Identification of Functional Segments within the \( \beta_2 \)-I-domain of Integrin \( \alpha_M \beta_2 \)*

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Yu-Mei Xiong‡, Thomas A. Haas§, and Li Zhang¶

From the ‡Department of Vascular Biology, American Red Cross Holland Laboratory, Rockville, Maryland 20855 and the §Department of Molecular Cardiology, Cleveland Clinic Foundation, Cleveland, Ohio 44195

The \( \alpha_M \beta_2 \) integrin plays an important role in leukocyte biology through its interactions with a diverse set of ligands. Efficient ligand binding requires the involvement of both the \( \alpha_M \) and \( \beta_2 \) subunits. Past ligand binding studies have focused mainly on the \( \alpha_M \) subunit, with the \( \beta_2 \) subunit being largely unexplored. Therefore, in this study we conducted homolog-scanning mutagenesis on the I-domain (residues 125–385) within the \( \beta_2 \) subunit. We identified four noncontiguous sequences (Arg\(^{144}\)-Lys\(^{148}\), Gln\(^{199}\)-Ala\(^{203}\), Leu\(^{225}\)-Leu\(^{228}\), and Gly\(^{305}\)-His\(^{309}\)) that are critical for fibrinogen and C3bi binding to \( \alpha_M \beta_2 \). Molecular modeling revealed that these four sequences reside within a narrow region on the surface of the \( \beta_2 \)-I-domain, in close proximity to three potential cation-binding sites. Among these sequences, Gln\(^{199}\)-Ala\(^{203}\), Leu\(^{225}\)-Leu\(^{228}\), and Gly\(^{305}\)-His\(^{309}\) are important for the binding of both ligands, whereas Arg\(^{144}\)-Lys\(^{148}\) is more critical for fibrinogen than for C3bi binding. These sequences within the \( \beta_2 \)-I-domain are directly involved in ligand binding, since 1) switching these segments to their corresponding \( \beta_1 \) sequences destroyed ligand binding; 2) loss of function was not due to a nonspecific gross conformational change, since the defective \( \alpha_M \beta_2 \) mutants reacted well with a panel of conformation-dependent mAbs; 3) mutation of these functional sequences did not affect Ca\(^{2+} \) binding; and 4) synthetic peptides corresponding to sequences Gln\(^{199}\)-Ala\(^{203}\) and Gly\(^{305}\)-His\(^{309}\) blocked ligand binding to \( \alpha_M \beta_2 \) and the peptides interact directly with fibrinogen and C3bi. Given the similarity among all integrin \( \beta \) subunits, our results may help us to understand the underlying mechanism of integrin-ligand interactions in general.

Integrins are heterodimeric cell surface receptors involved in diverse biological processes, including embryonic development, immune response, thrombosis and homeostasis, and wound healing. \( \alpha_M \beta_2 \) (mac-1, CR3, mo1, CD11b/CD18) is a member of the \( \beta_2 \) integrin subfamily, which includes \( \alpha_M \beta_2 \) (LFA-1, CD11a/CD18), \( \alpha_{M\beta_2} \) (p150,95, CD11c/CD18), and \( \alpha_M \beta_2 \). These leukocyte integrins are critical for the adhesion and transmigration of leukocytes through the endothelium, the activation of neutrophils and monocytes, the phagocytosis of foreign materials, and neutrophil apoptosis. \( \alpha_M \beta_2 \) recognizes a wide variety of protein and nonprotein ligands, including fibrinogen (Fg)\(^{1} \), intercellular adhesion molecule-1 (3), C3bi (4), zymosan (5), and neutrophil inhibitory factor (NIF), a specific \( \alpha_M \beta_2 \) antagonist isolated from canine hookworms (6).

