Dual Function for a Unique Site within the β2I Domain of Integrin αMβ2*

Driss Ehirchiou‡, Yu-Mei Xiong‡, Yang Li‡, Shelesa Brew§, and Li Zhang¶

From the Departments of ‡Physiology and §Biochemistry, University of Maryland School of Medicine, Rockville, Maryland 20855

Integrin activation has been postulated to occur in part via conformational changes in the I domain of the β subunit (the βI domain), especially near the F-α, loop, in response to “inside-out” signaling. However, direct evidence for a role of the F-α, loop (residues 344–358) within the βI domain has dual functions in ligand binding by αMβ2. On the one hand, it supports intercellular adhesion molecule 1 (ICAM-1) binding to αMβ2 directly as part of a recognition interface formed by five noncontiguous segments (Pro192–Glu197, Asn213–Glu220, Leu226–Leu229, Ser234–Thr238, and Glu344–Asp348) on the apex of the βI domain. On the other hand, it controls the open and closed conformation of the αMβ2 receptor, thereby indirectly affecting αMβ2 binding to other ligands. Switching the five constituent sequences of the ICAM-1-binding site within the βI domain to their β2 counterparts destroyed ICAM-1 binding but had no effect on the gross conformations of the receptor. Of the five ICAM-1-binding-defective mutants, four had normal or even stronger interaction with Fg and C3bi, as reported in our previous study. Synthetic peptides derived from the identified site inhibited αMβ2-ICAM-1 interaction and supported direct binding to ICAM-1. Most importantly, perturbation of the F-α, loop caused conformational changes within the βI domain, which was further propagated to other regions of αMβ2. Altogether, our data demonstrate that inside-out signaling could modulate ligand binding directly by changing the ligand-binding pocket per se and/or indirectly by inducing multiple conformational changes within the receptor.

Integrins are heterodimeric surface receptors that play essential roles in cell-cell and cell-matrix interactions (1, 2). One of the most prominent features of the integrin receptor is its ability to change conformation in response to intracellular activation signals (“inside-out” signaling). Based on the crystal structures of free and ligand-bound αβ, several models have been proposed recently to explain the molecular mechanisms underlying receptor activation, including the “switchblade” model (3), the “bell-rope” model (4), and the “deadbolt” model (5). Despite the many differences, these models all predict that the F-α, loop (corresponding to residues 344–358 of the βI domain) is critical to the transition of the βI domain from closed to open conformation. In particular, the deadbolt model hypothesizes that residues within the F-α, loop are in close contact with the residues in the extended CD loop of the βTD domain, such that the βTD domain acts as a deadbolt that freezes the movement of the F-α, loop. Movement of the βTD domain as a result of the inside-out signaling will disrupt its interaction with the F-α, loop and thus induce conformational changes within the βI domain and ultimately receptor activation (5). Although very attractive, the validity of this model needs to be tested experimentally.

The β2 integrin subfamily contains four distinct receptors (α1β2, αMβ2, α5β2, and α6β2) with their expression restricted primarily to leukocytes. α1β2 (LFA-1 or CD11a/CD18) recognizes counter receptors ICAM-1, ICAM-2, and ICAM-3 present on the surface of different cell types (6, 7), whereas αMβ2 (Mac-1, CR-3, mo1, or CD11b/CD18) recognizes a wide range of ligands in addition to cell surface receptors. For example, αMβ2 interacts with fibrinogen (Fg) (8), C3bi (9), ICAM-1 (10), neutrophil inhibitory factor (NIF) (11), and zymosan (12). It is of interest, although ICAM-1 is recognized by α1β2 and αMβ2, that different domains are involved. Among its five Ig-like domains (D1–D5), the first two (D1 and D2) interact with α1β2 (13), whereas the last three (D3, D4, and D5) bind αMβ2 (14). Mutational studies suggested that α1β2-ICAM-1 interaction is mainly mediated by the I domain within α1 (15, 16) and, more specifically, by seven amino acid residues (17). These mutagenic data have been confirmed by the recently published crystal structure of the α1I domain-D1D2 complex (18). Less is known about the molecular mechanism underlying the αMβ2-ICAM-1 interaction. It was reported that both the αM and β2 subunits are involved (19–21). However, the nature of the ICAM-1-binding site within either subunit is not well defined.

Recently, using the homolog-scanning mutagenesis approach (22), we have identified a region within the β2I domain (residues 125–385) that interacts directly with two αMβ2 ligands (Fg and C3bi) (23). Given that ICAM-1 behaves differently from C3bi in its interaction with αMβ2 (20), whether this same region is also involved in ICAM-1 binding is still unknown. In this study, we screened 17 αMβ2 receptors that contain individual substitutions within the β2I domain for ICAM-1 binding. The results from this study demonstrate that ICAM-1 recognizes a novel region on top of the β2I domain, which is well separated from the binding sites for C3bi and Fg. Most impor-

* This work was supported in part by NHLBI, National Institute of Health Grant R01 HL61589-01 and by American Heart Association Grant 0240208N. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ An established investigator of the American Heart Association. To whom correspondence should be addressed: Dept. of Physiology, University of Maryland School of Medicine, 15601 Crabbbs Branch Way, Rockville, MD 20855. Tel.: 301-738-0657; Fax: 301-738-0465; E-mail: ZHANGL@USA.REDCROSS.ORG.

