A central characteristic of integrin adhesion receptors is their capacity to become activated, thereby enhancing their affinity for ligands. Here, we report the identification of a discrete site within the I domain of integrin αMβ2, which modulates the adhesive activity of this receptor. Based upon the crystal structure, this region is composed of two short and spatially proximal loops, E166Q/LKKS and Q198NP/PR. Mutations in these loops yield receptors which are often mediated by spontaneous cell adhesion to fibrinogen, whereas mutation of an adjacent region and wild-type receptors require activation to adhere to this substrate. An activating monoclonal antibody enhanced the adhesive activity of one but not the other loop mutants, suggesting that the activation states of these two mutant receptors were not identical. Given that similar I domains exist in several other integrin α subunits and non-integrin proteins, and possibly in all integrin β subunits, these two loop segments may represent a universal target for controlling integrin activation and the function of other I-domain-containing proteins. In support of this hypothesis, several naturally occurring mutations that activate von Willebrand factor map to the same loops of its I(A) domain.

I(A) domains, inserted domains of ~200 amino acids, are common structural motifs in numerous proteins, including several integrin α-subunits, von Willebrand factor (vWF), and the complement components C2 and factor B (1). Interactions of these proteins with other proteins are often mediated by their I domains. As two examples pertinent to this study: 1) the I domain mediates the interaction of vWF with collagen and with its platelet receptor GPIb/IX, thereby allowing vWF to bridge platelets to the subendothelial matrix, and 2) contact sites for several αMβ2 ligands, including Fn (2), C3bi (3), and ICAM-1, and fibrinogen (Fg) (4) map to the I domain of the αM subunit (αM-I-domain). The crystal structures of the αM-I- and αI-I-domains recently have been determined (1, 5) and are very similar to α/β structures, composed of seven α-helices and five β-sheets. A cation binding site is formed at the vertex of the β-sheets, and the bound cation is coordinated in a “MIDAS” motif (1). Structural flexibility within the I domains allows them to exist in two distinct conformational states, which differ in the mode of cation coordination (5, 6). Common to many I domain-containing proteins is their capacity to be “activated” such that their affinities for other proteins are enhanced. vWF can be activated by botrocetin (7), a snake venom protein, to acquire the capacity to interact with GPIb/IX. αMβ2 can be activated on resting neutrophils and monocytes to recognize certain αM-I-domain ligands, including Fn (8) and C3bi (9), and to execute certain αMβ2-dependent functions, such as the adhesion to endothelium (10), the adhesion-dependent respiratory burst (11), and phagocytosis (12). Certain cytokines (11), bacterial products (lipopolysaccharides), and mAbs, such as KIM185 (13), can activate αMβ2. The inherent flexibility noted in the crystal structure of the αM-I-domain (5, 6) may be relevant to such activation.

Spontaneous binding of vWF to platelets is observed in type IIB VWF disease (14), which is associated with platelet aggregation and thrombocytopenia. This activation of vWF has been ascribed to several mutations which map to its A1 domain. Based upon primary sequence homology and the crystal structures of the αM-I- and αI-I-domains, these vWF mutations are clustered in a spatially proximal site composed of two loop (L) segments, which connect secondary structural elements: 1) the loop region between helix 1 and β-sheet B, termed L(1-B), and 2) the loop between helix 2 and 3, termed L(2-3), of the αI-domain. The recognition sequence in the A1 domain of vWF for botrocetin also is located in this region (7).

In view of the structural similarity between I domains and the clustering of activating mutations to a local region within this domain of vWF, we hypothesized that mutations within the L(1-B) and L(2-3) region of the αI-I-domain might influence the activation state of αMβ2. To test this hypothesis, these two segments were switched individually to their counterparts in αI, to minimize gross perturbations of structure. These mutations are, in fact, shown to differentially influence the activation state of αMβ2. Thus, a very precise region that may be generally involved in the activation of I-domain-containing proteins has been defined.

