The Fourth Blade within the β-Propeller Is Involved Specifically in C3bi Recognition by Integrin αMβ2*

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Interactions between the complement degradation product C3bi and leukocyte integrin αMβ2 are critical to phagocytosis of opsonized particles in host defense against foreign pathogens and certain malignant cells. Previous studies have mapped critical residues for C3bi binding to the I-domains of the αM and the β2 subunits. However, the role of the αM β-propeller in ligand binding remains less well defined, and the functional residues are still unknown. In the present study, we studied the function of the αM β-propeller in specific ligand recognition by αMβ2 using a number of different approaches, and we report four major findings. 1) Substitution of five individual segments (Asp398–Ala406, Leu412–Leu413, Tyr426–Met427, Phe435–Glu436, and Ser443–Thr451) within the W4 blade of the β-propeller with their homologous counterparts in integrin β2 subunit, whereas substitution of eight other segments outside this blade had no effect. 2) These five mutants defective in C3bi binding supported strong αMβ2-mediated and cation-dependent cell adhesion to fibrinogen, suggesting that the conformations of these five defective mutants were intact. 3) Polyclonal antibodies recognizing sequences within the W4 blade significantly blocked C3bi binding by wild-type αMβ2. 4) A synthetic peptide corresponding to Gln434–Gly440 within W4 interacted directly with C3bi. In conclusion, our data demonstrate that the W4 blade (residues Asp398 to Thr451) is involved directly with C3bi. In the present study, we studied the function of the αM β-propeller in specific ligand recognition by αMβ2 using a number of different approaches, and we report four major findings. 1) Substitution of five individual segments (Asp398–Ala406, Leu412–Leu413, Tyr426–Met427, Phe435–Glu436, and Ser443–Thr451) within the W4 blade of the β-propeller with their homologous counterparts in integrin β2 subunit, whereas substitution of eight other segments outside this blade had no effect. 2) These five mutants defective in C3bi binding supported strong αMβ2-mediated and cation-dependent cell adhesion to fibrinogen, suggesting that the conformations of these five defective mutants were intact. 3) Polyclonal antibodies recognizing sequences within the W4 blade significantly blocked C3bi binding by wild-type αMβ2. 4) A synthetic peptide corresponding to Gln434–Gly440 within W4 interacted directly with C3bi. In conclusion, our data demonstrate that the W4 blade (residues Asp398 to Thr451) is involved specifically in C3bi but not fibrinogen binding to αMβ2. Altogether, our study supports a model in which three separate domains of αMβ2 (the I-domain, the αM β-propeller, and the βI-domain) function together and contribute to the formation of the C3bi-binding site.

C3bi, a degradation product derived from complement activation, is the major opsonin facilitating phagocytosis of foreign pathogens by leukocytes. Recognition of C3bi by these cells is mediated by the C3bi receptor, which is expressed on neutrophils and macrophages. A prominent integrin expressed on neutrophils and macrophages (1) is αMβ2, along with αLβ1 (LFA-1, CD11a/CD18, αLβ2 (p150, 95, CD11c/CD18) and αMβ2, constitutes the β2 integrin subfamily (2, 3). The physiological functions of αMβ2 include roles in adhesion and transmigration of leukocytes through endothelium (4), activation of neutrophils and monocytes (5), and phagocytosis of foreign materials as well as neutrophil apoptosis (6). Genetic deletion of the αM subunit in mice leads to defective C3bi-dependent phagocytosis and increased bacterial infections (6, 7). The importance of the β2 integrin subfamily in general human physiology is underscored by the severe phenotype of individuals with congenital deficiencies of these integrins (8).

Like other integrins, αMβ2 is notorious in its ability to recognize multiple structurally unrelated ligands, including fibrinogen (Fg) (9), ICAM-1 (10), C3bi (1), and neutrophil inhibitory factor (NIF), a specific αMβ2 antagonist isolated from canine hookworms (11). Studies from our laboratory and others suggested that the underlying molecular mechanism for such broad ligand specificity of the receptor is several overlapping but not identical binding pockets within αMβ2. For example, both C3bi and Fg recognize an inserted region of 200 amino acids in the αM subunit termed the I-domain (I-domain) (12), but different residues are involved (13–15). Additionally, ligand binding by αMβ2 is mediated by other regions including the β-propeller region of αM (16, 17) and the I-domain region (residues 125–385) of β2 (18, 19).

In the past few years, we have used homolog-scanning mutagenesis (20) to map the ligand-binding sites in αMβ2 (13, 14, 21). The ligand-binding sites identified have been confirmed using a number of different complementary approaches, including gain-in-function mutations, synthetic peptides, and epitope mapping studies of function-blocking mAbs. Importantly, the recently published crystal structure of the collagen-αI-domain complex (the αI-domain has 47% homology with the αI-domain) agrees well with our results (22). Therefore, we chose to employ homolog-scanning mutagenesis in this study to identify critical regions within the αM β-propeller for C3bi binding. We report here that the W4 blade, containing residues Asp398 to Thr451, is involved specifically in C3bi but not Fg binding. Combined with our published studies on the C3bi-binding site within the other two domains of the αMβ2 receptor, we propose a working model where three individual domains (the I-domain, the αM β-propeller, and the βI-domain) reside proximally in space and contribute to the formation of a single functional C3bi-binding site.

