Identity of the Amino Acid Residues Involved in C3bi Binding to the I-Domain Supports a Mosaic Model To Explain the Broad Ligand Repertoire of Integrin $\alpha_M\beta_2$†

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ABSTRACT: Interactions between the complement degradation product C3bi and leukocyte integrin $\alpha_M\beta_2$ are critical for host defense against foreign pathogens and in tumor cell surveillance. To gain insight into the mechanism by which the α_MI-domain of the integrin interacts with C3bi, detailed mapping of the C3bi binding site was undertaken. Previous mutagenesis studies had implicated five small structural segments within the α_MI-domain in recognition of this ligand. Sets of three amino acids within the five implicated segments were mutated to the corresponding α_LI-domain residues. Then, within the affected mutants, single point mutations were introduced to precisely define the requisite residues. Ultimately, H148, F150, Q204, L205, R208, T211, T213, I256, P257 were identified as being critical for C3bi binding. A synthetic peptide approach confirmed the involvement of the specified residues with the complex midsegment, Q204−I215, in C3bi recognition. Furthermore, the α_DI-domain, which has a low intrinsic affinity for C3bi, acquired high affinity for the ligand when the implicated residues were inserted. The residues necessary to engage C3bi reside on or adjacent to the cation binding MIDAS site of the α_MI-domain. The amino acids involved in C3bi binding are distinct from those involved in interaction of previously mapped ligands with the α_MI-domain. This divergence supports a *mosaic model*, in which different ligands engage different amino acids to bind to α_MI-domain, accounting for the broad recognition capacity of integrin $\alpha_M\beta_2$.

Integrins are a large family of membrane glycoproteins that regulate numerous and fundamental cellular functions, including adhesion, migration, shape change, proliferation, and intracellular signaling (*1, 2*). These responses depend on the capacity of integrins to engage a variety of extracellular ligands (*3*−*6*). Among the integrins, $\alpha_M\beta_2$ (CD11b/CD18, CR3, Mac-1) is particularly notorious for its ability to recognize multiple ligands, which have no apparent structural similarities; more than 30 protein and nonprotein ligands bind to $\alpha_M\beta_2$ (*7, 8*). Three regions of the receptor mediate recognition of protein ligands by $\alpha_M\beta_2$: the α_MI-domain, the α_M β-propeller, and the β_2I-like domain. The latter two regions are highly conserved among integrins; and numerous studies, ranging from mutational analyses (*9, 10*) to structural studies (*11*−*14*), have implicated these regions in the engagement of most ligands by their cognate integrins. Nine of the 24 integrins contain I (A)-domains in their α subunits, and these regions have been shown to play a major role in ligand recognition (*15*−*17*). In $\alpha_M\beta_2$, it is the α_MI-domain that imparts the broad recognition capacity to the integrin. For example, fibrinogen (*18*), platelet membrane glycoprotein GPI bα (*19*), urokinase (*20*), plasminogen (*21, 22*), and neutrophil inhibitory factor (NIF) (*23*−*25*) all bind with relatively high affinity to the α_MI-domain expressed as a recombinant protein in the absence of other regions of the receptor. The α_MI-domain is a region of ∼200 amino acid residues inserted into the β-propeller domain of the α_M subunit. The α_MI-domain was the first of a number of different I-domains to be crystallized (e.g., see refs *17, 26, 27*). It conforms to a Rossman-type fold consisting of seven short α helices surrounding six interiorized β sheets. Connecting these structural elements are flexible loops, which also contribute the five residues that coordinate a divalent cation bound in a MIDAS[1] motif. The flexible loops and the MIDAS-bound cation are critical to the binding of ligands to the α_MI-domain (*28*−*30*).

C3bi is formed during the activation and subsequent inactivation of complement component C3. C3bi itself has distinct biological functions; most notably, it is the major opsonin facilitating phagocytosis of foreign pathogens by leukocytes. Recognition of C3bi by these cells is mainly mediated by $\alpha_M\beta_2$, which is expressed prominently on neutrophils and macrophages (*31*). The role of this integrin and its recognition of C3bi is important not only in the phagocytosis of foreign materials but also in tumor surveillance in which leukocyte opsonization of cancer cells is

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[1] Abbreviations: C3bi, a derivative of complement component C3; MIDAS, metal ion-dependent adhesion site; BSA, bovine serum albumin; SPR, surface plasmon resonance.

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mediated by recognition of C3bi on the surface of the transformed cells (*32*). Genetic deletion of the α_M subunit in mice leads to defective C3bi-dependent phagocytosis and increased bacterial infections (*33*, *34*).

$\alpha_M\beta_2$, along with $\alpha_L\beta_2$ (LFA-1, CD11a/CD18), $\alpha_X\beta_2$ (p150, 95, CD11c/CD18), and $\alpha_D\beta_2$, constitutes the β_2 subfamily of integrins (*35*). All β_2 subfamily members contain I-domains within their α subunits, which contribute prominently to their ligand binding properties (*36*, *37*). Several independent studies have demonstrated the importance of the α_MI-domain for recognition C3bi by $\alpha_M\beta_2$. These studies have included the analyses of C3bi binding to the expressed α_MI-domain (*38*) as well as introduction of point mutations in the α_MI-domain of the intact receptor (*10*, *39*).

