A New Functional Role of the Fibrinogen RGD Motif as the Molecular Switch That Selectively Triggers Integrin αIIbβ3-dependent RhoA Activation during Cell Spreading*

Received for publication, January 5, 2005, and in revised form, May 11, 2005. Published, JBC Papers in Press, June 13, 2005, DOI 10.1074/jbc.M500146200

Alexandre Salsmann1,2, Elisabeth Schaffner-Reckinger1, Fabrice Kable, Sébastien Plançon, and Nelly Kieffer1

From the Laboratoire de Biologie et Physiologie Intégrée (CNRS/GDRE-ITI), Université du Luxembourg, 162A Avenue de la Falencerie, L-1511 Luxembourg, Grand-Duchy of Luxembourg

A number of RGD-type integrins rely on a synergistic site in addition to the canonical RGD site for ligand binding and signaling, although it is still unclear whether these two recognition sites function independently, synergistically, or competitively. Experimental evidence has suggested that fibrinogen binding to the RGD-type integrin αIIbβ3 occurs exclusively through the synergistic γ400–411 sequence, thus questioning the functional role of the RGD recognition site. Here we have investigated the respective role of the fibrinogen γ400–411 sequence and the RGD motif in the molecular events leading to ligand-induced αIIbβ3-dependent Chinese hamster ovary (CHO) cell or platelet spreading, by using intact fibrinogen and well-characterized plasmin-generated fibrinogen fragments containing either the RGD motif (fragment C) or the γ400–411 sequence (fragment D), and CHO cells expressing resting wild type (αIIbβ3wt), constitutively active (αIIbβ3T562N), or non-functional (αIIbβ3D119Y) receptors. Our data provide evidence that the γ400–411 site by itself is able to initiate αIIbβ3 clustering and recruitment of intracellular proteins to early focal complexes, mediating cell attachment, FAK phosphorylation, and Rac1 activation, while the RGD motif subsequently acts as a molecular switch on the β3 subunit to trigger cell spreading. More importantly, we show that the premier functional role of the RGD site is not to reinforce cell attachment but, rather, to imprint a conformational change on the β3 subunit leading to maximal RhoA activation and actin cytoskeleton organization in CHO cells as well as in platelets. Finally, αIIbβ3-dependent RhoA stimulation and cell spreading, but not cell attachment, are Src-dependent and phosphoinositide 3-kinase-independent and are inhibited by the Src antagonist PP2.

Plasma fibrinogen is one of the most abundant soluble adhesion molecules present in blood vessels and serves as a ligand to a variety of vascular cells, including platelets, endothelial cells, and monocytes. Fibrinogen is primarily involved in the maintenance of hemostasis by mediating platelet aggregation, clot formation, and wound healing. In addition, together with thrombin-converted insoluble fibrin, fibrinogen also functions as a component of the extracellular matrix in non-hemostatic normal or pathological processes promoting placenta development, angiogenesis, atherosclerosis, metastasis, as well as a variety of vascular and renal diseases (1). Both fibrinogen and fibrin expose multiple interacting sites that serve as adhesion motifs for vascular cell receptors. Undoubtedly, the first and best characterized of these binding sites are those interacting with the β3 integrins, the platelet-specific αIIbβ3 fibrinogen receptor (2), and the αvβ3 vitronectin receptor (3).

Human fibrinogen contains three putative β3 integrin binding sites, two RGD motifs within the Aα chain, Aα95–98 (RGDF) and Aα572–575 (RGDS) (4), and a non-RGD dodecapeptide sequence in the γ chain (C-terminal γ400–411) (5). Although fibrinogen binding to αvβ3 relies essentially on the Aα572–575 RGD sequence (3), binding to αIIbβ3 involves, in addition to the RGD sites, non-RGD dodecapeptide γ400–411 sequence (5). Evidence for the functional importance of these fibrinogen motifs in β3 integrin ligand binding emerged from biochemical cross-linking and site-directed mutagenesis studies (6), as well as from genetic analysis of naturally occurring αIIbβ3 variants in patients with Glanzmann’s thrombasthenia (7), characterized by complete loss of fibrinogen binding due to single point mutations within the β pro-peller of the αIIb subunit or the Aα domain of the β3 subunit, that function as contact sites for the γ400–411 sequence and the RGD motif, respectively. With the recently published crystal structure of the extracellular domains of αvβ3 (8) and αIIbβ3 (9), the precise contact sites of the RGD motif with the β3 subunit have been defined with residue Asp ligated to a Mn2+ held in the metal ion-dependent adhesion site of the βA domain and stabilized by additional contacts with residues Tyr-122, Arg-214, and Asn-215 of the βA domain.

Despite the higher affinity of the RGDS peptide for αIIbβ3 as compared with the γ400–411 dodecapeptide, experimental evidence has suggested that neither of the two RGD sequences in fibrinogen significantly contributes to the binding of αIIbβ3 to either surface-bound or soluble fibrinogen (10–12), whereas in contrast, binding of αvβ3 to fibrinogen appears to rely essentially on the Aα572–575 RGDS sequence (3). Soluble or surface-bound fibrinogen interaction with αIIbβ3 is believed to be mediated primarily by the γ400–411 sequence (3, 5, 10, 11, 13–16), consistent with electron microscopy images showing αIIbβ3 associated with the distal ends of fibrinogen comprising the γ400–411 sequence (17). In addition, fibrinogen deleted of the γ400–411 AGDV terminal sequence on each γ chain produced in vitro (18) or in transgenic mice in vivo (19) is unable to support platelet aggregation, in contrast to fibrinogen mutated in the Aα chain RGD sites that promotes normal platelet aggregation (10). These data highlight the predominant role of the γ chain residues 408–411 in both αIIbβ3-mediated cell adhesion to surface-bound fibrinogen and αIIbβ3-mediated platelet aggregation and...
question the precise functional role of the \(\alpha_\text{IIb}\beta_3\) RGDS sequence in integrin \(\alpha_\text{IIb}\beta_3\) fibrinogen recognition.