1 The abbreviations used are: ICAM, intercellular adhesion molecule; DPBS, Dulbecco’s phosphate-buffered saline; Fg, fibrinogen; FACs, fluorescence activated cell sorting; mAb, monoclonal antibody; NIF, neutrophil inhibitory factor; BSA, bovine serum albumin; HRP, horseradish peroxidase.
tantly, the F-α7 loop is located within this identified ligand-binding site. Perturbation of the F-α7 loop could directly switch the conformation of the β1 domain as well as other critical regions within ααββ from closed to open state, thus providing the first experimental support for a role of the F-α7 loop of the β1 domain in receptor activation. Altogether, our data suggest that inside-out signaling could activate the integrin receptor either directly, by changing the shape of the ligand-binding pocket, and/or indirectly, by affecting the global conformation of the heterodimeric receptor.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human kidney 293 cells and the expression vector pCIS2M were gifts from Dr. F. J. Castellino (Notre Dame, IN). The cDNAs of CD11b and CD18 were obtained from Dr. B. Karan-Tamir (Agen, Thousand Oaks, CA). mAb 24 was provided by Dr. Nancy Hogg (Imperial Cancer Research Fund, London, UK); mAb CBRM1/5 was obtained from ebioscience (San Diego, CA); IB4, OMK1, and TS1/18 were obtained from the ATCC (Manassas, VA); MEM148 was obtained from Serotec (Raleigh, NC); mAb 67.2 was from Pharmingen (San Diego, CA); and mAb MEM-48 was obtained from Biodesign (Kennebunk, ME). The recombinant CA); and mAb MEM-48 was obtained from the ATCC (Manassas, VA); MEM148 was obtained from Biodesign (Kennebunk, ME). The recombinant pCIS2M were gifts from Dr. F. J. Castellino (Notre Dame, IN). The cDNAs of human IgG(H/L) F(ab')2 fragment (1:20 dilution) (Zymed Laboratory) and mAb 24 was provided by Dr. L. Medved (American Red Cross, Rockville, MD). All other reagents were the highest grade available from Sigma unless otherwise noted.

**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 the 17 ααββ mutants in human kidney 293 cells have been published (24). To create constitutively active ααββ domains, we changed residue Ile236 to Gly, which enables the ααββ domain to exist in an open conformation with constitutive ligand binding activity (25), and alternatively, we changed both Phe687 and Ala684 to Cys (the resulting Cys687-Cys684 linkage is compatible with an open conformation of the ααββ domain (26)), using mutagenic primers 5′-CGGGAGAAGGGCTTTGCGATCGAG-3′ and 5′-GATACGCTGTGCAAGGTGAATCTTTGAGTGTCTGAAGACCATT-3′, respectively. The plasmids of Fg were provided by Dr. L. Medved (American Red Cross, Rockville, MD). All other reagents were the highest grade available from Sigma unless otherwise noted.

**Preparation of ICAM-1-Fc Fusion Protein—** The cDNAs of human IgG1 and human ICAM-1 were inserted separately into the pCIS2M expression vector (27), and the resultant ICAM-1-Fc fusion protein was then expressed in Chinese hamster ovary cells by co-transfection with pH5SVEco using Lipofectamine (Invitrogen). Stable cells were established by selection with 800 μg/ml G418. The ICAM-1-Fc fusion protein was purified from the conditioned media by affinity chromatography using protein G-Sepharose, and the purity of the purified protein was confirmed by a single band of 140 kDa on 10% SDS-PAGE under reduced conditions.

**FACS Analysis—** A total of 10⁶ cells expressing wild type or mutant ααββ in Hanks' balanced salt solution containing 1 mM Mg²⁺ and 1 mM Ca²⁺ were incubated with 1 μg of mAb for 30 min at 4 °C, except for mAb 24, which were kept at 37 °C. A subtype-matched mouse IgG served as a control. After washing with phosphate-buffered saline, the cells were mixed with fluorescein isothiocyanate goat anti-mouse IgG(H+L) F(ab')2 fragment (1:20 dilution) (Zhyned Laboratory) and kept at 4 °C for another 30 min. The cells were then washed with phosphate-buffered saline and resuspended in 500 μl of DPBS. FACS analysis was performed using FACSscan (Becton-Dickinson), counting 10,000 events. Mean fluorescence intensities were quantified using the FACSscan program.

**Adhesion Assays—** Adhesion of the ααββ-expressing cells to ICAM-1 was conducted based on our published method (28), except that the cells were pretreated with an activating β1-specific mAb (MEM48) (29). Briefly, a 24-well polystyrene plate was coated with ICAM-1-Fc (50 μg/ml) and then blocked with 400 μl of 0.05% polyvinylpyrrolidone in DPBS. A total of 2 × 10⁷ cells in Hanks' balanced salt solution containing 1 mM Ca²⁺ and 1 mM Mg²⁺ in the presence or absence of 2 mg/ml synthetic peptides or 50 nM NIF (specific for the ααββ subunit) were added to each well and incubated at 37 °C for 20 min. Unbound cells were removed by three washes with DPBS, and adherent cells were quantified by cell-associated acid phosphatase as described previously (28).