EXPERIMENTAL PROCEDURES

Materials—Human kidney 293 cells and the expression vector, pCDNA3M, were gifts from Dr. F. J. Castellino (Notre Dame, IN). The cDNAs of αM and β2 were obtained from Dr. B. Karan-Tamir (Amgen, Thousand Oaks, CA), Dr. C. J. Lennartz (Johns Hopkins University, Baltimore, MD), Dr. J. L. Wingfield (University of Virginia, Charlottesville, VA), and the American Red Cross, Rockville, Maryland 20855.

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1 The abbreviations used are: Fg, fibrinogen; DPBS, Dulbecco’s phosphate buffered saline; EC3bi, C3bi-coated sheep erythrocytes; FACS, fluorescence activated cell sorting; HBSS, Hanks’ balanced salt solution; mAb, monoclonal antibody; NIF, neutrophil inhibitory factor; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin.
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RESULTS

Contribution of the αM β-Propeller to C3bi Binding—Previously, we showed that switching the β-propeller and the stalk region between αM and α1 (αMβ2 does not interact with C3bi) had no effect on C3bi binding (13), suggesting that the functional residues within the β-propellers of αM and α1 are well conserved. Therefore, we chose another I-domain containing integrin α subunit (αJ) in this study, which is less homologous to αM. The αM and αJ β-propellers have 52% homology, yet the αJ subunit does not recognize C3bii as its ligand. Because the αM I-domain plays a major role in ligand binding, we tested whether its ligand binding property could be affected by other domains of the receptor, including the β-propeller. Thus, we placed the αJ I-domain in the context of α1, by substituting the αJ I-domain with the α1 I-domain, and we expressed the chimeric receptor α1/αJβ2 on Chinese hamster ovary cells. FACS analyses showed that the chimeric α1/αJβ2 receptor was well expressed (Fig. 1A); it reacted with a mAb (31H4) that recognizes the αJ β-propeller (Fig. 1A, e) but not with a mAb (P1E6) that recognizes the α1 I-domain (Fig. 1A, d). The presence of the αM I-domain within α1/αJβ2 was confirmed by its reactivity toward the αM-specific mAb 44a (Fig. 1A, f), whose epitope is located within the αM I-domain (12, 13). As a control, α1β2 reacted with both αM-specific mAbs (31H4 and P1E6) but did not bind the αM-specific mAb 44a (Fig. 1A, a–c), whereas αJβ2 reacted with mAb 44a but not with mAbs 31H4 or P1E6 (data not shown). To test if the expressed chimeric receptor is functional, we incubated the Chinese hamster ovary cells expressing either wild-type αM β2 or the chimeric α1/αJβ2 receptor with biotinylated NIF, which recognizes specifically αM β2 (11, 24), and we then detected bound NIF with avidin-FITC by FACS analysis. As shown in Fig. 1B, wild-type αM β2 did not interact with biotinylated NIF significantly. However, insertion of the αJ I-domain in α1 conferred αJ β2 the ability to bind NIF. Compared with wild-type αM β2, NIF binding to α1/αJβ2 was increased 7-fold (mean fluorescence intensity 4 versus 28), demonstrating that the αJ I-domain, when placed in the context of the αM β-propeller, is still functional. Verifying specificity of the FACS analysis, NIF binding to either αM β2 or αJ/ αM β2 could be blocked by addition of 1 mM EDTA or 50-fold excess of non-labeled NIF (data not shown).

To see if the chimeric receptor α1/αJβ2 is capable of binding another αM β2 ligand C3bi, we carried out the C3bi binding assays with α1β1, α1/αJβ1, and αJβ2. As shown in Fig. 1C, whereas αJβ2 bound C3bi strongly, neither α1β1 nor αJ/αMβ1 had detectable C3bi binding, suggesting that 1) the αJ β-propeller did not support C3bi binding, and/or 2) efficient C3bi binding required the β2 subunit as well.

Homolog-scanning Mutagenesis of the αM β-Propeller—To localize functional residues within the β-propeller, we carried out homolog-scanning mutagenesis using our established methods (21). We focused our attention primarily on four individual blades (W4 to W7) of the αM β-propeller, which have 52% homology, yet the αJ β-propeller was well expressed (Fig. 1A, e). To obtain cell lines that express equivalent receptor numbers, we used homology. After 90 min at 37 °C, cells were washed twice in HBSS, containing 5 mM HEPES and 1 mM MgCl2, which have 52% homology. After incubation at 37 °C of incubation at 37 °C, the absorbance at 405 nm was determined.

Site-directed Mutagenesis and Establishment of Stable Cell Lines—The detailed procedures for homolog-scanning mutagenesis and establishment of stable cell lines expressing wild-type and mutation αβ2-expressing cells were seeded onto polylysine (50 μg/ml/96-well plate) and human kidney 293 cells have been published (21). Similar procedures were used to generate 13 αM β-propeller mutants that contain individual switches between the αM and αJ β-propellers, which have 52% homology. To obtain cell lines that express equivalent receptor numbers as wild-type αM β2, each mutant cell line was subcloned by cell sorting using αM-specific mAb 44a. Up to 20 colonies were picked and analyzed for C3bi binding by FACS. Cells expressing similar levels of receptor to those expressing wild-type αM β2 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.