Ligand binding sites within \( \alpha_M \beta_2 \) have been partially mapped to the \( \alpha_M \) subunit and, in particular, to its I-domain region (7–9). Recent studies from our laboratory and others’ have demonstrated the importance of the \( \beta_2 \) subunit for ligand binding, with the conserved DXSXS sequence (the MIDAS motif) within the \( \beta_2 \)-I-domain being identified as an essential motif (10, 11). Mutations in the MIDAS motif that destroyed cation binding to \( \beta_2 \) also resulted in a loss in ligand binding activity. To localize those sequences within the \( \beta_2 \)-I-domain that coordinate ligand binding, we recently performed homolog-scanning mutagenesis (12), which entailed switching 16 sequences that were 3–9 amino acids long within the \( \beta_2 \)-I-domain to their homologous sequences within the \( \beta_2 \)-I-domain. This approach was used because the \( \beta_2 \) and \( \beta_1 \)-I-domains are highly homologous (74%), but \( \beta_1 \) does not recognize Fg, NIF, or C3bi. When transfected together with wild-type \( \alpha_M \), all 16 \( \beta_2 \) mutants were expressed on the surface of the cell membrane as a heterodimer (13). We found that the region critical for ligand binding by \( \beta_2 \), Phe\(^{192}\)-Glu\(^{197}\), is dispensable for ligand binding by \( \beta_2 \) (14, 15, 16). Therefore, residues within \( \beta_2 \) and \( \beta_2 \) coordinating ligand binding are different.

In this study, we have screened the 16 existing \( \beta_2 \)-I-domain mutants and one new \( \beta_2 \)-I-domain mutant for ligand binding. We report here that four spatially proximal sequences within the \( \beta_2 \)-I-domain are directly involved in ligand binding by \( \alpha_M \beta_2 \), and usage of these sequences for Fg and C3bi binding is different. Our data demonstrated that both the \( \alpha_M \)-I-domain and the \( \beta_2 \)-I-domain are required for efficient ligand binding by the heterodimeric receptor. Therefore, the orientation of these two ligand binding sites within \( \alpha_M \beta_2 \) is critical and is a possible means by which the ligand binding activity of \( \alpha_M \beta_2 \) is modulated.

EXPERIMENTAL PROCEDURES

Materials—Human kidney 293 cells and the expression vector, pCIS2M, were gifts from Dr. F. J. Castellino (University of Notre Dame). The cDNAs of CD11b and CD18 were obtained from Dr. B. Karan-Tamir (Amgen, Thousand Oaks, CA); mAb HMH23 was obtained from Dako (Carpinteria, CA); IB4 and TS1/18 were from the ATCC (Manassas, VA); mAb 44 was from Sigma; CLB-LFA-1/1,54 (CLB54) was from RDI (Flanders, NJ); YFC118.3 and R3.3 from were maintained from Dako (Carpinteria, CA); H20A was from VMRD Inc. (Pullman, WA); and others’ have demonstrated the importance of the MIDAS motif that destroyed cation binding to \( \beta_2 \) also resulted in a loss in ligand binding activity. To localize those sequences within the \( \beta_2 \)-I-domain that coordinate ligand binding, we recently performed homolog-scanning mutagenesis (12), which entailed switching 16 sequences that were 3–9 amino acids long within the \( \beta_2 \)-I-domain to their homologous sequences within the \( \beta_2 \)-I-domain. This approach was used because the \( \beta_2 \) and \( \beta_1 \)-I-domains are highly homologous (74%), but \( \beta_1 \) does not recognize Fg, NIF, or C3bi. When transfected together with wild-type \( \alpha_M \), all 16 \( \beta_2 \) mutants were expressed on the surface of the cell membrane as a heterodimer (13). We found that the region critical for ligand binding by \( \beta_2 \), Phe\(^{192}\)-Glu\(^{197}\), is dispensable for ligand binding by \( \beta_2 \) (14, 15, 16). Therefore, residues within \( \beta_2 \) and \( \beta_2 \) coordinating ligand binding are different.

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† An established investigator of the American Heart Association. To whom correspondence should be addressed: Dept. of Vascular Biology, American Red Cross Holland Laboratory, 15601 Crabbs Branch Way, Rockville, MD 20855. Tel.: 301-738-0657; Fax: 301-738-0465; E-mail: zhout2@usa.redcross.org.
LISTNL segment in the β2I-domain to the corresponding sequence (SVSRNR) in the β1-domain. To obtain cell lines expressing the wild-type receptor, each mutant cell line was subcloned by cell sorting using an α2s-specific mAb 21D1. 185 to 20 colonies were picked and analyzed for integrin expression by FACS analysis. Cells expressing similar levels of receptor to those expressing wild-type α2β1 were selected and subcloned. To exclude the possibility of subcloning artifacts, all of our studies have been repeated using the original pool for every mutant.