**Solid Phase Binding Assays—** To test the interaction between the identified sequences of the β1 domain and ICAM-1, 96-well microtiter plates (Immulon 4B; 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. ICAM-1-Fc (50 μg/ml) in Tris-buffered saline (20 mM Tris, 150 mM NaCl, 1 mM CaCl², 1 mM MgCl², pH 7.4) was added to the wells and incubated for 2–3 h at 22 °C. After washing with Tris-buffered saline, bound ICAM-1-Fc was detected using protein A conjugated to horseradish peroxidase (HRP), and the HRP substrate 3,3′,5,5′-tetramethylbenzidine (KPL, Gaithersburg, MD). Alternatively, the peptides were biotinylated via their N-terminal Cys using the EZ-link PEO-Maleimide activated biotin kit, or their N-terminal Lys using sulfo-Succinimidyl 6-(biotinamido) hexa-oxo (Pierce), based on the product instructions. The plate was coated with ICAM-1-Fc (50 μg/ml) and post-coated with BSA. Biotinylated peptides were added and incubated with ICAM-1-Fc for 2 h at room temperature. The bound peptides were detected using avidin conjugated to HRP.

To detect real time interactions between ICAM-1 and the β1 domain peptides, a BIAcore 3000 SPR-based biosensor (BIAcore AB, Uppsala, Sweden) was used, and the protocols recommended by the manufacturer were followed. Briefly, the ICAM-1-Fc was covalently coupled to the CM5 sensor chips via primary amino groups, which was preactivated with N-ethyl-N-(3-dimethylaminopropyl)carbodiimide hydrochloride/N-hydroxysuccinimide. This protocol resulted in ~1000 resonance units of immobilized ICAM-1. A blank flow cell that had been N-ethyl-N-(3-dimethylaminopropyl)carbodiimide hydrochloride/N-hydroxysuccinimide-activated and ethanolamine-blocked was used as a control. The whole wells were then expressed by injecting various concentrations of the peptides over the ICAM-1-Fc surface at a flow rate of 5 μl/min. The experiments were conducted in a Tris buffer (20 mM Tris, 150 mM NaCl, pH 7.4) containing 1 mM MnCl₂. The chip surface was regenerated with 0.1 M HCl. All of the data were analyzed using the BIAevaluation 3.0 program, subtracting binding to the blank flow cell to account for any nonspecific binding. To analyze steady state peptide binding, 1 μl of mAb MEM48 was added to the flow cell in the presence of the peptides, and the steady state peptide concentrations were obtained by injecting various concentrations, which were then fit to a single-site model (Langmuir isotherm) to determine the dissociation constants (Kd). The specificity of peptide binding was further verified using the corresponding scrambled controls.

**RESULTS**

**Cell Adhesion to ICAM-1 by ααββ Is Activation-dependent—** Ligand binding by the β1 integrins depends on receptor activation (2, 4), especially for ICAM-1 binding by ααββ, which is of low affinity (10). Indeed, when expressed on 293 cells, ααββ interacted with 303b constitutively in the absence of receptor activation (28, 29), whereas it failed to do so with ICAM-1, unless activated by the β2-specific activating mAb MEM48 (Fig. 1), which recognizes the mid-region of β2 (29). Addition of 10 μg/ml of mAb MEM48 enhanced cell adhesion by 4–8-fold, which could be blocked completely by the addition of an ααββ-specific antagonist (NIF) or EDTA, confirming the specificity and cation dependence of the adhesion assay. Moreover, mock transfected cells did not exhibit significant adhesion to ICAM-1. These results demonstrated the feasibility of using mAb MEM48 as an activator of ααββ for ICAM-1 binding. Therefore, all of the subsequent cell adhesion assays to ICAM-1 were conducted in the presence of MEM48.

**A Unique Region on the Apex of the β1 Domain Is Critical to ICAM-1 Recognition by ααββ—** Previously, we have systematically substituted 17 segments that reside on the hydrated surface of the β1 domain with their counterpart sequences of the β1 domain and established stable cell lines for these 17 homolog-scanning ααββ mutants in 293 cells. We demonstrated that all 17 β1 domain mutants, when transfected together with wild type ααββ, were expressed at wild type levels on the cell surface. In addition, we have shown that these 17 β1 domain mutants possessed a normal intact conformation, based on a number of different criteria, including surface labeling and immunoprecipitation, as well as reactivity toward a panel of
conformation-dependent antibodies (24). Most importantly, we have studied these mutants using two conformation- and cation-dependent mAbs and showed that all 17 mutants exhibited similar cation-dependent conformations (23, 24). Thus, loss of function by these homolog-scanning mutants is most likely attributed to perturbations of the ligand-binding site by these mutations.