We have recently proposed a *mosaic model* to explain the capacity of so many unrelated ligands to bind to $\alpha_M\beta_2$ (*25*). Accordingly, different ligands bind to the same face, the MIDAS face, of the α_MI-domain but use different sets of residues on this face to contact and achieve productive binding to the integrin. This model was predicated on our findings that NIF binding to the α_MI-domain could be ablated by mutation of any of five noncontiguous amino acid residues on the MIDAS face; that the α_LI-domain acquired high affinity for NIF upon introduction of these five residues into its structure; and that the chimeric the α_LI-domain still was unable to bind C3bi. Left unresolved was how C3bi interacted with the α_MI-domain and if the mosaic model would, indeed, apply to other $\alpha_M\beta_2$ ligands. Yakubenko et al. (*8*) have recently suggested that a particular sequence, Lys(245)−Arg(261), serves as a "promiscuity loop" and multiple ligands bind to the α_MI-domain through this structure, providing an alternative to the mosaic model. In view of the biological importance of C3bi and to address these questions regarding the structure−function relationships of $\alpha_M\beta_2$, we have sought to identify the residues within the α_MI-domain critical for C3bi binding.

EXPERIMENTAL PROCEDURES

Materials. Monoclonal antibodies 44, 44a, and IB4 were obtained from the American Tissue Culture Collection (Rockville, MD), and CBL 189 from Chemicon (Temecula, CA). In most of the experiments, C3bi binding to the α_MI-domain was analyzed as described by Bilsland et al. (*40*) with certain modifications (*41*) using activated sheep erythrocytes as resource of C3bi. Purified human C3bi, purchased from Calbiochem (La Jolla, CA), also was used in some experiments.

Site-Directed Mutagenesis, Expression, and Purification of I-Domain Fusion Proteins. The cDNAs of α_MI-domain [678 nucleotides, Arg(131)−Ser(356)], α_LI-domain [633 nucleotides, Pro(145)−Ser(355)], and α_DI-domain [561 nucleotides, Met(149)−Val(335)] were cloned and inserted into the pGEX-5X-3 expression vector (*42*). All wild-type and mutant I-domains were expressed as glutathione *S*-transferase (GST) fusion proteins and purified by adsorption onto glutathione-Sepharose 4B as described (*25*). The quality of the fusion proteins was assessed by SDS−PAGE. Each preparation migrated as a single band of ∼49−52 kDa. The concentrations of I-domains were determined using the BCA protein assay (Pierce) or by measuring the absorbance at 280 nm.

For selected experiments, the α_MI-domain was cleaved from its GST fusion partner by incubation of the recombinant protein adsorbed onto glutathione-Sepharose 4B with factor Xa (10 μg/mL factor Xa per 200 μg/mL I-domain) for 4−18 h at room temperature with agitation. The cleaved α_MI-domain was recovered by washing the resin with 3 column volumes of TBS, and the eluate was concentrated by centrifugation through a Millipore membrane. SDS−PAGE indicated a single molecular weight band of ∼30 kDa, appropriate for the α_MI-domain.

For site-directed mutagenesis, the QuikChange site-directed mutagenesis kits from Stratagene (San Diego, CA) were used. The pGEX-5X-3 construct containing DNA encoding the I-domains was modified by site-directed mutagenesis using mutagenic primers containing the desired mutation and analyzed by sequencing. As a strategy, target residues in the α_MI- and α_LI-domains were changed to those present in the other I-domain. If the α_M and α_L residues were the same, the amino acid was mutated to that present in the corresponding position in the α_XI-domain; or if the residue was identical in all three I-domains, the amino acid was replaced with an Ala or Gly. This mutagenesis strategy has been used successfully to characterize the binding sites for other ligands in the α_MI-domain (*19*, *25*).

C3bi Binding to I-Domains. Sheep erythrocytes (Colorado Serum Co., Denver, CO) at 7×10^8 were activated by anti-sheep erythrocyte IgM antibody M1/87 (Accurate Chemical & Scientific Co., Westbury, NY) and human C5-deficient serum (Sigma, St. Louis, MO) (*25*). The coated erythrocytes were surface-labeled with sulfosuccinimidyl 6-(biotinamido)-hexanoate (Pierce, Rockford, IL). After washing twice with Hank's balanced salt solution, the activated erythrocytes were resuspended in 3 mL of the same buffer and disrupted with four 30-s cycles at 70 W in a Branson Ultrasonics sonciator (VWR Scientific). The insoluble cellular material was recovered by centrifugation at 25000*g* for 15 min at 4 °C and used as a source of C3bi after resuspension in Hank's balanced salt solution. The equivalent of $\sim 3 \times 10^6$ erythrocytes in 50 μL was added to 96-well plates (Immulon 4BX, Dynex Technologies Inc., Chantilly, VA), which had been precoated with the I-domains at 50 μg/mL for 1 h at 37 °C and postcoated with 2% BSA for 1 h at 37 °C as described (*42*). The bound C3bi was detected using avidin-alkaline phosphatase and *p*-nitrophenyl phosphate. As a control, the background reaction on BSA coated wells was subtracted. In certain experiments in which purified C3bi was used, the plates were coated with C3bi and postcoated with 2% BSA, and I-domain binding was detected using anti-GST (Upstate, Lake Placid NY), alkaline phosphatase conjugated anti-mouse IgG, and *p*-nitrophenyl phosphate. When I-domains cleaved from GST were used in such experiments, anti-α_M, 44 or 44a, following goat anti mouse IgG conjugated with alkaline phosphatase, were used for detection. To calculate binding constants from this assay C3bi titration data were fit to a single site model with the equation $[\text{C3bi}]_{\text{bound}} = B_{\max}[\text{C3bi}]/(K_d + [\text{C3bi}])$, where $B_{\max}$ is the maximal binding and K_d is its dissociation constant, using the SigmaPlot program (Jandel Co., San Rafael, CA). K_d values in the tables are presented as means ± SD.

