Binding of galectin-1 to $\alpha_{IIb}\beta_3$ integrin triggers "outside-in" signals, stimulates platelet activation, and controls primary hemostasis

Maria A. Romaniuk,* Diego O. Croci,† Maria J. Lapponi,* Maria V. Tribulatti,‡ Soledad Negrotto,* Francoise Poirier,§ Oscar Campetella,‡ Gabriel A. Rabinovich,†,1,2 and Mirta Schattner*,1,2

*Laboratory of Experimental Thrombosis, Institute of Experimental Medicine (IMEX), National Academy of Medicine, †Laboratory of Immunopathology, Institute of Biology and Experimental Medicine (IBYME), and ‡Institute of Biotechnological Investigations-Institute of Technology of Chascomús-National University of San Martín, Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET), Buenos Aires, Argentina; and §Jacques Monod Institute, Unité Mixte de Recherche (UMR)–Centre National de la Recherche Scientifique (CNRS) 7592, Paris Diderot University, Paris, France

ABSTRACT **Understanding noncanonical mechanisms of platelet activation represents an important challenge for the identification of novel therapeutic targets in bleeding disorders, thrombosis, and cancer. We previously reported that galectin-1 (Gal-1), a β-galactoside-binding protein, triggers platelet activation *in vitro*. Here we investigated the molecular mechanisms underlying this function and the physiological relevance of endogenous Gal-1 in hemostasis. Mass spectrometry analysis, as well as studies using blocking antibodies against the anti-α_{IIb} subunit of$\alpha_{IIb}\beta_3$ integrin or platelets from patients with Glanzmann's thrombasthenia syndrome ($\alpha_{IIb}\beta_3$ deficiency), identified this integrin as a functional Gal-1 receptor in platelets. Binding of Gal-1 to platelets triggered the phosphorylation of β_3-integrin, Syk, MAPKs, PI3K, PLCγ2, thromboxane (TXA_2) release, and Ca^{2+} mobilization. Not only soluble but also immobilized Gal-1 promoted platelet activation. Gal-1-deficient (*Lgals1*$^{-/-}$) mice showed increased bleeding time ($P<0.0002$, knockout *vs.* wild type), which was not associated with an abnormal platelet count. *Lgals1*$^{-/-}$ platelets exhibited normal aggregation to PAR4, ADP, arachidonic acid, or collagen but abnormal ATP release at low collagen concentrations. Impaired spreading on fibrinogen and clot retraction with normal levels of $\alpha_{IIb}\beta_3$ was also observed in *Lgals1*$^{-/-}$ platelets, indicating a failure in the "outside-in" signaling through this integrin. This study identifies a noncanonical mechanism, based on galectin-integrin interactions, for regulating platelet activation.—Romaniuk, M. A., Croci, D. O., Lapponi, M. J., Tribulatti, M. V., Negrotto, S., Poirier, F., Campetella, O., Rabinovich, G. A., Schattner, M. Binding of galectin-1 to $\alpha_{IIb}\beta_3$ integrin triggers "outside-in" signals, stimulates platelet activation, and controls primary hemostasis. *FASEB J.* 26, 2788–2798 (2012). www.fasebj.org**

Key Words: inflammation · glycobiology

ALTHOUGH OVERLOOKED FOR many years, lectin-glycan interactions have recently emerged as key players in a variety of biological processes through fine-tuning the activation and signaling threshold of cognate receptors (1, 2). Galectins (Gals), a family of endogenous lectins highly expressed in inflammatory and tumor microenvironments, control diverse cellular processes, including adhesion, migration, proliferation, and apoptosis (3). These highly conserved glycan-binding proteins control cellular signaling through multivalent interactions with specific glycan structures present on cell surface glycoproteins (3). Although the carbohydrate recognition domain (CRD) sequence of Gals is highly conserved, each individual member of the family recognizes diverse glycan structures and shows different

Abbreviations: Ab, antibody; ACD, acid-citrate dextrose; ADP, adenosine diphosphate; BSA, bovine serum albumin; CRD, carbohydrate recognition domain; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; Gal, galectin; GPIb, glycoprotein Ib; GT, Glanzmann's thromboasthenia; HRP, horseradish peroxidase; KO: knockout; MAPK, mitogen-activated protein kinase; MALDI-TOF, matrix-assisted laser desorption/ionization–time of flight; PAR4-AP, protease-activated receptor 4–activating peptide; PBS, phosphate-buffered saline; PECAM, platelet endothelial cell adhesion molecule; PFA, paraformaldehyde; PLCγ2, phospholipase Cγ2; PRP, platelet-rich plasma; RT, room temperature; TXA_2, thromboxane A2; VWF, von Willebrand factor; WP, washed platelet; WT, wild type

[1] These authors contributed equally to this work.

[2] Correspondence: M.S., Instituto de Medicina Experimental (IMEX), CONICET/ANM, Pacheco de Melo 3081, Buenos Aires (1425), Argentina. E-mail: mschattner@hematologia.anm.edu.ar; G.A.R., Instituto de Biología y Medicina Experimental, CONICET, Vuelta de Obligado 2490, Buenos Aires (1428), Argentina. E-mail: gabriel.r@ibyme.conicet.gov.ar

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binding affinities. Gal-1, a prototype member of the family, plays key roles in innate and adaptive immune response, cell trafficking, and angiogenesis. This multifunctional lectin is expressed by cells of the hematopoietic lineage as well as in endothelial cells, and its expression is regulated in physiological processes, such as cellular activation and differentiation, and in pathological processes, such as atherosclerosis and cancer (4–6).

Platelets contribute to normal blood circulation through the preservation of vascular integrity and prevention of hemorrhage after injury, playing a critical role in hemostasis and thrombosis (7). Moreover, platelets are now widely recognized as important effectors in the modulation of inflammation, immune responses, wound healing, tissue repair, antimicrobial host defense, and angiogenesis. Their function also extends to pathological conditions, such as atherosclerosis, chronic ischemia, or cancer (8).

The immobilization of platelets at sites of vascular injury requires specific platelet-vessel wall (adhesion) and platelet-platelet interactions (aggregation). The aggregation of platelets is characterized by the accumulation of platelets into a hemostatic plug. The central platelet receptor in this process is the $\alpha_{IIb}\beta_3$-integrin linking activated platelets through fibrinogen bridges (7). As an integrin, $\alpha_{IIb}\beta_3$ has the ability to transmit bidirectional signaling *via* "inside-out" (agonist-induced activation of integrin) or "outside-in" (postligand-occupied integrin) signaling events. Outside-in signaling leads to irreversible platelet adhesion, cytoskeletal reorganization required for platelet spreading, clot retraction, microvesicle formation, platelet aggregation, and subsequent thrombus growth (9, 10).

We recently found that two structurally different Gals (Gal-1 and Gal-8) trigger platelet activation (11, 12), suggesting that these endogenous lectins might play key roles in hemostatic/thrombotic processes. Gal-1 not only triggers platelet activation but also promotes platelet-leukocyte mixed aggregates through the expression of P-selectin (11). These observations reinforced the notion of Gal-1 as a modulator of the inflammatory response. In this regard, Gal-1 has been proposed to be involved in the pathogenesis of cardiovascular diseases in particular atherosclerosis, suggesting a role in platelet-dependent inflammatory settings. Because Gal-1 is expressed in atherosclerotic lesions, influences processes that are important for plaque growth and stability (13–15), and triggers platelet activation (12), this lectin has been proposed to play a key role not only in the fate of the atherosclerotic plaque but also as a potent thrombogenic molecule that is exposed after plaque rupture. Yet, despite considerable progress, the mechanisms underlying Gal-1-induced modulation of platelet function as well as the physiological relevance of this modulatory effect *in vivo* remain uncertain.