Here we have investigated the respective role of the \(\gamma_{34}^{400-411}\) sequence and the \(\alpha_\text{IIb}\beta_3\) RGDS site in the molecular events leading to \(\alpha_\text{IIb}\beta_3\)-dependent Chinese hamster ovary (CHO) cell or platelet spreading. Using intact fibrinogen and well characterized proteolytic fragments containing either the \(\gamma_{34}^{400-411}\) site (fragment D) or the \(\alpha_\text{IIb}\beta_3\) RGDS site (fragment C) as ligands and platelets as well as CHO cells expressing resting wild type (\(\alpha_\text{IIb}\beta_3\)wt), constitutively active (\(\alpha_\text{IIb}\beta_3\Delta626\)N), or non-functional (\(\alpha_\text{IIb}\beta_3\Delta119\)) receptors, we report a new functional role of the RGDF motif as a molecular switch that triggers an \(\alpha_\text{IIb}\beta_3\)-dependent signaling cascade leading to Src-dependent RhoA activation and cell spreading.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The following monoclonal antibodies were generous gifts: the anti-\(\beta_3\) mAbs P37 (J. Gonzalez-Rodriguez), AP5 (T. I. Kunicki), D3GP3 (L. Jennings), and MA-16/N7 (M. Hoylaerts); anti-\(\gamma_3\) mAb S222 (C. G. Ruan), anti-\(\alpha_\text{IIb}\beta_3\) mAb P2-73 (C. Kaplan), anti-\(\alpha_\text{v}\beta_3\) mAb 23C6 (M. Horton), anti-fibrinogen \(\gamma_{34}^{400-411}\) mAb 4A5 (G. Matsueda), and the anti-fibrinogen RGDS antibody 134B29 (Z. Ruggeri). All other monoclonal or polyclonal antibodies were purchased: blocking anti-\(\alpha_\text{v}\beta_3\) (LM609) (Chemicon International, Temecula, CA); anti-FAK (C-903) and anti-RhoA (sc-418) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-phosphoFAK (07-012) and anti-Rac1 (23A8) (Upstate Biotechnology, Lake Placid, NY); anti-\(\alpha_\text{IIb}\beta_3\) (PAC-1) (BD Biosciences, San Jose, CA); anti-phosphotyrosine (PY-20) and anti-paxillin (P13520) (BD Transduction, Lexington, KY); anti-talin rod domain (TD 77) (Research Diagnostics, Inc., Flanders, NJ); anti-vinculin (VIN-11-5) (Sigma). Wortmannin and prostaglandin \(E_1\) were purchased from Sigma and P2P and P3P from Calbiochem (Darmstadt, Germany). Plasmin-generated fibrinogen fragments D and C, a gift from Dr. D. R. Phillips (COR Therapeutics, South San Francisco, CA), were generated essentially as described by Strickland et al. (20), and were purified by conventional size exclusion chromatography.

**cDNA Constructs, Transfection, and Cell Culture**—The pTG2328-\(\beta_3\)D119Y plasmid was obtained from Dr. F. Lanza. The cDNA construct \(\beta_3\)T562N was generated by PCR mutagenesis using standard procedures and pcDNA3-\(\beta_3\)wt as a template. Expression of wild type, \(\alpha_\text{IIb}\gamma_{34}^{400-411}\beta_3\), \(\alpha_\text{IIb}\beta_3\), and \(\alpha_\text{IIb}\beta_3\) integrin in CHO cells was performed as described previously (21, 22). CHO transfectants were cultured under standard tissue culture conditions in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum.

**Cell Adhesion Assays**—Adhesion assays were carried out as described previously (23) with minor modifications. Briefly, for each experiment, 3 \times 10^4 washed cells were preincubated for 30 min at room temperature with or without inhibitors, then added to the ligand-coated wells (20 000 washed cells/well) and cell adhesion was allowed to occur at 37 °C. At given time points, the cells were microphotographed in the wells without prior washing of the plates or discharge of non-adherent cells. For all \(\alpha_\text{IIb}\beta_3\)-dependent cell adhesion assays, \(\alpha_\text{v}\beta_3\) receptor function was blocked by the addition of 10 \(\mu\)M of the \(\alpha_\text{v}\beta_3\) inhibitor RO65-5233/001 (Roche Applied Science). For cell adhesion inhibition assays, the cells were either preincubated with 10 \(\mu\)M wortmannin (PI3K inhibitor), 10 \(\mu\)M PP2 (Src inhibitor), or 10 \(\mu\)M PP3 (as a negative control for PP2), all dissolved in Me_2SO, or with Me_2SO alone, as a vehicle control (final concentration 0.1%).

**Immunofluorescence Microscopy and Flow Cytometry**—Cells adherent on glass coverslips precoated with 20 \(\mu\)g/ml fibrinogen, fragment D, or fragment C were fixed for 10 min at 4 °C with 3% paraformaldehyde, 2% sucrose in PBS, pH 7.4, rinsed twice with PBS, and permeabilized with 0.5% Triton X-100 in PBS, pH 7.4, containing 0.5% heat-denatured bovine serum albumin. Immunofluorescent staining was performed using specific primary antibodies and a fluorescein isothiocyanate-conjugated secondary antibody (CalTag Laboratories, Burlingame, CA).

**Polymerization of actin was stained with tetramethylrhodamine isothiocyanate (TRITC)-phalloidin (Molecular Probes, Leiden, The Netherlands). Microphotographs were taken using a Leica DC 300F digital camera and the Leica IM1000 1.20 software. Flow cytometry analysis of antibody binding to CHO transfectants or platelets was performed as previously described (23).