Ligand Binding to αβ2-expressing Cells—C3bi binding was performed with slight modification of the method of Bilandsk (23). Sheep erythrocytes coated with C3bi (EC3bi) were prepared using anti-sheep erythrocyte IgM antibody M1/87 and human C5-deficient serum (Sigma). Briefly, 7 × 109 sheep erythrocytes (Colorado Serum Company, Denver, CO) were washed twice in HBSS, containing 5 mM HEPES and 1 mM MgCl2, and coated with IgM as described (24). The coated erythrocytes were surface-labeled with biotin using 1 mg of sulfosuccinimidyl-6-(biotinamido) hexafluoropropane (Pierce) at 37 °C for 20 min. The biotinylated erythrocytes were suspended in 0.9 mM HBSS with 5 mM HEPES, 1 mM CaCl2, and 1 mM MgCl2, mixed with 100 μl of C5-deficient serum, and incubated at 37 °C for 60 min. After washing twice, the resulting EC3bi was resuspended in 2 ml of the above solution.

To perform the C3bi binding assays, a total of 2 × 105 αM β2-expressing cells were seeded onto polylals (50 μg/ml)/24-well non-tissue culture polystyrene plates (BD Biosciences) for 15 min at 37 °C, followed by addition of 2 × 105 EC3bi. After 60 min at 37 °C, unbound EC3bi were removed by washing with PBS. Bound EC3bi were fixed with 2% paraformaldehyde overnight, and excess paraformaldehyde was neutralized with 1% BSA at 37 °C for 2 h. Bound EC3bi were quantitated by addition of 300 μl of avidin-alkaline phosphatase conjugate (10,000 dilution) (Zymed Laboratories Inc., San Francisco, CA). After 90 min at 37 °C, the plates were washed three times with PBS, and 250 μl of 3 mg/ml p-nitrophenyl phosphate was added. After 15 min of incubation at 37 °C, the absorbance at 405 nm was determined.

Cell adhesion to the Fg γ-module was carried out as described previously (13, 14, 21). A total of 2 × 105 αM β2-expressing cells were added to 24-well non-tissue culture polystyrene plates, which were pre-coated with the 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 quantitated by cell-associated acid phosphatase activity.

Solid Phase Binding Assay—To test interactions between identified sequences of the αM β-propeller and the αJ β2 ligand C3bi, 100 μl of the synthetic peptides at different concentrations (0–5 mM in DPBS) were coated onto the center of a 24-well plate overnight at 4 °C. The efficiency of peptide coating was determined by labeling the N-terminal free sulphydryl (–SH) group within each peptide, using the EZ-link PEIO-maleimide-activated biotin kit (Pierce), based on the product instruction. After washing with PBS and blocking with BSA, the amount of the immobilized biotin group within each well was determined using an avidin-alkaline phosphate conjugate and p-nitrophenyl phosphate as the substrate and measuring the absorption at 405 nm. For C3bi binding, the peptide-coated plate was blocked with 1% BSA for 1 h at 22 °C, and then biotinylated EC3bi in 300 μl of HBSS containing 1 mM CaCl2 and 1 mM MgCl2 was added. After incubating for 1 h at 37 °C, non-adsorbed biomolecules were removed by washing. The plate was then blocked with 2% paraformaldehyde and blocked with 2% BSA. C3bi binding was measured with a conjugate of avidin-alkaline phosphatase as described above and measuring the absorbance at 405 nm.

FACS Analysis—A total of 1 × 106 cells in HBSS containing 1 mM MgCl2 were incubated with 5 μg of mAb for 30 min at 4 °C. A subtype-matched mAb IgG (as a control) was then washed with PBS, cells were mixed with FITC-goat anti-mouse IgG(Fc' )/2 fragment (Zymed Laboratories Inc.), kept at 4 °C for another 30 min, washed with PBS, and then resuspended in 500 μl of PBS. FACS analysis was then performed using FACSScan (BD Biosciences), counting 10,000 events. Mean fluorescence intensities were quantitated using the FACScan program, and the values were used to compare αM β2 expression levels and reactivity of the cells with various mAbs.
our previously published methods. Altogether, a total of 13 mutants was constructed, and their corresponding stable cell lines were established. We found that all 13 mutants could be well expressed on the cell surface and could react with both an \(\alpha_2\)-specific mAb (44a) and a \(\beta_2\)-specific mAb (IB4). In addition, we have used a panel of conformation-dependent mAbs to probe the folding of these \(\alpha_2\)-propeller mutants, and we found that reactivity toward these mAbs was preserved for all the 13 mutants (data not shown), suggesting that substitution of these individual sequences within \(\alpha_2\) did not significantly affect the gross structure of the \(\alpha_2\beta_2\) receptor. Furthermore, surface labeling and immunoprecipitation experiments using mAb 44a showed that all 13 mutants formed correct heterodimers with \(\beta_2\) on the cell surface and exhibited expected molecular weights as judged by SDS-PAGE (Fig. 2B).

