The tetraspanin superfamily member CD151 regulates outside-in integrin $\alpha_{IIb}\beta_3$ signaling and platelet function

Lai-Man Lau, Janet L. Wee, Mark D. Wright, Gregory W. Moseley, P. Mark Hogarth, Leonie K. Ashman, and Denise E. Jackson

The tetraspanin family member CD151 forms complexes with integrins and regulates cell adhesion and migration. While CD151 is highly expressed in megakaryocytes and to a lesser extent in platelets, its physiologic role in platelets is unclear. In this study, we investigate the physical and functional importance of CD151 in murine platelets. Immunoprecipitation/Western blot studies reveal a constitutive physical association of CD151 with integrin $\alpha_{IIb}\beta_3$ complex under strong detergent conditions. Using CD151-deficient mice, we show that the platelets have impaired “outside-in” integrin $\alpha_{IIb}\beta_3$ signaling with defective platelet aggregation responses to protease-activated receptor 4 (PAR-4) agonist peptide, collagen, and adenosine diphosphate (ADP); impaired platelet spreading on fibrinogen; and delayed kinetics of clot retraction in vitro. This functional integrin $\alpha_{IIb}\beta_3$ defect could not be attributed to altered expression of integrin $\alpha_{IIb}\beta_3$, CD151 deficient platelets displayed normal platelet alpha granule secretion, dense granule secretion, and static platelet adhesion. In addition, CD151-$^{-/-}$ platelets displayed normal “inside-out” integrin $\alpha_{IIb}\beta_3$ signaling properties as demonstrated by normal agonist-induced binding of soluble fluorescein isothiocyanate (FITC)-fibrinogen, JON/A antibody binding, and increases in cytosolic-free calcium and inositol 1,4,5 triphosphate (IP$_3$) levels. This study provides the first direct evidence that CD151 is essential for normal platelet function and that disruption of CD151 induces a moderate outside-in integrin $\alpha_{IIb}\beta_3$ signal defect.

© 2004 by The American Society of Hematology

Introduction

Integrin $\alpha_{IIb}\beta_3$ maintains an inactive conformation on the surface of resting platelets until it is converted to its high-affinity state by agonist induced “inside-out” signaling via G protein–coupled or tyrosine kinase–linked pathways. Once activated, integrin $\alpha_{IIb}\beta_3$ can bind its soluble ligands, fibrinogen, and von Willebrand factor (VWF). Subsequently, “outside-in” integrin $\alpha_{IIb}\beta_3$ signaling events induce integrin clustering that leads to cytoskeletal reorganization and postoccupancy events including clot retraction, platelet spreading, and platelet aggregation. The importance of integrin $\alpha_{IIb}\beta_3$ has been documented in Glanzmann thrombasthenia (GT) patients, who have bleeding complications despite a normal number of platelets. GT patients have dysfunctional platelets, which display quantitative or qualitative defects in integrin $\alpha_{IIb}$ or $\beta_3$. This gives rise to an integrin $\alpha_{IIb}\beta_3$-mediated platelet defect, where the platelets fail to aggregate, bind fibrinogen, or retract fibrin clots. This phenotype has been recapitulated in a complete $\beta_3$ knock-out (KO) mouse model, which shows the cardinal features of Glanzmann thrombasthenia.

The concept that transmembrane receptors including integrins can form multimolecular complexes with tetraspanin-4 superfamily members (TM4SF) was first demonstrated by the ability of integrin $\alpha_{IIb}\beta_3$ to associate with a tetraspanin superfamily member, CD9, in stimulated platelets. Subsequently, biochemical associations between tetraspanin superfamily members and other transmembrane receptors have been reported and clarified based upon associations in the presence of dextrans with differing stringencies.

The tetraspanin superfamily member CD151 (also known as platelet endothelial tetraspan antigen-3/PETA-3) is expressed on the surface of platelets, megakaryocytes, endothelial cells, activated T lymphocytes, Schwann cells, muscle cells, and epithelial cells. CD151 is found to be constitutively associated with a wide range of integrins including $\beta_3$ from immunoprecipitation studies using hematopoietic cell lines. It appears to have strong physical associations with integrins $\alpha_6$, $\alpha_6$, and $\alpha_7$ chains, and $\alpha_5\beta_3$ complexes. Biochemical studies have revealed that the CD151 associations with $\alpha_5$ and $\alpha_6$ integrins involve an integrin contact site, QRDHASNYKVE$^{204-205}$, located in the EC2 loop of CD151. Mutation of this integrin contact site resulted in loss of integrin association and disrupted integrin-dependent cell spreading.