**FAK Tyrosine Phosphorylation and GTPase Activity Assays**—Petri dishes were coated with 20 \(\mu\)g/ml of fibrinogen, fragment D, or fragment C, blocked with bovine serum albumin and finally washed twice with serum-free Iscove’s modified Dulbecco’s medium. CHO cells were either kept in suspension or added to the coated Petri dishes. Following incubation at 37 °C, adherent cells were chilled on ice and lysed in situ.

**Rho family GTPase activity determination was performed as described by Ren et al. (24). Briefly, adherent cells were lysed in 500 \(\mu\)l of lysis buffer containing 25 mM Heps, pH 7.3, 150 mM NaCl, 5 mM EDTA, pH 8.0, 10 mM NaF, 2 mM Na_3VO_4, 20 \(\mu\)g/ml pepstatin A, 10 \(\mu\)g/ml aprotinin and leupeptin, 50 \(\mu\)M 4-(2-aminoethyl)benzenesulfonyl fluoride. Phosphorylated FAK was detected by immunoblotting of cell lysates with a polyclonal antibody specific for the phosphorylated FAK residue Tyr-397. The blot was then stripped and reprobed with a FAK-specific polyclonal antibody to monitor total FAK loading. Quantification of FAK tyrosine phosphorylation was performed by densitometric scanning of the autoradiograms (HP ScanJet 5p Scanner and QuantiScan software, Biosoft).

**Platelet Preparation and Adhesion Assays**—Acid-citrate-dextrose-anti-coagulated blood (1:7, v/v) was obtained from healthy donors. After centrifugation at 200 \(\times\) g for 15 min at room temperature, the platelet-rich plasma was carefully removed, adjusted to pH 6.5 with acid-citrate-dextrose-anti-coagulated blood, and centrifuged at 1200 \(\times\) g for 15 min at room temperature in the presence of 1 \(\mu\)M prostaglandin \(E_1\). Platelets were then gently resuspended and subsequently washed twice in a buffer containing 35 mM citrate acid, pH 6.5, 5 mM glucose, 1 \(\mu\)M prostaglandin \(E_1\), 5 mM KCl, 2 mM CaCl_2, 1 mM MgCl_2, 100 mM NaCl.

For adhesion assays, washed resting platelets or platelets stimulated for 20 min at 37 °C with either 0.5 \(\mu\)M MnCl_2 or D3GP3 mAb were
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RESULTS

A Two-step Mechanism Underlies αIIbβ3-dependent CHO Cell Spreading on Fibrinogen—Integrins αvβ3 and αIIbβ3 mediate cell adhesion to immobilized fibrinogen through two distinct molecular mechanisms: while αvβ3-dependent cell adhesion to fibrinogen relies essentially on the C-terminal αvRGDS-572–575 RGDG recognition site in the fibrinogen α chain (15), αIIbβ3-mediated cell adhesion is mediated primarily through the γ chain C-terminal γ500–511 sequence (11, 15). This distinct receptor-ligand interaction could easily be demonstrated in the cell adhesion assay used here by studying the inhibitory effect of the RGDG peptide or various anti-β3 antibodies on CHO cell clones expressing similar amounts of either αvβ3 or αIIbβ3, as demonstrated by flow cytometry and Western blot analysis (Fig. 1A). The morphology of CHO cells during cell adhesion is shown in Fig. 1B. Non-adherent cells appeared as small round cells with a refringent peripheral rim, whereas attached cells were enlarged, flattened cells with small protrusions that had lost their peripheral refringency, and fully spread cells exhibited a fibroblastoid morphology.

Because of the presence of a chimeric hamster αv/human β3 integrin in CHO αIIbβ3 cells, control experiments were performed in the presence of 10 μM RO65-5233/001, a selective inhibitor of αvβ3 and αvβ5 integrins, or in the presence of the αvβ3-blocking antibody LM609. Whereas these inhibitors completely blocked adhesion of CHO αvβ3 cells, they had no effect on CHO αIIbβ3 cell adhesion (Fig. 1C). All subsequent αIIbβ3-dependent adhesion assays were therefore performed in the presence of RO65-5233/001. The effect of the RGDG peptide or the RGDG-containing mAb MA-16N7C2 on the cell morphology is shown in Fig. 1D. Pretreatment of the cells with RGDG or MA-16N7C2 totally inhibited CHO αvβ3-mediated cell spreading as well as cell attachment to intact fibrinogen, whereas these inhibitors prevented essentially CHO αIIbβ3wt cell spreading, but still allowed cell attachment. In addition, αvβ3-dependent cell adhesion was inhibited with antibodies directed against various epitopes of the β3 subunit, whereas these antibodies did not impair αIIbβ3-mediated cell adhesion to fibrinogen. Shown is a representative result obtained with the anti-β3 mAb P37, which prevented αvβ3-dependent CHO cell spreading by 80%, but had no major inhibitory effect on αIIbβ3-dependent CHO cell spreading (Fig. 1E).

Loss-of-function point mutations have been identified in the β3 integrin subunit of Glanzmann’s thrombasthenia patients that directly affect the RGD interaction with the β3 subunit A domain. Among these, the Asp-119 → Tyr mutation, which interferes with the β3 metal ion-dependent adhesion site, completely inhibits fibrinogen-dependent

RESUMEN

RESULTADOS

Un mecanismo en dos etapas subyace al role funcional del motivo RGD en αIIbβ3—Integrinas αvβ3 y αIIbβ3 mediaban la adherencia celular a fibrinógeno a través de dos mecanismos moleculares distintos: mientras que la adherencia celular αvβ3 dependiente a fibrinógeno depende principalmente del sitio de reconocimiento de la cadena αvRGDS-572–575 en el α cadena del fibrinógeno (15), la adherencia αIIbβ3 dependiente se medió principalmente por la secuencia terminal de la cadena γ C-terminal γ500–511 (11, 15). Este distinto intercambio receptor-ligando se pudo fácilmente demostrar en la prueba de adherencia celular utilizada aquí al estudiar el efecto inhibidor del peptide RGDG o de varias antibióticos anti-β3 en las clones de células CHO expresando cantidades similares de αvβ3 o αIIbβ3, como se demostró por flujo de citometría y análisis Western (Fig. 1A). El morfología de las células CHO durante la adherencia celular se muestra en la Fig. 1B. Las células no-adherentes aparecieron como células redondas pequeñas con un borde periférico refringente, mientras que las adherentes eran células ampliadas, planas con pequeñas proyecciones que habían perdido su borde periférico refringente, y las células extendidas completas exhibían una morfología fibroblastídica.

