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Modulation of CD11b/CD18 Adhesive Activity by Its Extracellular, Membrane-Proximal Regions

Yu-Mei Xiong,* Jian Chen,† and Li Zhang2*

The integrin receptor CD11b/CD18 is normally kept in a low adhesive state and can be activated by many different agents. However, the mechanism underlying receptor activation is not yet fully understood. We hypothesized that the extracellular, membrane-proximal regions of CD11b/CD18 are critically involved in modulation of its adhesive functions. To test our hypothesis, we perturbed the extracellular, membrane-proximal regions of individual CD11b and CD18 subunits and studied their effect on ligand binding, receptor clustering, and lipid raft association. We report here three major findings: 1) perturbation of the extracellular, membrane-proximal region of either subunit leads to enhanced adhesion, caused by changes in receptor conformation, but not the state of receptor clustering or lipid raft association; 2) the CD11b subunit plays a more important role in confining the receptor in an inactive state; and 3) upon modification of the extracellular, membrane-proximal region, the mutant CD11b/CD18 acquires the ability to respond to stimulation by “inside-out” signaling. Our results suggest that the extracellular, membrane-proximal region of the receptor plays an important role in integrin activation and therefore could be targeted by certain cell surface proteins as a conduit to control the integrin “inside-out” signaling process. The Journal of Immunology, 2003, 171: 1042–1050.

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from Dr. M. K. Robinson (Celltech, Slough, U.K.). IB4 and TS1/18 were supplied by American Type Culture Collection (Manassas, VA). mAb 44 was purchased from Sigma-Aldrich (St. Louis, MO). Human Fg was purchased from Enzyme Research Laboratories (ERL, South Bend, IN). The recombinant γ-module of Fg was provided by Dr. L. Messner, German Red Cross. All other reagents were the highest grade available and were purchased from Sigma-Aldrich unless otherwise noted.

Insertion of a short segment within CD11b/CD18 by site-directed mutagenesis

The detailed procedures for site-directed mutagenesis and establishment of stable cell lines expressing wild-type and mutant CD11b/CD18 have been published (19). To insert a segment containing two copies of the sequence GlyGlyGlyGlySer into CD11b, the mutagenic primer 5′-TTCTGGGTGCCCAGGAGGAGGTCTCTGGAGGAGGAGGAGGGCCGCTCATC-3′ and a reverse primer 5′-ATTGGTGATGCTATTGGTTATTTT-3′ were used in the first PCR to amplify a 357-bp fragment, which was then used as a mega-primer, together with a forward primer, 5′-AACCCAGGAGGAGGTCTCTGGAGGAGGAGGAGGAGGGCCGCTCATC-3′ and a reverse primer 5′-ATTGGTGATGCTATTGGTTATTTT-3′ were used in the first PCR to amplify a 357-bp fragment, which was then used as a mega-primer, together with a forward primer, 5′-ATGCCCCGAACCAACCAACAGT-3′, for the second PCR to generate a 906-bp fragment. This fragment was then transferred back to the cDNA of CD11b using KpnI and NotI sites. Site-directed mutagenesis procedures were used for the CD18(F) mutant, except that the primers used for the first PCR were 5′-AGCGGAGGTGTGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGC-3′ (the reverse primer recognizes the polylinker of the vector). The forward primer for the second PCR was 5′-TGGCTACATTGGGAAAAA-3′. The mutated CD18 fragment was transferred back to the cDNA of CD11b using XhoI and BglII sites. To express the wild-type and mutant CD11b/CD18 receptors in K562 cells, the corresponding CDNAs were transferred into the retroviral expression vector MGIN (20) using EcoRI and NorI sites. To prepare viral supernatants, individual MGIN vectors were transfected into the packaging cell line PG13 (21) using Lipofectamine (Invitrogen, Carlsbad, CA), and the transfected cells were selected with 800 μg/ml G418. For expression in K562 cells, the viral supernatants for both CD11b and CD18 were added to K562 cells in the presence of 8 μg/ml polybrene, and the mixture was then centrifuged at 3200 rpm (2000 × g) for 4 h at 37°C based on a published method (20). The infected cells were then cultured in the presence of 800 μg/ml G418, and the cell population expressing CD11b/CD18 was enriched by immunopanning on 100-mm non-tissue culture polystyrene dishes that were precoated with 20 μg/ml mAb IB4 and blocked with 3% BSA in PBS.