To further understand the role of Gal-1 in platelet biology, here we aimed to identify the elusive receptor for this lectin to examine the signaling pathways underlying Gal-1-mediated platelet activation and to elucidate the role of endogenous Gal-1 in the hemostatic process. Our data show that integrin $\alpha_{IIb}\beta_3$ acts as a functional receptor for Gal-1 in platelets, and this interaction triggers outside-in signaling through the activation and phosphorylation of the β_3 subunit of this integrin and the tyrosine kinases Syk, Akt, p38, and extracellular signal-regulated kinase (ERK) and phospholipase Cγ2 (PLCγ2). In addition, we identified, using Gal-1-deficient (*Lgals1*$^{-/-}$) mice, an important role for endogenous Gal-1 in normal primary hemostasis.

MATERIALS AND METHODS

Reagents

Human α-thrombin was purchased from Enzyme Research Labs (Swansea, UK); luciferin-luciferase, ATP, fibrinogen, Fluo 3AM, and TRITC-phalloidin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Collagen was from Nycomed Pharma (Unterschleibheim, Germany). fluorescein isothiocyanate (FITC)-conjugated anti-human CD62P, CD41, CD61, CD42b, FITC-conjugated irrelevant IgG_1, FITC-conjugated anti-mouse CD62P, CD61, and anti-actin antibodies (Abs) were obtained from BD Biosciences (San Jose, CA, USA). protease-activated receptor 4–activating peptide (PAR4-AP; AYPGKF) was obtained from Genbiotech (Buenos Aires, Argentina). Mouse anti-β_3, rabbit anti-phospho-PLCγ2 (Tyr753), and phospho-β_3 (Tyr773) were purchased from Abcam (Cambridge, UK). Rabbit anti-phospho Syk (Tyr525/526), rabbit anti-Akt, rabbit anti-Syk, and rabbit anti-PLCγ2, were purchased from Cell Signaling (Danvers, MA, USA). Mouse anti-phospho ERK1/2 (Tyr 204), rabbit anti-phospho p38, rabbit anti-p38, rabbit anti-ERK, rabbit anti-phospho Akt (Tyr473), and horseradish peroxidase (HRP)-conjugated anti-rabbit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-human CD41 (clones Hip-2 and Hip-11) were obtained from Abbiotec (San Diego, CA, USA). Recombinant human Gal-1 and rabbit anti-human Gal-1 polyclonal Abs were produced and purified as described previously (11, 16, 17). Lipopolysaccharide content of the purified samples was tested using a gel-clot *Limulus* test.

Preparation of human platelets

Blood samples were obtained from healthy donors who had not taken nonsteroidal anti-inflammatory drugs 10 d before sampling. This study was performed according to institutional guidelines (National Academy of Medicine, Buenos Aires, Argentina) and received the approval of the Institutional Ethics Committee and written consent from all the subjects. Blood was drawn directly into plastic tubes containing acid-citrate dextrose (ACD; 6:1) or 3.8% sodium citrate for aggregation in platelet-rich plasma (PRP). PRP from normal donors or from patients with Glanzmann's thrombasthenia (GT) was obtained by centrifugation of the blood samples (180 *g* for 10 min). For washed platelet (WP) suspensions, PRP was centrifuged in the presence of prostacyclin (PGI_2; 75 nM), and after being washed in washing buffer, WPs were resuspended in Tyrode's buffer (3×10^8/ml). Platelet suspensions were kept at room temperature (RT) for 30 min before use, and unless otherwise stated Ca^{2+} (1 mM) was added before platelet stimulation.

Affinity chromatography and mass spectrometry

Human platelets were lysed with 1% Triton X-100 in the presence of a protease inhibitor cocktail and passed through a Gal-1 affinity column made by coupling purified human recombinant Gal-1 to *N*-hydroxy succinimide-activated Hi-Trap columns (GE Healthcare, Uppsala, Sweden). Gal-1 binders were eluted with 150 mM lactose in phosphate-buffered saline (PBS) and concentrated by ultrafiltration. After alkylation with iodoacetamide, samples were resuspended in cracking buffer and solved by 10% SDS-PAGE. After Coomasie blue staining, discrete bands were cut out, in-gel digested with trypsin, and subjected to peptide mass fingerprinting by the mass spectrometry service of the Pasteur Institute (Montevideo, Uruguay). Mass spectra of digestion mixtures were acquired in a 4800 matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF)/TOF instrument (Applied Biosystems, Foster City, CA, USA) in reflector mode and were externally calibrated using a mixture of peptide standards (Applied Biosystems). Collision-induced dissociation MS/MS experiments of selected peptides were performed. Proteins were identified by searching the U.S. National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) database NCBInr with peptide m/z values, using the MASCOT program and the following search parameters: monoisotopic mass tolerance, 0.08 Da; fragment mass tolerance, 0.2 Da; and methionine oxidation as possible modifications; and one missed tryptic cleavage allowed. Protein scores were derived from ion scores as a nonprobabilistic basis for ranking protein hits. Protein score is $-10*\mathrm{Log}(P)$, where P is the probability that the observed match was a random event. Protein scores >84 were considered significant ($P<0.05$).

Gal-1 binding

WPs were incubated for 15 min with FITC-conjugated Gal-1 and platelets were then fixed with 1% paraformaldehyde (PFA). In selected experiments, platelets were preincubated with anti-CD41 or anti-IgG1a Abs (50 μg/ml) for 20 min at RT. The binding of Gal-1 to the platelet surface was analyzed by flow cytometry (FACSCalibur flow cytometer; BD Biosciences, San Jose, CA, USA).

Platelet aggregation and ATP release

Aggregation and ATP release were measured in a lumiaggregometer (Chrono-Log, Havertown, PA, USA). ATP levels were measured at the end of the assay by addition of a known amount of standard ATP. Where indicated, platelets were preincubated with anti-CD41 or anti-IgG1a Abs (50 μg/ml) for 20 min at RT.

Phenotypic analysis of human platelets from controls and patients with GT and from mouse platelets

To evaluate P-selectin exposure, WPs were stimulated, fixed, and stained with a FITC-conjugated CD62P Ab (anti-P-selectin) in PBS/0.1% FBS solution or an equivalent amount of isotype FITC-IgG_1 as a negative control. To evaluate platelet surface expression of CD41, CD61, and CD42b, GT and control platelets were incubated with a FITC-conjugated CD41, CD61, or CD42b Ab. For murine platelet analysis, WPs were stimulated, fixed, and stained with a FITC-conjugated anti-CD62P Ab in PBS/0.1% FBS solution or an equivalent amount of isotype FITC-IgG_1 as a negative control. Expression of CD61 was assessed by incubating platelets with a FITC-conjugated anti-mouse CD61 Ab (BD Biosciences). After fixation, cells were analyzed by flow cytometry in a FACSAria (BD Biosciences).