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 (Amon, Thousand Oaks, CA), and NIF was provided by Dr. M. Moyle (Corvas Intl., San Diego, CA). The αMβ2-activating mAb, KIM185, was a gift from Dr. M. Robinson (Celltech, Berkshire, UK). mAbs 2LP19c and HM2/M2 were from Dako (Carpinteria, CA); TS1/18, OKM1, M1/70, and LM2/1 were from ATCC (Rockville, MD). G418, Dulbecco’s modified Eagle’s medium/F-12, HBSS, DMSO, and restriction enzymes were from Life Technologies, Inc./BRL (Life Technologies, Inc.).
Site-directed Mutagenesis and Expression—All mutations were created by oligonucleotide-directed mutagenesis using uracil-containing single stranded M13mp18 DNA (15). To mutate M13KOCEFVST of helix 1 in α2 to its counterpart in α5 (ILDFMKD) to create α5[H(1)], a 51-mer primer (5’-TATTGCTCAGCTCAGCTTTATAGGACAGAGCCTTTCTGAGATCC-3’) (the underlined nucleotide represents the mutated bases) was used, and resultant plaques were screened with BanHI (GGATCC). Primer 5’-CTGGATCCAGGAGAGACTTGATGCGTTGGTCATTTGTTCACTCGAGGACTTTTG-3’ (57-mer) was used to create mutant α2[L(1-B)], in which the α2 segment of E162QLKKS-KTL was changed to the corresponding α5 segment of KKLNTSYQ. Primer 5’-TATTGCTCAGCAGTGTCGGCCTTCCATATTAAACGGACCTTTGGAAGTTG-3’ (54-mer) was used to create mutant α2[L(2–3)], in which the α2 segment of Q190NNPNPRSwas changed to the corresponding segment of VWKDPDA in α5. DNA sequencing of the entire I domain was conducted to confirm the desired mutations without alteration of the remaining sequence. The mutant I domains were reintroduced into α2 using previously introduced ClaI1645 and NheI1141 sites (16). The reconstructed cDNAs of α2 and β2 were then inserted separately into the expression vector pCIS2M by employing XhoI and XhoI sites. In this vector, α2 between residues 54 and β2 expression is under the control of human cytomegalovirus promoter and enhancer (15). Each expression vector was purified using CsCl gradients and transfected, together with pRSVneo (neomycin resistance gene), into 293 cells according to our established procedure (15). G418 (600 μg/ml) resistant colonies were pooled, and α2β2-expressing cells were sorted by FACS analysis (FACStar, Becton Dickinson, San Jose, CA) using an α2-specific mAb, OKM1, which recognizes an epitope outside of the I domain (17).

Ligand Binding to α2β2-expressing Cells—NIF binding assay was performed as described previously (16), using 0.05 to 1 μg of 125I-NIF and 106 α2β2-expressing cells in a total of 200 μl of HBSS containing 2.5 mM Ca2+. The procedure for C3bi binding was modified from the original method of Bilsland (18) as described previously (16). For adhesion, denatured ovalbumin (dOva) (19) and Fg (20) were used as substrates. The exact procedure has been described elsewhere (16).

Analytical Procedures—For FACS analyses, our previous method (16) was used. For immunoprecipitation, α2β2-expressing 293 cells were 125I-surface-labeled with lactoperoxidase according to a published method (21) and immunoprecipitated with 10 μg mAb as described (16). For protein modeling, the coordinates for αI I-domain were assigned according to the crystal structure of α1 I-domain (5) using the HOMOL-gram (Biosym Technologies, San Diego, CA).

RESULTS

α2β2 Mutants Are Cell-surface Expressed as Heterodimers—Based on the location of the vWF-activating mutations (14) and the crystal structures of the integrin I-domains (1, 5), three mutant αM were constructed using site-directed mutagenesis. The mutant αM[H(1)], αM[L(1-B)], or αM[L(2–3)] contained a switch of the (M13KOCEFVST) segment within the helix 1, the loop between Helix 1 and β-sheet B (E162QLKKS-KTL), and the loop between Helix 2 and Helix 3 (Q190NNPNPRSw), to their counterpart sequences in α5 (ILDFMKD, KKLNTSYQ, and VWKDPDA), respectively (see Fig. 5). Upon introduction of these mutants and wild-type αMβ2 into 293 cells, all were stably expressed. Immunoprecipitation of surface labeled cells with OKM1, a mAb to the αM subunit, or TS1/18, a mAb to the β2 subunit, showed two bands by polyacrylamide gel electrophoresis in sodium dodecylsulfate (nonreducing) of the same mobility as wild-type αMβ2 with estimated molecular weights of 165 and 95 kDa, indicating that all three mutants were correctly assembled into heterodimers on the cell surface. FACS analyses showed that the mean fluorescence intensities of the three mutants with any one of a panel of mAbs against αM (OKM1, 44, LM2/1, M170) or β2 (M1H23) differed by less than 15%, suggesting that the three mutants were expressed at similar levels as the wild-type receptor.