Surface labeling and immunoprecipitation

Cells expressing wild-type and mutant CD11b/CD18 were washed once with Dulbecco’s PBS, biotinylated with EZ-link Sulfo-NHS-LC-Biotin (sulfosuccinimidyl 6-biotinamido hexaone; Pierce, Rockford, IL) and lysed with a solution containing 20 mM Tris-HCl, 150 mM NaCl (pH 7.4), 1% Triton X-100, 1 mM PMSF, 10 mM benzamidine, 25 μg/ml soybean trypsin inhibitor, and 20 μg/ml leupeptin. The cell lysates were subjected to immunoprecipitation with a CD18 mAb (IB4). The immunoprecipitates were analyzed by 8% SDS-PAGE, and the surface-expressed CD11b/CD18 receptors in K562 cells were visualized by Western blot using a HRP-avidin conjugate. The exact procedure has been described previously (19).

Fg adhesion assays

The adhesive activity of CD11b/CD18 was assessed using Fg and its CD11b/CD18 recognition domain, the γ-module, as representative ligands (19, 22). Briefly, 100 μl of 10 μg/ml γ-module or 2 μg/ml Fg was coated onto the center of a 24-well non-tissue culture plate at room temperature for 2 h. The plate was then blocked with 0.1% polyvinylpyrrolidone and 3% BSA in PBS at 37°C for 1 h. The adhesion assay was performed using 2–106 cells/well in HBSS containing 1 mM Ca2+ and 1 mM Mg2+ with or without 100 nM PMA at 37°C for 60 min. The nonadherent cells were removed by washing the plate three times with HBSS, and the adherent cells were counted directly or quantitated indirectly using cell-associated acid phosphatase as described previously (19).

FACS analysis

A total of 106 cells expressing wild-type or mutant CD11b/CD18 in HBSS containing 1 mM Ca2+ and 1 mM Mg2+ were incubated with 1 μg of mAb for 30 min at 4°C, except that 37°C was used for mAb 24. A subtype-matched mouse IgG served as a control. After washing with PBS, cells were mixed with FITC-goat anti-mouse IgG (H+L) (Ab_1, 1/20 dilution; Zymed Laboratories, San Francisco, CA) and kept at 4°C for another 30 min. Cells were then washed with PBS and resuspended in 500 μl of Dulbecco’s PBS. The FACS analysis was performed using a FACSScan (BD Biosciences, Mountain View, CA), counting 10,000 events.

Confocal laser scanning fluorescence microscopy

The K562 cells expressing wild-type and three CD11b/CD18 mutants were stained with a CD11b-specific mAb OKM1 or a CD71-specific mAb M-A712 (BD Biosciences) and biotinylated cholera toxin subunit B (Sigma-Aldrich) at room temperature for 20 min, followed by staining with a rhodamine conjugate of goat anti-mouse IgG and an FITC conjugate of avidin. The stained cells were fixed with 1% paraformaldehyde, seeded on polylysine-coated slides, and then analyzed using a Radiance 2000 confocal Laser Scanning Fluorescence Microscope System (Bio-Rad, Hercules, CA) equipped with a Nikon Eclipse E800 Upright light microscope (Melville, NY).

Lipid raft preparation

Lipid rafts were prepared from K562 cells expressing wild-type and mutant CD11b/CD18, based on the published procedure (23). A total of 1×10⁸ cells from the above confocal laser scanning fluorescence microscopy experiments were lysed with 1 ml of MES-buffered saline (pH 6.5) containing 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 5 mM sodium orthovanadate, and 5 mM EDTA. The lysate was adjusted to 1.5×108 cells/ml in MES-buffered saline buffer and was mixed with equal volume of 80% sucrose (w/v). The resultant 3 ml of lysate with 40% sucrose was overlaid with 3 ml each of 30, 20, and 10% sucrose in a 12-ml clear centrifuge tube (Beckman, Palo Alto, CA) and was centrifuged at 200,000 × g for 4 h with a Beckman SW41 Ti rotor at 4°C for 18 h. A total of 12 1-ml fractions were collected from the top, and fractions 2–5 (raft) and fractions 6–12 (non-raft) were collected from the bottom. The raft and non-raft fractions were then examined for the presence of the raft (ganglioside GM1) and non-raft (CD71) markers by Western blot using biotinylated cholera toxin subunit B and an mAb (M-A712) specific for CD71.