Goodman and Bajt (20) reported that a mutation within the β1 domain had differential effects on ICAM-1 versus C3b binding to αMβ2, suggesting that the β1 domain may possess different binding sites for ICAM-1 and C3b. To locate critical sequences within the β1 domain for ICAM-1 binding, we conducted cell adhesion assays on all 17 αMβ2 mutants using ICAM-1 as the substrate, based on the conditions established in Fig. 1. The cell adhesion results are presented in Fig. 2. Most of the mutants supported cell adhesion to ICAM-1 and exhibited similar adhesive activities to the wild type receptor (less than 1.4-fold difference from wild type receptor). These functional mutants include αMβ2 (Arg144-Lys148), αMβ2(Cys297–Cys304), αMβ2(Asn181–Asp326), αMβ2(Gln189–Ala203), αMβ2(Pro247–Glu250), αMβ2(Ala262–Asp305), αMβ2(Asp290–Glu298), αMβ2(Gly305–His308), αMβ2(Thr334–Ile336), αMβ2(Val344–Asp356), and αMβ2 (His371-Lys375). Mutant αMβ2(Phe292–Glu197) had a modest loss of cell adhesion (~2.0-fold). Five mutants lost most of their ICAM-1 binding activity; compared with the wild type receptor, αMβ2(Glu162–Gly164), αMβ2(Pro192–Glu197), αMβ2(Asn231–Glu239), αMβ2(Leu225–Leu230), αMβ2(Thr232), and αMβ2(Glu344–Asp348) had 8.5-, 6.2-, 10.7-, 5.5-, and 8.7-fold reduction in cell adhesion, respectively. The specificity of ligand binding to each of the mutants that retained activity was verified by blocking experiments using the αMβ2 specific ligand NIF. These data suggest that six noncontiguous segments (Glu162–Gly164, Pro192–Glu197, Asn231–Glu239, Leu225–Leu230, Ser234–Thr239, and Glu344–Asp348) within the β1 domain contributed significantly to ICAM-1 binding by αMβ2. Fig. 3 shows the location of these critical residues within the β1 domain, which was constructed earlier (23) based on its homology to β2 (30). Of the identified six segments, sequences Pro192–Glu197, Asn231–Glu239, Leu225–Leu230, Ser234–Thr239, and Glu344–Asp348 reside proximally in space and form a common ligand-binding site that is distinct from the C3b-binding site we reported earlier (23). Therefore, we concluded that these five segments likely contributed directly to ICAM-1 binding. Segment Glu162–Gly164 resided at the bottom of the β1 domain that is far away from the identified ligand-binding site and thus less likely to contribute directly to ICAM-1 binding by αMβ2.

A major ligand-binding site is located within the αMβ2 domain, which exists in an equilibrium between the open and closed conformations (31, 32). Because activation of the αM domain depends critically on the β1 domain (33), it is possible that mutations of the β1 domain interfered with the activation of the αMβ2 domain, leading to defective ligand binding. To exclude this possibility, we created constitutively active αMβ2 domains by replacing the critical Ile316 within helix 7 of the αMβ2 domain with Gly, based on the study of Xiong et al. (25), and alternatively by creating a disulfide linkage between residues 297 and 304, which is compatible with an open conformation of the αMβ2 domain (26). The αMβ2(Glu162Gly164) domain was shown to exist in an open conformation, and their corresponding αMβ2 receptors interact with ligands constitutively (25). Thus, two different sets of αMβ2 mutants, that of αMβ2 and αMβ2β2(Leu225–Leu230) and that of αMβ2(Cys297–Cys304)β2 and αMβ2(Cys297–Cys304)β2(Leu225–Leu230), were prepared, all of which contain the αMβ2 domains with an open conformation. In addition, based on the report that the αMβ2 domain adopts an open conformation in the presence of Mn2+ (31), we also conducted ligand binding assays in the presence of Mn2+. Thus, should the β1 domain function solely to control the conformations of the αMβ2 domain, mutations within the β1 domain would have no effect on ligand binding by the active αMβ2 and αMβ2β2 receptors. On the other hand, if the β1 domain provides a direct ligand-binding site, mutations in β2 should abolish ligand binding even by the constitutively active αMβ2. As shown in Fig. 2 (B and D), cells expressing the αMβ2(Glu162) receptor or the αMβ2(Cys297–Cys304)β2 receptor both adhered constitutively to ICAM-1 (~7.4- to 7.7-fold higher than that of wild type, respectively) in the absence of Mn2+, whereas wild type αMβ2-expressing or mock transfected cells did not show significant adhesion, confirming that both αMβ2 and αMβ2β2 are constitutively active for ICAM-1 binding. As expected, when activated by Mn2+, the wild type receptor supported strong cell adhesion to ICAM-1 (Fig. 2C). Of interest, the αMβ2(Cys297–Cys304)β2 receptor exhibited differential binding activities, where it supported constitutive adhesion to ICAM-1 (Fig. 2D) but not to C3b (26), which is consistent with the different conformational requirements for αMβ2 recognition of these two ligands (28, 38). Most importantly, mutations within segment Leu225–Leu230, a component of the identified ICAM-1-binding site within the β1 domain, abolished ligand binding by the constitutively active αMβ2β2 receptor (Fig. 2B) and the constitutively active αMβ2(Cys297–Cys304)β2 receptor (Fig. 2D), regardless of Mn2+, suggesting that the β1 domain mutations destroyed ligand binding independent of the conformation of the αMβ2 domain.