**FACS Analysis**—A total of 10^6 cells expressing wild-type or mutant α2β1 in Hanks’ balanced salt solution (HBSS) containing 1 mM Mg^2+ and 1 mM Ca^2+ were incubated with 1 μg of mAb for 30 min at 4°C. A subtype-matched mouse IgG served as a control. After washing with PBS, cells were mixed with fluorescein isothiocyanate-labeled goat anti-mouse IgG (H + L) F(ab')_2 fragment (1:20 dilution) (Zymed Laboratory) and kept at 4°C for another 30 min. Cells were then washed with PBS and resuspended in 500 μl of DPBS. FACS analysis was performed using FACSScan (Becton-Dickinson), and fluorescent intensities were quantified using the FACScan program, and the values were used to compare α2β1 expression levels for the subclones of each mutant or the reactivity of different β2 mutants with various β2-specific mAbs.

**Solid Phase Binding Assays**—To test the interaction between the α2β1-domain and the α2β1 ligand Fg, 96-well microtiter plates (Immulon 4BX, Dynex Technologies Inc., Chantilly, VA) were coated with different synthetic peptides at 2 μg/ml overnight at 4°C and postcoated with 3% BSA for 2 h at room temperature. The Fg γ1 module (10 μg/ml) in DPBS was added to each well. After blocking with 400 μl of 0.05% polyvinylpyrrolidone in DPBS, a total of 2 × 10^4 cells in HBSS containing 1 mM Ca^2+ and 1 mM Mg^2+ in the presence or absence of 2 μg/ml synthetic mAbs or 20 μg/ml mAb 44a (against the αM subunit) were added to each well and incubated for 30°C at 15 min. Unbound cells were removed by washing three times with DPBS, and adherent cells were quantified by cell-associated acid phosphatase as described previously (11).

**Ligand Binding Assays for α2β1**—The ligand binding activity of the β2 mutants was assessed using two classic α2β1 ligands, C3bi and Fg, according to our published methods (11, 13). The adhesion of α2β1 expressing cells to Fg was assessed using the recombinant γ1-module, which is the principle binding site for α2β1 (14). 24-Well polystyrene plates were coated with the γ1-module (10 μg/ml). After blocking with 400 μl of 0.05% polyvinylpyrrolidone in DPBS, a total of 2 × 10^4 cells in HBSS containing 1 mM Ca^2+ and 1 mM Mg^2+ in the presence of 2 μg/ml synthetic mAbs or 20 μg/ml mAb 44a (against the αM subunit) were added to each well and incubated for 2 h at 4°C. After washing with PBS, bound γ1-module was detected using a sheep anti-mouse IgG conjugated to horseradish peroxidase, and the horseradish peroxidase substrate, 3,3’,5,5’-tetramethylbenzidine (KPL, Gaithersburg, MD). To examine the interaction between the β2I-domain peptides and C3bi, 80 μl of the synthetic peptides (2 mg/ml in DPBS) were coated onto 24-well plates at 4°C. After blocking with 1% BSA for 1 h at 22°C, biotinylated EC3bi in 300 μl of HBSS containing 1 mM Ca^2+ and 1 mM Mg^2+ were added. After incubating for 1 h at 37°C, nonadherent C3bi was removed by washing. The plate was then fixed with 50 μl of 3% glutaraldehyde and blocked with 2% BSA. C3bi binding was measured with a conjugate of avidin-alkaline phosphatase, and the amount of bound C3bi was determined by reaction with p-nitrophenyl phosphate, measuring the absorbance at 405 nm.

