Fibulin-1 Mediates Platelet Adhesion Via a Bridge of Fibrinogen

By Svetlana Godyna, Maribel Diaz-Ricart, and W. Scott Argraves

Fibulin-1 is a component of the extracellular matrix that surrounds vascular smooth muscle. This observation, along with the recent finding that fibulin-1 can bind fibrinogen (J Biol Chem 270:19458, 1995), prompted investigation into the potential role of fibulin-1 as a thrombogenic agent. In perfusion chamber assays, platelets in whole blood under flow conditions attached and spread on surfaces coated with fibulin-1. This adhesion was completely blocked by fibulin-1 antibodies. Platelets free of plasma did not attach to fibulin-1 coated surfaces; however, with the addition of fibrinogen, platelet adhesion to fibulin-1 took place. When detergent extracts of platelets were subjected to fibulin-1-Sepharose affinity chromatography, the integrin αIIbβ3 was selected. Solid phase binding assays using purified components showed that integrin αIIbβ3 could not bind directly to fibulin-1 but in the presence of fibrinogen the integrin bound to fibulin-1-coated surfaces. Monoclonal αIIbβ3 antibodies capable of blocking its interaction with fibrinogen completely blocked platelet adhesion to fibulin-1 in both whole blood perfusion and static adhesion assays. The results show that fibulin-1 can support platelet attachment via a bridge of fibrinogen to the platelet integrin αIIbβ3. When fibroblast monolayers containing extracellular matrix-incorporated fibulin-1 were used as adhesion substrates, platelet adhesion in the presence of fibrinogen could be inhibited by 30% using antibodies to fibulin-1. Following vascular injury, fibulin-1 present in the extracellular matrix of the vessel wall may therefore interact with plasma fibrinogen and promote platelet adhesion, leading to the formation of a platelet plug. Thus, fibulin-1 joins the list of matrix proteins including collagens I and IV and fibronectin that mediate platelet adhesion via a plasma protein bridge. This bridging phenomenon may represent a general mechanism by which platelets interact with exposed subendothelial matrices following vascular injury.

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Research Laboratories, Inc (South Bend, IN). Human plasma (FN) was isolated by sequential affinity chromatography on gelatin-Sepharose followed by heparin-Sepharose according to Miekka et al. Collagen type I was purchased from Chronolog Corp (Havertown, PA). The integrin gpIIb-IIIa/αIIbβ3(αIIbβ3) was purified by affinity chromatography according to Kirchhofer et al. Thrombospondin-1 (TSP1) was purified from human platelets by adsorption to barium citrate followed by heparin-agarose chromatography according to the method of Alexander and Dextler.

Radiolabeling. Integrin αIIbβ3 (20 μg) was radioiodinated with 0.5 μCi Na125I (Amersham Corp, Arlington Heights, IL) for 10 minutes at 4°C using 20 μg/ml Iodo-Gen reagent (Pierce, Rockford, IL) in 100 μL of 50 mMol/L Tris, pH 7.4, 150 mMol/L NaCl Tris-buffere d saline (TBS), containing 25 mMol/L octyl-β-D-glucoside (Calbiochem, La Jolla, CA), 2 mMol/L CaCl2, 2 mMol/L MgCl2. Radiolabeled protein was separated from free iodine by gel filtration on PD-10 columns containing Sephadex G-25 M (Pharmacia-LKB, Uppsala, Sweden). The integrity of radioiodinated protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography of the gels. Specific activity that was typically obtained ranged from 1 to 10 μCi/μg.

Antibodies. Mouse monoclonal anti-αIIbβ3 IgG containing ascites fluid, monoclonal antibody (MoAb) Edu3, was obtained from Dr Vitella (Hospital Clinic Provincial, Barcelona, Spain). Anti-αIIbβ3 IgG was selected from ascites fluid by affinity chromatography on protein G-Sepharose. Mouse monoclonal anti-αIIbβ3, 2G12 IgGs were selected.