CD151 monoclonal antibodies (mAbs) have proved to inhibit endothelial cell migration and modify angiogenesis in vivo. In addition, the CD151 protein was found to be involved in integrin-dependent neurite outgrowth, keratinocyte migration, neutrophil migration, and tumor metastasis. This implies that CD151 participates in transmembrane signaling pathways normally initiated by integrin ligation.

Like other members of the tetraspanin family, CD151 forms highly specific direct lateral interactions with other tetraspanin family members and transmembrane receptors including $\alpha_5$, $\alpha_6$, $\alpha_7$.
and αmβ3 integrins. Several studies have suggested that CD151 may not modulate integrin-dependent cell adhesion, but markedly affects integrin-dependent cell spreading and morphogenesis when cells are grown on a Matrigel basement membrane.\textsuperscript{17,18} These studies indicate that CD151 is capable of modulating the signaling properties of integrins. The role of CD151 in platelet function is unclear. Previous studies have implied a strong dependence between CD151 and Fcy receptor IIa (FcyRIIa) in platelet activation using Mab 14A2.H1, antihuman CD151 antibody.\textsuperscript{19} Mab 14A2.H1 was shown to recognize a restricted repertoire of CD151 excluding certain integrin complexes. More recent studies in human platelets have reported that CD151 functions independent of platelet FcyRIIa.\textsuperscript{21} Apart from CD151, platelets express several tetraspanins including CD9, CD63, and TSSC6.\textsuperscript{22-25} Of these, CD9 has probably been the only one whose platelet function has been examined in detail, however, the results are not conclusive. Several reports indicate that CD9 is functionally linked with the low-affinity immunoglobulin G (IgG) receptor, FcyRIIa, in human platelets,\textsuperscript{22,24} while others suggest that CD9 may regulate platelet function independent of FcyRIIa.\textsuperscript{25}

In light of new evidence indicating that CD151 is independent of platelet FcyRIIa, we hypothesized that CD151 may play a role in regulating integrin αmβ3-mediated platelet function. In order to address this hypothesis, we studied the platelet biology of mice that are genetically deficient in CD151. Since murine platelets are devoid of the low-affinity IgG receptor, FcyRIIa, this provided an ideal system to define the functional role of CD151 in platelets. We aimed to determine if CD151 was both physically and functionally associated with the major platelet integrin αmβ3 in murine platelets. We dissected the functional importance of CD151 by examining platelet responses dependent upon integrin activation and postligand-binding events. These studies provide the first evidence that integrin αmβ3 and CD151 are physically and functionally associated in murine platelets. More importantly, it demonstrates that CD151 plays an essential role in platelet function by modulating the outside-in signaling properties of the major platelet integrin αmβ3.

### Materials and methods

#### Antibodies

The following antibodies (Abs) were used in this study. Antihuman CD151 mAb 11B1.G4 has been previously described for detection of murine CD151\textsuperscript{26}; antihuman integrin αm mAb, antihuman integrin β3 mAb, fluorescein isothiocyanate (FITC)-conjugated antihuman P-selectin mAb, and FITC-conjugated antihuman CD3 mAb were purchased from BD Pharmingen (San Diego, CA). CD4 (murine IgG2a) was used as an isotype control for 11B1, and all other antibodies are of rat origin. FITC-conjugated antihuman CD44 mAb was obtained from Beckman Coulter (Brea, CA). JON/A–phycocerythrin (PE) mAb was purchased from Cemfret Analytics (Wurzburg, Germany). Normal mouse IgG\textsubscript{1} was obtained from Sigma Chemical (St Louis, MO). All human and mouse work was approved by the Austin Campus Human Research Ethics Committee and Animal Ethics Committee.

#### Mice

The construction of CD151-deficient mice on a C57BL/6 background has been recently described.\textsuperscript{27} These mice were housed in a pathogen-free facility at the Austin Research Institute Animal House Facility.

#### Preparation of washed platelets and PRP

Blood (700 μL) was taken into 100-μL 3.8% (wt/vol) trisodium citrate by cardiac puncture under anesthesia. Platelet-rich plasma (PRP) was obtained by centrifugation at 700g for 10 minutes at room temperature (RT). PRP was pooled from 2 to 3 mice for preparing platelet samples. In order to prepare washed platelets, PRP was centrifuged in the presence of protaglandin E\textsubscript{1} (PGE\textsubscript{1}, 50 ng/mL; Sigma Chemical) at 1500g for 10 minutes at room temperature without brake. Sedimented platelets were resuspended in Ringer citrate dextrose (RCD) buffer, pH 7.4 (106 mM NaCl, 38 mM KCl, 1.7 mM NaHCO\textsubscript{3}, 21.2 mM sodium citrate, 27.8 mM glucose, and 1.1 mM MgCl\textsubscript{2} · 6 H\textsubscript{2}O, with pH adjusted to 7.4), counted, and diluted to 150 × 10\textsuperscript{9}/L.