**RESULTS**

**Ligand Binding by the Homolog-scanning Mutants of α2β1**—Previously, using homolog-scanning mutagenesis, we systematically mutated residues that reside on the surface of the β1-domain and mapped the epitopes of several function-blocking mAbs. We demonstrated that all 16 β1-domain mutants, when transfected together with wild-type αM, were expressed at wild-type levels on the cell surface. In addition, five homolog-scanning mutants, which contain individual substitutions of the segments Leu^154–Glu^159, Pro^192–Glu^197, Asn^213–Glu^220, Glu^244–Asp^248, and His^254–Asn^258 that constitute the epitopes for nine function-blocking mAbs, were studied for ligand binding and found to bind to both αMβ2 substrates (Fg and C3bi) (13). Within the β2I-domain, 511SVSRNRDAPGGC^225 has been identified as a critical sequence for ligand recognition by β2 integrins (15). Most recently, crystallographic studies of the RGD-α2β1 complex have confirmed that the three β2 residues, 24RNR, were in direct contact with RGD (16). Therefore, to see if this sequence is also important in ligand binding by αMβ2, we constructed a new β2I-domain mutant by switching the corresponding sequence (522LISTNL^399) within the β2I-domain to its homolog (SVSRNR) within β2I (15), because this region is well conserved between β2 and β1. To access the functionality of these β2 mutants, we conducted a ligand binding experiment following our established assays (13). Two representative ligands of αMβ2, the γ1-module of Fg and C3bi, were chosen in this study, because they recognize overlapping but not identical binding sites within the receptor (11). As shown in Fig. 1A, most of the mutants, including the five mutants (αMβ2(Leu^154–Glu^159), αMβ2(Pro^192–Glu^197), αMβ2(Asn^213–Glu^220), αMβ2(Asp^248), and αMβ2(His^254–Asn^258)) that we studied earlier (13), supported cell adhesion to the γ1-module and exhibited similar adhesive activities to the wild-type receptor. Altogether, this group of mutants includes αMβ2(Leu^154–Glu^159), αMβ2(Leu^162–Glu^164), αMβ2(Asn^181–Asp^185), αMβ2(Pro^192–Glu^197), αMβ2(Asn^213–Glu^220), αMβ2(Pro^247–Glu^251), αMβ2(Ala^262–Asp^265), αMβ2(Asp^290–Glu^298), αMβ2(Ser^324–Thr^329), αMβ2(Thr^334–Ile^366), αMβ2(Thr^334–Glu^344–Asp^348), αMβ2(Asn^213–His^254–Asn^258), and αMβ2 (His^271–Lys^279), Two mutant cell lines, αMβ2 (Arg^144–Lys^148) and αMβ2 (Glu^305–His^309), had modest loss of cell adhesion to the γ1-module (~2.5-fold). The most dramatic changes in ligand binding (~10-fold different from the wild-type receptor) were observed for the following two β2I-domain mutants: αMβ2(Glu^399–Ala^403) and αMβ2(Leu^225–Leu^230). The specificity of ligand binding to each of the mutants that retained activity was verified by blocking experiments using an αM-specific mAb, 44a. Furthermore, we verified that the wild-type receptor, αMβ2(Leu^225–Leu^230), and the other 16 αMβ2 mutants had similar surface expression levels (~2-fold), as assessed by FACS analysis using αM-specific mAb 44a. In addition, to exclude possible clonal effects resulting in defective ligand binding, we repeated the above adhesion experiments using two additional independent clones of each defective mutant. These data suggest that the Fg binding pocket is composed of four segments (Arg^144–Lys^148, Glu^399–Ala^403, Leu^225–Leu^230, and Gly^305–His^309) within the β2I-domain.
FIG. 1. Ligand binding to the β\textsubscript{1}-domain homolog-scanning mutants. A, Fg adhesion. A total of 2 × 10\textsuperscript{5} α\textsubscript{3}β\textsubscript{2}-expressing cells were added to 24-well nontissue culture polystyrene plates, which were precoated with recombinant γ-module (10 μg/ml) and subsequently blocked with 0.05% polyvinylpyrrolidone in DPBS. After incubation at 37 °C for 20 min, the unbound cells were removed by three washes with DPBS, and the adherent cells were quantified by cell-associative acid phosphatase. The number of adherent cells expressing wild-type α\textsubscript{3}β\textsubscript{2} was taken as 1.0. Specificity was demonstrated by the addition of an α\textsubscript{3}β\textsubscript{2}-specific function-blocking mAb, 44a, *, a value of less than 1%. Data shown are the means ± S.D. of three independent experiments. B, C\textsubscript{3}bi binding. Biotinylated EC\textsubscript{3}bi (2 × 10\textsuperscript{5}) were added to 2 × 10\textsuperscript{5} cells expressing α\textsubscript{3}β\textsubscript{2}, which had been preseeded onto polylysine-coated 24-well plates. After 60 min at 37 °C, the number of bound EC\textsubscript{3}bi was determined using avidin-alkaline phosphatase and p-nitrophenyl phosphate, measuring the absorbance at 405 nm. The value for wild-type α\textsubscript{3}β\textsubscript{2} was taken as 1.0. Specificity was demonstrated by the addition of EDTA (1 mm), *; a value of less than 1%. Data are the means ± S.D. of 3–6 independent experiments. Note that the Fg adhesion and C\textsubscript{3}bi binding data for the six mutants α\textsubscript{3}β\textsubscript{2}(Arg\textsuperscript{144}–Lys\textsuperscript{148}), α\textsubscript{3}β\textsubscript{2}(Leu\textsuperscript{154}–Glu\textsuperscript{197}), α\textsubscript{3}β\textsubscript{2}(Pro\textsuperscript{192}–Glu\textsuperscript{197}), α\textsubscript{3}β\textsubscript{2}(Glu\textsuperscript{220}), α\textsubscript{3}β\textsubscript{2}(Glu\textsuperscript{220}–Asp\textsuperscript{227}), and α\textsubscript{3}β\textsubscript{2}(His\textsuperscript{254}–Asn\textsuperscript{259}) were taken from a previously published study (13).