Intracellular Ca^{2+} mobilization

Intracellular Ca^{2+} concentrations in fluo-3-loaded platelets were assessed under flow cytometry as described previously (12). In brief, platelets were labeled with 5 μM fluo-3-AM (Sigma) at 37°C for 15 min. After being washed, 3×10^6 platelets in 500 μl of Tyrode's buffer were subjected to flow cytometry analysis. After determination of baseline fluo-3 fluorescence for ~10 s, cell aspiration into the flow cytometer was briefly paused for agonist addition. The acquisition was then resumed, and changes in fluorescence *vs.* time were recorded. Results are expressed as percentage of positive cells and represent the events with FL1 values above the baseline.

Immunoblotting

Highly purified WP lysates were prepared by solubilizing aspirine-treated (0.5 mM) platelets (1×10^8) in loading buffer (62.5 mM Tris-HCl at pH 6.8, 25% glycerol, 2% SDS, 0.01% bromphenol blue, and 5% 2-mercaptoethanol). Equal amounts of proteins were electrophoresed on a 10% SDS-PAGE and electrotransferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). After blocking, the membranes were incubated overnight at 4°C with primary Abs followed by a HRP secondary Ab. Protein bands were visualized by using the enhanced chemiluminescence (ECL) reaction. Immunoblotting results were quantified using Gel-Pro Analyzer 3.1 software (Media Cybernetics, Inc., Bethesda, MD, USA), and the values were used for monitoring equal protein loads.

Platelet spreading

Glass slides were coated with recombinant Gal-1 (1 μM) or fibrinogen (100 μg/ml) and blocked with 2% bovine serum albumin (BSA) for 2 h. Then, human WPs or mouse PRP (5×10^7/ml) were plated and incubated for 20 min. Adhered platelets were fixed (4% PFA), permeabilized (0.1% Triton X-100), and stained with TRITC-Phalloidin. Images were analyzed using ImageJ [U.S. National Institutes of Health (NIH), Bethesda, MD, USA]. To test for β_3 integrin phosphorylation, after permeabilization, platelets were incubated with TRITC-phalloidin and anti-phospho β_3 Ab for 1 h, followed by incubation with FITC-conjugated anti-rabbit. After being mounted, spread platelets were visualized under confocal microscopy (Olympus FV-1000; Olympus, Tokyo, Japan).

Plate adhesion assay

A 96-well plate was coated with Gal-1 (1 μM), fibrinogen (100 μg/ml), or heat-inactivated BSA overnight at 4°C. The plate was blocked with heat-inactivated BSA, and aliquots of WPs (1×10^8/ml) were added to each well and incubated at RT for 60 min. After being washed, the substrate solution (0.1 M citrate buffer, pH 5.4, containing 5 mM *p*-nitrophenyl phosphate and 0.1% Triton X-100) was added and incubated for 1 h at RT. The reaction was stopped, and after the addition of 2 N NaOH, the absorbance at 405 nm was measured with a microplate reader (Dynatech MR 5000; Dynatech Laboratories).

Mouse studies

Gal-1-null (*Lgals1*$^{-/-}$) mice (C57BL/6) and their wild-type (WT) counterparts were bred at the animal facility of the

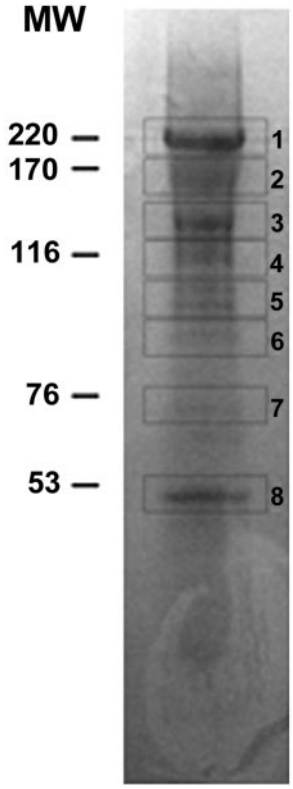

Figure 1. Gal-1-binding partners identified in human platelets by mass spectrometry. WPs were lysed and passed through a Gal-1 affinity column. Gal-1 binders were eluted in the presence of lactose and resolved in a 10% SDS-PAGE. Stained bands were cut out and subjected to MALDI-TOF mass spectrometry analysis. Table 1 details the proteins identified in each band. MW, molecular weight.

Institute of Biology and Experimental Medicine according to NIH-approved guidelines.

Tail bleeding assay

Tail bleeding assay was performed as described previously (18). Briefly, 8- to 12-wk-old mice were anesthetized by intraperitoneal injection of tribromoethanol (Avertin). After prewarming the tail for 5 min, the terminal 3 mm segment was amputated with a sharp sterile blade and immediately immersed into saline solution at 37°C. Tail bleeding time was defined as time required for bleeding to stop.

Mouse platelet preparation

Orbital blood from 8- to 12-wk-old Avertin-anesthetized mice was drawn from into plastic tubes containing 10% volume of 3.8% citrate. Whole blood was diluted 1:1 in Tyrode's buffer without calcium, and PRP was obtained by centrifugation (100 *g* for 5 min at RT). Platelet count was adjusted to 3×10^8/ml with platelet-poor plasma.

Clot retraction assay

Clot retraction was measured by mixing 200 μl of PRP from *Lgals1*$^{-/-}$ or WT mice with 350 μl of Tyrode's buffer, 10 μl of erythrocytes (to enhance clot contrast for photography), and 50 μl of thrombin (0.25 U/ml final concentration). A rod was placed in each glass test tube and incubated at RT for 1 h. Clot formation and subsequent clot retraction were recorded visually at various time intervals before photography.

Statistical analysis

Results are expressed as means ± SE. Student's paired *t* test was used to determine the significance of differences between the groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Gal-1 activates human platelets through binding to $\alpha_{IIb}\beta_3$ integrin

To identify potential candidate receptors for Gal-1 in platelets, we isolated binding partners by Gal-1-affinity chromatography and analyzed them by mass spectrometry. The proteins retained in the column were eluted with lactose and separated by SDS-PAGE resolution. Among all the bands detected (**Fig. 1** and **Table 1**), platelet endothelial cell adhesion molecule-1 (PECAM-1) and the subunits α_{IIb} and α_6 from $\alpha_{IIb}\beta_3$ and $\alpha_6\beta_1$ integrins, respectively, were found to be platelet surface membrane proteins, as identified by MALDI-TOF analysis. Since PECAM-1 activation and signaling are well known for the ability to dampen platelet function (19), PECAM-1 was disregarded as a potential functional receptor for Gal-1. Considering that $\alpha_{IIb}\beta_3$ is a major platelet protein involved in platelet activation (10), we hypothesized that this integrin could be an appropriate counterreceptor for this lectin.