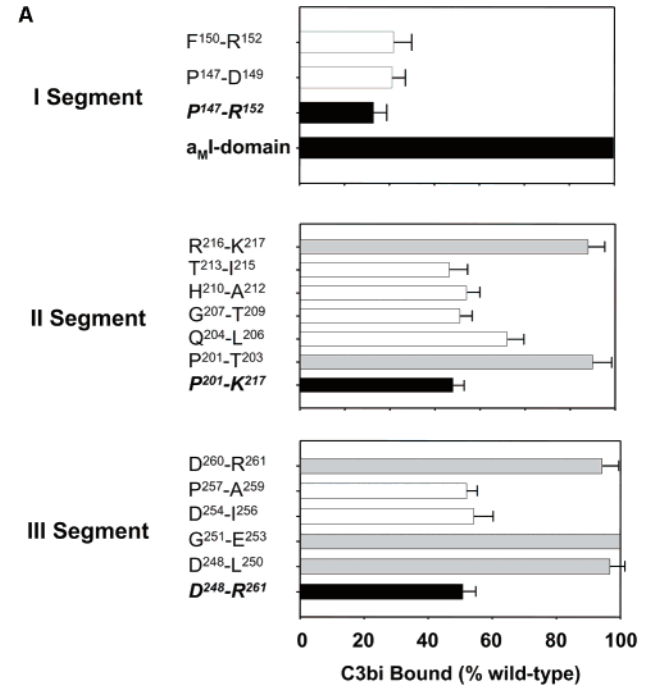
Surface Plasmon Resonance (SPR) Experiments. SPR experiments were carried out using a BIAcore 3000 instrument (Biacore, Inc., Uppsala, Sweden). Standard amine

coupling was employed for direct immobilization of I-domains or for anti-GST immobilization on research-grade CM5 sensor chip according to the manufacturer's procedure. The typical immobilization level was ~3000 RU (response units) for the I-domains. The purified GST fusion I-domains or recombinant GST was presented to the anti-GST chips at 5 μg/mL. The typical response obtained with immunocaptured GST-proteins was ~1500 RU. In the experiments, a chip with captured GST was used in the reference cell, and the signal obtained was subtracted as background from the experimental data set. To study the C3bi/I-domains interaction, different concentrations of C3bi, diluted in running buffer (HBS-P buffer containing 10 mM Hepes, pH 7.4, 150 mM NaCl, 0.005% Surfactant P20 and 60 μg/mL BSA with 2 mM $CaCl_2$, $MgCl_2$ or $MnCl_2$ as specified), were injected at a flow rate of 5 μL/min, and the sensograms that developed were analyzed. Each protein concentration was repeated at least twice, and samples were analyzed in random order. Data were fit to a simple 1:1 Langmuir interaction model, and equilibrium binding constants, K_d, were derived from the on and off rate constants using the instrument software package.

Molecular Modeling. The model building of α_MI-domain was based upon its crystal structure (*14*, *26*) using PDB deposited coordinates, mmdbId:23013.

RESULTS

C3bi Binding to "Triple Mutants" of the α_MI-Domain. In previous studies (*41*), we employed homologue scanning mutagenesis in which individual segments of a defined secondary structure, α helix, β sheet, and connecting loop, in the α_MI-domain were switched to the corresponding segment of the α_LI-domain, which is highly homologous but does not bind the same ligands as $\alpha_M\beta_2$. Altogether, seventeen "$\alpha_M\beta_2$ swap mutants" were created to cover the hydrated surface of the α_MI-domain, and five of these mutants lost their capacity to bind multiple $\alpha_M\beta_2$ ligands, including NIF, GPI bα, and C3bi (*24*). The five segments lay primarily within the connecting loops on the MIDAS face of the α_MI-domain, and two of these segments were contiguous, allowing us to define three continuous amino acid sequences that were critical to ligand binding: segment I, residues P(147)−R(152); segment II, residues P(201)−G(207) + R(208)−K(217); and segment III, residues D(248)−Y(252) + E(253)−R(261). To define the NIF binding residues with these sequences, a series of 13 *triple mutants* α_MI-domain were created. In each of these triple mutants, a set of three consecutive amino acids within the α_MI-domain was changed to the corresponding α_L residues. These same triple mutants, as well as wild-type α_MI-domain, were purified and immobilized onto microtiter wells, and their binding of biotinylated C3bi was assessed. As shown in Figure 1A, 8 of the 13 triple mutants, P(147)−D(149), F(150)−R(152), Q(204)−L(206), G(207)−T(209), H(210)−A(212), T(213)−I(215), D(254)−I(256), and P(257)−A(259), showed significant reductions in C3bi binding. Compared to wild-type α_MI-domain, the reductions in C3bi binding ranged from 35% to 70%. The residual activities of each of the affected triple mutants were similar to those of the mutants with the entire segments substituted. The positions of the eight affected triple mutants are located on the crystal structure of the α_MI-domain in Figure 1B. Four of the triple mutants, Q(204)−L(206), G(207)−T(209), H(210)−A(212), and T(213)−I(215), lie on the MIDAS face, and the other four, P(147)−D(149), F(150)−R(152), D(254)−I(256), and P(257)−A(259), lie at its edges.