Leu\textsuperscript{256}–Leu\textsuperscript{260} and Gly\textsuperscript{305}–His\textsuperscript{309}, with one exception; segment Arg\textsuperscript{144}–Lys\textsuperscript{148} is a critical sequence for α\textsubscript{3}β\textsubscript{2}-mediated cell adhesion to Fg but not for C\textsubscript{3}bi binding (13). Thus, the ligand binding sites with the β\textsubscript{1}-domain for Fg and C\textsubscript{3}bi are overlapping but not identical.

The β\textsubscript{1}-domain Mutants Exhibited Correct Conformations—To exclude the possibility that the defective ligand binding by the β\textsubscript{1}-domain mutants resulted from gross alterations of the structures of the mutant α\textsubscript{3}β\textsubscript{2} receptors, we conducted a number of experiments. First, as reported previously, all mutant α\textsubscript{3}β\textsubscript{2} receptors were expressed on the cell surface. Surface labeling and immunoprecipitation experiments conducted on cells expressing these α\textsubscript{3}β\textsubscript{2} mutants showed that all mutant α\textsubscript{3}β\textsubscript{2} had similar molecular sizes to that of the wild-type receptor (13). Second, the four defective mutants could be recognized by a panel of conformation-dependent β\textsubscript{2}-specific mAbs, including MEM48, 6.7, 7E4, TS1/18, and CLB54, judged by FACS analysis (13). In particular, the epitopes of TS1/18 and CLB54 are composed of at least three noncontiguous segments within the β\textsubscript{1}-domain and therefore could only recognize correctly folded α\textsubscript{3}β\textsubscript{2} (13, 17). Third, using mAbs (YFC 118.3 and CLB54) that depend on both a correct conformation and a functional Ca\textsuperscript{2+} binding site for their optimal recognitions of the β\textsubscript{1}-domain (13), we probed the integrity of the Ca\textsuperscript{2+}-binding site within the β\textsubscript{2} subunit for the above four defective mutants. Antibody binding to the four defective mutants was determined by FACS analysis in the presence of different concentrations of Ca\textsuperscript{2+}. Since mutation of segment Arg\textsuperscript{144}–Lys\textsuperscript{148} destroyed the epitope for mAb YFC118.3 (13), we used mAb CLB54 for evaluating the Ca\textsuperscript{2+} binding affinity of mutant α\textsubscript{3}β\textsubscript{2}(Arg\textsuperscript{144}–Lys\textsuperscript{148}). Like mAb YFC118.3, mAb CLB54 recog-
**DISCUSSION**

In this work, we have studied the ligand binding site within the \( \beta_2 \) domain using the homolog-scanning mutagenesis approach. We report three major findings. 1) Fg and C3bi recognize overlapping but nonidentical binding sites within the \( \beta_2 \) domain. The ligand binding site is composed of four noncontiguous sequences: Arg\(^{144}\)-Lys\(^{148}\), Gln\(^{199}\)-Ala\(^{203}\), peptide-Fg interaction, we coated the \( \beta_2 \) peptides onto 96-well microtiter plates, and the binding of the Fg \( \gamma \)-module to the peptides was assessed. As shown in Fig. 4A, only peptide P6 bound the \( \gamma \) module effectively. The other three peptides as well as a scrambled control peptide (sP6) did not bind the \( \gamma \) module. To see whether the synthetic peptides could directly bind C3bi, we coated these \( \beta_2 \) peptides onto microtiter plates. Binding of the biotinylated EC3bi to these peptides was then determined. As shown in Fig. 4B, two peptides, P6 and P11, bound C3bi strongly, whereas peptides P1 and P17 had no detectable binding. Similarly, all controls were negative.