**A Critical Role of the W4 Blade in C3bi Binding to \(\alpha_2\beta_2\)** — To evaluate the impact of the homolog-scanning mutations of the \(\alpha_2\)-propeller on ligand binding function of \(\alpha_2\beta_2\), we con-

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**Fig. 1. Ligand binding to the chimeric \(\alpha_2(\alpha_4)\beta_1\) integrin receptor.** A, surface expression of the chimeric receptor. The wild-type \(\alpha_2\beta_1\) and chimeric \(\alpha_2(\alpha_4)\beta_1\) receptor were transfected into Chinese hamster ovary cells and stable cell lines were established. A total of \(1 \times 10^6\) cells expressing \(\alpha_2\beta_1\) (a–c) or \(\alpha_2(\alpha_4)\beta_1\) (d–f) were stained with a mAb (P1E6) against the \(\alpha_2\)-domain (a and d), a mAb (31H4) against the \(\alpha_2\)-propeller (b and e), and a mAb (44a) against the \(\alpha_1\)-domain (c and f). Bound antibodies (filled lines) were detected with a FITC conjugate of goat anti-mouse IgG by FACS analysis. An isotype matched IgG (open lines) was used as a control. B, NIF binding. Biotinylated NIF was added to \(1 \times 10^6\) cells expressing either \(\alpha_2\beta_1\) (left panel) or \(\alpha_2(\alpha_4)\beta_1\) (right panel) in the presence of \(1 \text{ mM Ca}^{2+}\) and \(1 \text{ mM Mg}^{2+}\). After washing, bound NIF (filled lines) was detected with an avidin-FITC conjugate. Verifying specificity of NIF binding, addition of \(1 \text{ mM EDTA}\) completely blocked NIF binding by \(\alpha_2(\alpha_4)\beta_1\) (open lines). C, C3bi binding. Biotinylated EC3bi (\(2 \times 10^9\)) was added to \(2 \times 10^5\) cells expressing wild-type \(\alpha_2\beta_2\), the chimeric receptor \(\alpha_2(\alpha_4)\beta_1\), or \(\alpha_2\beta_2\), which had been pre-seeded onto polylysine-coated 24-well plates. After 60 min at 37°C, the amount of bound EC3bi was determined using avidin-alkaline phosphatase and p-nitrophenyl phosphate, measuring the absorbance at 405 nm. Specificity was demonstrated by addition of \(1 \text{ mM EDTA}\) (gray bars). Data are the means ± S.D. of two independent experiments.
ducted ligand binding assays using two representative ligands of $\alpha_{M}\beta_2$, Fg and C3bi. As shown in Fig. 3A, all 13 homolog-scanning mutants interacted with the $\gamma$-module (a major recognition domain within Fg for $\alpha_{M}\beta_2$ (13, 14, 21)) in a cation-dependent manner very similar to the wild-type receptor, indicating that 1) all 13 mutants exhibited correct conformations capable of ligand binding, and 2) switching these sequences did not alter the cation-dependent nature of the $\alpha_{M}\beta_2$ ligand interaction. Verifying the specificity of the cell adhesion assay, mock-transfected cells did not adhere to the $\gamma$-module, and for all 13 mutants, cell adhesion could be completely blocked by addition of 10 mM NIF (data not shown). We next evaluated C3bi binding activity of the 13 mutant $\alpha_{M}\beta_2$ receptors using our established procedures (15). As shown in Fig. 3B, wild-type $\alpha_{M}\beta_2$-expressing but not the mock-transfected cells reacted well with C3bi, which could be blocked by addition of 1 mM EDTA. Of the 13 $\alpha_{M}\beta_2$-propellor mutants, 8 mutants reacted well with C3bi, and the mean fluorescence intensity did not vary by more than 1.5-fold compared with wild-type $\alpha_{M}\beta_2$-bearing cells. Most importantly, these five mutants exhibited similar cell adhesion to Fg (Fig. 3A). Thus, differences in receptor expression level or global conformation of the receptors were not responsible for the loss of C3bi binding by these five mutants.

**Inhibition of C3bi Binding by Antibodies Specific for the W4 Blade**—To confirm the importance of the W4 blade in C3bi binding, we prepared polyclonal antibodies against the functional sequences of the W4 blade. One of the polyclonal antibodies, recognizing the sequence NMTRVDSDMNDAYL at the beginning of W4, gave a high titer in enzyme-linked immuno-

