Bidirectional Transmembrane Modulation of Integrin αIIbβ3 Conformations*

(Received for publication, September 14, 1998, and in revised form, January 13, 1999)

Tina M. Leisner‡, June D. Wencel-Drake§§, Wei Wang‡, and Stephen C.-T. Lam‡¶

From the §Department of Pharmacology and ¶School of Biomedical and Health Information Sciences, University of Illinois, Chicago, Illinois 60612

Activation of blood platelets by physiological stimuli (e.g. thrombin, ADP) at sites of vascular injury induces inside-out signaling, resulting in a conformational change of the prototype integrin αIIbβ3 from an inactive to an active state competent to bind soluble fibrinogen. Furthermore, ligand occupancy of αIIbβ3 initiates outside-in signaling and additional conformational changes of the receptor, leading to the exposure of extracellular neoepitopes termed ligand-induced binding sites (LIBS), which are recognized by anti-LIBS monoclonal antibodies. To date, the mechanism of bidirectional transmembrane signaling of αIIbβ3 has not been established. In this study, using our newly developed anti-LIBS cy1 monoclonal antibody, we showed that extracellular ligand binding to αIIbβ3 on blood platelets induces a transmembrane conformational change in αIIbβ3, thereby exposing the LIBS cy1 epitope in the αIIb cytoplasmic sequence between Lys1004 and Asp1003. In addition, a point mutation at this site (P998A/P999A) renders αIIbβ3 constitutively active to bind extracellular ligands, resulting in fibrinogen-dependent cell-cell aggregation. Taken collectively, these results demonstrated that the extracellular ligand-binding site and a cytoplasmic LIBS epitope in integrin αIIbβ3 are conformationally and functionally coupled. Such bidirectional modulation of αIIbβ3 conformation across the cell membrane may play a key role in inside-out and outside-in signaling via this integrin.

Interaction of adhesive proteins with transmembrane integrin adhesion receptors is essential for diverse biological processes including embryogenesis, angiogenesis, immune response, and hemostasis (1, 2). It is generally agreed that inside-out signaling processes regulate the affinity state of integrins for binding extracellular ligands (2, 3). Thus, upon activation of blood platelets by physiological stimuli (e.g. thrombin or ADP) at sites of vascular injury, the prototype integrin αIIbβ3 undergoes a conformational change from an inactive to an active state competent to bind soluble fibrinogen (1–5). The cytoplasmic domain of αIIbβ3 appears to play a regulatory role in αIIbβ3 activation, since truncations of the entire cytoplasmic sequence of either the αIIb or β3 subunit, including their membrane-proximal regions, were found to increase the ligand binding affinity of the mutant receptor expressed on Chinese hamster ovary (CHO) cells (6, 7). More recently, a potential salt bridge hinge formed between the αIIb and β3 cytoplasmic sequences has been suggested to maintain αIIbβ3 in a default low affinity state; disruption of this structure may result in receptor activation (8).

It is well established that binding of adhesive ligands to integrins initiates outside-in signaling processes that mediate post-ligand binding events including cytoskeleton reorganization, receptor clustering, and gene transcription (2, 3, 9). Although the mechanisms regulating outside-in signaling of integrins remain elusive, the binding of cytoskeletal proteins and signaling molecules to the receptor’s cytoplasmic domain as well as the receptor’s conformational state have been implicated to play an important role in this process. In this regard, it has been suggested that ligand occupancy of the αIIbβ3 integrin may induce a transmembrane conformational change of the receptor, thereby unmasking specific regions in the receptor cytoplasmic domain mediating cytoskeletal attachment, which ultimately leads to receptor localization to focal contacts (10). However, to date, ligand-induced transmembrane conformational changes of an integrin receptor have not been demonstrated.

It has previously been shown that ligand binding to αIIbβ3 induces further conformational changes of the receptor extracellular domain, resulting in the exposure of neoantigenic sites termed ligand-induced binding sites (LIBS), which are recognized by anti-LIBS monoclonal antibodies (mAbs) (11–16). Furthermore, certain anti-LIBS mAbs were found to activate αIIbβ3 to bind soluble fibrinogen (14, 15). In this study, we postulated that bidirectional conformational changes of αIIbβ3 transducing through the receptor’s transmembrane segment occur as a result of cellular activation and ligand binding. To test this possibility, we examined whether extracellular ligand binding induces the exposure of LIBS epitope(s) in the cytoplasmic domain of αIIbβ3. In addition, we evaluated the functional role of a putative cytoplasmic LIBS epitope in regulating αIIbβ3 ligand binding affinity.