**REFERENCES**

1. [Reference 1]
2. [Reference 2]
3. [Reference 3]
The ligand binding sites within the β1-domain were identified initially using a homolog-scanning mutagenesis approach (12), which we successfully used in our previous studies (14, 18, 19). This approach is based on the sequence similarity (74%) but functional disparity between the β1- and β2-integrins. To preserve the gross structure of the β1-domain, none of the mutations in this study involved residues that either played an essential role in protein folding, or provided a coordination site for cations, such as Asp134, Ser136, Ser138, and Glu234 or Asp264 (equivalent to Thr209 and Asp242 of the α2I-domain, respectively) (20). Our earlier studies strongly demonstrated that the homolog-scanning mutations, including those that destroy ligand binding, did not significantly change the overall structure of the intact receptor (14, 18, 19). Therefore, the loss of function was attributed to perturbations in the ligand recognition site. Indeed, the ligand binding sites we identified earlier within the α2I-domain fit very well with the data obtained from the crystal structure of the collagen-α2I-domain complex (the α2I-domain is a homolog of the α5I-domain) (21). Given such a success and the similarity between the I-domains of the integrin α and β subunits (20, 22), we conducted similar homolog-scanning mutagenesis on the β1-domain by substituting the sequences of β2 with their homologous counterparts of β1 (13). A total of 17 homolog mutants were constructed for this study, and four of these mutations had significant effects on ligand binding, suggesting that the ligand binding site is composed of these four segments. This conclusion is supported by the following observations: 1) all negative α4β2 mutants could be expressed well on the cell surface as correct heterodimers and were recognized by a panel of conformation-dependent mAbs (13); 2) the four defective mutants still possessed an intact and high affinity Ca2+-binding site located within the β2 subunit (Fig. 2); 3) one peptide (P6), located within the identified ligand binding site, could effectively compete with the intact α4β2 receptor for ligand binding; and 4) when immobilized on the plastic surface, peptides P6 and P11 could directly bind the γ-module and C3bi. Although peptides P1 and P17 did not exhibit detectable ligand binding activity, the negative results do not exclude a role for these peptides in ligand binding. The immobilized peptides may simply not adopt the appropriate conformation for recognition by the ligands. The ligand binding site we determined in this work agrees well with the observation by Goodman et al. (17) that residues Asp235 and Glu235, which reside within the identified ligand binding site, are critical to Fg and C3bi binding by α4β2.

To locate these four functional segments within the β1-domain, we modeled its three-dimensional structure based on the recently published crystal coordinates of integrin α5β1 (16). As shown in Fig. 5, the four segments (Arg144–Lys148, Gln199–Ala203, Leu225–Leu230, and Gly305–His309) reside in the same region on the apex of the β1-domain, supported by the underlying β-strands. The proximity of these identified segments in the three-dimensional structure is consistent with a role of these sequences in ligand recognition. Furthermore, this identified binding site contains the conserved DXXSXS sequence and the predicted Mg2+ and Ca2+-binding sites, which are essential to α5β2-ligand interactions (23). Based on the data from our earlier study (13) and from the recently published crystal structure of the RGD-α5β2 complex (16), the principle function of the DXXSXS sequence is likely to provide a scaffold for high affinity binding of divalent cations to the β1-domain. These bound cations, in turn, help to maintain correct conformations of the β1-domain (13), and at the same time, they also contribute directly to ligand binding by contacting with the acidic residues of the ligand (16). Thus, the location of our identified ligand binding site agrees well with the critical roles of the DXXSXS sequence and these divalent ions in ligand recognition.