Preparation of polyclonal Abs

Two peptides corresponding to the cytoplasmic tails of CD11b (CYKLG-FFKRQYKDDMMSEGGPGGPAEQP) and CD18 (CWKALIHLSDLREYRFFEKELKSQWNND) were synthesized by SynPeP (Dublin, CA) and then conjugated to keyhole limpet hemocyanin using the Maleimide Immunogen Conjugation kit (Pierce). Rabbit polyclonal Abs specific for these two peptides were prepared by Cocalico Biologicals (Reamstown, PA), and their titers were determined by ELISA using their respective OVA conjugates. For Western blots, the antiserum was used directly at a 1/2000 dilution and were detected with an HRP conjugate of goat anti-rabbit IgG.

Results

Perturbation of the extracellular membrane-proximal regions of CD11b/CD18

Previous studies of “inside-out” signaling have focused mainly on the cytoplasmic domain (16, 17) and the ligand binding domain of integrins (24, 25). However, the role of the transitional region between these two domains, especially the extracellular, membrane-proximal region of the integrin receptor, in controlling integrin functions has not been probed. In this study we hypothesized that these membrane-proximal regions of the receptor play a critical role in integrin activity modulation. Our focus on this particular region of the integrin receptor is based on the following observations. 1) A number of mAbs increase integrin binding activity by interacting with the extracellular, membrane-proximal regions of the receptor (13, 14, 18). 2) Alternatively, proteolytic cleavage of this region within the CD41 (αm) chain results in activation of the CD41/CD61 (αmβ2) receptor (26). To test our hypothesis, we chose to perturb the extracellular regions that are adjacent to the predicted transmembrane domains of CD11b and CD18 by inserting a 10-residue flexible spacer containing the sequence GGGS GGGS (Fig. 1A). The insertion site in CD11b is one residue above the predicted transmembrane domain, and the insertion site in CD18 is adjacent to the functionally important residue Cys695 (C695 to A mutation activates the receptor) (27) and four residues away from the transmembrane domain. The GGGSGGGGG sequence adopts a random coil conformation in solution (28) and
thus is unlikely to cause gross disruption of the three-dimensional structure of the CD11b/CD18 receptor.

Expression of the CD11b/CD18 mutants in K562 cells

To evaluate the effect of perturbing the extracellular, membrane-proximal regions of CD11b/CD18 on ligand binding and receptor activation, we expressed the wild-type and three mutant CD11b/CD18 receptors, CD11b(F)/CD18, CD11b(FL)/CD18(F), and CD11b(F)/CD18(F), on K562 cells (these cells do not express endogenous CD18 or CD11b) based on the report that the CD18 integrins expressed on K562 cells exist in an inactive state (17, 29). To achieve higher gene expression of the receptor, we used the retroviral expression vector MGIN (20) and prepared viral particles using the packaging cell line PG13 (21). The stable cell lines expressing the wild-type and three CD11b/CD18 mutants were established by G418 selection and immunopanning. All four stable cell lines had similar surface expression of CD11b/CD18 based on FACS analyses using either a CD11b-specific mAb (OKM1; Fig. 1B) or a CD18-specific mAb (MHHM23; data not shown). Among the four cell lines, the wild-type receptor had the highest expression level, which is ~1.8- and 1.9-fold higher than those of CD11b(FL)/CD18(F) and CD11b(F)/CD18(F), and 8-fold higher than that of CD11b(F)/CD18. In addition, when surface labeling and immunoprecipitation experiments were repeated on these stable K562 cell lines, two bands of ~95 and 165 kDa were observed on 8% SDS-PAGE for the wild type and all three mutant receptors (Fig. 1C), but not for mock-transfected cells (data not shown).

Cells expressing the CD11b/CD18 mutants display distinct morphology

The nontransfected and mock-transfected K562 cells behaved like typical suspension cells. Similarly, the wild-type CD11b/CD18-expressing cells also existed as a suspension and did not attach to the tissue culture flask. In sharp contrast, the K562 cells expressing the CD11b/CD18 mutants, especially mutant CD11b(F)/CD18, acquired certain characteristics of adherent cells and attached spontaneously to the bottom of the tissue culture flask. The percentage of the attached cells in the total cell population is ~10% for CD11b(FL)/CD18(F), 20% for CD11b(F)/CD18, and 15% for CD11b(F)/CD18(F).