MATERIALS AND METHODS

Peptides and Antibodies—Peptides, represented as sequences of single letter amino acid codes (17), were synthesized by solid-phase synthesis using an ABI model 431 peptide synthesizer or were purchased from Research Genetics, Inc. (Huntsville, AL). The amino acid composition of each peptide was consistent with its desired sequence. The anti-αIIbβ3 antibodies PMI-1 (18), anti-V41 (19), anti-LIBS1 (13), and mAb 15 (13) were from Dr. M. H. Ginsberg, and AP-2 (20) was from Dr. T. J. Kunicki of the Scripps Research Institute (La Jolla, CA). Anti-Chinese hamster ovary (CHO) ligand-induced binding site(s); mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; LPC, lysophosphatidylcholine; FITC, fluorescein isothiocyanate.
αιβ, C, an antipeptide polyclonal antibody raised in rabbits against the αιβ cytoplasmic sequence Pho969-Glu1030 was a generous gift of Dr. X. Du of the University of Illinois (Chicago, IL).

Production of Anti-LIBS cyt—For the production of antipeptide mAbs against the αιβ cytoplasmic sequence, the full-length P2b vector (CRK-VGFVKRIIPPLEEDDEGE) was coupled to keyhole limpet hemocyanin- m-maleimidohexahydroxy-N-hydroxysuccinimide ester and used as immunogen for BALB/c mice. Isolated spleenocytes were fused with P3-X63Ag8.653 myeloma cells. Hybridomas were grown in selective media (hygloxantine/aminopterin/thymidine), and their supernatants were tested in an ELISA for the presence of antibodies reactive with RGD affinity-purified αιββ3 (13, 21). A positive hybridoma 3F5, which secreted anti-αιβ, antibodies belonging to the IgG1 subclass, was subcloned at limiting dilutions of 0.5 cell/well. The antibody was produced as ascites and purified by chromatography on protein A-Sepharose CL-4B (Amersham Pharmacia Biotech).

Immunoprecipitation—Gel-filtered platelets were surface-labeled with Na125I and solubilized in lysis buffer containing 50 mM octyl glucoside (21). Cell lysates were incubated with GRGDSP, GRGESP, or vehicle buffer for 30 min at 37 °C. Antibodies were then added and incubated overnight at 4 °C. The immunoprecipitated proteins were collected on protein G-Sepharose, electrophoresed on SDS-7% polyacrylamide gels under nonreducing conditions, and analyzed by autoradiography.

Indirect Immunofluorescent Microscopy—Washed human platelets resuspended in Tyrode’s solution (2.5 × 108 cells/ml) were incubated with the indicated reagents (see legend of Fig. 2) at 37 °C for 30 min and subsequently fixed with 1% paraformaldehyde on ice for 1 h. After blocking unreacted aldehyde with Tris-buffered saline (20 mM Tris, pH 7.4) containing 0.5 M NaCl, cells were allowed to settle onto polylysine-coated glass coverslips and incubated with 0.2 mg/ml lysothosphatidylcholine (LPC) for 5 min to render them permeable. Permeabilized cells were rinsed with Tris-buffered saline containing 0.1% bovine serum albumin and incubated with anti-LIBS cyt1 followed by rhodamine-conjugated goat anti-mouse IgG. Samples were mounted with a droplet of FITC guard, and platelets were viewed with a Jenaval microscope (Eppendorf) and photographed with Eastman Kodak Tri-X panchromatic film (22).

Competitive ELISA—Microtiter wells were coated with the full-length P2b peptide (5 μg/well) and blocked with 3% bovine serum albumin. Anti-LIBS cyt1 was incubated with 10 μM inhibitory peptides at 37 °C for 30 min and added to the P2b-coated wells. Antibody binding to the adsorbed P2b proceeded at 37 °C for 1 h. The wells were washed, and bound antibody was detected with horseradish peroxidase-conjugated anti-mouse IgG using o-phenylenediamine as substrate (12). Absorbance at 490 nm (A490) was measured, and percentage inhibition was calculated relative to control without inhibitor.