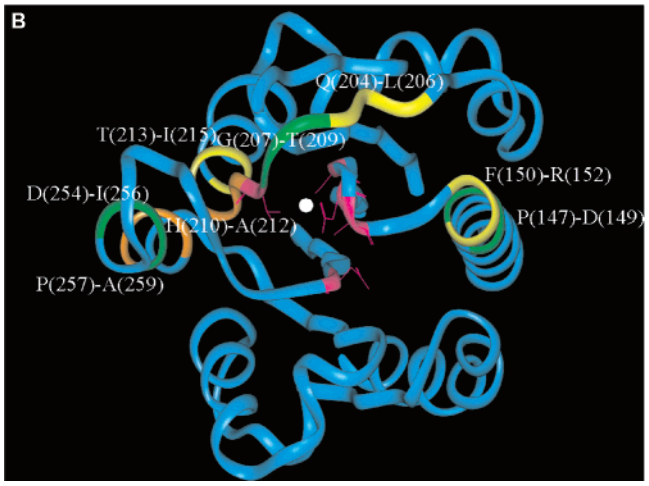


FIGURE 1: (A) C3bi binding to α_MI-domain triple mutants. The series of 13 *triple mutants* were created to span segment I, P(147)−R(152); segment II, P(201)−K(217); and segment III, D(248)−R(261) in recombinant α_MI-domains. In each triple mutant, a set of three consecutive amino acids was changed to the corresponding α_L residues. Mutant α_MI-domains were expressed as GST fusion proteins. Each GST fusion I-domain was reacted with immobilized C3bi, and binding was quantified with alkaline phosphatase conjugated anti-GST. Each data set is the mean ± SEM of at least three independent experiments. (B) Location of the triple mutants within the crystal structure of the α_MI-domain. The structure is modeled according to the crystal coordinates (*26*) of the α_MI-domain using PDB coordinates, mmdbId:23013. The divalent cation bound in the MIDAS motif is shown in white. The coordinated the metal amino acid residues (D140, S142, S144, T209, and D242) are in red (*26*).

C3bi Binding to Single-Point Mutants of the α_MI-Domain. Within the eight triple mutants with defective C3bi binding, each of the three amino acids was mutated individually to the corresponding residue in α_LI-domain. Thus, a total of

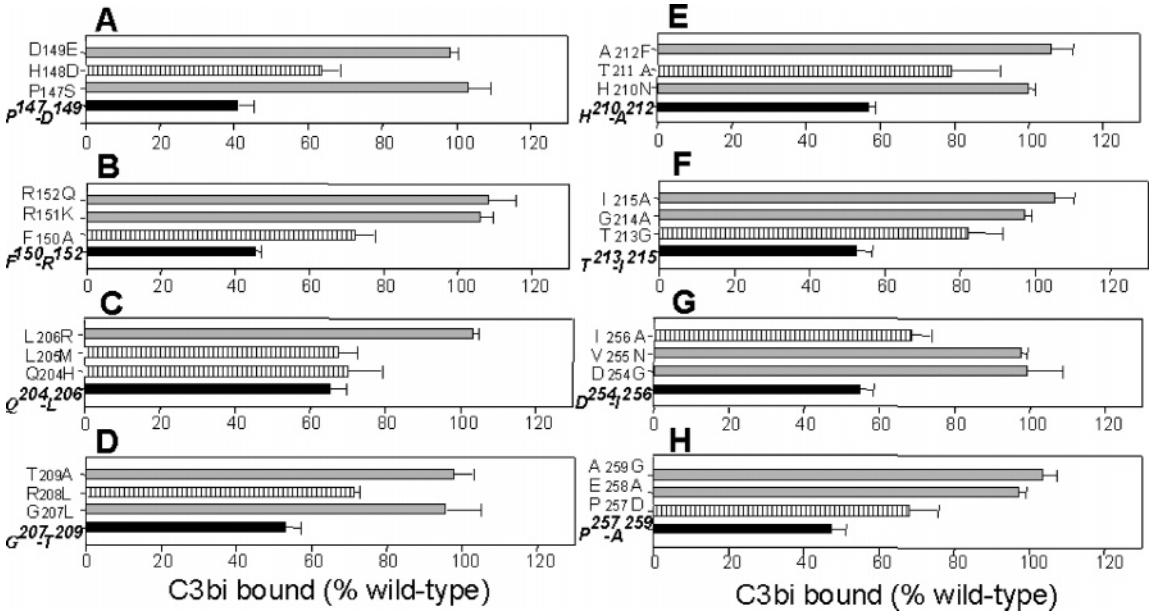


FIGURE 2: C3bi binding to α_MI-domain single mutants. Within the eight triple mutants with defective C3bi binding (see Figure 1), each of the three amino acids was mutated individually to the corresponding residue in α_LI-domain. A total of 24 *single mutants* were tested for their binding to immobilized C3bi using alkaline phosphatase conjugated anti-GST for detection.