Perturbation of the extracellular, membrane-proximal regions of CD11b/CD18 promotes cell adhesion

The above observation that K562 cells expressing the three mutant CD11b/CD18 integrins attached spontaneously to the tissue culture flasks strongly suggests that these mutant receptors are constitutively active. To test this hypothesis, we performed adhesion experiments on the wild-type and the three mutant receptors using both Fg and its CD11b/CD18 recognition domain, the γ-module (22). As shown in Fig. 2A, the wild-type-expressing K562 cells did not adhere to the γ-module in the presence of 1 mM Ca2+ plus 1 mM Mg2+, but did adhere in the presence of 0.5 mM Mn2+. These data suggest that wild-type CD11b/CD18 exists in an inactive state and requires activation for adhesion. In contrast, cells expressing the three mutants adhered to the γ-module without the need for activation, suggesting that perturbation of the extracellular, membrane-proximal region of either CD11b or CD18 resulted in constitutive receptor activation. The specificity of the adhesion assays was verified by the following experiments: mock-transfected K562 cells did not have any detectable adhesion (data not shown), and a CD11b-specific mAb (44a) completely blocked cell adhesion mediated by the wild-type and three mutant receptors (Fig. 2A).

Differential ability of CD11b and CD18 in confining CD11b/CD18 in an inactive state

In accordance with our earlier observation that the percentage of the attached cells differed among the three mutants, the ability of the three mutant receptors to support cell adhesion is also different. Among these three mutant CD11b/CD18 receptors, CD11b(F)/CD18 is most adhesive (~3- to 10-fold higher than mutant CD11b(FL)/CD18; Fig. 2, A and B) despite its much lower surface expression (~8-fold lower than the wild-type receptor and 4-fold lower than CD11b(FL)/CD18(F)). Consistent with the lesser degree of cell adhesion by CD11b(FL)/CD18(F)-expressing K562 cells, addition of Mn2+ could further enhance the adhesive ability of CD11b(FL)/CD18(F), but had no effect on mutants CD11b(F)/CD18 and CD11b(F)/CD18(F) (Fig. 2A), implying that the latter two mutant receptors had reached maximum adhesion toward the γ-module.

Similar observations were made when the CD11b/CD18-expressing K562 cells were treated with a CD18–specific activating mAb (KIM127) (13). As shown in Fig. 2B, adhesion to the γ-module of the wild-type-expressing cells was enhanced 8-fold by KIM127, as was adhesion of the CD11b(FL)/CD18(F)-expressing cells. The other two cell lines (CD11b(F)/CD18 and CD11b(F)/CD18(F)) had higher basal adhesion to the γ-module, which did

### Figure 1

**FIGURE 1.** Surface expression of the CD11b/CD18 receptors on K562 cells. A, Schematic drawing of the CD11b/CD18 mutants. A flexible segment, containing two copies of the GlyGlyGlyGlySer sequence, is inserted between residues Asn1106 and Pro1108 in CD11b and between Val606 and Ala697 in CD18. The insertion site in CD11b is one residue away from the predicted transmembrane domain, and the insertion site in CD18 is located between Cys1199 and the predicted transmembrane domain. For both CD11b and CD18, the protein sequence was numbered from the initiation methionine. B, FACS analysis. CD11b/CD18-expressing K562 cells (10⁶) were incubated with 1 μg of a CD11b-specific mAb (OKM1; thick lines). After three washes with PBS, the cells were stained with FITC-goat anti-mouse IgG and analyzed, counting 10,000 events. An isotype-matched nonimmune IgG was used as a control (thin lines). C, Surface labeling and immunoprecipitation. The K562 cells expressing the wild-type and mutant CD11b/CD18 receptors were labeled with biotin and subsequently lysed. The biotinylated proteins were immunoprecipitated with the CD18-specific mAb IB4 and then separated on 7% SDS-PAGE. The exact procedure has been published (19). Lane 1, Wild-type CD11b/CD18; lane 2, CD11b(FL)/CD18; lane 3, CD11b(FL)/CD18(F); lane 4, CD11b(F)/CD18(F).
not increase further in response to KIM127 treatment. Altogether these data suggest that the abilities of the CD11b and CD18 subunits to confer the CD11b/CD18 receptor in an inactive state are not equivalent; perturbation of CD11b results in maximum activation of the CD11b/CD18 receptor, whereas similar modification of CD18 only elevates the receptor to an intermediate activation state, which is still responsive to stimulation by either Mn\(^{2+}\)/H11001 or the activating mAb KIM127.