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**Table I**

| Mutagenic primers used in the homolog-scanning mutagenesis of $\alpha_{M}\beta_2$ |
|--------------------------|--------------------------|
| **Position** | **From** | **To** |
| 1 | 398–402 | DMNDA | LQRDRHSS | ACCAGAGTGTGATCAGGACCCAGCGAATCATCCGACTTGTTGATCCTG |
| 2 | 412–419 | LHRNVRQSL | STGESTHF | GGTCCGCCCATATCCTCCAGAAAGGGACACATCGTTCGTTGGGGC |
| 3 | 426–434 | YQHYLGVL | ANYTGQIVL | GTTCGGGTCGACCTCGAGGAGATATTATGCTGCGTGATACGATC |
| 4 | 435–443 | PRQNTGMEW | YSVNENGT | GCCCTGAGGACGATGCTACTCGCGAGAATGGCCGACACATCCCAAGC |
| 4 | 444–451 | SNANVKT | VQHRGDG | AGGACGAAACCTGGACACATGGGTGCTAGGGTACAGGACCC |
| 4 | 452–461 | SGHASG | VQHRGDG | AGGAGACGAAACCTGGACACATGGGTGCTAGGGTACAGGACCC |
| 5 | 483–491 | YEYQTRGGQV | MSLDKKEG | GGGCGCCCATACATGAGGAGAAGGGGAGATCGCCGTTGAGCTTC |
| 6 | 497–505 | PRGQRQARW | PTKKGIL | TCCGGTTCGCTTGCCTTATCAGAAAGAGATGCTGAGCCCTT |
| 7 | 511–519 | YGQECGWQP | ELEFTG | TGATGCTGTCGTTGACGGAGCAACAGGATGAAATACCTTGCTT |
| 8 | 545–553 | AGPEEIRD | SPLQENS | GACGTCGGCAGGATGTTCCCGGAGAATCCAAATTCGCGTCGCTC |
| 9 | 559–567 | TSGSHGSP | HTWIRTKY | TACCTGTTCAGCAGCCAACAGAAAGAAGAATAGACCCAAGC |
| 10 | 576–584 | KLSPR | DQGFRSH | CGGATGACAGGATCGCCAGCTTTCAGGCTCTCATGCATGTTT |
| 11 | 589–598 | SGQDLTMDG | DGYGDLNGDS | CAGTCAGTGGGTGACGGGTCCAGCTGTGTCGCTGCTATCAGT |
nosorbent assays. In addition, an antibody specific for a sequence on the upper face of the W2 blade (QEIVAN-QRGLYQ) was prepared as a control. This antibody gave a similar titer as the antibody specific for the W4 sequence. The ability of these two antibodies to react with $\alpha_{\text{M}$-$\beta_2$ was confirmed by FACS analysis (data not shown). To see if the two antibodies were capable of blocking C3bi binding, we pre-incubated these two antibodies with the wild-type $\alpha_{\text{M}$-$\beta_2$-expressing cells, and we then performed the C3bi binding assays. Fig. 4 shows that addition of the W4-specific antibody reduced C3bi binding significantly ($p < 0.01$), whereas neither a rabbit IgG control nor the W2-specific antibody displayed significant inhibition. As the $\alpha_{\text{M}$-I-domain plays an important role in C3bi binding by $\alpha_{\text{M}$-$\beta_2$, we expected that the W4-specific antibody would exert stronger inhibition when combined with NIF, a high affinity antagonist of $\alpha_{\text{M}$-$\beta_2$ that is specific for the $\alpha_{\text{M}$-I-domain (13, 24). Indeed, addition of NIF (10 nM) alone reduced C3bi binding to $\alpha_{\text{M}$-$\beta_2$ by 60%, and combining NIF with the W4-specific antibody resulted in more than 90% inhibition of C3bi binding. In contrast, addition of the W2-specific antibody or a rabbit IgG control did not produce any further inhibition over NIF alone (Fig. 4). These data demonstrated that the W4 blade of the $\beta$-propeller, along with the $\alpha_{\text{M}$-I-domain, contributed significantly to C3bi binding by $\alpha_{\text{M}$-$\beta_2$.

C3bi Binding to the W4 Peptides—As an ultimate proof for the direct involvement of the W4 blade in C3bi binding, we tested whether C3bi would bind directly to peptides derived from the W4 blade. Based on the three-dimensional model of the $\alpha_{\text{M}$-$\beta$-propeller and our above mutagenesis data, we synthesized four peptides pN$^{399}$-L$^{404}$ (C$^{399}$NMRVDSMDNDA-YL$^{404}$), pL$^{411}$-V$^{420}$ (C$^{411}$RLRVRSVL$^{420}$), pP$^{424}$-G$^{440}$ (C$^{424}$PRYHQHGLVAFRQNTG$^{440}$), and pM$^{441}$-G$^{454}$ (C$^{441}$MWESANVKGTTIQC$^{454}$), and their corresponding scrambled controls. In our preliminary experiments, we tested the ability of these synthetic peptides to support direct C3bi binding, when immobilized on the surface of a 24-well plate. All four peptides were immobilized readily on the plastic surface with similar coating efficiencies (~0.3 nmol of each peptide were immobilized per well at 100 $\mu$M of the peptide used for coating). Of the four synthetic peptides, one peptide pP$^{424}$-G$^{440}$ bound C3bi well. The other three peptides did not support detectable C3bi binding (data not shown), possibly due to their less optimal conformations in the absence of the structural constraints of the $\beta$-propeller. Subsequently, we conducted further experiments on this peptide. As shown in Fig. 5A, peptide pP$^{424}$-G$^{440}$ bound C3bi in a dose-dependent and saturable manner, whereas its scrambled control (CRLQPRHMYFQGATIN) did not show significant binding. The titration data could be fit to a single binding site model using non-linear regression analysis. The binding constant ($K_d$) was estimated to be around 20 $\mu$M, which is in the same range as one of the most active $\alpha_{\text{M}$-I-domain-derived peptides (A7, residues...
except that the biotinylated EC3bi were mixed with different soluble peptides (500 μM) or its scrambled control (RLGPIRHMVYFQQGATN) and BSA. Then biotinylated EC3bi in HBSS containing 1 mM Ca2+ and 1 mM Mg2+ was added and incubated for 1 h at 37 °C. After washing, fixation, and blocking, the amount of bound EC3bi was determined using an avidin-alkaline phosphatase conjugate and pNPP-nitrophenyl phosphate as the substrate, measuring the absorbance at 405 nm. Data shown are the means ± S.D. of three independent experiments. B, inhibition of C3bi binding by soluble peptides. A 24-well non-tissue culture plate was coated with 80 μl of 100 μM pP424-G440 at the center of each well. Binding of C3bi to the immobilized peptide was performed as in A, except that the biotinylated EC3bi was mixed with different αMβ2-propeller peptides (500 μM) (black bars) or β2 I-domain peptides (1 μM) (gray bars) prior to addition to the Pro424-G440-coated wells. The amount of EC3bi was determined as above. Data shown are the means ± S.D. of three independent experiments. Verifying specificity, scrambled pP424-G440 had no inhibitory activity, and no specific C3bi binding was observed on BSA-coated wells.