Site-directed Mutagenesis—The expression constructs encoding wild type αιβ (CD2b) and β3 (pc3A) have been previously described (23, 24). To generate the pc2b construct encoding wild type αιβ, a 3.3-kilobase fragment of αιβ containing the entire coding sequence and the 3′-untranslated region was excised from CD2b by digestion with XhoI and ligated into the expression vector pcDNA3. The resultant construct was designated as pc2b. Both pc2b and pc3A were kindly provided by Dr. J. C. Loftus at the Mayo Clinic (Scottsdale, AZ). The p998A/p999A mutation in αιβ was generated by splice overlap extension mutagenesis (25). Overlapping fragments containing this mutation were first made by polymerase chain reaction amplifications on pc2b using the following oligonucleotide pairs: (a) 5′-CACCCGAGATGACAGAGACTTCTAATTGGCTC-3′ and 5′-CTTCCAGCAAGGCTC-5′; (b) 5′-CACCCGAGATGACAGAGACTTCTAATTGGCTC-3′ and 5′-CTTCCAGCAAGGCTC-5′; (c) 5′-GAACTGACCGGATTGAGCTGCTGACGTTCCGCTG-3′; and (d) 5′-GCAAGGATTGAGCTGCTGACGTTCCGCTG-3′. The overlapping fragments were combined, denatured by heating at 94 °C for 5 min, and renatured by cooling to 55 °C. The ends were filled in with Pfu, and the double-stranded fragments were then amplified by polymerase chain reaction using the oligonucleotide pair a and d. The amplified product was digested with BamHI and XhoI and reinserted into a BamHI–XhoI-digested pc2b vector fragment. The mutant construct was identified by automated DNA sequencing, purified by chromatography on QIAGEN Tip-100, and co-transfected with the wild type β3 construct (pc3A) into CHO-K1 cells (ATCC, Rockville, MD) by liposome-mediated transfection as described (7). Surface expression of mutated αιββ3 was analyzed by flow cytometry using FITC-conjugated AP-2. Stable cell lines were selected in medium containing 0.75 mg/ml G418 (Sigma), and single cell sorting was performed to obtain stable clonal lines, which were high expressors of the mutant αιββ3.

RESULTS AND DISCUSSION

Ligand Binding Induces A Transmembrane Conformational Change of αιββ3—To examine the possibility that ligand binding induces the exposure of LIBS epitopes in the cytoplasmic domain of αιββ3, we developed anti-peptide mAbs against the receptor’s cytoplasmic sequences and screened for antibodies that preferentially bind to the ligand-occupied conformer of αιββ3. In the present study, we focused on the αιβ cytoplasmic tail. Initially, mAbs reactive with RGD affinity-purified αιββ3 in an ELISA system were further characterized by immunoblotting and immunoprecipitation studies. The mAb obtained from clone 3F5 recognizes a ligand-induced binding site in the cytoplasmic domain of αιββ3 (LIBS cyt), and therefore this mAb is designated as anti-LIBS cyt1. Fig. 1A shows that anti-LIBS cyt1 specifically immunoblotted the 140-kDa nonreduced αιβ subunit in RGD affinity-purified αιββ3 (lane 1) and in a detergent extract of platelet proteins (lane 2). Upon reduction of purified αιββ3 and proteins in the platelet lysate, anti-LIBS cyt1 immunoblotted the 27-kDa light chain of αιβ, which contains its cytoplasmic sequence (lanes 3 and 4). To determine whether the interaction of anti-LIBS cyt1 with nondenatured αιββ3 is dependent on ligand occupancy, we performed immunoprecipitation experiments using lysates of surface-oxidized platelets in the presence and absence of an RGD peptide. As judged by densitometric scanning of the immunoprecipitated 125I-labeled protein bands, incubation of platelet lysates with GRGDSP caused a 7.2-fold increase in the amount of αιββ3 immunoprecipitated by anti-LIBS cyt1 (Fig. 1B). In contrast, the variant GRGESP peptide was much less effective (1.5-fold). As controls, we used the well characterized anti-LIBS1 mAb (13), which demonstrated a similar effect in RGD-dependent immu-
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**Fig. 2.** Indirect immunofluorescent microscopy studies to detect the exposure of the anti-LIBS<sub>cyt1</sub> epitope in ligand-occupied αIIbβ3 of permeabilized platelets. A, platelets were treated with 1 μM GRGDSP, 1 μM GRGESP, or vehicle buffer prior to fixation and permeabilization with LPC. B, resting or activated platelets (10 μM ADP) were incubated with or without fibrinogen (3 μg/ml), fixed, and permeabilized with LPC (original magnification, ×400).