#### Platelet aggregation studies

PRP derived from 2 to 3 wild-type C57BL/6 and CD151+/− mice was diluted in 53 μM Na\textsubscript{3}HPO\textsubscript{4}–12 μM KH\textsubscript{2}PO\textsubscript{4} buffer to adjust platelet count to 100 × 10\textsuperscript{9}/L. Then, 250 μL diluted wild-type or CD151+/− PRP together with 100 μg/mL fibrinogen and 1 mM CaCl\textsubscript{2} were placed into glass aggregometer cuvettes in the platelet aggregometer (Chronolog; Edward Keller, Hallam, Australia) with constant stirring (1000 rpm) at 37°C. Diluted platelet-poor plasma (PPP) 1:2 from wild-type and CD151+/− mice was used to set baseline for platelet aggregation runs. Once a baseline was obtained, different concentrations of various agonists (5 U/mL thrombin, 500 μM protease-activated receptor 4 (PAR-4) agonist peptide, 20 μg/mL collagen, 20 μg/mL Ca ionophore A23187, and 10 μM adenosine diphosphate (ADP); Sigma) were used to initiate platelet aggregation profiles with both wild-type and CD151+/− PRP. For each platelet aggregation run, the slope and amplitude were determined.

#### Clot retraction assay

Clot retraction was essentially performed as previously described.\textsuperscript{28}

#### Static platelet adhesion assays

Static platelet adhesion assays were performed using a modified method of Yuan et al.\textsuperscript{29} Whole mouse blood from wild-type and CD151+/− mice was labeled with 50 μg/mL 3,3’-diethyloxacarbocyanine iodide (DiOC\textsubscript{6}) fluorescent dye in dimethyl sulphoxide (Molecular Probes, Eugene, OR) for 10 minutes at RT in the presence of PGE\textsubscript{1} (50 ng/mL). Washed labeled platelets (100 × 10\textsuperscript{9}/L) were isolated and then incubated with the desired extracellular matrix over time (0-60 minutes) at 37°C to allow platelet adhesion and spreading to occur. Unbound platelets were washed away and adherent platelets were fixed with 3.7% (vol/vol) paraformaldehyde for 10 minutes at RT and viewed at × 100 magnification using a Leitz DMRB2E fluorescence microscope (Leica, Hawthorn East, Australia). Images were captured using a Leica DC200 digital camera.

#### Platelet spreading on fibrinogen

Platelets (100 × 10\textsuperscript{9}/L) were added to 100 μg/mL fibrinogen-coated coverslips and incubated over time (0-60 minutes) at 37°C. Nonadherent platelets were removed and adherent platelets fixed with 3.7% (vol/vol) paraformaldehyde (ICN Biochemicals, Aurora, OH) for 30 minutes at RT, then permeabilized with 0.1% (vol/vol) Triton X-100 (Sigma) for 30 minutes at RT. Platelets were stained with tetramethylrhodamine isothiocyanate (TRITC)–phalloidin (2 μg/mL; Sigma) for 30 minutes at RT and viewed at × 100 magnification using a Leitz DMRB2E fluorescent microscope (Leica). Images were captured using a Leica DC200 digital camera.

For scanning electron microscopy (EM) analysis, the adherent platelets were fixed with 2.5% (vol/vol) glutaraldehyde (Sigma) in 0.1 M sodium cacodylate buffer, pH 7.3 (Sigma) containing 2% (wt/vol) sucrose for 15 minutes at RT. The fixed platelets were washed and then processed for dehydration, gold labeling, and capturing of scanning EM images using a Philips 515 scanning electron microscope (5 kV; Philips, Andover, MA).

#### Integrin β3 expression

For surface expression of integrin β3, 100 μL diluted PRP (platelet count adjusted to 150 × 10\textsuperscript{9}/L) from wild-type and CD151+/− mice was suspended in 500 μL RCD buffer, pH 6.5, and then labeled with 50 μg/mL FITC-conjugated antihuman β3 antibody (BD Pharmingen) for 30 minutes at RT in the dark.
Platelets were fixed in fluorescence-activated cell sorter (FACS) Fix (16 g/L glucose, 40% [vol/vol] formaldehyde, and 0.1% [wt/vol] azide prepared in phosphate-buffered saline, pH 7.4) for 30 minutes at RT in the dark before samples were washed twice at 2000 g for 5 minutes at RT. Platelet pellets were resuspended in 0.5 mL RCD buffer, pH 6.5, containing 0.2% (wt/vol) bovine serum albumin (BSA). Flow cytometry (FACS) analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, North Ryde, Sydney, Australia) formatted for a platelet-specific FITC protocol with forward scatter (FSC), side scatter (SSC), and fluorescent parameters in log scale. Analysis of flow profile was performed using CellQuest Version 1.2.2 software (Becton Dickinson, San Jose, CA). The main platelet population was selected and gated before acquisition of data.