Por la presencia de un híbrido de camello αv/humano β3 integrina en CHO αIIbβ3, se realizaron experimentos de control en presencia de 10 μM RO65-5233/001, un inhibidor selectivo de αvβ3 y αvβ5 integrinas, o en presencia del inhibidor anti-β3 bloqueador LM609. Whereas these inhibidores completamente bloqueaban la adherencia de CHO αvβ3, ellos no mostraron ningún efecto en CHO αIIbβ3 celular adherencia (Fig. 1C). Todas las siguientes pruebas de adherencia αIIbβ3 dependientes fueron, por tanto, realizadas en presencia de RO65-5233/001. El efecto del peptide RGDG o el peptide anti-β3 RGDG-containing mAb MA-16N7C2 en el morfología de la célula se muestra en la Fig. 1D. Pretratamiento de las células con RGDG o MA-16N7C2 totalmente inhibió la adherencia celular αvβ3 como también la adhesión celular a fibrinógeno intacto, mientras que estos inhibidores evitaron completamente la adherencia cellular αIIbβ3wt, pero todavía permitían la adhesión celular. En adición, la adherencia αvβ3 dependiente fue inhibida con antibióticos dirigidos contra varios epitopos del subunidad β3, mientras que estas antibióticos no impidieron la adherencia αIIbβ3-mediada a fibrinógeno. Se muestra el resultado representativo obtenido con el anti-β3 mAb P37, que evitó la adherencia cellular αvβ3 por 80%, pero no tuvo ningún efecto inhibidor mayor en la adherencia αIIbβ3-dependiente a la población celular (Fig. 1E).

MUTACIONES DE PUNTO perdieron función han sido identificadas en el subunidad β3 integrina de los pacientes con trombastenia de Glanzmann que directamente afectan el intercambio RGD con el subunidad A dominio β3. Entre estas, la Asp-119 → Tyr mutación, que interfiere con el sitio de adhesión dependiente de ion metal β3, completamente inhibió la adherencia fibrinógeno-dependiente...
platelet aggregation (27). Here we have tested whether CHO cells expressing this mutant αIIbβ3D119Y receptor with a non-functional RGD recognition site were still able to interact with immobilized fibrinogen. As expected, CHO αIIbβ3D119Y mutants failed to spread on immobilized fibrinogen (28) but underwent attachment, suggesting that the initial step of cell adhesion is unaffected by this mutation and relies on the αIIb subunit interaction with the \( \gamma^{400-411} \) sequence (Fig. 1F). These results provide evidence that, in contrast to αβ3, the αIIbβ3-fibrinogen interaction is a two-step mechanism involving two distinct contact sites in fibrinogen that function independently, the first step mediating cell attachment and relying on the \( \gamma^{400-411} \) sequence, and the second step leading to cell spreading, and mediated through RGD recognition.

CHO αIIbβ3wt Cells Plated on Fragment D Are Blocked at the Cell Attachment Stage—Previous studies have shown that resting αIIbβ3 in platelets is able to mediate cell adhesion to intact fibrinogen as well as plasmin-generated fibrinogen fragment D (16, 29). To determine the specific involvement of the fibrinogen \( \gamma^{400-411} \) recognition site in the two-step adhesion process, we have used fibrinogen fragment D, which contains the \( \gamma^{400-411} \) site but is devoid of the RGD motifs, and fragment C, which corresponds to the C-terminal part of the fibrinogen α chain and only contains the A\( \varepsilon^{572-575} \) RGDS sequence, as αIIbβ3 ligands (Fig. 2, A and B). When plated on fragment D, CHO αIIbβ3wt cells attached but were unable to spread, in contrast to cells plated on intact fibrinogen. On fragment C, the cells failed to attach and spread, indicating that resting αIIbβ3 expressed in CHO cells is unable to interact with the RGDS site exposed in fragment C in the absence of receptor activation through the αIIb-\( \gamma^{400-411} \) interaction (Fig. 2C). A time-course experiment performed over a period of 4 h further showed that the cells plated on fragment D were blocked at this attachment stage (data not shown). These findings support the concept that the fibrinogen \( \gamma^{400-411} \) site in fragment D initiates cell attachment but cannot mediate irreversible cell spreading in the absence of the RGD site.

Attachment of CHO αIIbβ3wt Cells to Fragment D Induces αIIbβ3 Clustering and Recruitment of Intracellular Cytoskeletal and Adaptor Proteins into Focal Complexes—To determine whether CHO αIIbβ3wt
cell attachment to fibrinogen fragment D merely corresponded to receptor-ligand interaction or whether this interaction by itself was able to initiate outside-in signaling events leading to αIIbβ3 clustering. We investigated the subcellular localization of αIIbβ3 in cells plated on fragment D or fragment C. Interestingly, in CHO αIIbβ3wt cells attached to fragment D, αIIbβ3 was already well organized in small adhesion contacts at the cell periphery, giving the cells a star-like morphology (Fig. 3A). However, these cells were essentially devoid of actin stress fibers and exhibited submembranous actin staining, in contrast to cells plated on intact fibrinogen that displayed a fibroblastoid morphology with well-structured stress fibers connected to mature focal adhesions. When plated on fragment C, the diffuse staining of β3 and the absence of actin filaments confirmed the absence of specific αIIbβ3-dependent adhesion structures in these cells.