**Fig. 1.** Ligand binding to ICAM-1 by αMβ2 is activation-dependent. A total of 2 x 10⁶ αMβ2-expressing cells in the presence or absence of the β2-activating mAb MEM48 were added to 24-well non-tissue culture polystyrene plates, which were precoated with ICAM-1-Fc (50 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-associated acid phosphatase. Verifying specificity, mock transfected 293 cells did not adhere significantly to ICAM-1, and adhesion by the αMβ2-expressing cells could be completely blocked by the addition of the αM-specific antagonist NIF. The data shown are the means ± S.D. of three independent experiments.
these soluble peptides to block wild type $\alpha_M\beta_2$-mediated cell adhesion to ICAM-1 was tested, and the results are shown in Fig. 4. Among these five peptides, P7 and P12 showed the strongest inhibition, and addition of these two peptides reduced the number of adherent cells by 17- and 24-fold, respectively. Peptide P14 exhibited small but significant inhibition (3-fold), whereas peptides P5 and P17 had no significant effect on cell adhesion. To verify the specificity of peptide inhibition, we synthesized two scrambled control peptides sP7 (KQNLNNFVETTGSQ) and sP12 (CKTRTLVEVESTWKY), corresponding to the two most active peptides P7 and P12, respectively. Both control peptides exhibited some, but significantly weaker, inhibition (~1.5-fold) than their corresponding peptides (17-fold for P7 and 24-fold for P12). Furthermore, the addition of NIF blocked more than 95% cell adhesion, and no adhesion was observed for mock transfected cells, confirming the specificity of the adhesion assay. These data suggest that the $\beta_2$I domain peptides were effective soluble inhibitors of the $\alpha_M\beta_2$-ICAM-1 interaction, most likely by binding directly to the ligand ICAM-1.

**Direct Interactions between the $\beta_2$I Domain Peptides and ICAM-1**—To see whether peptides P7, P12, and P14 inhibited cell adhesion by interacting with ICAM-1, we conducted direct binding assays using two different formats. First, we coated the synthetic peptides onto 96-well microtiter plates, and binding of soluble ICAM-1-Fc to the peptides was assessed. To make sure that these peptides had similar coating efficiencies, we measured the amount of immobilized peptides by labeling the free SH group (for peptides P12 and P14) or the NH$_2$ group (for peptides P5, P7, and P17) present on these peptides with biotin and then quantified the amount of immobilized biotin using an HRP conjugate of avidin. We found that similar amounts of peptides were present in each well (data not shown). The binding data are shown in Fig. 5A. Parallel to their inhibitory activity in the above cell adhesion assays, peptides P7 and P12 bound ICAM-1 effectively, which were significantly higher than their corresponding scrambled control peptides. The other three peptides, P5, P14, and P17, also exhibited significant
A Dual Role for the $\beta_2$I Domain in Ligand Binding by $\alpha_M\beta_2$

Fig. 3. Ligand-binding sites within the $\beta_2$I domain. The structure of the $\beta_2$I domain is modeled according to the crystal coordinates of integrin $\alpha_6\beta_1$ (Protein Data Bank code 1L5G) (30). Molecular modeling was carried out using InsightII modules: Biopolymer, Homology, and Discover (Accelrys Inc.) (23). The backbone of the $\beta_2$I domain is shown with the $\beta$-sheets in light blue and $\alpha$-helices in red, with the bound Ca$^2+$ cations as gray spheres. The Connolly surface of the ligand recognition sites generated using a probe size of 1.4 Å are highlighted in blue (residues 192–197), green (residues 213–220), dark orange (residues 225–230), pale green (residues 324–329), and dark green (residues 344–348). The F-7 loop is located within residues 344–358 and contains part of the ligand-binding site (residues 344–348 shown in dark green). This figure was prepared using the program MOLMOL (41).

Fig. 4. Inhibition of $\alpha_M\beta_2$ ligand binding by synthetic peptides derived from the $\beta_2$I domain. Inhibition of $\alpha_M\beta_2$-mediated cell adhesion to ICAM-1 by synthetic peptides derived from the functional sequences identified within the $\beta_2$I domain was conducted similarly as in Fig. 1, except that 2 mg/ml peptides were added in the assay mixture. The number of the adherent cells in the absence of the peptides was taken as 1.0. Verifying specificity of the assay, cell adhesion to ICAM-1 could be blocked by the addition of NIF, and the scrambled peptides sP7 and sP12 did not significantly inhibit ICAM-1 binding by $\alpha_M\beta_2$. The data shown are the means ± S.D. of four to eight independent experiments.