FITC-fibrinogen and JON/A-PE mAb binding
FITC-fibrinogen labeling and binding assays were performed essentially as described.30 JON/A-PE mAb (recognizes the active conformation of murine integrin αIIbβ3; 1:50 dilution) binding was analyzed by flow cytometry.

Platelet alpha granule secretion monitored by P-selectin exposure
Washed platelets (50 μL, 150 × 10^9/L) derived from wild-type and CD151−/− mice were labeled with 10 μg/mL FITC-conjugated antibody to P-selectin (BD Pharmingen) and stimulated with human thrombin (0-10 U/mL) and PAR-4 agonist peptide (0-500 μM) for 5 minutes at RT and platelet pellet was resuspended in 0.5 mL RCD buffer, pH 6.5, containing 0.2% (wt/vol) BSA prior to analysis by flow cytometry.

Platelet dense granule secretion monitored by serotonin release
Dense granule secretion was performed essentially as described.31

Calcium mobilization and inositol 1,4,5 triphosphate (IP3) measurement
Calcium mobilization and IP3 levels were performed essentially as described.32

Immunoprecipitation and Western blot studies
Immunoprecipitation and Western blot studies were performed essentially as described.21 Chemical cross-linking with 3,3′-dithiobis(sulphosuccinimidyl propionate) (DTSSP) was essentially as described.31

Statistics
The statistical significance of differences between means was evaluated using Student t test for paired samples, and P values of less than .05 (*) and .005 (**) were considered significant. Results are expressed as mean ± standard error of the mean (SEM).

Results
Physical association of integrin αIIbβ3 complex with CD151 in human and murine platelets
In order to examine a potential physical association of integrin αIIbβ3 complex with CD151 in human and murine platelets, immunoprecipitation studies were performed using antibodies directed against isotype control mAb, integrin αIIbβ3 mAb, platelet endothelial cell adhesion molecule 1 (PECAM-1) mAb, and CD151 (11B1 mAb) in the presence of either 1% Triton X-100 or with 1% Brij-96, 1% Thesit, and 1% CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propane-sulfonate). The only exception was with murine platelets, where chemical cross-linking with DTSSP was performed. Following immunoprecipitation, protein complexes were resolved on sodium dodecyl sulfate–polyacrylamidigel electrophoresis (SDS-PAGE) under nonreducing conditions, and the presence of CD151 was detected by Western blotting. As shown in Figure 1A, glycosylated (30 kDa) and predominantly nonglycosylated (28 kDa) forms of CD151 were detectable only in human integrin αIIbβ3 (P2) and anti-human CD151 immunoprecipitates. As shown in Figure 1B, unglycosylated CD151 was detectable in murine integrin αIIbβ3 immunoprecipitates and in murine CD9 and CD151 immunoprecipitates, but not in the isotype control or PECAM-1 immunoprecipitates. This CD151–integrin αIIbβ3 association was stabilized by cross-linking with DTSSP in the presence of 1% (vol/vol) Triton X-100. As a positive marker of CD151, 11B1 immunoprecipitates of resting murine and human platelets were included (Figure 1B, right panel). These results are consistent with a type I direct interaction.