Interaction of Mutant αMβ2-bearing Cells with NIF, C3bi, and dOva Does Not Require Activation—The interactions of NIF, C3bi, and dOva with the αMβ2 mutants were examined. Varying quantities of 125I-NIF were added to the transfectants. The binding isotherms obtained with all the αMβ2-bearing cells were very similar (Fig. 1A), providing evidence for saturable binding. Specificity was supported by the failure of mutants-transfected 293 cells to bind NIF and the abolition of 125I-NIF binding (>98% inhibition) by excess unlabeled NIF or EDTA. From the binding curves, the following Kd values were calcu-
labeled: 7.0 ± 1.6 nM for wild-type; 11.4 ± 3.4 nM for αMβ2L(1-B); and 5.0 ± 1.9 nM for αMβ2L(2–3)]. The similarity in binding parameters for the three mutant and wild-type αMβ2 indicates that the mutated sites are not directly required for NIF recognition (αMβ2 does not bind NIF) and that overall conformation and function of the I domain in the αMβ2 receptors had been preserved.

EC3bi binding (Fig. 1B) and adhesion to dOva (Fig. 1C) also were not altered by the mutations. Verifying the specificity of EC3bi binding, uncoated or IgM-coated erythrocytes did not interact with the three mutant and wild-type αMβ2-transfectants; mock-transfected 293 cells did not rosette with EC3bi; and EDTA abolished binding (Fig. 1B). Thus, EC3bi bound to the mutants without a requirement for activation, consistent with the reports in the literature (16, 22). The interactions of the mutants with dOva were completely inhibited by 0.5 mM EDTA, indicating divalent cation dependence, or 10 μg/ml NIF, indicating αMβ2 dependence. Mutation of the H(1) segment of αM had no effect on adhesion, whereas the L(1-B) and L(2–3) mutants exhibited a 2-fold increase in adhesion.

The H(1), L(1-B), and L(2–3) Mutants Map the Epitope for the Function Blocking mAb 2LPM19c—a mAb 2LPM19c, which recognizes a conformational epitope in the I domain (23), blocks many αMβ2-mediated functions including ICAM-1, Fg, and C3bi binding and neutrophil aggregation (17). Consistent with these reports, 2LPM19c inhibited the adhesion of wild-type αMβ2 to dOva (Fig. 2A). However, the mAb had no effect on the adhesion of αM(L(1-B)), or αM(L(2–3)) to this substrate. One explanation for this result is that the mutated regions of the receptor contributed to the epitope of 2LPM19c. FACS analyses supported this interpretation. As shown in Fig. 2B, while 2LPM19c reacted well with wild-type αMβ2-bearing cells, it failed to bind to the three mutant αMβ2 lines.

Mutants ααMβ2L(1-B) and ααMβ2L(2–3) Exhibit Constitutively Active Adhesion to Fibrinogen and Display Distinct Morphologies—Adhesion of wild-type αMβ2-expressing cells to Fg requires activation of the receptor such as with mAb KIM185 (16). This is verified in Fig. 3A, as was also the case for mutant αM(H(1)). In contrast, αM(L(1-B)) and αM(L(2–3)) mutants adhered aggressively to Fg in the absence of KIM185. Compared to wild-type αMβ2, the adhesion of the αMβ2L(1-B)β2 and αMβ2L(2–3)β2 increased by 9- and 8-fold, respectively. Addition of 10 μg/ml NIF (Fig. 3A), 1 mM EDTA, or 5 μg/ml of the blocking mAb M1M23 (not shown) abrogated adhesion of all the αMβ2-transfectants by more than 95%, confirming that the enhanced adhesion of the loop mutants remained αMβ2- and cation-dependent.