binding to ICAM-1. As a negative control, we did not detect significant ICAM-1 binding to BSA-coated wells. In a reverse format, we coated 96-well microtiter plates with ICAM-1-Fc and then blocked with BSA. The biotinylated peptides were added and bound peptides were detected using an HRP conjugate of avidin. In a separate experiment, we have verified that all five peptides had similar biotinylation efficiencies (data not shown). As shown in Fig. 5B, peptide P7 exhibited specific binding to the immobilized ICAM-1 that was significantly higher than its corresponding scrambled control. Peptide P12 did not exhibit specific binding in this format, and the other three peptides, P5, P14, and P17, exhibited modest binding. As negative controls, we found that none of these peptides bound specifically to BSA-coated surfaces. To study peptide binding to ICAM-1 in real time, we conducted additional experiments using surface plasmon resonance technology (BIACore), where ICAM-1 was covalently coupled to the hydrophilic surface of the CM5 sensor chip, and peptide P5 or its scrambled control was added over the ICAM-1 surface at a flow rate of 5 µl/min. As shown in Fig. 5C, P5 bound to ICAM-1 dose-dependently, and the calculated $K_d$ is 14.1 µM. In comparison, sP5 bound ICAM-1 weakly with a $K_d$ of 5.7 mM (data not shown). Furthermore, analysis of steady state binding (i.e. the peak response level) achieved at different concentrations of peptide P5 supported a single-binding site model with a $K_d$ of 34 µM (Fig. 5D), whereas no $K_d$ could be obtained for sP5. Collectively, these results demonstrated that the peptides derived from the identified ICAM-1-binding site interacted directly with ICAM-1 and therefore could provide a direct contact interface for ICAM-1 recognition.

A Dual Function for the F-\alpha7 Loop of the $\beta_2$I Domain in Ligand Binding—One of the functional segments within the identified ICAM-1-binding site is Glu344–Asp348, which resides in the F-\alpha7 loop (residues 344–358) of the $\beta_2$I domain. The F-\alpha7 loop undergoes large movement upon ligand engagement to the $\beta$I domain and thus was proposed to play a key role in integrin activation (30). Specifically, it was hypothesized that residues within the F-\alpha7 loop interacted with residues within the extended loop of the $\beta$TD domain in an inactive receptor (the deadbolt model) (5), and disruption of this interaction leads to receptor activation. Hence, we speculated that mutations of this loop would directly break such interaction and thereby result in conformational changes of the $\beta_2$I domain and receptor activation. To test this hypothesis, we first evaluated the conformational states of mutant $\alpha_M\beta_2$(Glu344–Asp348), in which the sequence of the F-\alpha7 loop has been altered, using three different mAbs CBRM1/5, mAb 24, and mAb MEM148, which recognize activation-dependent neo-epitopes within the $\alpha_M$I domain (34), the $\beta_2$I domain (35), and the $\beta_2$ hybrid domain (36), respectively. Representative FACS analyses with mAb 24 were shown in Fig. 6A. Compared with the wild type receptor, the mutant bound mAb 24 much better, approaching its maximal binding activity, which was obtained in the presence of Mn$^{2+}$. Verifying specificity, no mAb 24 binding was observed for either wild type or mutant in the presence of EDTA. To quantify the extent of receptor activation, we determined the mean fluorescence values for these three mAbs and then normalized surface expression based on mean fluorescence of two activation-independent mAbs, the $\alpha_M$-specific OKM1 (for mAb CBRM1/5) and the $\beta_2$-specific 6.7 (for mAb 24 and mAb MEM148). As shown in Table I, little binding (for mAb 24 and MEM148), and weak binding (for mAb CBRM1/5) were observed for wild type $\alpha_M\beta_2$. However, when the sequence within the F-\alpha7 loop was mutated, significant increases (2.5-, 2.9-, and 2.8-fold, respectively) in the mean fluorescence intensity was observed for mAbs CBRM1/5, 24, and MEM148, indicating that conformational changes within the $\beta_2$I domain had been propagated into at least two other regions within $\alpha_M\beta_2$ (the $\alpha_M$I domain and the $\beta_2$ hybrid domain). Thus, mutations of the F-\alpha7 loop disrupted potential interactions between the F-\alpha7 loop and the $\beta$TD domain, resulting in global conformational changes and receptor activation. In support of this notion, the $\alpha_M\beta_2$(Glu344–Asp348) mutant exhibited higher adhesive activity toward another $\alpha_M\beta_2$ ligand (the Fg $\gamma$-module) (Fig. 6B). In this assay, a 293 cell clone that expresses wild type $\alpha_M\beta_2$ at a level equivalent to that of $\alpha_M\beta_2$(Glu344–Asp348) was used in parallel adhesion assays. As shown in Fig. 6B, cells expressing the mutant receptor exhibited stronger adhesion toward the $\gamma$-module across a wide range of concentrations (5–40 µg/ml), indicating that this mutant receptor is more active than wild type $\alpha_M\beta_2$. The specificity of this assay was confirmed by the
ability of NIF to completely abrogate cell adhesion (data not shown).

DISCUSSION

A well known feature of the integrin receptor is its ability to change conformation in response to intracellular activation signals (inside-out signaling). Although the detailed molecular mechanism underlying integrin inside-out signaling still remains elusive, several models have been recently proposed (3–5). A key element that is shared among these models is the assumption that the F-α7 loop within the βI domain (residues 344–358 of β2) is critical to the conformational transition from the closed to the open state of the integrin receptor. Yet, no study to date has directly mutated this loop and studied its impact on ligand binding. Therefore, this work provides the first experimental support for a critical role of this F-α7 loop in controlling the open and closed conformations of the βI domain as well as the other two regions within the αMβ2 receptor. In addition, we found that the F-α7 loop is also part of a novel binding interface for ICAM-1 within the βI domain. Thus, we conclude that the F-α7 loop has dual functions in ligand binding by αMβ2.