CD151 knock-out platelets have abnormalities in integrin αIIbβ3-mediated function
The initial characterization of the CD151 knock-out mice revealed that the mice are healthy and viable with normal Mendelian inheritance. These mice show normal development of hematopoiesis, including platelet production and a moderate in vivo bleeding defect associated with a tendency to rebleed.27 Based upon this in vivo bleeding defect, the CD151 knock-out mice have either an underlying endothelial or platelet defect contributing to the unstable hemostasis. In order to investigate the possibility that CD151 knock-out platelets have a functional platelet abnormality, we tested the kinetics of fibrin clot retraction of wild-type versus CD151−/− platelets. Clot retraction is an essential part of platelet thrombus consolidation and is dependent upon an active integrin αIIbβ3 complex. In this assay, wild-type and CD151−/− PRP (normalized platelet counts) were initiated to form clots at 37°C by the addition of 2.5 U/mL thrombin. These clots were observed every 10 minutes over an hour period and digital images captured every 20 minutes. As shown in Figure 2A, a delay in the kinetics of clot retraction was observed in CD151 knock-out compared with wild-type platelets. Wild-type platelets started retracting at 20
In order to assess whether deletion of CD151 modulates integrin α<sub>m</sub>β<sub>3</sub>-mediated adhesive events, we examined the static platelet adhesion responses of DiOC<sub>6</sub> fluorescence labeled wild-type and CD151<sup>−/−</sup> platelets to immobilized extracellular matrix (ECM) proteins including fibrinogen (100 μg/mL), bovine VWF (20 μg/mL) in the presence of botrocetin (1 μg/mL), and type I fibrillar collagen (20 μg/mL) over time (0-60 minutes). Following washing, fluorescently labeled platelets bound to various matrices were examined and quantitated for evidence of adhesion by fluorescence microscopy. As shown in Figure 4, wild-type and CD151<sup>−/−</sup> platelets showed similar kinetics of adhesion to fibrinogen under static conditions. This pattern of static adhesion for CD151<sup>−/−</sup> platelets was observed for immobilized bovine VWF and type I fibrillar collagen. Taken together, these static platelet adhesion profiles suggest that the absence of CD151 does not affect the platelet adhesive properties involving inactive integrin α<sub>m</sub>β<sub>3</sub> binding to the immobilized ligands, fibrinogen, or VWF, and collagen receptors (integrin α<sub>m</sub>β<sub>3</sub> and glycoprotein VI [GPVI]) to the immobilized ligand, collagen.

Restricted cytoskeletal reorganization of CD151<sup>−/−</sup> platelets

Once adherent to fibrinogen, platelets become activated and undergo substantial cytoskeletal remodeling, leading to platelet shape change and spreading. Outside-in integrin α<sub>m</sub>β<sub>3</sub> signaling has been demonstrated to play a major role in this process. We investigated the role of CD151 in modulating the platelet spreading using 2 different experimental systems to monitor the spreading of wild-type and CD151<sup>−/−</sup> platelets on fibrinogen. Firstly, the spreading of platelets on fibrinogen over time was investigated by staining of the actin cytoskeleton using rhodamine-labeled phalloidin. Secondly, the formation of platelet filopodia and spreading on fibrinogen over time was monitored using scanning EM.

As shown in Figure 5A-B, wild-type mouse platelets were seen to form a wide network of filopodia on fibrinogen over time with

---

**Figure 2.** Delayed clot retraction in CD151-deficient platelets in the presence of normal integrin α<sub>m</sub>β<sub>3</sub> expression. (A) Photographs showing the kinetics of in vitro clot retraction using platelet-rich plasma (PRP) (normalized platelet counts) from wild-type and CD151<sup>−/−</sup> mice. Samples were treated with 10 nM thrombin. Red blood cells (5 μL) were added to enhance color contrast for photography. Each photograph is representative of 3 independent experiments. (B) Surface expression of integrin β<sub>3</sub> on platelets was determined by staining with a buffer control, isotype control FITC-CD3 mAb, positive control FITC-CD44 mAb, FITC-CD9, and FITC-integrin β<sub>3</sub> mAb for both wild-type and CD151<sup>−/−</sup> platelets. FITC-labeled samples were analyzed on a FACS Calibur analyzer. Results are representative of 3 independent experiments. The data represent the mean fluorescence intensity (MFI) ± SEM.

---

**Figure 3.** CD151<sup>−/−</sup> platelets show reduced platelet aggregation responses. Aggregation responses of PRP (platelet count adjusted to 100 x 10<sup>9</sup>/L) for wild-type and CD151<sup>−/−</sup> mice. (A) PAR-4 agonist peptide, 500 μM; (B) calcium ionophore A23187, 20 μg/mL; (C) collagen, 20 μg/mL; and (D) ADP, 10 μM. Data are representative of 4 experiments.
Figure 4. Static platelet adhesion to extracellular matrices. Anticoagulated whole blood from CD151+/+ and CD151−/− mice was labeled with DiOC6 (50 μg/mL) and washed platelets were isolated. Labeled platelets/mL (1 × 10^9 platelets/μL) were then allowed to adhere to collagen-coated (2.5 mg/mL), fibrinogen-coated (100 μg/mL), or bovine VWF-coated (20 μg/mL) coverslips in the presence of botrocetin (1 μg/mL) for 15 to 60 minutes at 37°C. Adherent platelets were fixed at different time points and visualized using fluorescence microscopy. Quantitation of adherent CD151+/+ and CD151−/− platelets was determined by analysis of images acquired using × 100 objective. These data represent the mean ± SEM from 3 independent experiments. HPF indicates high power field.