In concert with their ability to adhere spontaneously to Fg, both αM(L(1-B)) and αM(L(2–3)) acquired a distinct morphology (Fig. 3B). More than 90% of the two mutant cell lines that adhered to Fg also spread upon the substratum, forming lamellipodia extensions and developing a very flattened appearance. In contrast, the few wild-type αMβ2- or αM(H(1))β2-bearing cells which did adhere remained round and rarely spread (<5% of adherent cells).

The Adhesive Activity of αMβ2L(2–3), but Not αMβ2L(1-B), Can Be Further Activated by KIM185—The ability of KIM185 to further activate the two loop mutants was examined (Fig. 4). Addition of 2 μg/ml of KIM185 significantly enhanced the adhesion of wild-type αMβ2 (11-fold) and the αMβ2L(2–3) mutant (2.5-fold) to Fg. However, the mAb had little effect on the adhesion of αM(L(1-B))L(1,1-1,1) but, as judged by FACS analysis (data not shown). Despite the fact that the unstimulated adhesion of αMβ2L(2–3) was lower than that of αMβ2L(1-B), its adhesive activity exceeded that of αMβ2L(1-B) upon further activation with KIM185, suggesting...

**Fig. 2. Interaction of wild-type and mutant αMβ2 receptors with mAb 2LPM19c.** A, adhesion: transfected cells (2 × 10^6) were incubated with 5 μg/ml 2LPM19c at 4°C for 30 min and then added to wells coated with dOva. After incubation at 37°C for 25 min, non-adherent cells were removed by three washes with PBS, and the adherent cells were quantitated as in Fig. 1C. Data are the means ± S.D. of three independent experiments. B, FACS, αMβ2-expressing cells (10^6) were incubated with 5 μg of 2LPM19c (solid line) or an isotype matched control (dotted line) at 4°C for 30 min. After three washes with PBS, the cells were stained with FITC-goat anti-mouse IgG and analyzed using FACS, counting 10,000 events. A, wild-type; B, αMβ2L(1-B); C, αMβ2L(2–3); D, αMβ2L(2–3)].

that the mechanisms of activation of these two mutants are distinct.

**DISCUSSION**

In the A domain of vWF, activating mutations, which lead to spontaneous binding to platelets, cluster in two noncontiguous regions, M546ARMRR15QKVRV and P2745SELR (14) (see Fig. 5A). These segments correspond to the loop regions between helix 1 and β-sheet B and between helix 2 and 3 of the αMβ2 domain (see Fig. 5B). We hypothesized that mutation of these two loop regions might control the activation state of αMβ2 and, accordingly, created two mutants, αM(L(1-B)) and αM(L(2–3)), in which the loop regions between helix 1 and β-sheet B (E512Q, K553K, K553K, helix 2 and helix 3 (Q1809N, NPNNP), were switched to counterparts sequences in αL (based on their...
FIG. 3. Adhesion and morphology of wild-type and mutant \( \alpha_{\text{m}} \beta_2 \) transfectants on fibrinogen. A, adhesion: 24-well microtiter plates were coated in the center of each well with 100 \( \mu \)l of 50 \( \mu \)g/ml Fg. Transfected cells (\( 2 \times 10^6 \)) were added to each well in the presence (solid bar) or absence (open bar) of 10 \( \mu \)g/ml NIF. Adherent cells were quantitated as in Fig. 1C. Data are the means \( \pm \) S.D. of three independent experiments. B, morphology: \( \alpha_{\text{m}} \beta_2 \)-expressing cells were seeded onto coverslips at 37 °C overnight. The cells were fixed with 2% paraformaldehyde and photographed under phase contrast.