The involvement of α_{IIb} integrin in Gal-1-induced platelet responses was initially investigated using a mixture of specific blocking mAbs (Hip-2 and Hip-11) directed against α_{IIb} (CD41) or control IgG before stimulation with Gal-1. This mAb mixture did not inhibit adenosine diphosphate (ADP)-induced (data not shown) or thrombin-induced platelet aggregation (**Fig. 2*B***), suggesting lack of interference with fibrinogen binding to the RGD domain of the $\alpha_{IIb}\beta_3$ integrin. In addition, these mAbs did not alter $\alpha_{IIb}\beta_3$-independent platelet responses, such as ATP release and P-selectin expression induced by thrombin (Fig. 2*C*, *D*). However, pretreatment of platelets with the mixture of mAbs decreased not only Gal-1 binding to the platelet

TABLE 1. *Proteins identified in each band in Fig. 1*

Band	Identified proteins
1	Thrombospondin-1, talin, filamin Aα
2	P-selectin
3	Integrin α2b, integrin α6
4	PECAM-1
5	Coagulation factor XIII
6	Glycoprotein V precursor
7	Fermitin family homolog 3
8	Tubulin β-1

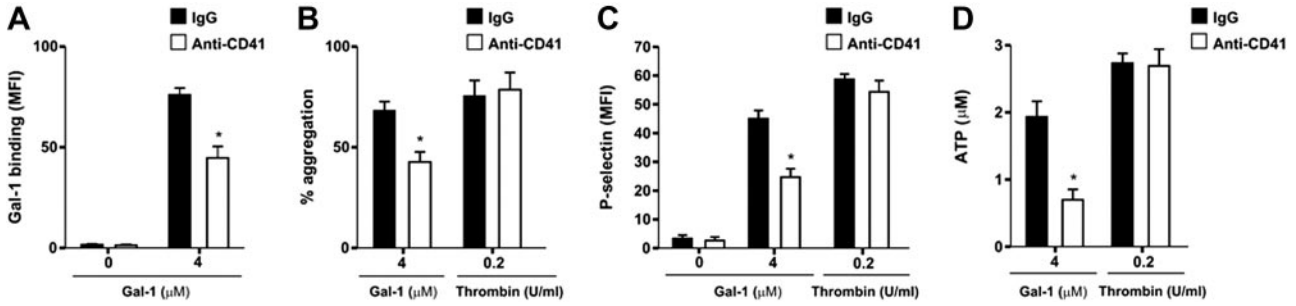

Figure 2. Prevention of Gal-1 binding and platelet activation by anti-$\alpha_{IIb}\beta_3$ integrin blocking mAb. WPs were incubated for 15 min with IgG or with a mixture of anti-CD41 mAbs (clones HIP-2 and HIP-11). *A*) Gal-1 binding to platelet surface was determined by flow cytometry using different concentrations of FITC-coupled Gal-1. *B*) Platelet aggregation was monitored using an aggregometer. *C*) Surface expression of P-selectin induced by Gal-1 or thrombin (Thr) was detected by flow cytometry. *D*) ATP release was measured using a lumiaggregometer. $*P < 0.05$ *vs.* IgG.

surface, but also aggregation and granule release triggered by Gal-1 (Fig. 2). The failure of mAbs to fully block Gal-1 binding and platelet activation could be associated with the fact that Gal-1-mediated platelet activation is dependent on carbohydrate recognition and these mAbs may recognize nonglycosylated sites in the α_{IIb} subunit. To further verify the role of $\alpha_{IIb}\beta_3$ as a potential Gal-1 receptor in platelets, we performed functional studies using platelets from 2 patients with GT (GT1 and GT2), who were deficient in $\alpha_{IIb}\beta_3$ integrin but had normal levels of glycoprotein Ib (GPIb), another major surface protein involved in platelet function (ref. 20 and **Fig. 3*A***). Binding of Gal-1 to GT platelets was substantially reduced compared with control samples, with the decrease in GT2 platelets being more pronounced than in GT1 platelets (Fig. 3*B*). Platelet aggregation and granule secretion are independent phenomena. Although thrombasthenic platelets fail to aggregate due to the absence of $\alpha_{IIb}\beta_3$ integrin, strong agonists, including thrombin and Ca^{2+} ionophores, can induce the release of their dense and α-granule content (21, 22). In agreement with these data, we found that thrombin failed to induce aggregation of platelets from patients GT1 and GT2 but efficiently promoted ATP release and P-selectin exposure (as a measure of dense and α-granule, respectively; Fig. 3*C–E*). Remarkably, Gal-1 did not trigger aggregation or granule secretion from GT platelets (Fig. 3*C–E*). Thus, prevention of Gal-1-induced platelet activation through blockade or absence of α_{IIb} subunit indicates that this integrin is not only a binding partner, but also a functional receptor for Gal-1 in platelets.

Signaling pathways activated on platelet stimulation with Gal-1

The observation that Gal-1 triggers platelet activation through direct binding to $\alpha_{IIb}\beta_3$ was unexpected, as in nonstimulated platelets $\alpha_{IIb}\beta_3$ integrin is present in a resting conformation, which has low affinity for its ligands (8, 24). The binding of fibrinogen and von Willebrand Factor (vWF), the two main $\alpha_{IIb}\beta_3$ ligands,

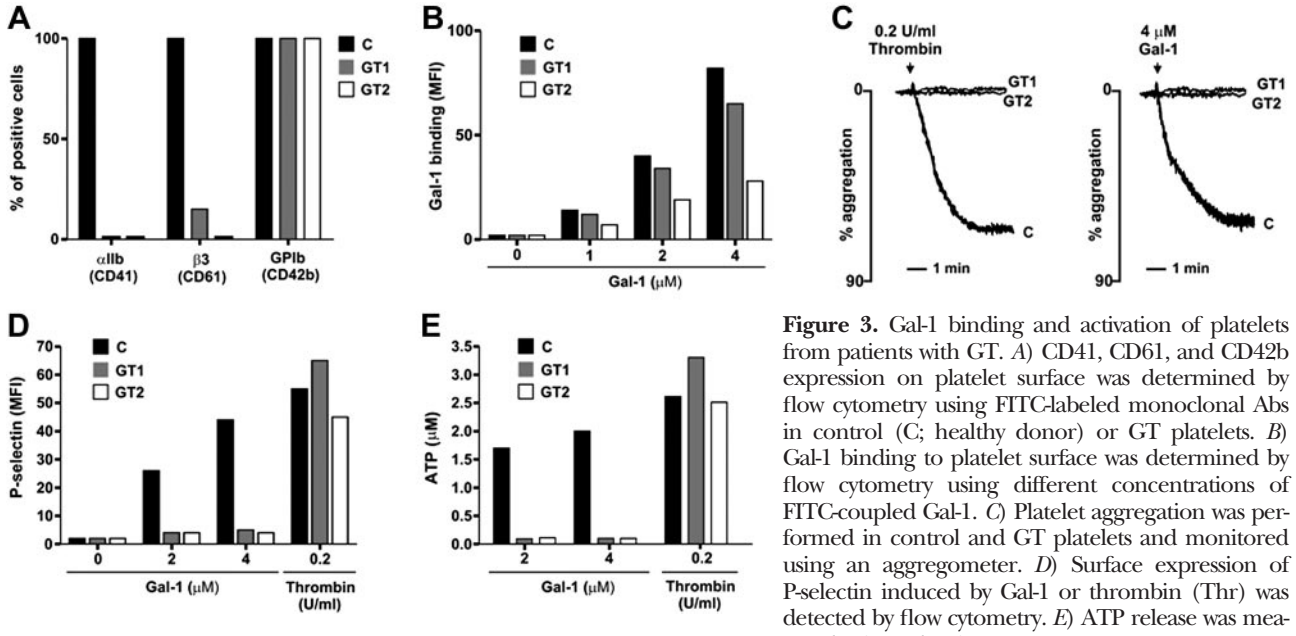

Figure 3. Gal-1 binding and activation of platelets from patients with GT. *A*) CD41, CD61, and CD42b expression on platelet surface was determined by flow cytometry using FITC-labeled monoclonal Abs in control (C; healthy donor) or GT platelets. *B*) Gal-1 binding to platelet surface was determined by flow cytometry using different concentrations of FITC-coupled Gal-1. *C*) Platelet aggregation was performed in control and GT platelets and monitored using an aggregometer. *D*) Surface expression of P-selectin induced by Gal-1 or thrombin (Thr) was detected by flow cytometry. *E*) ATP release was measured using a lumiaggregometer.