24 *single mutants* were created. After confirmation of the DNA sequence of these single mutants, each was expressed as a GST fusion protein in *Escherichia coli* and purified. The capacity of the 24 single mutants to bind C3bi is summarized in Figure 2. Nine of the 24 single mutants exhibited reduced C3bi binding. These were H(148) and F(150) in segment I; Q(204), L(205), R(208), T(211), and T(213) in segment II; and I(256) and P(257) in segment III. In eight of the nine triple mutants, a single amino acid substitution accounted for the loss of function of the triple mutants. The one exception was the Q(204)−L(206) mutant, in which mutation of either Q(204) or L(205) caused a substantial and similar loss of function.

Peptide Analyses of Segment II. The above results suggest that the recognition of C3bi with α_MI-domain is complex, particularly within segment II, where mutation of any one of five residues perturbed the interaction. A synthetic peptide analysis was undertaken as an independent approach to confirm the role of these multiple residues in segment II in C3bi recognition. A peptide corresponding to segment II was synthesized, purified, and tested as an inhibitor of the α_MI-domain−C3bi binding reaction. As shown in Figure 3A, the 17 amino acid peptide corresponding to the naturally occurring α_M sequence of segment II caused a dose-dependent inhibition of C3bi binding to immobilized α_MI-domain, and fully blocked the interaction at 100−200 ng/mL. In contrast, a scrambled peptide had no effect, even at the highest concentration tested. Next, a series of 17-mer α_L peptides were synthesized containing a single substitution of an α_M residue. This substituted residue was one of the five segment II residues that was identified to be important for C3bi binding from the mutant α_MI-domains. For example, the mutant α_MI-domain containing the H(204)Q substitution reduced C3bi recognition (Figure 2); hence, the α_L segment II peptide containing the Q(204)H substitution was synthesized. Five such α_L segment II mutant peptides were synthesized with the Q(204)H, L(205)M, R(208)L, T(211)A, and T(213)G substitutions. In addition, a chimeric α_L II segment peptide was synthesized carrying all five substitutions. As shown in Figure 3B, wild-type α_L segment II peptide did not inhibit α_MI-domain binding to C3bi. How-

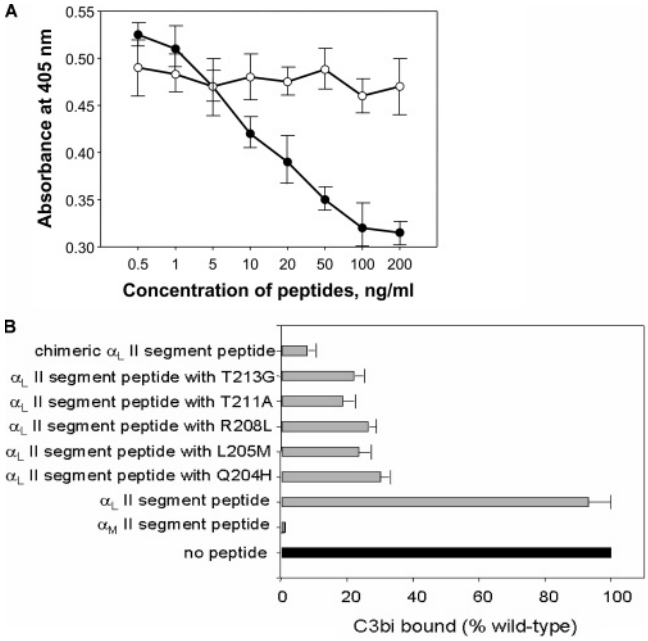


FIGURE 3: Inhibition of α_MI-domain binding to C3bi by segment II peptides. (A) Different concentrations of a synthetic peptide corresponding to segment II, P(201)−K(217) (●), or the scrambled peptide (○) were added together with the α_MI-domain-GST fusion protein to microtiter wells containing immobilized C3bi. Binding was quantified using an anti-GST conjugated to alkaline phosphatase. (B) The α_MI-domain and C3bi interaction was measured in the presence of variant α_L peptides, corresponding to either the naturally occurring sequence of the α_MI-domain segment II, the corresponding α_LI-domain peptide, or the α_LI-domain peptide containing the indicated single amino substitution of an α_M residue. The peptides are all composed of 17 amino acids. Values are presented as the means ± SEM of at least three separate experiments. The inhibition induced by no peptide and by the α_M peptide was assigned as 0% and 100% inhibition and used to calculate the inhibition induced by 400 ng/mL of each peptide.