Figure 5. CD151−/− platelets have a defect in integrin-dependent cytoskeletal reorganization. (A) Washed platelets were placed on coverslips coated with fibrinogen in the presence of PGE2, for different time points at 37°C. Platelets were fixed, permeabilized, and stained for F-actin using rhodamine-conjugated phalloidin. Representative pictures of rhodamine-conjugated phalloidin-stained platelets spread on a fibrinogen surface for wild-type and CD151-deficient mice. The slides (panel A) were viewed under confocal microscopy using a Leitz DMRBE microscope (Leica, Hawthorn East, Victoria, Australia). Images were captured using a Leica DC200 digital camera. Magnification × 100. (B) Quantitation of platelet spreading per high-powered field was analyzed on confocal images from 8 random fields and expressed as a percentage of the total adherent platelets from CD151+/+ and CD151−/− platelets. *P < .005. (C) Washed CD151+/+ and CD151−/− platelets were allowed to adhere for 60 minutes at 37°C to a fibrinogen matrix. Adherent platelets were fixed and examined by scanning electron microscopy. Representative CD151+/+ and CD151−/− platelet spreading EM images are shown. (D) The number of filopodia per platelet were analyzed on scanning EM images from 8 random fields and expressed as a percentage of the total adherent platelets from CD151+/+ and CD151−/− platelets. *P < .005.

evidence of profound platelet spreading by 30 minutes. The majority of CD151−/− platelets underwent a far less pronounced change in morphology and maintained a more rounded morphology forming a smaller number of filopodia, and this was accompanied by at least a 10-fold reduction in spreading over time (91% versus 7% at time = 60 minutes, P < .005). Examination of platelet spreading on fibrinogen over time using scanning EM revealed a similar kinetic profile as observed in the rhodamine-labeled phalloidin staining. As shown in Figure 5C-D, CD151−/− platelets adhere as efficiently to fibrinogen as wild-type platelets, however, CD151−/− platelets exhibit a significant reduction in their capacity to extend filopodia. Specifically, CD151−/− platelets were characterized by a lack of filopodia, with fewer having in excess of 3 filopodia (P < .005; n = 4). These results indicate that CD151−/− platelets have restricted cytoskeletal reorganization when adhered to immobilized fibrinogen.

CD151−/− platelets show normal inside-out integrin αMβ3 signaling properties

As CD151−/− platelets have defects in cytoskeletal reorganization, clot retraction, and platelet aggregation, we wanted to test the possibility that platelet secretion may also be altered. In order to test this possibility, we examined the exposure of P-selectin and serotonin release as indicators of platelet alpha and dense granule secretion. As shown in Figure 6A-C, the surface expression of P-selectin on stimulated platelets and dense granule secretion derived from wild-type and CD151−/− platelets were equivalent over a range of thrombin (0-10 U/mL) and PAR-4 (0-500 μM) concentrations. Therefore, based upon these results, it would appear that CD151−/− platelets have normal alpha and dense granule secretion.

In the assessment of CD151−/− platelet aggregation responses, it was noticed that the primary phase of platelet aggregation (an event dependent on inside-out integrin αMβ3 signaling) was essentially intact, while the secondary phase of platelet aggregation (an event dependent on outside-in integrin αMβ3 signaling) was severely affected. Agonists that act through either G protein–coupled or nonreceptor tyrosine kinases are the main platelet activators used to assess the inside-out integrin αMβ3 signaling properties. For this purpose, we chose thrombin as a potent agonist of G protein–coupled receptors in murine platelets and phorbol 12-myristate 13-acetate ester (PMA), ADP, and ADP + epinephrine that have been previously shown to induce the binding of soluble FITC-fibrinogen by integrin αMβ3 on platelets. In order to test integrin αMβ3 activation on CD151−/− platelets, we examined the ability of wild-type and CD151−/− platelets to bind to soluble FITC-fibrinogen under resting conditions and following thrombin stimulation, PMA, ADP, and ADP + epinephrine. The binding of
soluble FITC-fibrinogen was assessed by flow cytometry. As shown in Figure 7A-F, agonist-induced stimulation of wild-type and CD151<sup>−/−</sup> platelets demonstrated equivalent ability to bind soluble FITC-fibrinogen, to bind JON/A mAb, and to increase cytosolic-free calcium and IP<sub>3</sub> levels of wild-type versus CD151<sup>−/−</sup> platelets. These results indicate that CD151<sup>−/−</sup> platelets display normal inside-out integrin α<sub>IIb</sub>β<sub>3</sub> signaling properties.