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obtained from Dr Virgil Woods (University of California, San Diego). Rabbit anti-human fibulin-1 serum (rb2954) was prepared using fibulin-1 isolated by immunoaffinity chromatography using MoAb 3A11-Sepharose and immunization methodology described previously.20 Rabbit polyclonal anti-fibulin-1 IgG (rb2954) was selected from the antisera by affinity chromatography on protein G-Sepharose and then on fibulin-1-Sepharose. F(ab')2 fragments of anti-fibulin-1 IgG and preimmune rabbit IgG were prepared by pepsin digestion (1:100 wt/wt, pepsin:IgG) for 16 hours at 37°C in 0.1 mol/L sodium citrate buffer, pH 3.5. Monovalent Fab fragments were generated by reduction of F(ab')2 with 2-mercaptoethanol for 1 hour at 37°C and alkylation with iodoacetamide for 8 hours at 22°C in the dark according to Parham.21 MoAb against human FG was provided by Dr Bryan Butman (PerImmune, Rockville, MD).

Platelet adhesion under flow conditions. Platelet adhesion under flow conditions was performed using a parallel plate perfusion chamber as previously described.22 Purified proteins were sprayed onto plastic microscope coverslips (18 x 18 mm²) using an airbrush (50 μg/coverslip for fibulin-1, bovine serum albumin (BSA) and type I collagen and 15 μg/coverslip for FG, FN, and TSP-1). Pairs of protein-coated coverslips were inserted into the chamber and using a peristaltic pump, 15 mL of either whole blood or reconstituted blood (discussed later), prewarmed to 37°C, was recirculated through the chamber at shear rates of 300 s⁻¹, 800 s⁻¹, and 1,300 s⁻¹, for 5 minutes each. The coverslips were subsequently removed from the chamber, rinsed with PBS, pH 7.4, fixed for 2 hours in 0.5% glutaraldehyde, stained in 0.02% solution of toluidine blue and mounted on glass slides. Platelet adhesion was evaluated by light microscopy and quantified using a computerized image analysis system (Image-1; Universal Imaging Corp, West Chester, PA). Platelet adhesion is expressed as a percentage of the total coverslip surface covered by platelets (%CS).

Fresh human blood, anticoagulated with a 1:7 vol of citrate-phosphate dextrose solution (CPD-A1), (Baxter Healthcare Corp, Deerfield, IL) was obtained from the American Red Cross Washington Regional Blood Services (Washington, DC). The blood was centrifuged at 120g for 10 minutes at 22°C to pellet red blood cells. The resulting platelet-rich plasma supernatant (PRP) was reconstituted to remove residual red blood cells. Platelets from the PRP were recovered by centrifugation at 1,800g for 20 minutes at 22°C. Following the centrifugation the supernatant (platelet-poor plasma [PPP]) was removed and the pelleted platelets washed twice by resuspension in citrate-citric dextrose buffer (93 mmol/L sodium citrate, 7 mmol/L citric acid, pH 6.5, 140 mmol/L dextrose, containing 5 mmol/L adenosine and 3 mmol/L theophylline).23 Washed platelets were finally resuspended in Hanks’ buffer solution, pH 7.3.

Reconstituted blood was prepared by combining PPP with washed red cells, and washed platelets as previously described.2324 Washed red cells from the previously mentioned 120g pellet were prepared by repeated resuspension in saline, containing 0.1% glucose and 3.000g centrifugation (10 minutes, 22°C). To deplete PPP of FN, it was passed over a column of gelatin-Sepharose 4B. When required, the FN that was affinity selected on the gelatin-Sepharose 4B column was added to reconstituted blood to a final concentration of 0.3 mg/mL.