Perturbation of the extracellular, membrane-proximal regions of the receptor leads to conformational changes within its distal ligand binding domain

The enhanced adhesive activity of the three mutant receptors could result from conformational changes and/or receptor clustering or surface redistribution. To determine whether the enhanced adhesion is due to conformational changes, we performed FACS analyses using two activation-dependent mAbs (mAb 24 and mAb MEM148) on the wild-type and three mutant receptors. mAb 24 recognizes a neo-epitope within the I-like domain (30), a candidate ligand-binding site within the CD18 subunit (19, 31). Binding of mAb 24 to the CD18 integrins requires receptor activation by either intracellular signaling or addition of Mn\(^{2+}\) (32, 33). Therefore, mAb 24 reports the presence of an active conformation within the ligand binding domain of the CD11b/CD18 receptor. mAb MEM148 also recognizes activated receptors. However, its epitope is located within the C-terminal portion of the CD18 subunit. Activation of monocytes by PMA leads to enhanced cell adhesion and concomitant exposure of the MEM148 neo-epitope (34). To determine whether the three mutant receptors had acquired similar active conformations, we conducted FACS analyses on wild-type and mutant CD11b/CD18 receptors using mAb 24 (Fig. 3) and mAb MEM148 (Table I). The extent of

Table I. Conformational changes within the mutant CD11b/CD18 receptors

<table>
<thead>
<tr>
<th>Mutants</th>
<th>mAb 24</th>
<th>MEM148</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>CD11b(F)/CD18</td>
<td>123</td>
<td>86</td>
</tr>
<tr>
<td>CD11b/CD18(F)</td>
<td>71</td>
<td>38</td>
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<tr>
<td>CD11b(F)/CD18(F)</td>
<td>71</td>
<td>37</td>
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Binding of the conformation-dependent mAb 24 and mAb MEM148 to K562 cells stably expressing the wild-type and three CD11b/CD18 mutants was determined by FACS analysis. Mean fluorescence staining of mAb 24 and mAb MEM148, after subtraction of the background fluorescence, is expressed as the percent mean fluorescence of mAb OKM1, which recognizes an activation-independent epitope located outside the I domain of CD11b.
receptor activation was also quantified using the mean fluorescence value obtained with these two mAbs and was expressed in the percent mean fluorescence obtained with mAb OKM1, which recognizes an activation-independent epitope outside the I domain of CD11b (35). As shown in Table I, there was little binding of mAb 24 or MEM148 to wild-type CD11b/CD18. However, perturbation of the extracellular, membrane-proximal regions of CD11b/CD18 significantly increased the expression of both activation neo-epitopes. Among the three mutants, CD11b(F)/CD18 displayed the highest binding to mAb 24 and mAb MEM148 (Table I). While binding of mAb 24 to the wild-type receptor was dependent on Mn$^{2+}$, binding of this mAb to the three mutants did not increase further upon addition of Mn$^{2+}$ (Fig. 3). Similar increases in mAb MEM148 binding were observed. The reactivity of mAb MEM148 toward the four different CD11b/CD18 receptors was WT < CD11b(F)/CD18(F) = CD11b(F)/CD18(F) < CD11b(F)/
CD18 (Table I), which parallels their adhesive activities (Fig. 2). These results strongly suggest that modification of the extracellular, membrane-proximal regions of the receptor causes conformational changes within the ligand binding domain, leading to enhanced adhesive activity of the CD11b/CD18 mutants.

Activation of CD11b/CD18 was not caused by changes in receptor clustering or lipid raft association