**Discussion**

This study provides the first report to demonstrate that the tetraspanin superfamily member CD151 is functionally as well as physically associated with the major platelet integrin α<sub>IIb</sub>β<sub>3</sub>. We demonstrate a physical association of integrin α<sub>IIb</sub>β<sub>3</sub> with CD151 in resting murine and human platelets. As no naturally occurring examples of CD151 deficiency are available in humans, the availability of the CD151 knock-out mouse model has provided us with a unique opportunity to study the functional importance of CD151 in platelet biology. Our studies demonstrate that CD151-deficient platelets have a defect in postoccupancy events of integrin α<sub>IIb</sub>β<sub>3</sub> as indicated by delayed kinetics in clot retraction, reduced platelet aggregation responses, and reduced platelet spreading on fibrinogen. Finally, CD151-deficient platelets showed normal levels of integrin α<sub>IIb</sub>β<sub>3</sub> expression, normal alpha and dense granule secretion, and normal ability to bind to soluble FITC-fibrinogen, to bind JON/A mAb, and to cytosolic-free calcium and IP<sub>3</sub> levels upon agonist stimulation. Taken together, these results support the identification of an outside-in integrin α<sub>IIb</sub>β<sub>3</sub> signaling defect in CD151-deficient platelets.
In this study, we show that the physical proximity of CD151 and integrin αmβ3 in platelets is likely to be of fundamental importance to mediate a close functional relationship between receptor signaling pathways. Tetraspanin protein interactions have been subclassified into 3 different levels of biochemical interaction based upon the stability of the physical interaction with detergents of varying stringencies. Based upon these criteria, our results support the concept that the physical association of integrin αmβ3 with CD151 in platelets is a first-level direct interaction. This interaction is resistant to disruption by strong hydrophobic detergents including Triton X-100.

Previous studies have focused on the role of CD151 in other cell types particularly involving transfected cell systems. A major focus of the CD151 studies has involved examining the integrin αmβ3, where it retains a stable association with approximately 90% of cellular αmβ3 integrins. CD151 would appear to be important in the modulation of extracellular matrix–driven cell migration, neurite outgrowth, and cell spreading but not αv or αm integrin–mediated cell adhesion. CD151 has been reported to exert an influence on αmβ3 integrin–dependent morphogenesis when fibroblasts and endothelial cells are grown on Matrigel. Tetraspanins are thought to modulate integrin-dependent signaling leading to alteration of cytoskeletal reorganization. Based upon these earlier studies, it has been proposed that tetraspanins are likely to influence the outside-in signaling properties of integrins. However, many of the molecular details remain elusive and have awaited studies in murine models where tetraspanin genes are deleted either singly or in combination.

Based upon this study, CD151 appears to be important in modulating the outside-in integrin αmβ3 signaling properties in platelets; it would suggest that CD151 participates in transmembrane signaling pathways. The N- and C-termini of CD151 are believed to be intracellular. These short amino acid sequences do not contain any enzymatic activity to confer autoposphorylation properties of their cytoplasmic regions. Therefore, it is likely that either phosphorylation or nonphosphorylation events may mediate the recruitment of signaling molecules or cytoskeletal proteins. At this stage, there is very little known about the signaling properties of CD151 and tetraspanin family members in general. Recent studies have suggested that CD151 can be communoprecipitated with protein kinase C (PKC) and phosphatidylinositol 4-kinase. These studies provide evidence that integrins αmβ3 and αmβ1 that are strongly associated with tetraspanins are also found in association with protein kinase C. In these PKC-TM4SF integrin complexes, integrin αm and αv tails are phosphorylated in a PKC-dependent manner. Whether these are direct or indirect interactions with either the N- or C-terminus of CD151 is currently not known, although it has been proposed that PKC may be involved in a direct interaction with TM4SF members. At present, there is no evidence that PKC-CD151 integrin complexes exist in platelets. Recent studies have highlighted that the C-terminal of CD151 (SLKLEHY) is important for modulating integrin αmβ3 adhesion strengthening by affecting integrin clustering. This finding is supported by the observation that the short C-terminal cytoplasmic domain of CD151 is required in αmβ3 integrin–mediated spreading, migration, and cellular cable formation on Matrigel.