Our results suggest that sequences Arg144–Lys148, Gln199–
Ala^{203}, Leu^{225}–Leu^{230}, and Gly^{305}–His^{309} contribute to the formation of ligand binding sites within the $\beta_2$-I-domain. Interestingly, this identified ligand binding site resembles very much the ligand binding pocket within the $\alpha_M$-domain, in that both ligand binding pockets encompass a broad region within their respective I-domains, containing the conserved DXXXS sequence and one or more cation binding sites (Fig. 5) (18, 19). In particular, we have reported that NIF (an $\alpha_M$-$\beta_2$-specific ligand) and C3bi recognize overlapping but nonidentical binding sites with the $\beta_2$-I-domain (11). As shown in Fig. 5F, the region shared by these two ligands is composed of three segments (Pro^{147}–Arg^{152}, Pro^{201}–Lys^{217}, and Asp^{248}–Arg^{261}), whereas residue Lys^{245} is recognized differentially by C3bi and NIF (18, 19). Although the Fg binding site within the $\alpha_M$-domain has not been fully delineated, our earlier studies show that it overlaps with the NIF and C3bi binding sites and contains yet another segment (261-RLENTI) that resides outside the identified NIF and C3bi binding pockets (11), indicating that the Fg binding site occupies an even broader region than those of NIF and C3bi. Similar results were obtained for the $\beta_2$-I-domain, where Fg and C3bi recognize an overlapping region (Gln^{199}–Ala^{203}, Leu^{225}–Leu^{230}, and Glu^{305}–His^{309}) and a differentially recognized region (Arg^{144}–Lys^{146}). Finally, the ligand binding site we identified here for the $\beta_2$-I-domain agrees well with the recently determined ligand binding site within the $\beta_2$-I-domain, containing residues Tyr^{225} and 241-RNR^{216} (16), which are equivalent to residues located within the $\beta_2$ peptides P1 and P17, respectively. Interestingly, compared with the $\beta_2$-I-domain, the $\beta_2$-I-domain appears to utilize a much broader region for binding of its ligands, which may reflect the different requirements for recognition of protein versus peptide ligands or different mechanisms for ligand recognition by the $\beta_2$ versus $\beta_3$ integrins.

Our finding that both $\alpha_M$ and $\beta_2$ are involved directly in ligand recognition also implies that the proper alignment of the two binding domains will be critical to the formation of a high affinity ligand binding site within the $\alpha_M$-$\beta_2$ heterodimer. Therefore, the $\beta_2$ subunit may also play a regulatory role in ligand binding. In support of such a notion, we have reported that recognition of Candida albicans by $\alpha_M$-$\beta_2$ is mediated mainly by the $\alpha_M$ subunit, and the $\beta_2$ subunit influences ligand binding by modulating the activity of $\alpha_M$ (24). Similarly, it was reported that the $\beta_2$ subunit plays an indirect regulatory role in ligand binding by $\alpha_M$-$\beta_2$ (25). In addition, it was proposed recently that the $\beta_2$-I-domain may actually recognize the $\alpha_M$ subunit as a ligand and thereby controls ligand binding by $\alpha_M$-$\beta_2$ (26). Altogether, these data suggest that the $\alpha_M$ and $\alpha_I$-I-domains provide major ligand contact sites, whereas the $\beta_2$ subunit contributes directly and/or indirectly to ligand binding, depending on the nature of the individual ligands.

In summary, using a combination of different approaches, we have demonstrated that the $\beta_2$ subunit contributes directly to ligand binding by $\alpha_M$-$\beta_2$. The recognition sites for the two representative ligands (Fg and C3bi) reside in a narrow region, composed of four segments, Arg^{144}–Lys^{146}, Gln^{199}–Ala^{203}, Leu^{225}–Leu^{230}, and Gly^{305}–His^{309} on the apex of the $\beta_2$-I-domain, with the first segment playing a more prominent role in Fg binding than in C3bi binding. The three potential cation binding sites (Mg^{2+} and Ca^{2+}) within the $\beta_2$-I-domain are located inside this identified region, suggesting that these three ions may contribute directly to ligand binding by the $\alpha_M$-$\beta_2$ receptor. Since both the $\alpha_M$ and $\beta_2$ subunit contribute directly to ligand recognition, alignment of the two binding domains within the heterodimeric $\alpha_M$-$\beta_2$ receptor will be critical to the formation of a proper ligand binding pocket, thus providing a potential mechanism for modulation of integrin activities. Given the similarity among all integrin $\beta$ subunits, our results may help us to understand the underlying mechanism of integrin-ligand interactions in general.