Previously, we reported our establishment and characterization of 17 αMβ2 homolog-scanning mutants that contain individual segment switches between the homologous β1 and β2 I domains, based on the premise that the β1 and β2 integrins are very similar in protein sequence (74% identity for the βI domains) yet recognize completely different sets of ligands. We showed that the homolog-scanning mutants, including those that were defective in C3bi and Fg binding, possessed intact conformations, judged by their reactivity toward a panel of conformation-dependent mAbs and by their ability to respond normally to Ca²⁺ (24). Therefore, the loss of ligand binding function was most likely caused by direct perturbations of the ligand recognition site per se rather than indirectly by conformational changes within the mutant receptors. In this study, we screened these 17 αMβ2 mutants for ICAM-1 binding using our established adhesion method (28) with an additional activation step by a β2-activating mAb (MEM48). Consistent with reports in the literature (20), we found that receptor activation was required for ICAM-1 binding by αMβ2 (Fig. 1). Based on the adhesion assays, we found five noncontiguous segments (Pro192–Glu197, Asn213–Glu220, Leu225–Leu230, Ser324–Thr329, and Glu344–Asp348) on the apex of the βI domain that were critical to ICAM-1 recognition (Fig. 2). That these segments are involved directly in ligand binding was supported by the obser-
that recognizes the FACS analysis using the activation-dependent mAb 24 (dashed line).

The F-adhesion to ICAM-1. Conformation of WT assay. Cells expressing either wild type (thin line).

B. Perturbation of the F-adhesion to ICAM-1. Conformation of WT assay. Cells expressing either wild type (thin line) or mutant αMβ2(Glu344–Asp348) (○) that existed in a constitutively active conformation were allowed to adhere to different concentrations of a representative αMβ2 ligand, the Fg γ-module. After washing, the number of adherent cells was determined as for Fig. 1.

TABLE I

| Binding of the conformation-dependent mAb CBRM1/5, mAb 24, and mAb MEM148 to the 293 cells expressing the wild-type and mutant αMβ2 determined by FACS analysis | Mean fluorescence staining of mAb CBRM1/5, mAb 24, or mAb MEM148 as expressed as the percentage of mean fluorescence of mAb OKM1 (for mAb CBRM1/5) or mAb 6.7 (for mAbs 24 and MEM148), which recognizes an activation-independent epitope located within either αM or β2, respectively. |
|---|---|---|
| | CBRM1/5 | mAb 24 | MEM148 |
| Wild type | 71 | 4.6 | 2.8 |
| αMβ2(Glu344–Asp348) | 175 | 13 | 7.9 |

vation that four of the five defective mutants in ICAM-1 binding interacted with Fg and C3bi as well as the wild type receptor, and these five mutants all exhibited correct Ca2+-dependent conformations (23). In addition, using constitutively active αMβ2, we demonstrated that inhibition of ligand binding by the β2I domain mutations was independent of the conformational states of the αM2I domain, because mutating a functional segment (Leu229–Leu230) abolished ligand binding by constitutively active αMβ2 receptors, which were generated by changing the critical Ile316 residue or by creating a disulfide linkage between residues 297 and 304 within the αM2I domain (25) and by the addition of Mn2+ (31) (Fig. 2). Furthermore, we showed that synthetic peptides derived from these sequences, but not scrambled control peptides, were able to inhibit the interaction between wild type αMβ2 and ICAM-1 when present in solution (Fig. 4) and to directly bind soluble ICAM-1 when coated on microtiter plates (Fig. 5A). Similar interactions between ICAM-1 and these β2I domain-derived peptides were observed using a reverse format where ICAM-1 was immobilized on the plates, and the peptides were added in solution, providing further support that these sequences represent a direct ICAM-1-binding interface within the β2I domain (Fig. 6). Interestingly, several peptides supported ICAM-1 binding when immobilized on the microtiter plate but did not interact with ICAM-1 well in solution, suggesting that these peptides may adopt different conformations depending on the environment. Similar phenomena have been well documented in the literature for large proteins such as fibrinogen, vitronectin, and von Willebrand factor (37).