One of interesting aspects of the CD151 KO platelet phenotype is that it does not correlate with features of the complete β3 knock-out mice, which display cardinal features of naturally occurring Glanzmann thrombasthenia platelets. Platelets derived from complete β3 knock-out mice have profound defects with absent platelet aggregation, inability to bind fibrinogen, absent fibrin-mediated clot retraction, and prolonged bleeding times. Despite having normal platelet production, these mice have a profound platelet defect that results in spontaneous bleeding complications predominantly involving cutaneous and gastrointestinal systems. The phenotype observed in integrin β3 knock-out mice is more severe than the CD151 KO phenotype reported in this study. The CD151 mice are viable and healthy without spontaneous bleeding complications. Indeed, our findings more closely resemble a mouse model that expresses integrin αmβ3, in which the tyrosines in the integrin cytoplasmic domain have been mutated to phenylalanine (Y747759F). Like the CD151 KO mice, these mice were found to have selectively impaired outside-in integrin αmβ3 signaling with normal integrin expression, but with defective platelet aggregation, delayed clot retraction responses in vitro, and an in vivo bleeding defect, which is characterized by a tendency to rebleed. One of the contrasting features in the diYF integrin mutant platelets is evidence of disaggregation in the secondary phase of platelet aggregation using G protein–coupled agonists, ADP, and thrombin.

No naturally occurring examples of the diYF integrin β3 mutant have been described. However, examination of the variant group of Glanzmann thrombasthenia patients has revealed several mutations in the β3 cytoplasmic domain that give rise to bidirectional integrin αmβ3 signaling defects. These include Ser752 → Pro β3 and Arg724 → Ter β3 mutations. Platelets from a thrombasthenic patient having a Ser752 → Pro mutation within the cytoplasmic domain of β3 fail to bind soluble fibrinogen after stimulation with either ADP or thrombin, indicating abnormal inside-out β3 signaling. Subsequent studies on the effects of this integrin β3 cytoplasmic domain mutation on cytoskeletal interactions and signal transduction have primarily been performed using transfected cells. The introduction of the Ser752 → Pro β3 mutant into Chinese hamster ovary (CHO) cells together with a constitutively active chimeric integrin α-subunit abolished ligand binding and reduced αmβ3-mediated cell spreading on immobilized fibrinogen, clot retraction, and focal adhesion. These studies highlight a bidirectional integrin αmβ3 signaling defect, indicating that integrin activation and postoccupancy events of integrin αmβ3 are affected in this Ser752 → Pro β3 mutant.

By examination of CD151-deficient platelets, we observed evidence of platelet aggregation responses with reduced amplitude and slope with a range of agonists. This feature is often associated with a defect in inside-out as well as outside-in integrin αmβ3 signaling. On occasion, we found evidence of disaggregation in the secondary phase of only PAR-4–induced platelet aggregation, but not ADP- or collagen-induced platelet aggregation (data not shown). Based upon repetition of numerous platelet aggregation assays, we have concluded that platelet disaggregation is not a general feature of CD151-deficient platelets. In fact, a recent report of an outside-in integrin αmβ3 signaling defect in calpain-deficient murine platelets has reported reduced platelet aggregation responses to thrombin, ADP, and collagen, but with no evidence of platelet disaggregation. We explored the possibility that CD151−/− platelets may have an underlying bidirectional integrin αmβ3 signaling defect. Based upon normal agonist-induced binding of soluble FITC-fibrinogen, JON/A binding, cytosolic-free calcium, and IP3 levels, it would appear that CD151−/− platelets do not have an inside-out integrin αmβ3 signaling defect. Taken together, these results support the notion that CD151 plays an important role in platelet function by acting as a modulator of outside-in integrin αmβ3 signaling in platelets.
In conclusion, in this study we have demonstrated a physical and functional association of the major platelet integrin αIIbβ3 with the tetraspdan family member CD151 in platelets. Furthermore, we show that absence of murine CD151 leads to an outside-in integrin signaling defect in platelets. Future studies will examine the signaling properties of CD151, the tetraspanin family member CD151 and integrin signaling pathways, and the role of CD151 in regulating platelet thrombus formation both in vitro and in vivo.

Acknowledgments

We would like to thank Dr YuPing Yuan for supplying the bovine VWF used in this study and for technical advice in platelet adhesion assays. We thank Dr Anna Friedhuber for performing the scanning electron microscopy analysis.

References

31. Chen YP, OToole TE, Ylanne J, Rosa JP, Glns- berg MH. A point mutation in the integrin αIIbβ3 cytoplasmic domain (Ser752→Phe) impairs bi-direc- tional signalling through αIIbβ3 (platelet glycoprotein IIb-IIIa). Blood. 1994;84:1867-1865.
The tetraspanin superfamily member CD151 regulates outside-in integrin $\alpha_{\text{IIb}}\beta_{3}$ signaling and platelet function

Lai-Man Lau, Janet L. Wee, Mark D. Wright, Gregory W. Moseley, P. Mark Hogarth, Leonie K. Ashman and Denise E. Jackson