We next analyzed recruitment of typical components of focal adhesions to these αIIbβ3-dependent adhesion structures generated on fibrinogen fragment D. To demonstrate that all the immunostained adhesion contacts were also positive for αIIbβ3, we used CHO cells expressing an autofluorescent αIIbβ3 GFP or αIIbβ3 GFP integrin (22). A perfect colocalization of immunostained paxillin and autofluorescent αIIbβ3 GFP is shown in Fig. 3B, demonstrating that on fibrinogen fragment D, only integrin αIIbβ3 is engaged in adhesion structures. An identical result was also obtained with cells expressing αIIbβ3 GFP (data not shown). For further experiments, CHO cells expressing wild type αIIbβ3 were used. Interestingly, the cytoskeletal proteins talin and vinculin, as well as tyrosine-phosphorylated proteins were found in these adhesion structures (Fig. 3C). These results suggest that the αIIbβ3 γ600-411 interaction induces first wave signaling events leading to integrin clustering and to the recruitment of cytoskeletal scaffolding, and signaling proteins into adhesion structures that resemble focal complexes (30). However, in the absence of an RGD site, the cells are blocked at this attachment stage, suggesting that a synergistic αIIbβ3-RGD interaction is required to trigger additional signaling events allowing full cell spreading.

The Spontaneously Active αIIbβ3T562N Mutant Mediates CHO Cell Spreading on Fibrinogen Fragment D—Ligand-induced outside-in signaling relies on the long range propagation of conformational changes in the β3 integrin subunit that are transmitted from the ligand binding pocket to the cytoplasmic tail, necessary for the formation of mature focal adhesions. These ligand-induced conformational changes can be monitored with well characterized monoclonal antibodies, which identify ligand-induced binding site (LIBS) neoeptopes (31), exposed on the β3 integrin subunit following RGD or fibrinogen binding and not present on the unoccupied resting receptor. To determine whether αIIbβ3 outside-in signaling necessary for cell spreading requires a physical interaction of the RGD recognition motif with the β3 subunit or whether it can be brought about by a conformational change within the β3 subunit independent of the RGD interaction, we investigated the adhesive properties of CHO cells expressing the constitutively active αIIbβ3T562N receptor (32). As shown in Fig. 4, CHO αIIbβ3T562N cells spontaneously bound the fibrinogen-mimetic mAb PAC-1 and bound the LIBS-specific anti-β3 antibody AP5 in the absence of ligand stimulation, confirming the constitutive high affinity state of the mutant receptor and the conformational change of the β3T562N subunit described by Kashwagi and coworkers (32). An identical result was also obtained with two other anti-LIBS antibodies (data not shown). The most exciting observation, however, was the capacity of CHO αIIbβ3T562N cells to undergo full cell spreading on fibrinogen fragment D with the presence of αIIbβ3-containing focal adhesions and well organized actin stress fibers (Fig. 4C). A similar result was also obtained with CHO αIIbβ3wt cells following activation of αIIbβ3 with the activating mAb D3GP3 or AP5 (data not shown). Because, in the absence of the RGD site, a reinforced adhesion due to the RGD-β3 interaction and necessary for cell spreading could be excluded, we conclude that the structural change in the β3T562N subunit mimics the conformational change normally induced following RGD binding to the β3 subunit and that this conformational change is sufficient to initiate

**FIGURE 4.** The constitutively active mutant αIIbβ3T562N mediates cell spreading on fragment D independent of RGD recognition. A, flow cytometry histograms depicting the binding of the ligand-mimetic mAb PAC-1 to CHO αIIbβ3wt or CHO αIIbβ3T562N cells in the absence (green) or in the presence of the activating mAb D3GP3 (blue) or in the presence of 1 mM RGDS used here as an inhibitory peptide (purple). Total β3 expression was monitored by labeling the cells with the β3-specific mAb P37 (red), whereas irrelevant mouse IgG was used to monitor nonspecific mAb binding (black). The ordinate depicts the number of cells per channel, and the abscissa depicts the relative fluorescence intensity in arbitrary units (log scale). B, the binding of the LIBS-specific mAb AP5 to CHO αIIbβ3wt or CHO αIIbβ3T562N cells in the presence (blue) or absence (green) of 1 mM RGDS is depicted. C, microphotographs of CHO αIIbβ3wt and CHO αIIbβ3T562N cells plated for 2 h at 37°C on intact fibrinogen or on fragment D. The bar equals 20 μm. Immunolocalization of human β3 and staining of the actin cytoskeleton in CHO αIIbβ3T562N cells plated for 2 h at 37°C on intact fibrinogen or fragment D. The microphotographs show the same cells labeled with the β3-specific mAb P37 and phalloidin to visualize actin stress fibers. The bar equals 20 μm.
intracellular signaling events leading to actin stress fiber organization and focal adhesion formation.