It has been shown that under certain conditions, integrins can cluster or redistribute on the cell surface, which can facilitate cell adhesion (36–38). To determine whether changes in receptor clustering or surface redistribution are responsible for the enhanced adhesive activity of the mutants, we examined surface distribution of the wild-type and the three CD11b/CD18 mutants by confocal laser scanning fluorescence microscopy using a CD11b-specific mAb (OKM1). In addition, lipid raft was visualized by staining with the B subunit of cholera toxin. Fig. 4A shows that when expressed on K562 cells, the wild-type CD11b/CD18 receptors formed large clusters on the cell surface. Similar degrees of receptor clustering were observed for the three mutants. Moreover, a majority of the wild-type CD11b/CD18 receptors resided within the lipid raft portion of the membrane. Likewise, the three CD11b/CD18 mutants were colocalized with lipid rafts, and no significant difference was observed between the wild-type and the three mutant receptors. As a negative control, we studied receptor clustering and surface distribution of a ubiquitous cell surface protein (CD71). As shown in Fig. 4A, the CD71 receptor present on K562 cells did not cluster significantly and resided mostly outside the lipid rafts. To corroborate our confocal laser scanning fluorescence microscopy data, we separated the raft and non-raft membranes using the sucrose gradient method (23), and then examined both raft and non-raft fractions by Western blot using rabbit polyclonal Abs against the cytoplasmic tails of either CD11b or CD18. Fig. 4C shows that similar percentages (30–50%) of the CD11b/CD18 receptors were present in the rafts for all four CD11b/CD18 receptors. No significant difference was observed between the wild-type receptor and any of the three mutants. The quality of the isolated lipid rafts was confirmed by the complete separation of the two commonly used markers GM1 (for rafts) and CD71 (for non-rafts). Taking these data together, we conclude that the degree of receptor clustering and surface redistribution did not change significantly upon modification of the membrane-proximal regions, and thus it is less likely to play an important role in the enhanced adhesive activities of the mutant receptors.

Inside-out signaling by the three CD11b/CD18 mutants

It was reported that wild-type CD11a/CD18, when expressed on K562 cells, does not increase its adhesive activity in response to PMA stimulation (39). To determine whether this is also the case for CD11b/CD18, we stimulated the K562 cells expressing wild-type CD11b/CD18 with PMA, a commonly used PKC agonist, and performed adhesion assays on Fg. Fig. 5A shows that the wild-type CD11b/CD18 receptor expressed on K562 cells behaved like the CD11a/CD18 receptor, in that it did not respond to the stimulation and remained nonadherent in the presence of PMA. As the three CD11b/CD18 mutants existed in certain activated conformational states, we anticipated that they may acquire some degree of responsiveness to inside-out signaling. Indeed, we found that all three mutant receptors responded to PMA stimulation and exhibited higher adhesive activity (Fig. 5A). Among the three mutants, the less active CD11b/CD18(F) responded better to PMA stimulation, and its adhesive activity was increased ~4-fold. The degree of enhancement for CD11b/CD18(F) is comparable to that observed for the endogenous CD29 integrins on K562 cells. For ex-
ample, Bleijs et al. (29) reported a 2-fold enhancement by PMA on CD49e/CD29 adhesion to fibronectin. The other two mutants, CD11b(F)/CD18 and CD11b(F)/CD18(F), that had higher basal adhesive activity were less responsive, suggesting that their adhesive activity may have reached a maximum and therefore could not be increased further. To determine whether the addition of 0.1 mM Mn$^{2+}$ would allow the wild-type integrin to support significant adhesion in response to PMA stimulation, we repeated the above adhesion assays in the presence of 0.1 mM Mn$^{2+}$. As shown in Fig. 5B, even in the presence of 0.1 mM Mn$^{2+}$ and 100 nM PMA, the K562 cells expressing the wild-type receptor had <5% maximum adhesion. Under similar conditions, the three mutant receptors, including the less active CD11b/CD18(F), exhibited strong adhesion, which could not be enhanced further by PMA. These results demonstrate that perturbation of the extracellular membrane-proximal regions of CD11b/CD18 conferred upon the receptor the ability to respond to PKC activation. To exclude a potential role of receptor clustering or surface redistribution in the enhancement of cell adhesion by PMA, we analyzed surface distribution and lipid raft association for the different CD11b/CD18 receptors in the presence of 100 nM PMA. As shown in Fig. 4B, confocal laser scanning fluorescence microscopy did not reveal a significant difference between the wild-type and mutant CD11b/CD18 receptors in either receptor clustering or lipid raft association. Thus, increased cell adhesion upon PMA stimulation did not arise from increased receptor clustering or association with lipid rafts. It has been reported that PMA stimulation of peripheral leukocytes leads to cleavage of the C-terminal region of the CD18 subunit (34). To determine whether activation of the three mutant receptors is due to cleavage of their extracellular domains, we examined the integrity of the CD11b/CD18 receptors on the cell membrane, which was isolated from the total cell lysate by sucrose gradient, using our rabbit polyclonal Abs specific for the cytoplasmic tails of CD11b and CD18. As shown in Fig. 5C, only a single band of the expected molecular mass (95 kDa for CD18 and 165 kDa for CD11b) was observed for all three mutant receptors. No cleavage product was detected. In addition, similar intensities among the wild-type and three mutant receptors were observed for both subunits. Occasionally, we observed an extra band for the wild-type receptor, which may represent the precursor form of CD18. These data suggested that activation of the three mutant receptors in this study was not caused by cleavages within either the inserted sequence or any other region of the CD11b/CD18 receptors.