noprecipitation of αIIbβ3. However, using the control mAb 15, whose binding to αIIbβ3 is not markedly influenced by ligand occupancy (13), we observed that GRGDSP incubation induced only a slight (1.5-fold) increase in the immunoprecipitation of αIIbβ3. Thus, these results suggest that interaction of GRGDSP with the extracellular ligand binding site of αIIbβ3 induces a conformational change in the receptor’s cytoplasmic domain. In support of our finding that the cytoplasmic domain of αIIbβ3 can exist in different conformational states, the cytoplasmic sequences of αIIb and β3 have been shown to interact with each other, and at least two docking models with different tertiary structures of the αIIbβ3 cytoplasmic domain have been proposed (26–28).

To examine whether RGD occupancy also induces transmembrane conformational changes of αIIbβ3 in situ, we performed indirect immunofluorescence microscopy using whole platelets preincubated with or without GRGDSP followed by paraformaldehyde fixation and LPC permeabilization to allow antibody access. As shown in Fig. 2A, incubation of platelets with GRGDSP (panel a) resulted in significant intracellular staining of anti-LIBS<sub>cyt1</sub> as opposed to control platelets incubated with vehicle buffer (panel b) or GRGESP (panel c). Furthermore, the observed rim staining pattern with GRGDSP-treated permeabilized platelets is suggestive of anti-LIBS<sub>cyt1</sub> localization to the inner face of the plasma membrane, since minimal staining was observed with nonpermeabilized cells (not shown). To investigate whether binding of the physiological ligand fibrinogen to αIIbβ3 on activated platelets also induces the exposure of LIBS<sub>cyt1</sub>, we performed indirect immunofluorescence studies with ADP-stimulated platelets in the presence and absence of exogenous fibrinogen. Again, anti-LIBS<sub>cyt1</sub> staining was performed following fixation and cell permeabilization. The addition of fibrinogen to ADP-stimulated platelets dramatically increased anti-LIBS<sub>cyt1</sub> staining as compared with activation of platelets with ADP alone (Fig. 2B, panels a and b). In control samples, resting platelets failed to stain for anti-LIBS<sub>cyt1</sub> in the presence and absence of fibrinogen (Fig. 2B, panels c and d). Therefore, these results demonstrate that anti-LIBS<sub>cyt1</sub> recognizes the ligand-occupied but not the activated unoccupied conformer of αIIbβ3. By immunogold staining with AP6, an anti-LIBS mAb directed against the β3 extracellular domain, Nurden et al. (16) previously reported that a pool of αIIbβ3 in the α-granules of unactivated platelets exist in the ligand-occupied state. However, using anti-LIBS<sub>cyt1</sub>, we failed to detect immunofluorescent staining of αIIbβ3 in the α-granules of resting platelets. This may be due to the association of αIIbβ3 with cytoskeletal components that mediate internalization and transport of the fibrinogen-αIIbβ3 complex to the platelet α-granules (22, 29, 30), thus blocking interaction of anti-LIBS<sub>cyt1</sub> with the cytoplasmic domain of ligand-occupied αIIbβ3 in the α-granule membranes. Nonetheless, the observation that fibrinogen binding to αIIbβ3 on the platelet surface induces a transmembrane conformational change of the receptor provides a possible mechanism by which ligand occupancy of αIIbβ3 mediates a variety of post-ligand binding function of blood platelets including clot retraction, receptor internalization, and cytoskeletal attachment.

To identify specific residues within the αIIb cytoplasmic sequence mediating interaction with anti-LIBS<sub>cyt1</sub>, we performed competitive ELISA analyses using peptides corresponding to the full-length or partial sequences of the αIIb cytoplasmic tail. As shown in Table I, the full-length P2b peptide, as well as the truncated 15-mer (KVGGFKRNRPPELEDEEGE) effectively blocked anti-LIBS<sub>cyt1</sub> binding to immobilized P2b peptide. Moreover, using two overlapping peptides, we further localized the anti-LIBS<sub>cyt1</sub> epitope to the KRNRPPLEED sequence. Molecular modeling suggests that this region in both αIIb and β3 subunits would form a tight β-turn (28, 31). Since Pro<sup>989</sup>Pro<sup>990</sup> may facilitate this β-turn formation, we tested the ability of KRNRAALEED to inhibit anti-LIBS<sub>cyt1</sub> binding. The inhibitory effect of the peptide was found to be significantly diminished by substitution of the two proline residues with alanine, indicating that anti-LIBS<sub>cyt1</sub> recognizes a structural motif dependent on these two proline residues.