**DISCUSSION**

C3bi binding by leukocyte integrin αMβ2 plays critical roles in host defense functions. The functional domains within αMβ2 involved in C3bi recognition have been mapped to the I-domains of the αM and the β2 subunits (13, 19, 26). Recently, Yalamanchili et al. (17) have presented evidence for a critical role of the αM β-propeller in C3bi binding by αMβ2. However, the exact location of the C3bi-binding site within the β-propeller is still unknown. In this study, using a combination of different approaches, we have identified the W4 blade within the αM β-propeller, containing segments Asp150–Ala202, Leu412–Leu419, Tyr426–Met434, Phe435–Glu443, and Ser444–Thr451, as a major contact site for C3bi. Our results indicate that the W4 blade is specifically involved in C3bi but not Fg recognition, as the five mutants that were defective in C3bi binding exhibited normal cell adhesion to Fg. A direct involvement of the W4 blade in C3bi recognition was demonstrated by the ability of a synthetic W4 peptide Pro424-Gly440 to bind C3bi, when immobilized on the surface of a microtiter plate.

The critical roles of the β-propeller in ligand binding have been well documented in the literature, especially for those integrins which do not contain an I-domain in their α subunits (non-I-domain integrins). Using different approaches, the ligand-binding sites have been mapped predominantly to the W2 and W3 blades for a number of integrins, including αV, αMβ2, and α5, etc. (25, 27–29). Indeed, the recently published crystal structure of the αMβ2–RGD complex reveals that Asp150 within the W4 blade is specifically involved in C3bi but not Fg recognition, as the five mutants that were defective in C3bi binding exhibited normal cell adhesion to Fg. A direct involvement of the W4 blade in C3bi recognition was demonstrated by the ability of a synthetic W4 peptide Pro424-Gly440 to bind C3bi, when immobilized on the surface of a microtiter plate.
an I-domain in its α subunit is still capable of C3bi binding (17), implicating a role of the β-propeller in C3bi binding by αMβ2, although such binding could also be mediated in part by the β2 subunit. Therefore, a definitive role of the β-propeller in ligand binding needs to be tested. Complicating this matter further, the ligand binding surface identified in the non-I-domain integrins is disrupted by insertion of the I-domain (between W2 and W3). Thus, the exact location of the ligand-binding site within the β-propeller of the I-domain integrins is unclear. Moreover, as the I-domain plays a dominant role in ligand recognition (12, 15, 21, 24, 26, 30–32), the function of the β-propeller within the intact heterodimeric receptor remains to be defined. To address these issues, we used several complementary approaches in this study. Our results demonstrate that the β-propeller is involved directly in C3bi recognition. In addition, we found that the ligand binding property of the I-domain could be affected by the β-propeller. For example, the αM I-domain within the αMβ-propeller recognized both NIF and C3bi, but it interacted only with NIF when placed in the α2 β-propeller (Fig. 1, B and C). These data suggested that the β-propeller could contribute to ligand binding in two different ways as follows: by participating directly in C3bi recognition, and by modulating the properties of the αM I-domain.

The novel C3bi-binding site we identified in this study resides within the W4 blade of the β-propeller (Fig. 6). Surprisingly, this C3bi-binding site contained the entire W4 blade, including residues located in both the upper and lower faces of the β-propeller. This is in sharp contrast to the ligand-binding sites identified within several non-I-domain integrins, where all the functional residues were shown to reside on the upper face of the β-propeller (25, 28, 33). That the W4 blade of the αM β-propeller is involved directly in C3bi recognition is supported by several observations. 1) Substitution of the five individual segments (Asp399–Ala402, Leu412–Leu419, Tyr426–Met434, Phe435–Glu443, and Ser444–Thr451) within W4 abrogated C3bi binding, whereas segment substitutions within the other three blades (W5, W6, and W7) had no effect (Fig. 3B). Loss of C3bi binding by these W4 mutants did not result from alterations of gross conformations of the mutant αMβ2 receptors, as all 13 β-propeller mutants were expressed well on the cell surface, formed correct heterodimers with the β2 subunit (Fig. 2), and reacted with a panel of conformation-dependent αMβ2 mAbs (data not shown). Most importantly, all 13 β-propeller mutants, including the five defective receptors, supported cation-dependent cell adhesion to Fg in a manner similar to wild-type αMβ2 (Fig. 3A). 2) Antibodies specific for the W4 blade, but not the W2 blade, blocked C3bi binding by the wild-type αMβ2 receptor (Fig. 4). 3) A synthetic peptide (Pro424–Gly440) corresponding to sequences within W4, but not its scrambled control, bound C3bi directly with a $K_d$ of 20 μM (Fig. 5A). Although the other three peptides did not show significant binding of C3bi, they may simply adopt a less optimal conformation for ligand recognition, when existing outside the structural constraints of the β-propeller. Additionally, we found that the two C3bi-binding peptides identified within the β2 I-domain (19) did not compete with peptide Pro424–Gly440 for C3bi binding (Fig. 5B), suggesting that either the β-propeller peptide Pro424–Gly440 and the two β2 I-domain peptides recognized different regions of C3bi or that the β2 I-domain peptides bound C3bi much weaker and therefore were unable to compete with the β-propeller peptide. The importance of W4 in ligand binding has also been demonstrated in other integrins. For example, a region within the αV β-propeller (Asp219 in W4), which is homologous to the critical segment Asp298–Ala302 found in this study, was shown to contact its ligand (RGD) directly in the crystal structure (25). In addition, Dickeson et al. (34) have reported that efficient colla-gen binding to α2β1 required both the I-domain and the W3 to W5 blades of the α2 β-propeller, suggesting that the regions surrounding the W4 blade may play important roles in direct ligand recognition for other integrin subfamilies as well.