**aIIB3 Clustering upon Cell Attachment to Fragment D Triggers FAK Tyrosine Phosphorylation**—To determine the signaling events that occur during aIIB3-dependent cell attachment versus cell spreading, we investigated FAK tyrosine phosphorylation on residue Tyr-397 during cell adhesion using an anti-phosphoTyr-397 pAb. As shown in Fig. 5A, FAK phosphorylation increased over a time course of 2 h when CHO aIIB3wt cells were plated on immobilized fibrinogen, whereas only background FAK tyrosine phosphorylation was observed in CHO aIIB3wt cells kept in suspension. We next compared FAK tyrosine phosphorylation in CHO cells expressing aIIB3wt, aIIB3D119Y, aIIB3T562N, or in mock transfected cells. As shown in Fig. 5B, CHO aIIB3wt cells plated for 2 h on fragment D triggered FAK tyrosine phosphorylation, corresponding to ~65% of that observed when cells were plated on native fibrinogen. In addition, weak but specific FAK tyrosine phosphorylation could also be observed in CHO aIIB3D119Y cells attached to fibrinogen, when compared with mock transfected CHO cells plated on fibrinogen or CHO aIIB3wt cells kept in suspension. More importantly, when CHO aIIB3T562N cells were tested, the amount of phosphorylated FAK was essentially identical when cells were plated on fragment D or on intact fibrinogen, in line with the immunofluorescence data showing similar receptor clustering in the cells spread on either substrate (Fig. 5C). Altogether, these data provide evidence that clustering of aIIB3 upon cell attachment to fragment D is sufficient to trigger significant FAK tyrosine phosphorylation and that, under all experimental conditions investigated, FAK phosphorylation correlated closely with the extent of aIIB3 clustering.

**RhoA, but Not Rac1 Activation, Is Dependent on the RGD Interaction with the β3 Subunit**—Previous studies have shown that cytoskeletal dynamics correlate closely with RhoA activity, with low levels of RhoA activity observed in cells with small focal complexes at the cell periphery and devoid of actin stress fibers, in contrast to high levels of RhoA activity in cells exhibiting stress fibers and focal adhesions (24). Likewise, in human platelets RhoA activation following integrin aIIB3 engagement leads to actin reorganization (33). Here we have investigated the activity of the small Rho family GTPases Rac1 and RhoA during aIIB3-mediated cell adhesion to intact fibrinogen and fragment D. In accordance with data shown by others (24), RhoA activity increased over a time course of 2 h on intact fibrinogen, whereas high levels of Rac1 activity could already be detected at early time points (Fig. 6A). To determine whether maximal RhoA activation relied on a conformational change induced following the RGD interaction with the β3 subunit, we next compared RhoA and Rac1 activity in CHO aIIB3wt or aIIB3T562N cells following a 2-h incubation on intact fibrinogen or fragment D. As shown in Fig. 6B, in CHO aIIB3wt cells plated on fragment D, the amount of precipitated active RhoA was low as compared with cells plated on intact fibrinogen. More importantly, in CHO aIIB3T562N cells, identical amounts of active RhoA were present on either intact fibrinogen or fragment D. On the other hand, Rac1 activity was very similar in CHO aIIB3wt cells attached to fragment D or spread on fibrinogen. These results demonstrate that complete RhoA activation requires an interaction of integrin aIIB3wt with the fibrinogen RGD motif, whereas Rac1 activity is independent of such an interaction and appears to rely solely on the aIIB3—β3 subunit recognition. And finally, as expected, the constitutively active mutant aIIB3T562N was able to circumvent the requirement for an aIIB3-RGD interaction and to trigger maximal RhoA activity on fragment D.

**Src, but Not PI3 Kinase, Is Involved in aIIB3-dependent Cell Spreading and RhoA Activation on Immobilized Fibrinogen**—An important event in aIIB3-dependent outside-in signaling is the selective and dynamic recruitment of signaling molecules that ultimately lead to Rho activation and cell spreading. Among these, phosphoinositide 3-kinase (PI3K) (34) as well as tyrosine kinases have been shown to be directly involved (35). Specific tyrosine kinases such as members of the Src family are of particular interest, because they function in proximity of aIIB3 by directly interacting with the cytoplasmic tail of the β3 subunit (36). Here we have used the pharmacological inhibitors wortmannin and PP2 to investigate the potential involvement of PI3K or Src in the aIIB3 signaling cascade leading to RhoA activation. As shown in Fig. 7A, aIIB3-dependent cell spreading, but not cell attachment, was completely blocked by the Src inhibitor PP2, and not by PP3 used as a negative control for PP2. Interestingly, the PI3K inhibitor wortmannin had no inhibitory effect. This result correlates with the strongly decreased generation of active RhoA in the cells treated with PP2, whereas RhoA activity was similar in the presence or absence of PP3 or wortmannin (Fig. 7B and C). Surprisingly however, when CHO aIIB3T562N cells were tested, complete cell spreading and RhoA activation occurred despite the presence of PP2. These results demonstrate the important role of Src in the signaling cascade downstream of the RGD-β3 interaction and necessary for RhoA activation. However, they also show that the constitutively active aIIB3T562N receptor can initiate cell spreading independent of active Src.

**Platelets Are Able to Spread on Fibrinogen Fragment D following Activation by Mn2**—Finally, to further assess our concept (a) that the aIIB3-fibrinogen interaction is a two-step mechanism and (b) that a physical interaction of the RGD motif with the β3 subunit, required for RhoA activation and actin stress fiber organization, can be substituted by Mn2+ activation of platelet aIIB3, we analyzed attachment and spreading as well as actin polymerization in resting and Mn2+-activated platelets when plated on either intact fibrinogen or fragment D. As expected, when plated on fragment D, resting platelets attached but failed to spread at early time points, whereas on native fibrinogen plate-
let spreading was rapid and almost complete after 5 min with platelets exhibiting a complete reorganized actin cytoskeleton (Fig. 8, A and B). At a later time point, however, platelet spreading on fragment D did also occur due to the release of α-granular fibrinogen and dense granule ADP, which has been shown to stimulate phosphatidylinositol 3-kinase independent of αIIbβ3 engagement (37, 38). When Mn²⁺ stimulated platelets were tested, spreading on both native fibrinogen and fragment D occurred at early time points, and this result was also obtained when platelets were treated with the αIIbβ3-activating mAb D3GP3 (data not shown). Fig. 8C shows that platelets stimulated with either Mn²⁺ or D3GP3 bound the fibrinogen-mimetic mAb PAC-1, in contrast to non-stimulated platelets, thus confirming the high affinity state of platelet integrin αIIbβ3 in stimulated platelets. All together, these results, which are in perfect agreement with our data obtained with αIIbβ3-expressing CHO cells, are also in accordance with the previously published data for the role of RhoA in αIIbβ3-dependent signaling following platelet spreading on native fibrinogen (33).