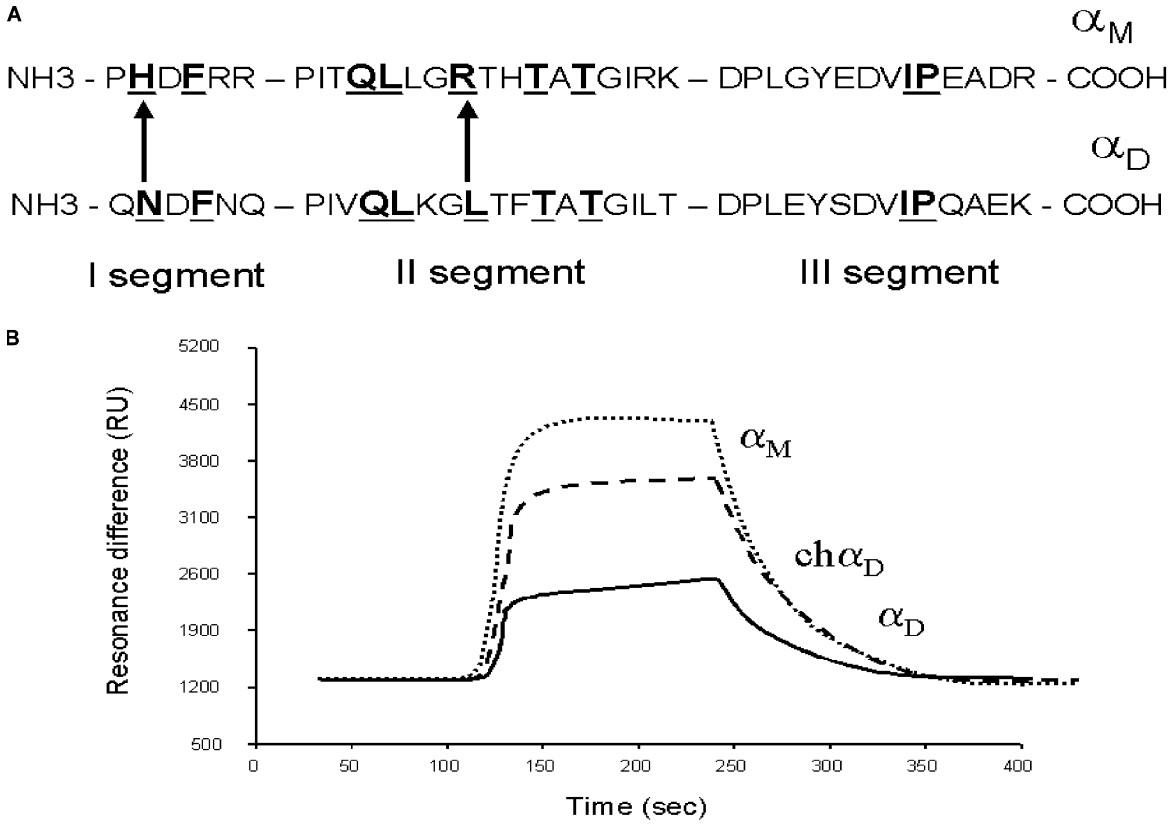


FIGURE 4: (A) Sequence alignments of the α_M and α_D I-domains. The amino acid residues 146 to 264 for the α_MI-domain are aligned with the corresponding sequences in the α_DI-domain (the numbering is based on the entire protein sequence of the α_M subunit including the signal peptide). The identified residues are lined above. (B) Analyses of the I-domains binding to C3bi by SPR. Representative profiles of the SPR responses of C3bi (20 μM) in the mobile phase binding to α_MI-domain (dotted line), α_DI-domain (solid line), and chimeric α_DI-domain (dashed line) coupled to chip surface. The chimeric α_DI-domain was mutated to contain H at N(148) and R at L(208) and, thereby, contains all 9 residues implicated in the binding of C3bi to the α_MI-domain. Experiments were performed in the presence of 2 mM Mg^{2+}. The data are representative of results obtained at various C3bi concentrations.

ever, each of the α_L segment II mutant peptides inhibited the interaction. Finally, the chimeric α_L segment I peptide showed inhibitory activity comparable to the wild-type α_M segment II peptide. Thus, these data fully support the mutational data implicating Q(204), L(205), R(208), T(211), and T(213) in C3bi binding.

Development of a Chimeric C3bi Binding I-Domain. Together, the above results lead to the predictions that I-domains with different residues at any of the nine positions should bind C3bi poorly and that the presence of the nine implicated α_M residues in another I-domain should impart C3bi binding function. To test these hypotheses, we choose the α_DI-domain as our template since it is the most divergent in sequence of four α subunits that partner with the common β_2 subunit in leukocyte subfamily member. The α_MI- and α_DI-domains are 60% identical at an amino acid level; but, as shown in Figure 4A, 7 of the 9 residues implicated in C3bi binding to the α_MI-domain are conserved in the two I-domains. Thus, the α_DI-domain should bind C3bi poorly; and, to create a chimeric α_DI-domain with C3bi binding activity, mutation of only N(148) to H and L(208) to R should be sufficient. The chimeric I-domain, as well as authentic α_DI-domain, was expressed as GST fusion proteins and purified, and their C3bi binding properties were evaluated by real-time SPR. The α_MI-domain, α_DI-domain, and chimeric α_DI-domain, as GST fusion proteins, were immobilized onto sensor chips by amino coupling, and their binding to a series of C3bi concentrations in the mobile phase was tested. Representative sensograms developed with 20 μM C3bi in the presence of 2 mM Mg^{2+} and the three I-domains are shown in Figure 4B, and the average K_d values derived from the various C3bi concentrations are summarized in Table 1. The α_DI-domain exhibited only weak C3bi binding (K_d = 330 μM) whereas the α_MI-domain bound the ligand with substantially higher affinity (K_d = 8.8 μM). Mn^{2+} also failed to support binding of C3bi to the α_DI-domain (data not shown). The chimeric α_DI-domain bound C3bi with an affinity that approached that of the α_MI-domain (K_d = 21.2 μM). Additional SPR analyses were performed with the I-domains either immobilized directly onto the sensor chips or captured onto immobilized anti-GST. The estimated K_d values, together with those derived from microtiter plate binding assays, are included in Table 1. Thevalues calculated