Results from this study and others in the literature suggest that αMβ2 possesses at least three independent ligand binding domains: the αM I-domain, the β2 I-domain, and the αM β-propeller (15, 17, 19, 26). How these individual domains work together to form a functional ligand-binding site within the heterodimeric αMβ2 receptor is currently unknown. Given that the W4 blade of the β-propeller is located in close proximity to another C3bi recognition site we identified previously in the β2 I-domain (19), it is highly possible that these individual binding sites may function together to form a composite C3bi binding pocket. Accordingly, we propose that three distinct regions within αMβ2, including the αM I-domain, the β2 I-domain, and the αM β-propeller, reside proximally within the receptor and contribute directly to the formation of the ligand binding pocket. The fact that the W4 blade is non-essential to Fg recognition suggests that the Fg/αMβ2 interaction is likely mediated mainly through the two I-domains within the αM and β2 subunits, whereas C3bi binding depends on three domains (the αM and β2 I-domains and the αM β-propeller). Based on this model, we predict that the MIDAS motif of the αM I-domain should reside closely to both the W4 blade of the β-propeller and the β2 I-domain. In support of this model, we found that C3bi binding to intact αMβ2 was inhibited more effectively if the binding sites within the αM I-domain and the αM β-propeller were both blocked (Fig. 4). In addition, Yalamanchili et al. (17) reported that C3bi binding to the I-domain-less αMβ2 could be inhibited completely by a β-propeller antibody (CBRM1/32), suggesting that the two binding surfaces (the αM β-propeller and the β2 I-domain) within the I-domain-less αMβ2 are located relatively close to each other in space (<70 Å of a typical antigen-binding site within a Fab’ fragment), such that the blocking mAb could occupy the two binding sites simultaneously, leading to complete inhibition. Alternatively, as mAb CBRM1/32 recognizes a region (corresponding to segment Ala444–Arg456) in the upper face of the W6 blade (35), which itself is not involved directly in C3bi binding (Fig. 3), it is also possible that CBRM1/32 could function as a wedge to alter the relative orientation between the αM β-propeller and the β2 I-domain, resulting in disruption of the optimal conformation for C3bi binding. Altogether, our model would expect that 1) the αM I-domain plays a major role in C3bi binding to the intact receptor; 2) among the three individual domains that form the composite C3bi-binding site, the β-propeller and the β2 I-domain reside relatively close to each other in space, whereas the αM I-domain is located farther apart; and 3) high affinity ligand binding to αMβ2 requires optimal orientations among all three ligand binding domains, and therefore spatial changes relative to each other could affect ligand binding and thereby provide a potential means to control the affinity state of the integrin receptor ("inside-out" signaling). Two other models have been proposed in the literature, in which the MIDAS motif of the αM I-domain was shown to project away from the W4 blade of the β-propeller and the β2 I-domain (36, 37). Further studies will be needed to test the validity of these different models.

In conclusion, using several different approaches, we have identified a novel C3bi-binding site within the W4 blade of the αM β-propeller. Our data demonstrate that the W4 blade is involved differentially in αMβ2 binding to its two physiological ligands C3bi and Fg. When compared with the ligand-binding site within α2β1, which is located on the upper face of the β-propeller (25), the C3bi-binding site identified in this study encompasses residues on both the upper and lower faces of the
\(\alpha_\text{M}\) \(\beta\)-propeller. Altogether, our studies support a model in which three individual domains of \(\alpha_\text{M}\beta_2\), the \(\alpha_\text{M}\)-domain, the \(\alpha_\text{M}\) \(\beta\)-propeller, and the \(\beta_\text{L}\)-domain, reside together in space and contribute to the formation of a common C3bi-binding site.