DISCUSSION

We have used intact fibrinogen and well characterized proteolytic fragments of fibrinogen containing either the α400–411 site (fragment D) or the α572–575 RGDS site (fragment C) as ligands and platelets as well as CHO cells expressing resting wild type (αIIbβ3wt), constitutively active (αIIbβ3T562N), or non-functional (αIIbβ3D119Y) receptors, to dissect the molecular events leading to αIIbβ3-dependent cell spreading and to further determine the functional role of each of the two fibrinogen binding sites during the adhesive process. Our major findings are as follows: the initial stage of cell attachment appears to rely essentially on the fibrinogen α400–411 sequence interacting with the αIIb integrin subunit, because fibrinogen fragment D, devoid of the RGD recognition sites, is able to support αIIbβ3-dependent cell attachment. This α400–411-αIIb interaction generates preliminary signaling events, which
lead to clustering of αIIbβ3 in early focal complexes, FAK tyrosine phosphorylation, as well as Rac1 activation. In the absence of the RGD binding site, αIIbβ3-mediated cell adhesion is blocked at this cell attachment stage and cannot proceed further, suggesting the requirement for a second signaling event promoting cell spreading. This second signaling event is generated through the subsequent RGDS-β3 interaction, which relies on receptor activation necessary for fibrinogen RGD recognition. Finally, the observation that the constitutively active αIIbβ3T562N receptor, which spontaneously exposes a β3 conformation normally induced following RGD binding, is able to mediate complete cell spreading on fibrinogen fragment D, suggests that the premier functional role of the RGDS-αIIb3 interaction is to imprint a conformational change on the β3 integrin subunit, necessary to initiate second wave intracellular signaling events, such as maximal RhoA activation, required for cell spreading. Finally, we show that these second wave intracellular signaling events are Src-dependent but PI3 kinase-independent. The results reported here thus support a two-step adhesion mechanism responsible for αIIbβ3-dependent cell spreading on surface-bound fibrinogen and provide evidence for a sequential interaction of the γ<sup>400–411</sup> site and the Α<sub>572–575</sub> RGDS site with αIIbβ3 that act synergistically to promote cell spreading. Our results are in good agreement with the recently published crystal structure of αIIbβ3 (9), which clearly highlights two distinct contact sites for fibrinogen binding, the specificity determining loop of the β3 I domain that interacts with the RGD sequence, and the αIIb cap subdomain comprising four insertions in the β-propeller, which is necessary for macromolecular recognition of fibrinogen.

The role of the αIIb-fibrinogen γ<sup>400–411</sup> interaction in mediating αIIbβ3-dependent platelet adhesion to intact fibrinogen and to fragment D has been reported by several authors (16, 39). Here we show for the first time that CHO αIIbβ3wt cells attached to fibrinogen fragment D exhibit numerous short protrusions with discrete αIIbβ3 clusters at the tips of the protrusions and colocalization of major cytoskeletal, adaptor, and signaling proteins such as talin, vinculin, or paxillin. These β3 clusters, which also contain tyrosine-phosphorylated proteins, are similar to focal complexes previously described (30). Our data thus provide evidence that the initial αIIb-fibrinogen γ chain interaction induces intracellular signaling events, independent of RGD-mediated signaling. It has to be emphasized that these results could only be observed in a cell adhesion assay devoid of washing steps. Indeed, the rather loose and reversible cell attachment mediated through the αIIb-fibrinogen γ<sup>400–411</sup> interaction was disrupted in standard cell adhesion assays including washing steps (16).

Integrin αIIbβ3 is an allosteric receptor that can switch from a resting to an active ligand binding receptor (40). In platelets, resting αIIbβ3 interacts with immobilized fibrinogen (29, 41), whereas activation of αIIbβ3 is required for interaction with soluble fibrinogen (42, 43). We and others have previously shown that resting αIIbβ3 in platelets or CHO cells promotes cell adhesion to native fibrinogen and to fibrinogen fragment D, provided that the γ<sup>400–411</sup> sequence is available for interaction (16, 44). These data underscore the synergistic role of the γ<sup>400–411</sup> site in the activation process of integrin αIIbβ3, necessary for fibrinogen RGD recognition. Indeed, based on data showing that binding of a high affinity cyclic γ<sup>400–411</sup> site analog to integrin αIIbβ3 results
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in increased LIBS1 antibody binding to the β3 subunit (45), we conclude that the αIIb-γ400–411 interaction most likely induces in trans conformational changes within the β3 subunit, allowing its high affinity interaction with the fibrinogen RGD site. Furthermore, biochemical studies have suggested that the RGD binding site becomes more accessible upon receptor activation (46).

The most exciting result, however, concerns integrin αIIbβ3T562N-mediated cell spreading on fibrinogen fragment D, devoid of the RGD recognition site. Because it is commonly accepted that both the synergistic site and the RGD site are necessary for integrin-dependent anchorage of cells to RGD ligands, our results provide evidence that irreversible αIIbβ3-dependent cell spreading can be achieved in the absence of the RGD site, provided that αIIbβ3 is in the active state and has undergone an additional conformational change. As previously reported, introduction of the T562N mutation into the β3 subunit induces spontaneous PAC-1 binding as well as constitutive exposure of LIBS neoepitopes normally only exposed following RGD binding. By mediating CHO cell spreading on fragment D, the αIIbβ3T562N receptor circumvented the requirement for a β3-RGD interaction, suggesting that the conformational change introduced into the β3 subunit by the T562N mutation most probably mimicked the conformational change normally imprinted on β3 following RGDs binding, and required for the initiation of the second wave of signaling events responsible for stress fiber organization and focal adhesions formation.