requires prior activation of the integrin through inside-out signals generated by interaction of classical platelet agonists with their specific receptors. Ligand-binding to $\alpha_{IIb}\beta_3$ integrin then activates intracellular pathways allowing cytoskeletal arrangements and eventually cell retraction in a process called outside-in signaling (7, 23). Because Gals in general have the ability to establish multivalent interactions with glycosylated cell surface receptors, we hypothesized that Gal-1 binding to platelet surface results in the clustering of $\alpha_{IIb}\beta_3$ molecules, a phenomenon known to trigger outside-in signaling (9, 23). In fact, stimulation of platelets by Gal-1 resulted in the activation of the major signaling pathways involved in the outside-in signaling, including phosphorylation of the β_3 cytoplasmic tail and activation of the tyrosine kinases Syk and Akt and the PLCγ2 (**Fig. 4*A*, *B***). Moreover, preincubation of platelets with blocking anti-CD41 mAbs (Hip-2 and Hip-11) inhibited protein phosphorylation induced by Gal-1, providing further evidence that integrin $\alpha_{IIb}\beta_3$ is the signaling receptor for Gal-1 in platelets (Fig. 4*C*).

The activation of PLCγ2 catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to release inositol trisphosphate and diacyglycerol, which activates Ca^{2+} mobilization and protein kinase C (PKC; refs. 23–25). We have previously demonstrated that the formation of microparticles and microaggregates induced by Gal-1 was inhibited by EDTA-chelation of extracellular Ca^{2+} (11). We further extended the role of this second messenger and showed that Ca^{2+} levels are also elevated on platelet stimulation with Gal-1 (Fig. 4*D*). Elevation of Ca^{2+} activates multiple signaling events, including thromboxane A2 (TXA_2) synthesis (23), which is also dependent on p38 activation (26). In accordance with the observed Ca^{2+} mobilization, platelet stimulation by Gal-1 resulted in the phosphorylation not only of p38 but also of other kinases, such as ERK1/2, in a time-dependent manner (Fig. 4*B*). In addition, Gal-1 favors formation of TXB_2, the stable analog of TXA_2 (Fig. 4*E*). These data strengthen the notion that Gal-1 activates platelets through activation of outside-in $\alpha_{IIb}\beta_3$ integrin-dependent signaling pathways.

Gal-1 induces platelet adhesion and spreading

Irreversible platelet adhesion and cytoskeletal reorganization required for platelet spreading are among the major biological outcomes of $\alpha_{IIb}\beta_3$ integrin outside-in signaling (9). To examine the ability of Gal-1 to trigger these cellular responses, platelets were allowed to spread over Gal-1-coated surfaces. Whereas platelets did not adhere to BSA-coated slides, they effectively attached, spread, and displayed lamellipodia formation when assayed on immobilized Gal-1-coated surfaces (**Fig. 5*A***). The number of adhered platelets and their area were similar to the area of platelets attached to fibrinogen-coated slides. However, Gal-1 attached to platelets displayed a differential actin pattern (Fig. 5*A*). Similar to suspensions of platelets stimulated by Gal-1, β_3 phosphorylation was observed in platelets spread on Gal-1-coated slides (Fig. 5*B*). These data not only

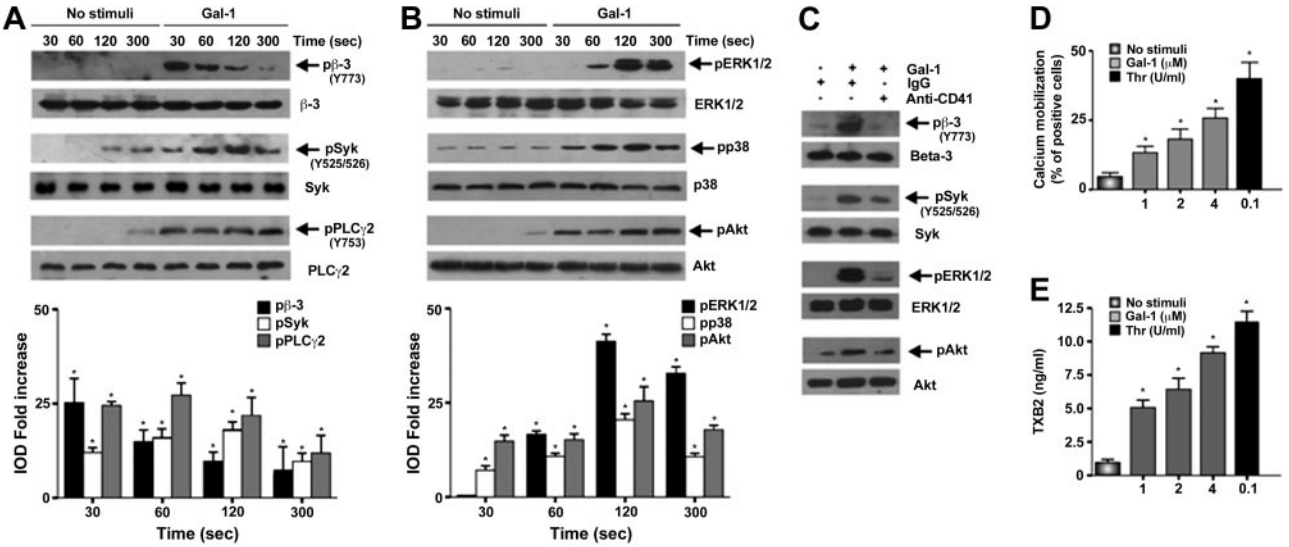

Figure 4. Signaling triggered by Gal-1 binding to $\alpha_{IIb}\beta_3$ integrin in human platelets. *A*, *B*) WPs preincubated with aspirin (0.5 mM) were stimulated with Gal-1 (4 μM) at 37°C without stirring, and the reaction was stopped by adding loading buffer. Integrin β_3, Syk, PLCγ2, ERK, p38, and Akt phosphorylations were determined in cell lysates. Total expression levels of unphosphorylated proteins were used to monitor equal protein loading in all lanes. Images represent 3 independent experiments. *C*) WPs preincubated with aspirin and with IgG or with a mixture of anti-CD41 mAbs (clones HIP-2 and HIP-11) for 15 min. Platelets were stimulated with Gal-1 (4 μM) at 37°C for 60 s without stirring, and the reaction was stopped by adding loading buffer. Integrin β_3, Syk, ERK, and Akt phosphorylations were determined in cell lysates. Total expression levels of unphosphorylated proteins were used to monitor equal protein loading in all lanes. Images represent 3 independent experiments. *D*) Intracellular Ca^{2+} concentration was determined at 37°C by flow cytometry using fluo-3-AM (n=4.) *E*) WPs were stimulated with Gal-1 in an aggregometer. After 5 min of stimulation, supernatants were collected, and TXB_2 levels were determined by ELISA (n=3). *$P < 0.05$ *vs.* unstimulated.