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REFERENCES

1. Wright, S. D., and Silverstein, S. C. (1983) J. Exp. Med. 158, 2016–2023
2. Arnaout, M. A., Pierce, M. W., Dana, N., and Clayton, L. K. (1987) Methods Enzymol. 156, 602–615
3. Plew, E. F., and Zhang, L. (1997) J. Clin. Invest. 99, 1145–1146
4. Springer, T. A. (1995) Annu. Rev. Physiol. 57, 827–872
5. Shappell, S. B., Toman, C., Anderson, D. C., Taylor, A. A., Entman, M. L., and Smith, C. W. (1990) J. Immunol. 144, 2702–2711
6. Coxon, A., Rieu, P., Barkalew, F. J., Askari, S., Sharpe, A. H., Von Andrian, U. H., Arnaout, M. A., and Mayadas, T. N. (1996) Immunity 5, 653–666
7. Lu, H., Smith, C. W., Ferrard, J., Bullard, D. C., Tang, L., Entman, M. L., Beaudet, A. L., and Ballantyne, C. M. (1997) J. Clin. Invest. 99, 1340–1350
8. Crowley, C. A., Curnutte, J. T., Rosin, R. E., Andre-Schwartz, J., Gallin, J. I., Klemperer, M., Snyderman, R., Southwick, F. S., Stossel, T. P., and Bahler, B. M. (1980) N. Engl. J. Med. 302, 1163–1168
9. Altieri, D. C., Agbanyo, F. R., Pliescia, J., Ginsberg, M. H., Edgington, T. S., and Plew, E. F. (1990) J. Biol. Chem. 265, 12119–12122
10. Diamond, M. S., Staunton, D. E., de Fougerolles, A. R., Stacke, S. A., Garcia-Aguilar, J., Hibbs, M. L., and Springer, T. A. (1996) J. Cell Biol. 111, 3129–3139
11. Joyce, M., Pastor, D. L., McGrath, D. E., Brown, S. M., Larche, Y., De Meutter, J., Bogovitz, C. A., Fried, V. A., and Ely, J. A. (1994) J. Biol. Chem. 269, 10008–10015
12. Diamond, M. S., Garcia-Aguilar, J., Bickford, J. K., Corbi, A. L., and Springer, T. A. (1993) J. Cell Biol. 126, 1031–1043
13. Zhang, L., and Plow, E. F. (1999) Biochemistry 38, 8064–8071
14. Ugarova, T. P., Solorjov, D. A., Zhang, L., Loukinov, D. I., Yee, V. C., Medved, L. V., and Plow, E. F. (1998) J. Biol. Chem. 273, 22519–22527
15. Zhang, L., and Plow, E. F. (1996) J. Biol. Chem. 271, 18211–18216
16. Springer, T. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 65–72
17. Yalamanchili, P., Lu, C., Ovig, C., and Springer, T. A. (2000) J. Biol. Chem. 275, 21877–21882
18. Goodman, T. G., and Bajt, M. L. (1996) J. Biol. Chem. 271, 23729–23736
19. Xiong, Y. M., Haas, T. A., and Zhang, L. (2002) J. Biol. Chem. 277, 46639–46644
20. Cunningham, B. C., Jhurani, P., Ng, P., and Wells, J. A. (1989) Science 243, 1330–1336
21. Zhang, L., and Plow, E. F. (1997) J. Biol. Chem. 272, 17558–17564
22. Emsley, J., Knight, C. G., Farndale, R. W., Barnes, M. J., and Liddington, R. C. (2000) Cell 101, 47–56
23. Bilsland, C. A., Diamond, M. S., and Springer, T. A. (1994) J. Immunol. 152, 4582–4589
24. Muchowski, P. J., Zhang, L., Chang, E. R., Soule, H. R., Plow, E. F., and Moyle, M. (1994) J. Biol. Chem. 269, 20419–20423
25. Xiong, J. P., Stehle, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S. L., and Arnaout, M. A. (2002) Science 296, 151–155
26. Ueda, T., Rieu, P., Brayer, J., and Arnaout, M. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10680–10684
27. Mould, A. P., Askari, J. A., and Humphries, M. J. (2000) J. Biol. Chem. 275, 20324–20336
28. Irie, A., Kamata, T., and Takada, Y. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7198–7203
29. Puzon-McLaughlin, W., Kamata, T., and Takada, Y. (2000) J. Biol. Chem. 275, 7285–7292
30. Randi, A. M., and Hogg, N. (1994) J. Biol. Chem. 269, 12395–12398
31. Huang, C., and Springer, T. A. (1995) J. Biol. Chem. 270, 19008–19016
32. Calderwood, D. A., Tuckwell, D. S., Eble, J., Kuhn, K., and Humphries, M. J. (1997) J. Biol. Chem. 272, 12311–12317
33. Mould, A. P., Burrows, L., and Humphries, M. J. (1998) J. Biol. Chem. 273, 25664–25672
34. Dickerson, S. K., Walsh, J. J., and Santoro, S. A. (1997) J. Biol. Chem. 272, 7661–7668
35. Lu, C., Ovig, C., and Springer, T. A. (1998) J. Biol. Chem. 273, 15138–15147
36. Wang, Q., Lu, C., Huang, C., Takagi, J., and Springer, T. A. (2002) J. Biol. Chem. 277, 22202–22212
37. Alonso, J. L., Essafi, M., Xiong, J. P., Stehle, T., and Arnaout, M. A. (2002) J. Biol. Chem. 277, 21877–21882
38. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723