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REFERENCES

1. Springer, T. A. (1995) Annu. Rev. Physiol. 57, 827–872
2. Altieri, D. C., Bader, R., Mannucci, P. M., and Edgington, T. S. (1988) J. Cell Biol. 107, 1893–1900
3. Diamond, M. S., Staumton, D. E., de Fougerolles, A. R., Stackar, S. A., Garcia-Aguilar, J., Hibbs, M. L., and Springer, T. A. (1990) J. Cell Biol. 111, 3129–3139
4. Wright, S. D., Rao, P. E., Van Voorhis, W. C., Craigmyle, L. S., Iida, K., Talle, M. A., Westerg, E. F., Golde, G., and Silverstein, S. C. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5699–5703
5. Ross, G. D., Cain, J. A., and Lachmann, P. J. (1985) J. Immunol. 134, 3307–3315
6. Moyle, M., Foster, D. L., McGrath, D. E., Brown, S. M., Larson, Y., De Meutter, J., Bogowitz, C. A., Fried, V. A., and Ely, J. A. (1994) J. Biol. Chem. 269, 10008–10015
7. Diamond, M. S., Garcia-Aguilar, J., Rockford, K. J., Corbi, A. L., and Springer, T. A. (1993) J. Cell Biol. 120, 1031–1043
8. Michishita, M., Viden, V., and Arnaout, M. A. (1993) Cell 72, 857–867
9. Muschowski, P.-J., Zhang, L., Chang, E. R., Soile, H. R., Plow, E. F., and Moyle, M. (1994) J. Biol. Chem. 269, 26419–26423
10. Bajt, M. L., Goodman, T. G., and McGuire, S. L. (1995) J. Biol. Chem. 270, 94–98
11. Zhang, L., and Plow, E. F. (1996) J. Biol. Chem. 271, 18211–18216
12. Cunningham, B. C., Chaturi, P., Ng, P., and Wells, J. A. (1989) Science 243, 1330–1336
13. Xiong, Y. M., and Zhang, L. (2001) J. Biol. Chem. 276, 19340–19349
14. Ugarova, T. P., Soloviev, D. A., Zhang, L., Kuzkinov, D. I., Yee, V. C., Medved, L. V., and Plow, E. F. (1998) J. Biol. Chem. 273, 23519–23527
15. Bajt, M. L., Ginsberg, M. H., Fredinger, A. L., III, Bertans, M. C., and Lofus, J. C. (1992) J. Biol. Chem. 267, 3738–3794
16. Xiong, J. P., Stehle, T., Zhang, L., Joachimiak, A., Frech, M., Goodman, S. L., and Arnaout, M. A. (2002) Science 296, 151–155
17. Goodman, T. G., and Bajt, M. L. (1996) J. Biol. Chem. 271, 23729–23736
18. Zhang, L., and Plow, E. F. (1997) J. Biol. Chem. 272, 17558–17564
19. Zhang, L., and Plow, E. F. (1999) Biochemistry 38, 8964–8971
20. Xiong, J. P., Stehle, T., Dienßenbach, B., Zhang, R., Dunker, R., Scott, D. L., Joachimiak, A., Goodman, S. L., and Arnaout, M. A. (2001) Science 294, 329–345
21. Emesley, J., Knight, C. G., Fandralle, R. W., Barnes, M. J., and Liddington, R. C. (2000) Cell 101, 47–56
22. Lee, C. J., Rieu, P., Bajt, M. L., Goodman, T. G., and Lu, C. (1998) Annu. Rev. Physiol. 57, 631–638
23. Plow, E. F., Haas, T. A., Zhang, L., Loftus, J. C., and Smith, J. W. (2000) J. Biol. Chem. 275, 21785–21788
24. Parry, C. B., Plow, E. F., and Zhang, L. (1998) J. Immunol. 161, 6198–6205
25. Lu, C., Shimaoka, M., Zang, Q., Takagi, J., and Springer, T. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2393–2398
26. Alonso, J. L., Essah, M., Xiong, J. P., Stehle, T., and Arnaout, M. A. (2002) Curr. Biol. 12, R340–R342
