation of the receptor (41), increased binding of mAb LIBS1 is evidence of receptor-ligand interaction. In the presence of Ro 43-5054, there was an increase in the binding of anti-LIBS1 to cells expressing the chimeras v2b(1–4C) even in the absence of ligand. This result suggested that these two chimeras possessed a structure that is slightly altered from that of the wild type receptors. Although the anti-LIBS1 epitope was exposed on these two chimeras, additional data (see below) indicate that their ability to bind ligand was not impaired.

To test whether the chimeric receptors containing substitutions of the cation binding repeats possessed an intact RGD ligand recognition function and to test their capacity to distinguish between the RGD and fibrinogen γ chain sequence, the ligand binding function of the recombinant receptors was analyzed by affinity chromatography (Fig. 6). Detergent lysates of radiolabeled, transfected cells were applied to an RGD affinity matrix. CHO cells stably expressing the wild type α1β3 or chimeric α1β10 receptors were radiolabeled and lysed, and the extract was applied on an GRGDSPK-Sepharose 4B column. After incubation and washing, the bound proteins were sequentially eluted with 1.5 mM K16, followed by 5 mM EDTA. The eluted fractions were immunoprecipitated with the anti-αv mAb LM142. The immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis on 7% nonreducing acrylamide gels and detected by autoradiography.

To determine the capacity of the chimeras containing substitutions of the divalent cation repeats to bind small activation-independent ligands specific for α1β3, we examined the capacity of the α1β3-selective peptidomimetic Ro 43-5054 (38, 51) to increase the binding of mAb anti-LIBS1 by flow cytometry. Since the mAb anti-LIBS1 binds preferentially to the occupied conformation of the receptor (41), increased binding of mAb LIBS1 is evidence of receptor-ligand interaction. In the presence of Ro 43-5054, there was an increase in the binding of anti-LIBS1 to cells expressing α1β3 but not to cells expressing α3β3 (Fig. 5). Similarly, Ro 43-5054 failed to stimulate the binding of mAb anti-LIBS1 to cells expressing the chimeras α1β2(2–3C) or α1β2(3–4C), indicating lack of binding to the receptor. Unexpectedly, mAb anti-LIBS1 bound maximally to cells expressing the chimeras α1β2(1–2C) or α1β2(1–4C) even in the absence of ligand. This result suggested that these two chimeras possessed a structure that is slightly altered from that of the wild type receptors. Although the anti-LIBS1 epitope was exposed on these two chimeras, additional data (see below) indicate that their ability to bind ligand was not impaired.

To test whether the chimeric receptors containing substitutions of the cation binding repeats possessed an intact RGD ligand recognition function and to test their capacity to distinguish between the RGD and fibrinogen γ chain sequence, the ligand binding function of the recombinant receptors was analyzed by affinity chromatography (Fig. 6). Detergent lysates of radiolabeled, transfected cells were applied to an RGD affinity matrix. CHO cells stably expressing the wild type α1β3 or chimeric α1β10 receptors were radiolabeled and lysed, and the extract was applied on an GRGDSPK-Sepharose 4B column. After incubation and washing, the bound proteins were sequentially eluted with 1.5 mM K16, followed by 5 mM EDTA. The eluted fractions were immunoprecipitated with the anti-αv mAb LM142. The immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis on 7% nonreducing acrylamide gels and detected by autoradiography.

Lanes 1, immunoprecipitate of K16-eluted material; lanes 2, immunoprecipitate of subsequent EDTA-eluted material.
**Fig. 7. Direct binding of an α11β3-selective peptidomimetic.** The binding of the α11β3-selective peptidomimetic SC52012 (37) to stably transfected cell lines expressing α11β2 or β3, or the indicated chimeric α1/a11β3 receptor was determined by incubating transfected cells with [3H]SC52012 (500 nM) at room temperature. After 40 min, bound ligand was separated from free ligand by centrifugation through 20% sucrose. The pellet associated counts were determined by liquid scintillation spectrometry. Background subtraction was measured in the presence of 5 mM EDTA. Shown are representative results of three separate assays. Results shown are mean ± S.D. of triplicates.

α2b(1–2C) (3.4%), α2b(2–3C) (7.5%), or α2b(3–4C) (13%) were poorly eluted by the K16 peptide from the RGD affinity matrix (Fig. 6). Each of these receptors bound to the RGD matrix and was readily eluted from the matrix by EDTA. Both wild type α11β2 and α1β3 receptors and all the chimeras were readily eluted from the affinity matrix by RGD peptide (data not shown). The fact that the chimeras α2b(1–4C) and α2b(1–2C) bound to the RGD affinity matrix and were specifically eluted by EDTA or RGD peptide indicates that the alteration in structure reported by anti-LIβS1 did not affect the ligand binding function of these receptors. These data show that all chimeras containing substitutions of the cation binding motifs can recognize the RGD sequence, but that substitution that all chimeras containing substitutions of the cation binding function of these receptors. These data show that all chimeras containing substitutions of the cation binding motifs can recognize the RGD sequence, but that substitution of the α1I b divalent cation binding regions with the corresponding regions from α11b was not sufficient to change the ligand binding specificity of α1β3 to that of α11β3.