Table 1: K_d Values for Binding of C3bi to α_M I-Domain, α_D I-Domain, and Chimeric α_D I-Domain

	SPR[a]		
I-domain	amine coupling	GST capture	microtiter plate[a]
α_D I-domain	3.3×10^{-4}	5.5×10^{-4}	9.8×10^{-3}
chimeric α_D I-domain	2.12×10^{-5}	6.8×10^{-5}	9.1×10^{-5}
α_M I-domain	8.8×10^{-6}	9.1×10^{-6}	6.1×10^{-6}

[a] K_d values were derived from SPR with the I-domain immobilized either with amino coupling or with anti-GST. In the microtiter plate assay, the I-domains were also immobilized. In all three assays, C3bi was in the fluid phase.

by the three approaches are similar and establish that the chimeric α_DI-domain acquired C3bi activity approaching that of the α_MI-domain.

DISCUSSION

In this study, we have identified amino acid residues within the α_MI-domain that are involved in C3bi binding. The approach of homologue scanning mutagenesis had previously identified five segments, as providing key contact sites for C3bi recognition (*41*). To define the contact residues for C3bi binding, triple mutants were developed in which sets of three amino acid residues within the implicated segments were changed to the corresponding α_LI-domain residues (or to Ala, or Gly residues if conserved in α_L), and the capacity of the resulting mutants to bind C3bi was assessed. Of the 13 triple mutants, eight lost their ability to bind C3bi. Four of them, Q(204)−L(206), G(207)−T(209), H(210)−A(212), and T(213)−I(215), are in the loop containing T(209), which forms one of the MIDAS cation coordinating sites (see Figure 1B). The other triple mutants reside on the sides proximal to the MIDAS face but are spatially distant from MIDAS cation. Then, within these eight triple mutants, the individual amino acids were mutated. The outcome of these data is the identification of residues His(148), Phe(150), Gln(204), Leu-(205), Arg(208), Thr(211), Thr(213), Ile(256), and Pro(257) within the α_MI-domain as being critical to C3bi binding. Mutation at any one of these positions reduced C3bi binding to the point that interaction was markedly reduced.

To determine if mutation of these individual positions altered C3bi binding by perturbing the conformation of the α_MI-domain, the two different approaches were taken. The first was a peptide inhibition strategy. Peptides coinciding to the segment II in α_LI-domain were synthesized with single amino acid substitutions at the implicated α_MI-domain residues Gln(204), Leu(205), R(208), Thr(211), and Thr(213). These substituted peptides, as well as ones corresponding to the wild-type sequences of the α_L and the α_MI-domains, were tested as inhibitors of the interaction between α_MI-domain and C3bi. The wild-type α_M segment II peptide, residues Q(204)−I(215), inhibited the interaction in a dose dependent manner and was capable of producing complete inhibition at high concentrations, whereas the corresponding α_L peptide was noninhibitory. Each of the singly substituted peptides expressed some inhibitory activity, and α_L peptides bearing the 5 substitutions expressed inhibitory activity comparable to the wild-type α_M segment II peptide. These results corroborate the direct involvement of the segment II residues, Q(204)H, L(205)M, R(208)L, T(211)A, and T(213)G of the α_MI-domain in C3bi recognition. The second approach was a gain-in-function strategy. To create a chimeric α_DI-domain with the nine residues implicated in C3bi binding to the α_MI-domain required only two substitutions, N(148)H and L(208)R, as the other positions were conserved in the two I-domains. By SPR, the wild-type α_DI-domain exhibited a very low affinity for C3bi, whereas the chimeric α_DI-domain displayed substantial C3bi binding activity. The increase in affinity of the chimera compared to wild-type α_DI-domain in the presence of Mg^{2+} and Mn^{2+}, a divalent cation that should activate both I-domains, was more than 200-fold and was only 4-fold lower than authentic α_MI-domain, despite the fact that 76 out the 192 residues in the two I-domains were still different. The differences in the affinities of the

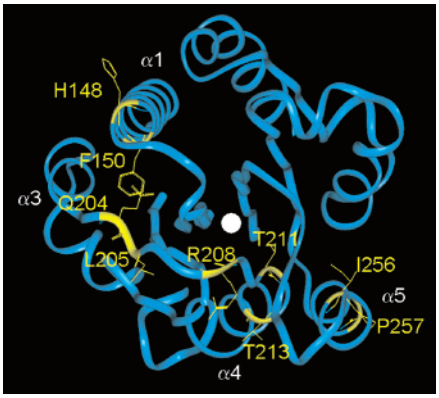


FIGURE 5: Location within the crystal structure of the distinct amino acid residues implicated in C3bi binding to the α_MI-domain. The crystal structure displayed is that of the α_MI-domain (*26*). The residues implicated in C3bi binding are highlighted in yellow. The divalent cation bound in the MIDAS motif is shown in white.

chimeric α_DI-domain and the α_MI-domain for C3bi indicate that recognition was not fully transferred. This may reflect that there is involvement of still additional α_MI-domain residues in C3bi binding, that certain α_DI-domain residues may clash with C3bi and reduce affinity, or that the activation states of the two I-domains were not identical.