Discussion

It has been well established that integrin functions are controlled dynamically by intracellular signaling events (inside-out signaling) (40, 41). However, the molecular mechanism underlying integrin activation remains ill defined. Studies over the past several years have been focused mainly on the cytoplasmic domain and the ligand binding domain of the integrin receptor (16, 17, 24, 25), whereas the region that bridges these two functional domains has not been well studied. In this work we investigated the role of this transitional region, specifically the extracellular, membrane-proximal regions of CD11b/CD18, on receptor activation, receptor clustering, and cell surface distribution. We report three major observations: 1) perturbation of the extracellular membrane-proximal region of either CD11b or CD18 leads to enhanced adhesive activity of the CD11b/CD18 receptor, which is attributed to changes in receptor conformation, but not changes in the state of receptor clustering or lipid raft association; 2) CD11b plays a major role in confining the receptor in a low adhesive state; and 3) the three CD11b/CD18 mutants acquired the ability to respond to inside-out signaling.

Several groups have shown that interactions between the cytoplasmic domains of the integrin $\alpha$ and $\beta$ subunits constrain the receptor in an inactive conformational state (16, 17). One example of such interactions is demonstrated by Hughes et al. (16) to involve a salt bridge between residue D$^{23}$ within the LITIHD sequence of CD41 and residue R$^{995}$ within the GFFKR sequence of CD61 (the hinge model). Mutation of either of the two residues or deletion of the entire cytoplasmic domain leads to receptor activation (16, 17). In support of this work, Lu et al. (17) showed that replacement of the CD11a and CD18 cytoplasmic domains with two basic peptides, but not one basic and one acid peptide, activates the CD11a/CD18 receptor via conformational changes. In addition, Takagi et al. (42) reported that linking the C-termini of the CD49e ($\alpha_5$) and CD29 ($\beta_1$) extracellular domains with a 17-residue linker plus a disulfide bond (GGLENLYFQGGKNAQC-CQA) confines the CD49e/CD29 integrin in an inactive state. Removal of such a linker sequence by specific protease cleavage leads to receptor activation, which was shown to result from conformational changes, but not receptor clustering. By and large, our results agree with the above model of integrin activation. However, there are significant differences. First, as perturbations introduced in this study were restricted to the extracellular regions of the receptor, the salt bridge between the CD11b and CD18 cytoplasmic tails should still be intact. Therefore, the receptor activation observed here could not be caused by disruption of the salt bridge between the two cytoplasmic domains. Second, the inserted sequence (a 10-residue spacer) within the three CD11b/CD18 mutants is much shorter than the sequence (a 17-residue linker plus a disulfide bond) that is sufficient to confine the CD49e/CD29 integrin in an inactive state (42). Thus, activation of the mutant CD11b/CD18 receptors in this work cannot be attributed to the opening of the C-terminal domains of the receptor. Third, the above models of integrin activation would predict that the CD11b and CD18 cytoplasmic domains have equivalent roles in controlling the C-terminal opening of the CD11b/CD18 complex. However, our data suggested the opposite. We found that CD11b and CD18 play different roles in controlling receptor activation (Fig. 2). Specifically, our data demonstrate that the CD11b subunit plays a much more important role than the CD18 subunit in constraining the CD11b/CD18 receptor in an inactive state. Perturbation of the extracellular, membrane-proximal region of CD11b resulted in larger conformational changes and stronger cell adhesion. In contrast, similar modification of CD18 only caused intermediate conformational changes and modest cell adhesion that could be further enhanced by PMA stimulation (Fig. 5A) or by addition of Mn$^{2+}$ or activating mAb (Fig. 2). Such a property of CD18 may allow a greater flexibility in controlling receptor activation. Consistent with such a concept, most of the cytoskeletal and intracellular signaling proteins that regulate integrin functions recognize the cytoplasmic domains of the integrin $\beta$ subunits (43). Taken together, the results presented in this study suggest that activation of the three CD11b/CD18 mutants was accomplished via a different mechanism than that of the CD49e/CD29 receptor (42). Further study is needed to elucidate the underlying mechanism for activation of the CD11b/CD18 receptors.