**Direct Binding of an α11β3-selective Peptidomimetic to Chimeric Integrins**—To directly test the ability of the chimeras to bind small activation-independent ligands and further verify that the chimeric receptors α2b(L1-Q549)β3 and α2b(L1-P334)β3 had acquired the capacity to bind α11β3-specific ligands, we examined the binding of the peptidomimetic SC52012 to cells expressing chimeric receptors. SC52012 is a high affinity RGD mimetic that inhibits ADP-induced platelet aggregation with an IC50 of 42 nM (37). SC52012 is also highly selective for α11β2 versus α1β3 (Fig. 7). All of the cell lines were assayed by flow cytometry prior to the binding assay to confirm that all cell lines expressed similar numbers of receptors. Direct binding assays with [3H]SC52012 demonstrated specific binding to cells expressing α11β2 and the chimeras α2b(L1-Q549)β3 and α2b(L1-P334)β3. The number of molecules SC52012 bound to cells expressing α11β2 was within the number of receptors (138,000–440,000 sites/cell) previously determined for this cell line (30). No specific binding of [3H]SC52012 was observed to cells expressing α1β3 or to any of the other chimeras. This result confirms that the chimeras α2b(L1-Q549)β3 and α2b(L1-P334)β3 exhibit a ligand binding specificity identical to α11β3.

**DISCUSSION**

The major findings of the present study are as follows. 1) Ligand recognition specificity of β3 integrins is regulated by the amino-terminal one-third of the α subunit. Substitution of the amino-terminal portion of αv with the corresponding 334 amino acid residues of α11b switched the ligand recognition specificity of α1β3 to that of α11β3. This change in ligand specificity was observed with an activation-dependent ligand mimetic antibody, an activation-independent ligand mimetic antibody, and small activation-independent ligands. 2) Neither the amino-terminal region or the cation binding repeats alone is sufficient to control ligand specificity. Chimeras that omit the amino-terminal 140 residues or first two divalent cation binding repeats of α11b fail to change ligand specificity. Thus, the ligand binding pocket of α11β3 is a structure that contains elements of both the α and β subunits.

Previous studies have suggested that the regions that control ligand binding to α11β3 reside in the amino-terminal portion of α11β3, but the minimal structures identified in these studies encompassed more than one half of constituent subunits (28, 29). In the present study, we have mapped the regions that regulate ligand specificity to a smaller region of α11b. The chimera designated α2b(L1-Q549) contained the amino-terminal portion and all four divalent cation repeats of α11b and reacted with several α11β3 complex-specific mAbs. In addition, this chimera specifically bound small activation-independent α11β3-specific peptidomimetics and both activation-dependent (PAC1) and activation-independent (OPG2) ligand mimetic mAbs. The chimera α2b(L1-P334) retains the amino-terminal portion of α11b but contains only the first two divalent cation repeats of α11b. This chimera also exhibited a ligand binding phenotype consistent with that of α11β3 in that it bound specific peptidomimetics, the ligand mimetic mAbs PAC1 and OPG2, and several α11β3-specific mAbs. These results indicate that the ligand specificity of α11β3 can be reconstituted with the first 334 amino acid residues of α11b and does not require the third or fourth divalent cation repeats of α11b.

Chimeras that omit the 140 amino-terminal residues or the first two divalent cation motifs of α11b fail to change the ligand specificity of α1β3 to that of α11β3. The chimera αv2b(1–4C) contains a substitution of the entire divalent cation repeat region of αv with the corresponding region of α11b. This chimera was expressed on the cell surface and could bind ligand as demonstrated by its ability to bind to an RGD affinity matrix. However, this chimera was poorly displaced from the matrix by a fibrinogen γ chain peptide and did not bind the ligand mimetic mAbs PAC1 and OPG2 or an α11β3-specific peptidomimetic. These data indicate that substitution of the divalent cation repeats alone is not sufficient to change the ligand binding specificity. Similarly, the chimera α2b(R140-P334) did
not bind the ligand mimetic mAbs PAC1 and OPG2 or the \( \alpha_{11}\beta_3 \)-specific peptide mimetic. This chimera contains the first two divalent cation repeats of \( \alpha_2 \) but is missing the first 140 amino-terminal residues of mature \( \alpha_{11} \). This result suggests a requirement for residues near the amino terminus and indicates that an extended portion of the receptor is required for ligand specificity.

The approach of homolog-scanning mutagenesis (52, 53) is of general use for the identification of functional domains. A recent report used this technique to localize the putative ligand binding domains of \( \alpha_2 \) by mapping the epitopes for function blocking antibodies to the amino-terminal portion, but not the divalent cation repeats of \( \alpha_2 \) (19). In the present study, we demonstrated that the mAb AP2, which blocks ligand function (42), binds strongly to the chimera \( \alpha_{2b}(1-1-F-223) \). However, a ligand binding domain cannot be ascribed to this region since this chimera did not bind the ligand mimetic mAbs PAC1 and OPG2 and did not bind an \( \alpha_{11}\beta_3 \)-specific peptide mimetic. Thus, our results based on the interaction of true ligand mimetics demonstrates the inherent limitations of relying solely on the localization of the epitopes of function blocking mAbs to map ligand binding sites.

Previous studies have clearly demonstrated a role for the \( \beta \) subunit in ligand binding to \( \alpha_{11}\beta_3 \). Single amino acid substitutions in a highly conserved region of \( \beta_3 \) completely block the ligand binding function of \( \alpha_{11}\beta_3 \) (9, 10). This loss of ligand binding is not due to an effect on the activation state of the receptor as the mutations also block the activation-independent binding of mAb OPG2 and small ligand mimetics. However, our present results demonstrate that the specificity for the binding of PAC1, OPG2, and specific peptide mimetics to \( \alpha_{11}\beta_3 \) is controlled by the first 334 amino acid residues of the \( \alpha \) subunit. Together, these results indicate that ligand recognition requires cooperation between elements in both the \( \alpha \) and \( \beta \) subunits and indicates that the ligand binding pocket is a topographical structure that is assembled from regions of both the \( \alpha \) and \( \beta \) subunits.

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