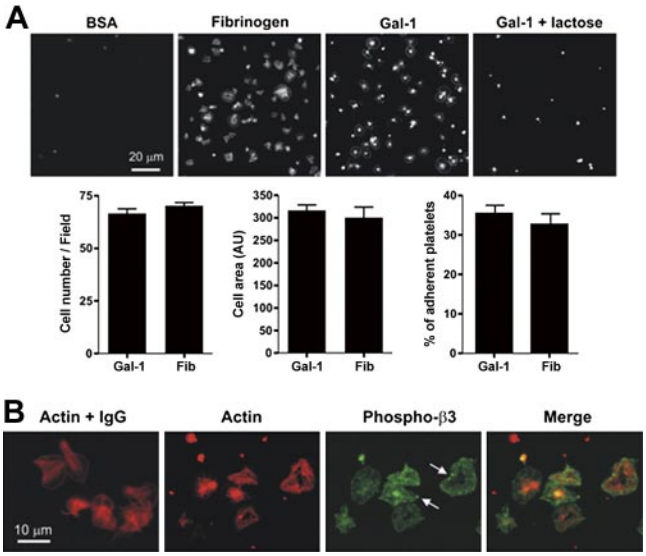

Figure 5. Immobilized Gal-1 promotes platelet adhesion, spreading, and β_3 integrin phosphorylation. *A*) WPs (5×10^7/ml) were plated on 1 μM Gal-1 or 100 μg/ml fibrinogen-coated slides for 30 min. Platelets were fixed and stained with TRITC-phalloidin. Platelet spreading was visualized by confocal microscopy (Olympus FV1000). Images represent 5 independent experiments. Percentage of adhered platelets was determined by a quantitative plate adhesion assay that determines acid phosphatase activity (n=4). *B*) WPs (5×10^7/ml) were plated on 1 μM Gal-1-coated slides for 30 min. Platelets were fixed and double stained with TRITC-phalloidin (red) and an anti-phospho-β_3 integrin (Y773) Ab (green). Platelets were visualized by confocal microscopy (Olympus FV1000). Arrows indicate intense stained areas, and they appear to be in focal contacts. Images represent 3 independent experiments.

support the role of $\alpha_{IIb}\beta_3$ integrin as a functional Gal-1 receptor in human platelets but also identify Gal-1 as a unique platelet agonist with the ability to activate platelets in both soluble and immobilized conformations.

Prolonged bleeding time in *Lgals1*$^{-/-}$ mice

To understand the contribution of endogenous Gal-1 to the hemostatic process *in vivo*, we explored the effect of Gal-1 on primary hemostasis by comparing the bleeding times of *Lgals1*$^{-/-}$ and WT mice. Although *Lgals1*$^{-/-}$ mice exhibited no evidence of spontaneous bleeding or hemorrhage, the median time to bleeding cessation was significantly prolonged (P<0.0002) in *Lgals1*$^{-/-}$ mice compared with WT mice of the same genetic background (104±7.8 *vs.* 65±2.8 s, respectively; **Fig. 6*A***). This effect was not attributed to alterations in platelet count, as *Lgals1*$^{-/-}$ mice had similar platelet numbers as WT animals [$623\pm52\times10^6$ *vs.* $645\pm38\times10^6$ platelets/ml for knockout (KO) and WT mice, respectively; n=14].

Defective platelet outside-in signaling in *Lgals1*$^{-/-}$ mice

Quantitative or qualitative alterations in platelets are among the most important defects underlying prolonged bleeding time (26). As *Lgals1*$^{-/-}$ mice exhibited normal platelet counts (this study) and endogenous Gal-1 contributes to platelet aggregation (11), we next explored *in vitro* activation responses of Gal-1-deficient platelets. Similar to human platelets, murine platelets expressed substantial amounts of Gal-1, which was absent in platelets from *Lgals1*$^{-/-}$ mice (Fig. 6*B*). As shown in Fig. 6*C*, Ca^{2+} rises induced by PAR4 were similar in platelets expressing or lacking Gal-1. In fact, *Lgals1*$^{-/-}$ and WT platelets showed a similar pattern of response when aggregation was induced by ADP, PAR4-AP, arachidonic acid (AA), or collagen. Representative WT and KO platelet aggregation profiles are shown in Fig. 6*D*. However, discrete differences were observed when platelet ATP release was analyzed in response to these agonists. In fact, while the amount of ATP released by AA or PAR4-AP was similar in platelets expressing or lacking Gal-1, it was significantly reduced after activation of *Lgals1*$^{-/-}$ platelets with low collagen concentrations (almost 50% of those released by WT platelets; **Fig. 7*A***). However, the amount of ATP release was similar in either Gal-1 KO or WT mice when platelet activation was triggered with higher collagen concentrations (data not shown). Moreover, the secretion of α-granules, reflected by the exposure of P-selectin, was not altered in *Lgals1*$^{-/-}$ platelets (Fig. 7*B*). Overall, these data indicate that Gal-1 deficiency is associated with minor alterations in dense granule secretion.

Prolonged bleeding times have been associated with an impaired platelet $\alpha_{IIb}\beta_3$ outside-in signaling (27,

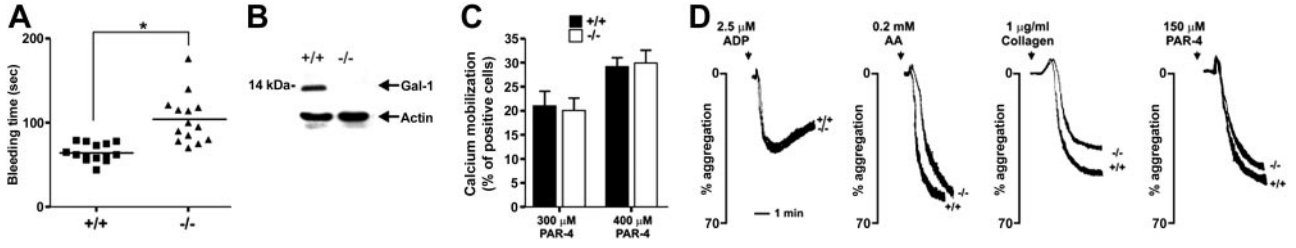

Figure 6. Bleeding time and platelet function in WT and *Lgals1*$^{-/-}$ mice. *A*) Terminal 3-mm segment of the tail was cut, and immediately immersed into saline solution at 37°C. Tail bleeding time was defined as the time required for bleeding to stop. *B*) Resting platelets from *Lgals1*$^{-/-}$ and WT mice were purified and lysed, and the expression of Gal-1 was determined by Western blot. *C*) Intracellular Ca^{2+} concentration was determined by flow cytometry at 37°C using fluo-3-AM (n=4). *D*) PRP was stimulated with the indicated agonists. Representative tracings of 4 separate experiments are shown.