**A Site-directed Mutation of the Anti-LIBS<sub>cyt1</sub> Epitope Acti-
vates αIIbβ3 to Bind Extracellular Ligands—The ability of certain anti-LIBS mAbs to activate αIIbβ3 (14, 15) suggests that these LIBS epitopes may regulate the ligand binding affinity state of the receptor. Therefore, we evaluated the functional significance of Pro998→Pro999 in the regulation of extracellular ligand binding to αIIbβ3. In these studies, a double P998A/P999A mutation in αIIb was generated by splice overlap extension mutagenesis (25), and the mutant αIIb construct was co-transfected with a wild type β3 construct into CHO cells. A stable clonal cell line (G4) expressing the mutant αIIbβ3 was established, and comparative analyses were performed with the control A5 cell line expressing wild type αIIb and mutated β3 heterodimers failed to bind soluble fibrinogen and undergo aggregation (not shown). These results indicate that a structural change in the anti-LIBScyt1 binding site in the β3 cytoplasmic tail induces a transmembrane conformational change of αIIbβ3, mimicking receptor activation due to inside-out signaling. 

It has previously been shown that ligand binding to integrin αIIbβ3 induces long range conformational changes in the extracellular domains of both αIIb and β3 subunits (12, 35). Our results demonstrated that such conformational changes transduce through the cell membrane to the cytoplasmic domain of the receptor. Besides αIIbβ3, several other integrins such as α5β1 and α6β1 have been shown to undergo extracellular conformational changes upon ligand occupancy (13, 36); therefore, it is tempting to speculate that ligand-induced conformational changes also occur in the cytoplasmic domains of other integrins. Since ligand binding to integrins results in cytoskeletal rearrangement and the generation of intracellular signals (2, 3, 9), the conformational state of integrin cytoplasmic domains may play a regulatory role in the assembly of cytoskeletal proteins and/or signaling molecules. In this regard, it has been shown that antibody-induced clustering of the αβ3 integrin in the absence of ligand occupancy is sufficient for the intracellular accumulations of tension and at least 20 signal transduction molecules (e.g., RhoA, Rac1, Ras, Raf, MEK, extracellular signal-regulated kinase, e-Jun N-terminal kinase, and focal adhesion kinase) (37, 38). In contrast, both ligand occupancy and clustering of αIIbβ3 are required for transmembrane accumulations of several cytoskeletal proteins (e.g., talin, vinculin, and α-actinin). In light of these findings, our present data suggest that ligand-induced conformational changes of integrin cytoplasmic domains may play an essential role in the intracellular assembly of cytoskeletal proteins found in focal adhesions. 

Emerging evidence has implicated ligand-induced oligomerization and/or conformational changes of transmembrane receptor complexes as potential mechanisms for receptor-mediated signal transduction. Specifically, it has been demonstrated that following ligand binding and dimerization of the platelet-derived growth factor receptor, there is a phosphorylation-dependent conformational change in the receptor cytoplasmic domain (39–41). Although integrin αIIbβ3 on platelets becomes tyrosine-phosphorylated as a result of ligand binding and cell aggregation, the monovalent RGD peptide has been shown to block receptor phosphorylation (42). Inasmuch as GRGDSP binding to αIIbβ3 is capable of inducing the exposure of the anti-LIBScyt1 epitope, receptor phosphorylation is Apparently not required for the observed effect. Thus, our results provide the first evidence of a direct effect of ligand occupancy.
on the conformation of the cytoplasmic domain of an intact integrin receptor. Additionally, site-directed mutation of the identified LIBSα1 epitope resulted in an increase of ligand binding affinity of αIIbβ3, indicating that the extracellular ligand-binding site and the cytoplasmic LIBS epitope of the receptor are functionally coupled. In sum, these findings suggest a bidirectional modulation of αIIbβ3 conformations across the cell membrane. Such conformational regulation may provide a novel mechanism for transmembrane receptor-mediated signal transduction.

Acknowledgments—We thank Drs. X. Du, A. L. Frelinger III, M. H. Ginsberg, L. F. Lau, and J. C. Lofts for helpful discussions and critical comments on the manuscript. We also thank Dr. T. E. O'Toole for providing Chinese hamster ovary cell lines expressing wild type and mutant receptor.

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