Although the newly identified ICAM-1-binding site, like those for Fg and C3bi, resides on the apex of the β2I domain, two important differences separate ICAM-1 binding from C3bi and Fg binding to αMβ2. First, we and others have shown that αMβ2-expressing 293 cells interact with C3bi constitutively (28, 38). In contrast, activation of αMβ2 by a β2-specific activating mAb (MEM48) is required for αMβ2-mediated cell adhesion to ICAM-1 (Fig. 1), suggesting that ICAM-1 recognizes a different conformation of αMβ2 than C3bi does. Second, mutations of the β2I domain had differential effects on αMβ2 binding to ICAM-1 and C3bi. Among the 17 mutations within the β2I domain, six of them, including Glu162–Gly164, Pro192–Glu197, Asn213–Glu220, Leu225–Leu230, Ser234–Thr320, and Glu344–Asp348, destroyed significant ICAM-1 binding by αMβ2 (Fig. 2). Yet most of these β2I domain mutants, including αMβ2(Glu162–Gly164), αMβ2(Pro192–Glu197), αMβ2(Asn213–Glu220), αMβ2(Ser234–Thr320), and αMβ2(Glu344–Asp348), had normal or even better binding activity (1.5–5-fold) than the wild type receptor for C3bi and Fg (23). Only one mutation, Leu225–Leu230, destroyed αMβ2 binding activity for all three ligands (ICAM-1, C3bi, and Fg). Thus, the ICAM-1-binding site is distinct from those of C3bi and Fg. The only overlapping region between the ICAM-1 and the C3bi- and Fg-binding sites is the region surrounding sequence Leu225–Leu230, which is involved in the formation of the metal ion-dependent adhesion site motif. Consistent with our data, Goodman and Bajt (20) reported that the Glu235 residue, which is located between segments Asn213–Glu220 and Ser234–Thr320 in space, is critical to ICAM-1 binding. Moreover, our mutagenesis results are consistent with the strong inhibitory activity of mAbs IB4 and MHHM23 on ICAM-1 binding to αMβ2 (39). Both mAbs recognize segment Pro192–Glu197 of the β2I domain (24). The critical sequence Glu162–Gly164 was not believed to be part of the binding site, because it resided at the bottom of the β2I domain, far from the other five segments. The mechanism by which it abolishes ICAM-1 binding is unclear. However, considering its proximity to the β2 hybrid domain (30), it is likely that perturbation of this region may alter the relative orientation between the β2I domain and the hybrid domain, thus affecting receptor activation by mAb MEM48. More studies will be needed to test this hypothesis.

Another important finding from this work is that sequence
Glu$^{344}$–Asp$^{348}$ located within the F-α2 loop of the β2I domain, exhibited dual activities in ligand binding; not only was it capable of controlling the open and closed conformations of the receptor, it also contributed directly to the formation of the ICAM-1-binding site. Our finding is consistent with the deadbolt model for integrin activation recently proposed by Xiong et al. (5). In this model, the authors proposed that the residues within the F-α2 loop of the βI domain form a close contact with the residues in the elongated CD loop of the βTD domain, which then acts as a deadbolt to prevent the flip of the F-α2 loop that is required for conversion of closed to open conformation of the β2I domain. Interruption of this interaction releases the deadbolt from the βI domain, leading to conformational change and receptor activation. Our observation that mutation of residues within the F-α2 loop activated αMβ2 is consistent with this hypothesis. Furthermore, our data demonstrated that conformational changes caused by perturbation of the F-α2 loop could be transmitted throughout the heterodimeric receptor, causing multiple structural rearrangements, including the ligand-binding pocket within the αM domain that is recognized by mAb CBMR1/5 (34) and the hybrid domain within the β2 subunit that is recognized by mAb MEM148 (36). Thus, the conformations of different regions within the receptor are intimately connected. One of the potential mechanisms that could link together the αM and βI domains is likely mediated by interaction between Glu$^{220}$ of αM, which resides in between the two I domains, and the metal ion-dependent adhesion site motif of the βI domain (33). Thus, mutation of Glu$^{220}$ could potentially disrupt the flow of conformational changes from β2 to αM, as well as alter the relative orientation between these two ligand-binding sites, both resulting in defective ligand binding. In further support of our model, it was reported that the αM and βI domains exist in both open and closed conformations, and integrin activation may require activations of the individual I domains, as well as changes of the secondary and tertiary structures among these ligand binding domains (32, 40).

In summary, the results from this study strongly support our original model that the β2I subunit plays a direct role in ligand binding by αMβ2 (23). Specifically, our data demonstrated that there exist several discrete subdomains on the upper surface of the βI domain that allow it to interact with multiple unrelated ligands. Surprisingly, the ICAM-1 binding site is much broader than those for C3bi and Fg and is composed of five noncontiguous segments Pro$^{192}$–Glu$^{197}$, Asn$^{213}$–Glu$^{220}$, Leu$^{225}$–Leu$^{230}$, Ser$^{254}$–Thr$^{255}$, and Glu$^{344}$–Asp$^{348}$. The ICAM-1 recognition site is well separated from the Fg- and C3b-binding sites (23), although they did share the common metal ion-dependent adhesion site motif that is located within segment Leu$^{259}$–Leu$^{260}$. The three potential cation-binding sites within the βI domain are located inside this identified region, suggesting that these three sites may also contribute directly to ICAM-1 binding by αMβ2. Most importantly, as the identified ligand-binding site contains a segment that is involved in modulating and closing conformations of the receptor in response to integrin inside-out signaling, our study raises an interesting possibility that inside-out signaling could directly modify the shape of the ligand-binding site, in addition to its ability to change the global conformation of the receptor. Given the similarity among all integrin β subunits, our results may help us understand the underlying mechanism of integrin-ligand interactions in general.