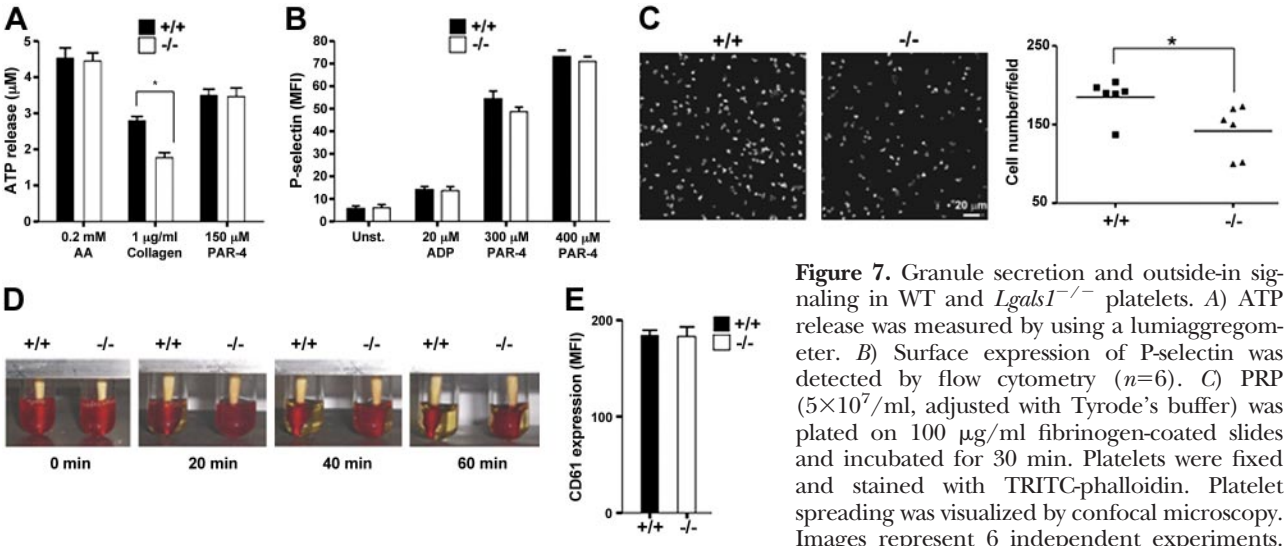

Figure 7. Granule secretion and outside-in signaling in WT and *Lgals1*$^{-/-}$ platelets. *A*) ATP release was measured by using a lumiaggregometer. *B*) Surface expression of P-selectin was detected by flow cytometry (n=6). *C*) PRP (5×10^7/ml, adjusted with Tyrode's buffer) was plated on 100 μg/ml fibrinogen-coated slides and incubated for 30 min. Platelets were fixed and stained with TRITC-phalloidin. Platelet spreading was visualized by confocal microscopy. Images represent 6 independent experiments. *D*) Clot retraction was determined using PRP in Tyrode's buffer with 2 mM Ca^{+2}. Platelets were stimulated with thrombin (0.25 U/ml) and monitored for 60 min (n=4). *E*) CD61 expression on platelet surface was determined by flow cytometry using FITC-labeled mAbs. *$P < 0.05$.

28). To evaluate whether $\alpha_{IIb}\beta_3$ integrin signaling was defective in platelets from *Lgals1*$^{-/-}$ mice, we investigated major platelet responses linked to this signaling mechanism, such as platelet adhesion, spreading, and clot retraction (9). As shown in Fig. 7*C*, *Lgals1*$^{-/-}$ platelets exhibited a significant reduction in their capacity to adhere to fibrinogen-coated slides compared with their WT counterparts. Clot retraction is a critical event in thrombus consolidation and is dependent on $\alpha_{IIb}\beta_3$ signaling (7, 9). We found that *Lgals1*$^{-/-}$ platelets displayed a delayed kinetics in clot retraction. In fact, while WT platelets started to retract at 20 min, and the clot was completely retracted after 60 min of thrombin addition, Gal-1-deficient platelets did not show retraction at 20 min, and after 60 min they were only partially retracted (Fig. 7*D*). These defects were not associated with reduced expression of $\alpha_{IIb}\beta_3$ integrin on the surface of Gal-1-deficient platelets, as demonstrated by flow cytometry studies (Fig. 7*E*). These data suggest that Gal-1 contained in platelets may play an important role in modulating $\alpha_{IIb}\beta_3$ integrin outside-in signaling.

DISCUSSION

Platelet surface $\alpha_{IIb}\beta_3$ integrin is a functional counterreceptor for Gal-1

Gals have recently emerged as nontraditional regulators of platelet physiology and mediators of platelet-dependent pathologies, including atherosclerosis and thrombosis (5, 11, 12). We previously demonstrated that soluble Gal-1 promotes several platelet activation responses, such as aggregation, granule secretion, and the formation of platelet microparticles and mixed leukocyte-platelet aggregates (11). Here we demonstrate that Gal-1 is a distinct platelet agonist that acts in both soluble or immobilized conformation and directly signals through the α_{IIb} subunit of $\alpha_{IIb}\beta_3$.

Different cell surface glycoconjugates, as well as extracellular matrix glycoproteins, appear to be primary ligands for Gals. Among them, integrins are known to be involved in Gal-mediated biological responses (29, 30). Interestingly, using mass spectrometry studies, we found that Gal-1 binds the subunit α_{IIb} from the $\alpha_{IIb}\beta_3$ integrin as well as $\alpha_6\beta_1$ integrin. Since activation of $\alpha_{IIb}\beta_3$ integrin is a major signaling mechanism involved in most platelet responses, we focused on the role of this integrin as a candidate Gal-1 counterreceptor in platelets. The inhibitory effect of preincubation with a mixture of anti-α_{IIb} mAbs or the use of GT platelets on Gal-1 binding to platelets, together with the significantly reduced platelet aggregation, P-selectin expression, and absence of ATP release, strongly suggest that $\alpha_{IIb}\beta_3$ is the functional counterreceptor involved in Gal-1-induced platelet activation. It could be argued that failure of Gal-1 to trigger platelet secretion in GT platelets was due to the fact that it is acting as a weak agonist, such as ADP or adrenaline, which requires $\alpha_{IIb}\beta_3$ integrin activation to trigger granule secretion (31). However, the ability of Gal-1 to induce both P-selectin expression under nonaggregating conditions (Fig. 2*C*) in normal platelets and the aggregation of aspirinized platelets (11) suggests that this lectin may act as a strong agonist like thrombin or collagen.

The observation that Gal-1 binding to either mAb-treated or thromboasthenic platelets was not completely suppressed together with the detection of platelet surface $\alpha_6\beta_1$ integrin and PECAM-1 by MALDI-TOF studies suggests that these proteins might also contribute together with $\alpha_{IIb}\beta3$ integrin to Gal-1 binding to the platelet surface. These results are in agreement with the ability of most Gals to establish Gal bivalent or multivalent interactions with *N*- and *O*-glycans, which enables

recognition of multiple binding partners and activation of distinct signaling pathways (3).

Interestingly, we have recently demonstrated that Gal-8, a member of the tandem-repeat subfamily of Gals, is a potent platelet agonist that only transduces Gal-8 platelet activation signals through the GPIb-V-IX complex (12). Thus, extracellular Gal-1 and Gal-8 may induce platelet activation by engaging distinct cell surface receptors. Whether this phenomenon implies a synergism between different members of the Gal family to trigger platelet activation requires further investigation.