In addition to conformational changes, integrin activation may also depend on receptor clustering or redistribution on the cell surface (37, 38). Lub et al. (36) reported that activation of PBL leads to uncoupling of CD11a/CD18 from the cytoskeleton and subsequent clustering of the CD11a/CD18 receptor on the cell surface, which, in combination with conformational changes that are
induced by inside-out signaling, results in enhanced ligand binding. Our results are in good agreement with their observations. Specifically, we found that CD11b/CD18 on K562 cells existed in the clustered form regardless of cell activation (Fig. 4). As a result of such receptor clustering, we found that CD11b/CD18-mediated adhesion by K562 cells was mainly controlled by the degree of conformational changes within the three different mutants (Fig. 3). Furthermore, our data suggested that such a unique property is specific for CD11b/CD18, as CD71 stained more uniformly on the same K562 cells (Fig. 4). In addition, we found that the wild-type and the three mutants resided in the lipid rafts. As a negative control, the ubiquitously expressed CD71 was found to reside mainly outside the lipid rafts. Thus, the enhanced adhesive activity of the three CD11b/CD18 mutants cannot be attributed to changes in lipid raft association. Taken together, these data demonstrate that the CD11b/CD18 receptors expressed on K562 cells existed in large clusters and resided within the lipid rafts. Consequently, their adhesive activity is mainly controlled by the conformational changes within the CD11b/CD18 receptor.

Although the underlying mechanism for inside-out signaling remains elusive, the conformational changes within the CD11b/CD18 receptor that occur as a result of cell activation have been well documented (33, 34, 44, 45). Relevant to this study, it was reported that PMA stimulation of peripheral leukocytes leads to the expression of a neo-epitope on CD11b/CD18, which can be probed by an activation-dependent mAb, MEM148 (34). Drbal et al. (34) proposed that the expression of such an activation neo-epitope is due to cleavage of the C-terminal region located between the I domain and the transmembrane domain of the CD18 subunit, most likely by membrane-anchored proteases, such as secretase, membrane-type matrix metalloproteinase, type II transmembrane serine protease, the uPA/uPAR system, etc. (46). These authors show that expression of the MEM148 neo-epitope correlates well with the adhesive activity of CD11b/CD18. Given the similarity between the three mutant receptors and the activated CD11b/CD18 receptor on peripheral leukocytes in their reactivity toward mAb MEM148, the three active CD11b/CD18 mutants may exhibit certain intermediate conformation that exist temporarily along the physiological activation pathway of the CD11b/CD18 receptor. Moreover, several other mechanisms could also play important roles in integrin activation, such as disulfide bond exchange within the cysteine-rich region of the β subunit (47), association between the α subunit Cαt-2 and the β subunit βTD domains (48), the hinge model (16), etc. As the inserted sequence (GGGSGGGGGS) in our study exists in a random flexible conformation in solution (28), we also speculated that the rigidity of the extracellular membrane-proximal regions of the CD11b/CD18 receptor is critical to modulation of integrin activity by inside-out signaling. Therefore, understanding the molecular mechanism that leads to activation of these CD11b/CD18 mutants may help us better understand the complex process of integrin inside-out signaling. Finally, given the importance of the extracellular, membrane-proximal regions of the CD11b/CD18 receptor, it is very likely that certain cell surface proteins could use this region as a conduit to control the inside-out signaling of integrin by altering the conformation of the receptor. Candidates for such regulatory proteins may include uPAR (49), integrin-associated protein (50), and certain tetraspan proteins (51), among others.

In summary, we have demonstrated that perturbation of the extracellular, membrane-proximal region of either CD11b or CD18 results in conformational changes within the ligand binding domain of the receptor. Our data suggest that the α and β subunits of CD11b/CD18 play differential roles in controlling receptor activation. We also found that the CD11b/CD18 receptor expressed on K562 cells is associated with the lipid rafts, which may explain our above observation that activation of the CD11b/CD18 receptor is mainly accomplished by conformational changes, rather than by changes in receptor clustering or surface distribution. Finally, the results presented in this work suggest that certain cell surface proteins or proteases could modulate the adhesive activity of the integrin receptor by interacting with its extracellular, membrane-proximal regions.

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