FIG. 4. Stimulation of adhesion of \( \alpha_{\text{m}} \beta_2 \) transfectants by KIM185. \( \alpha_{\text{m}} \beta_2 \)-expressing cells were incubated without mAb (open bar) or with the activating mAb KIM185 (5 \( \mu \)g/ml) + PMA (0.16 \( \mu \)M) (solid bar) and added to Fg-coated plates for 25 min at 37 °C. After three washes, the adherent cells were quantitated as in Fig. 1C. The number of wild-type \( \alpha_{\text{m}} \beta_2 \) cells adherent to Fg in the absence of KIM185 was assigned a value of 1. The means \( \pm \) S.D. of two independent experiments are shown. * \( t \) test (\( p \leq 0.01 \)).

\( \alpha_{\text{m}} \beta_2 \) into a constitutively active receptor; i.e. compared to wild-type \( \alpha_{\text{m}} \beta_2 \), the adhesive of the two mutants to Fg increased by about 10-fold. A similar increment in adhesion of neutrophils to Fg is observed upon activation of the cells (24). 2) The two mutant cell lines acquired a distinct morphology. They adhered and spread upon coverslips, formed lamellipodia extensions, and displayed a very flattened morphology. These changes indicate that the activating mutations could transmit outside-in signals, such that the cytoskeletal network within the cell reorganized. 3) The 2LPM19c mAb to \( \alpha_{\text{m}} \) blocked functions of wild-type but not the two mutant receptors which lacked the 2LPM19c epitope as assessed by FACS analysis. Thus, the inhibitory effects of the mAb is likely to arise from its allosteric effect on the receptor conformation. This explanation suggests that the mAb has similar recognition requirements but the opposite effect of botrocetin on vWF activation. 2LPM19c recognizes H(1), L(1-B), and L(2–3), and these segments are homologous to two critical regions in vWF for botrocetin recognition (residues 539–553 and 569–583) (7) (Fig. 5). Thus, the binding sites for 2LPM19c and botrocetin correspond to overlapping regions although the sites are not precisely identical. The alternative possibility that 2LPM19c sterically blocks ligand binding cannot be excluded but seem unlikely in view of the distance of the epitope from the “MIDAS” motif of the receptor (1).

In addition to the two loop regions of the \( \alpha_{\text{m}} \)I-domain, it is likely that other spatially adjacent segments also might influence receptor activation. In particular, the region composed of amino acids 117–130 of \( \alpha_{\text{m}} \) is a reasonable candidate. In the crystal structure of the \( \alpha_{\text{m}} \)I-domain, this segment, which lies outside the I domain and in front of the \( \beta \)-sheet A, is in close spatial proximity to the L(1-B) and L(2–3) segments (Fig. 5B). Mutations in the homologous region of vWF induce its activation, including two vWF type IIb mutations, Pro\(^{563} \rightarrow \)Leu (25) and His\(^{585} \rightarrow \)Asp (14).

The mechanism by which the loop mutations in \( \alpha_{\text{m}} \beta_2 \) or in
activation of integrin α₃β₂

Activation of Integrin α₃β₂ 29957

![Diagram](image)

**Fig. 5. Sequence and molecular model of the α₃I-domain.** A, sequence alignments: the sequence of α₃I-domain shown starts at residue 134 and the A1 domain of vWF starts at residue 514. The amino acids in boldface type are the activating mutations in vWF type IIB patients. The positions of the activating mutations in α₃I[L1-B] and α₃I[L2–3]) are shown on top of the wild-type α₃I sequence. B, molecular model of the structure of α₃I-domain. The structure of α₃I-domain is modeled from the coordinates of MI-domain shown starts at residue 134 and the A1 domain of vWF starts at residue 514. The amino acids in boldface in superadhesion” receptor. Mutation of a residue in the β₂ subunit of α₃Iβ₂ also has been shown to produce a “superintegrin” (26). The distinction between the two loop mutants in response to KIM185 suggests the existence of different activation states of the α₃β₂. In our study, short amino acid segments were altered to generate these different activation states. By analogy to vWF, point mutations or polymorphisms also may lead to α₃β₂ receptors with different functional properties. Recently, it has been shown that a polymorphism in the β₂ subunit is associated with a thrombotic phenotype (27). Mutations which alter the activation states of α₃β₂ could have broad ranging consequences including altered susceptibility to infection. We are currently searching for such naturally occurring activating mutations.

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