On the basis of the crystal structure (*26*), all of the nine amino acids implicated in C3bi binding reside at the hydrated surface of the α_MI-domain; three of the 8 lie on the top of the MIDAS face, and others lie at proximity, upper edges (see Figure 5B). Gln(204), Leu(205), and Arg(208) reside in loops that are in close proximity to the cation bound in the MIDAS motif, and this proximity is consistent with crystal structures showing that ligands bound to I-domains coordinate with the bound cation and with residues within it vicinity (*13*, *43*). Consistent with involvement of the MIDAS cation, mutation of Asp(140) and Asp(242), which disrupts cation binding to the MIDAS, destroyed C3bi binding activity (*44*). Of the other residues implicated in our study, His(148) and Phe(150) reside in the α1 helix; Thr-(211) and Thr(213) in the α4 helix; and Ile(256) and Pro-(257) in the α5 helix. Consistent with our map, F(246) and G(247) were previously shown not to be important for C3bi binding (*10*). Previous studies had also implicated K(245), contained within a three residue insertion in the α_MI-domain that is absent in the α_LI-domain, in C3bi binding (*38*, *41*). Furthermore, regions outside of the I-domain have been implicated in C3bi binding to $\alpha_M\beta_2$. Residues Asp(134), Ser-(136), and Ser(138), which contribute to the MIDAS site in the β_2 subunit, as well as sequences Gln199−Ala203, Leu225−Leu230, and Gly305−His309 within the β_2 subunit, have been implicated in C3bi binding (*45*), and residues Asp-(398) to Thr(451) of α_M β-propeller were involved specifically in C3bi recognition (*46*). The involvement of such non-I-domain regions is consistent with the binding of C3bi to a mutant $\alpha_M\beta_2$ in which the I-domain was deleted. This I-domain-less $\alpha_M\beta_2$ bound to iC3b but with a lower apparent affinity than wild-type $\alpha_M\beta_2$ (*11*). Together, these data indicate that the binding interface of C3bi within the I-domain and within $\alpha_M\beta_2$ is very complex, substantially more so than that of other ligands, NIF, GPIbα, and fibrinogen, that have been mapped by mutational strategies.The binding sites for C3 on the surface of other target proteins also appear to be complex. For example, at least

three regions of plasma factor H have been implicated in its interaction with C3b (*47*−*49*).

Another β_2 integrin, $\alpha_X\beta_2$, also binds C3bi (*40*). Sequence alignment of the α_X and α_M I-domains indicate that 7 of the 9 identified amino acid residues are conserved. His(148) and Arg(208) in the α_M I-domain correspond to Arg(146) and Phe(206) in the α_X I-domain. Vorup-Jensen et al. (*17*) reported an affinity for the α_XI-domain by SPR of 400 μM^{-1} while our measurement of the affinity of the α_MI-domain by this technique was 10 μM^{-1}. The differences at the two positions may contribute to the differences in affinity of the two I-domains for the ligand.

While previous studies have shown that the binding sites for ligands to $\alpha_M\beta_2$ have certain common requirements, such as functional MIDAS sites in both of α_M and β_2 subunits, our detailed mapping studies demonstrate that these binding sites are not identical. Asp(149), Arg(151), Gly(207), Tyr(252), and Glu(258) within the I-domain are critical for NIF binding (*25*); Phe(246), Asp(254), and Pro(257) for fibrinogen binding (*18*); and Thr(213) and Arg(216) for GPI bα binding (*19*). The present study adds His(148), Phe(150), Gln(204), Leu(205), Arg(208), Ile(256), and Pro(257) to the list of I-domain residues that engage ligands. All of these residues lie within segments I, II, or III, which circumscribe 19% of the total α_MI-domain residues. Within the 37 residues in the three segments, 17 have now been implicated in the binding of the various ligands analyzed, and only three residues are used by more than one ligand. Pro(257) is utilized for C3bi and fibrinogen; Thr(211) and Thr(213) are utilized by C3bi and GPIbα. In the latter case, Thr(211) is conserved in both the α_M and α_LI-domains so that it appears that the T(211)A mutations must clash or perturb C3bi binding rather than being directly required for ligand binding. This explanation also is a formal possibility at other implicated positions; i.e., it may be the presence of a residue in α_L that clashes with the ligand and prevents binding rather than the absence of an α_M residue. While this ambiguity remains, the data clearly support a mosaic model, in which different ligands require different sets of residues for a productive interaction with the α_MI-domain. Inherent in this model is the possibility that different ligands may induce different responses upon engagement of $\alpha_M\beta_2$. While the binding of many different ligands to integrins induces similar common cellular responses, engagement of a single integrin by different ligands also can induce different responses. For example, binding of two peptide ligands to $\alpha_{IIb}\beta_3$ has distinct effects on membrane fluidity and induction of epitopes recognized by mAbs (*50*); and two fibrinogen peptides, both of which react with the α_MI-domain (*18*), exert differential effects on $\alpha_M\beta_2$-mediated cell migration (*51*). With the broad role of $\alpha_M\beta_2$ in physiological and pathological responses, the mosaic model suggests that the interactions and/or functional consequences of the binding of specific ligands to $\alpha_M\beta_2$ can be selectively antagonized. This extrapolation has interesting therapeutic implications in view of the broad physiological and pathological roles of $\alpha_M\beta_2$.

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