By using platelets as a more physiological cell model relevant to hemostasis and thrombosis, we have been able to confirm the data observed with CHO transfectants. Indeed, resting platelets attached but did not spread onto fibrinogen fragment D, at an early time point, whereas antibody or Mn2+ activation of αIIbβ3 was sufficient to induce platelet spreading and actin reorganization. However, these results could only be observed at early time points, because the obvious platelet release of dense and α-granule constituents such as ADP and fibrinogen circumvented the signaling events initiated through αIIbβ3-fibrinogen fragment D interaction.

The CHO cell model used here thus provides an excellent tool to precisely dissect the signaling events that specifically occur during αIIbβ3-mediated cell attachment and cell spreading. Using FAK tyrosine phosphorylation assays as well as intracellular immunofluorescence labeling of phospho-FAK (data not shown), we provide evidence that FAK phosphorylation on residue Tyr-397 already occurred in the early focal complexes observed in αIIbβ3wt-expressing CHO cells attached to fragment D. These data indicate that integrin clustering is sufficient to mediate the recruitment of FAK, allowing phosphorylation of residue Tyr-397 in FAK by a transautophosphorylation mechanism, necessary for the recruitment of Src family tyrosine kinases (47, 48) and other signaling and cytoskeletal molecules. Full FAK Tyr-397 autophosphorylation was observed when cells were spread on intact fibrinogen. Thus, FAK tyrosine autophosphorylation correlated with integrin clustering, in agreement with our earlier results showing a strong correlation between the extent of mutant β3 clustering and the level of β3-triggered FAK tyrosine phosphorylation (21).

Integrin-dependent rearrangement of the cytoskeleton is highly influenced by the activity of the Rho family GTPases RhoA, Rac1, and Cdc42 (49). For example, RhoA promotes the formation of actin stress fibers and focal adhesions, whereas Rac1 promotes the formation of lamellipodia and membrane ruffling, and Cdc42 causes actin microspike formation and filopodia development. In contrast to Cdc42 and Rac1, the regulation of RhoA during cell adhesion is biphasic with a rapid and transient inhibition of RhoA activity upon integrin engagement, followed by a subsequent reactivation leading to the assembly of contractile actin-myosin filaments (stress fibers) associated with mature focal adhesions (24). Although the mechanisms linking integrins to the regulation of RhoA are still not fully understood, it has been shown that the extracellular domain of the integrin β subunits is critical in stimulating RhoA activity (50, 51). In human platelets, evidence has been provided that RhoA is not required for the adhesion of resting platelets (33) but plays an important role in regulating the stability of integrin αIIbβ3 adhesion contacts under high shear stress (52). Here we show for the first time that the RGD-β3 interaction functions as a molecular switch and triggers maximal RhoA activation, most likely by imprinting a conformational change on the β3 integrin subunit, whereas the αIIb-γ400–411 interaction mainly induces Rac1 activation. Our results further demonstrate that the signaling cascade initiated through the RGD-β3 subunit interaction and leading to RhoA activation and cell spreading is dependent on active Src, but not PI3K, suggesting an important role of Src in the downstream activation of RhoA.

These results are in line with the recent data reported by the group of Shattil, showing that Src activation occurs through its direct binding to the C-terminal part of the cytoplasmic tail of the β3 subunit of αIIbβ3 and thus becomes a major regulator of integrin-dependent outside-in signaling (36, 53, 54). Because Src is also a known regulator of the downstream guanine nucleotide exchange factors (GEFs) of Rho, such as Vav GEFs that regulate RhoA (55), Src signaling appears to link integrins to RhoA, as shown here with the Src inhibitor PP2, which strongly reduced the generation of active RhoA in CHO αIIbβ3 cells seeded on intact fibrinogen.

Finally, based on our data, we suggest that the two-step mechanism described here for αIIbβ3-mediated cell adhesion to fibrinogen could serve as a general model applicable to RGD-type integrins that rely on a synergistic site for full receptor function: one apparent function of the synergistic site would be to allow RGD-type integrins to bind a ligand preferentially over other RGD-containing proteins (56–58) and to mediate ligand-specific attachment resulting in the activation of the receptor necessary for subsequent RGD recognition. The major functional role of the RGD site would be to initiate additional outside-in signaling events necessary for full cell spreading.

Acknowledgments—We thank Dr. D. R. Phillips for the generous gift of plasmin-generated fibrinogen fragments; Drs. J. Gonzalez-Rodríguez, M. Horton, M. Hoylaerts, L. Jennings, C. Kaplan, T. J. Kunicki, G. Matsueda, C. G. Ruun, and Z. Ruggeri for monoclonal antibodies; Drs. J. G. Collard and M. A. Schwartz for providing the GST-PAR1B PBD and GST-Rho-kinase RBD plasmid constructs; Dr. F. Lanza for the pTG2328-B3D119Y plasmid; and Dr. S. Reigner for the αvβ3 integrin R065-5233/001. We are indebted to Dr. M. Aumaillé and G. Chometon for their precious help with Rho family GTPase activity assays. We are also grateful to the staff of the clinical laboratory of the Centre Hospitalier of Luxembourg for their help in collecting fresh blood samples for platelet isolation.

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A New Functional Role of the Fibrinogen RGD Motif as the Molecular Switch That Selectively Triggers Integrin αIIbβ3-dependent RhoA Activation during Cell Spreading

Alexandre Salsmann, Elisabeth Schaffner-Reckinger, Fabrice Kabile, Sébastien Plançon and Nelly Kieffer

J. Biol. Chem. 2005, 280:33610-33619. doi: 10.1074/jbc.M500146200 originally published online June 13, 2005

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