Gal-1-$\alpha_{IIb}\beta_3$ integrin interaction triggers platelet activation through outside-in signaling

Similar to other integrins, the function of platelet $\alpha_{IIb}\beta_3$ can be mediated by inside-out or outside-in signaling. Inside-out signaling occurs in response to binding of one or more platelet agonists (7, 23) and leads to the conversion of $\alpha_{IIb}\beta_3$ from a low-affinity/avidity receptor to a high-affinity/avidity receptor, thereby allowing engagement of soluble multivalent adhesive ligands, such as fibrinogen and vWF. Ligand binding to the integrin triggers another cascade of signals known as outside-in, which leads to irreversible adhesion and aggregation (23, 32). In this context, soluble Gal-1 may act as a peculiar agonist as it is capable of triggering platelet activation through outside-in signaling *via* direct binding to $\alpha_{IIb}\beta_3$ integrin. This intrinsic property of Gal-1 may be associated to a general feature of Gals, which have been proposed to form ordered arrays of supramolecular structures composed of multivalent glycans and lectins (often termed "lattices") on the cell surface (3). Thus, it is conceivable that Gal-1 binding to $\alpha_{IIb}\beta_3$ integrin forms lattices that induce integrin clustering that leads to platelet signaling and activation. Although the molecular pathways involved in this bidirectional signaling of $\alpha_{IIb}\beta_3$ are still not fully understood, we found that downstream signals involved in Gal-1 binding to platelets include the most common signals elicited by soluble or adhesive agonists that induce activation of $\alpha_{IIb}\beta_3$ integrin, such as Ca^{2+} mobilization and phosphorylation of PLCγ, Syk, mitogen-activated protein kinases (MAPKs), Akt, and β_3 integrin.

Tyrosine phosphorylation of the integrin tyrosine cytoplasmic motif of the β_3 subunit is one of the most proximal and specific signaling events occurring after ligand binding to $\alpha_{IIb}\beta_3$ integrin and it is evident *in vitro* only under stirring conditions that allow integrin clustering or on platelet adhesion to immobilized fibrinogen (33, 34). Notably, Gal-1-induced phosphorylation of β_3 integrin occurs rapidly on addition of Gal-1 to platelet suspensions. Among the different known physiological ligands for $\alpha_{IIb}\beta_3$, only the soluble form of CD40L has been shown to trigger β_3 phosphorylation under these conditions (33). Moreover, rapid tyrosine phosphorylation of Syk, PLCγ2, and Akt (signaling events involved in platelet outside-in signaling) was also observed after platelet exposure to soluble Gal-1. Interestingly, phosphorylation of these kinases has been described on platelet stimulation with concanavalin A, a plant lectin with different glycan specificity but similar conformation and topology (*i.e.*, jelly-roll fold; ref. 35), that triggers platelet activation by inducing clustering of $\alpha_{IIb}\beta_3$ integrin molecules (36). Collectively, our data indicate that Gal-1 is a primary platelet agonist and that platelet stimulation is induced on direct binding to $\alpha_{IIb}\beta_3$, triggering outside-in signaling *via* tyrosine phosphorylation of β_3, Syk, Akt, and PLCγ2.

Not only soluble but also immobilized Gal-1 triggers platelet activation

Platelet adhesion and spreading, together with cytoskeletal rearrangements, are functional responses that occur as a consequence of outside-in signaling on ligand binding to $\alpha_{IIb}\beta_3$ and integrin oligomerization (9, 37). So far, these *in vitro* responses can be triggered by adhesion of platelets to fibrinogen or sCD40L-coated slides (20, 34, 38). Here we demonstrate that immobilized Gal-1 is an efficient substrate for platelet adhesion, filopodia, and lamellipodia formation in the absence of additional platelet agonists. Moreover, like soluble Gal-1, binding of platelets to Gal-1-coated surfaces resulted in strong tyrosine phosphorylation of the β_3 subunit of $\alpha_{IIb}\beta_3$ integrin, which further reinforces the role of this integrin as the signaling receptor for Gal-1. These data also show that platelet adhesion to Gal-1 under static conditions offers, in addition to fibrinogen and sCD40L, another experimental model to study outside-in signaling. Remarkably, although Gal-1 shares many features with both agonists, Gal-1 has unique properties. For example, while soluble fibrinogen does not transduce platelet signaling under nonstirring conditions (7), Gal-1 is a strong activator of most of the classical platelet activation signaling pathways. On the other hand, whereas CD40L selectively induces platelet activation (spreading and microparticle formation; ref. 39), Gal-1 effectively triggers the whole spectrum of platelet functional responses (11).

As Gal-1 functions as a matricellular protein and its expression is increased in activated endothelium, tumor cells, and tumor-associated stromal cells (4, 6), the interaction of platelets with immobilized Gal-1 could be relevant in different clinical settings, including vascular disorders, inflammation, and cancer.

Gal-1 plays a role in normal primary hemostasis

To understand the pathophysiologic relevance of endogenous Gal-1 in the hemostatic process, we evaluated primary hemostasis and platelet function in *Lgals1*$^{-/-}$ and WT mice. Although equivalent platelet numbers were observed in WT and *Lgals1*$^{-/-}$ mice, Gal-1 deficiency resulted in prolonged bleeding time. Taking into account that the bleeding time depends on normal platelet and vascular function and that Gal-1 is highly represented in both platelets and activated endothelium (4, 11), it might

be possible that the prolonged bleeding time observed was a consequence of Gal-1 deficiency in both endothelial cells and/or platelets. However, our findings showing that Gal-1-deficient platelets have a restricted adhesion to immobilized fibrinogen and a delayed clot retraction indicate an impaired outside-in signaling of platelet integrin $\alpha_{IIb}\beta_3$ as a major cause of the altered bleeding time in mice lacking Gal-1. The addition of exogenous Gal-1 did not restore platelet activation responses, suggesting that endogenous platelet-derived Gal-1, which is not released to the extracellular medium, has a distinct role from that of the extracellular lectin (data not shown). Interestingly, many of the hemostatic alterations present in mice with Gal-1 ablation recapitulate the phenotypes observed in mice devoid of the tetraspanin superfamily member TSSC6 (27) or in mice in which the integrin cytoplasmic tyrosine motif has been mutated to phenylalanine (28), which all exhibited impaired outside-in signaling.

Pathophysiologic relevance of Gal-1-induced platelet activation

Our study extends the list of biological activities of Gal-1 from immune responses and tumor growth to hemostasis and thrombosis. Gal-1 could be found intracellularly or even in the extracellular medium and plasma in its soluble form. Increased cell surface expression as well as circulating Gal-1 levels occurs in many different tumor types (6, 40). In addition, increased Gal-1 serum levels have been reported in sera from patients with type 2 diabetes (41). Interestingly, cancer and diabetes are well-known diseases where thrombosis is a common clinical complication (42, 43). Whether platelet-Gal interactions are involved in the pathogenesis of thrombotic episodes and whether Gal-1 expression represents a risk factor for thrombosis still remain to be investigated.

Inflammation is currently known as a major trigger that initiates and/or mediates the progression of the atherosclerotic disease (44), and Gal-1 is increasingly recognized as a key player during inflammatory reactions (45, 46). Although many platelet stimuli might contribute to long-term vascular pathologies, Gal-1 appears to be particularly relevant as it behaves as a bifunctional protein exerting not only proaggregant but also proinflammatory responses due to its ability to promote platelet-leukocyte aggregates (11). As Gal-1 also has significant anti-inflammatory properties by suppressing leukocyte trafficking, promoting T-cell apoptosis, and/or inducing tolerogenic dendritic cells (47, 48), the findings presented here are worthwhile to be discussed in terms of the overall effects of this lectin at different stages of the inflammatory cascade. Interestingly, the demonstration that immobilized Gal-1 is as efficient as its soluble form in triggering platelet activation suggests that this lectin could have a direct role not only in the progression of atherosclerotic lesions but also in acute thrombosis occurring during plaque rupture. Understanding the mechanisms involved in platelet-Gal interactions and unraveling their biological roles *in vivo* will have critical implications not only in hemostasis and thrombosis but also in cardiovascular diseases, chronic inflammation, and cancer, the pathophysiology of which involves platelet activation and signaling. FJ

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