Platelet adhesion under static conditions. Protein A purified IgG (in TBS) were coated onto the wells of 96-well flat-bottomed plates (Immulon; Dynatech Labs Inc, Chantilly, VA) for 18 hours at 4°C and unoccupied sites blocked with 1% BSA, TBS-glucose buffer for 1 hour, rt. To isolate platelets for use in adhesion assays, blood was drawn in CPD-A1 and PRP prepared as previously described. Prostaglandin E₃ (PGE₃) at a concentration of 1 μg/mL was added to the PRP, the pH adjusted to 6.5 with citric acid and the platelets sedimented by centrifugation at 1,800g for 10 minutes. The pelleted platelets were washed in 5.5 mmol/L glucose, 120 mmol/L NaCl, 4.26 mmol/L NaH₂PO₄, 7.46 mmol/L Na₂HPO₄, 4.77 mmol/L trisodium citrate, 2.35 mmol/L citric acid, pH 6.5 (citrate wash buffer), and resuspended in 10 mmol/L Tris, pH 7.4, 140 mmol/L NaCl, 5.5 mmol/L glucose (TBS glucose buffer) at 1 x 10⁸ platelets/mL for radiolabeling according to Tandon et al.23 Typically, 50 μCi/mL of...
Na$_{35}$CrO$_4$ (New England Nuclear, Dupont, Boston, MA) were added to 1 mL of platelets and incubated for 1 hour at rt. The platelets were then washed three times with citrate wash buffer and resuspended at 2 x 10$^9$/mL in TBS-glucose buffer, containing 2 mmol/L CaCl$_2$, 2 mmol/L MgCl$_2$, 1% BSA (adhesion buffer). Fifty microliters of $^{35}$Cr-labeled platelet solution were added to microtiter wells and incubated for 1 hour at rt. In those experiments that tested the effects of exogenously added FG or FN on platelet adhesion, each protein was added to the platelet-containing adhesion buffer to a final concentration corresponding to its concentration in human blood (FG, 3 mg/mL; FN, 0.33 mg/mL). After 1 hour, the nonadherent platelets were removed by aspiration and wells washed 3 times with adhesion buffer. The adherent platelets were lysed with 2% SDS and the $^{35}$Cr content within the lysates measured by using scintillation counting.

Affinity chromatography of surface radiolabeled platelet extracts. Platelets, prepared as described in the previous section, were radiolabeled using a lactoperoxidase method. Typically, 1 mCi of carrier-free Na$^{125}$I (Amersham Corp., Arlington Heights, IL) was added to 1 mL of the platelet suspension (1 x 10$^9$ platelets/mL) in TBS-glucose buffer with 20 mmol/L glucose, 0.4 U/mL glucose oxidase (Sigma Chemical Co., St Louis, MO), and 200 μg/mL lactoperoxidase (Boehringer Mannheim, Indianapolis, IN) and incubated for 20 minutes at rt. The labeled platelets were washed twice in citrate wash buffer (as previously described), exposed to thrombin (3 U/mL) for 2 minutes at 37°C and extracted in a solution of 200 mmol/L octyl-$\beta$-D-glucopyranoside (OG), 1 mmol/L CaCl$_2$, 1 mmol/L MgCl$_2$, 2 mmol/L phenylmethylsulfonyl fluoride (PMSF), 100 μg/mL leupeptin, 3 μg/mL pepstatin in TBS for 30 minutes at 4°C. The lysate was then centrifuged at 50,000g for 20 minutes at 4°C, the supernatant absorbed on Sepharose CL-4B and the preabsorbed lysate applied to a column of fibulin-1 coupled to CNBr-activated-Sepharose (8 mg protein/mL resin), equilibrated with 25 mmol/L OG, 1% BSA, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L PMSF, 1 mmol/L CaCl$_2$, 1 mmol/L MgCl$_2$ in TBS (OG buffer). The column was washed with OG buffer and the bound protein eluted with 20 mmol/L EDTA in OG buffer or with the synthetic peptide GRGDSP (1 mg/mL) in OG buffer.

**Solid-phase binding assays.** Homologous ligand displacement assays were performed according to methods previously outlined by Godnya et al.$^{26}$ Briefly, removable microtiter wells (Immulon 2; Dynatech Laboratories Inc., Chantilly, VA) were coated with fibulin-1, FG, or BSA (each at 5 μg/mL) in TBS, pH 8.0 for 18 hours at 4°C and unoccupied binding sites were blocked for 1 hour with 3% BSA, 2 mmol/L CaCl$_2$, 2 mmol/L MgCl$_2$, TBS, pH 7.4. The coated wells were then incubated with $^{125}$I-$\alpha_{IIb}β_3$ (2 to 5 μmol/L) in the previous buffer plus 0.05% Tween-20 and 25 mmol/L OG and vary-

<table>
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<th>Proteins Coated</th>
<th>300 Sec $^{-1}$ (% CS)</th>
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<td>Fibulin-1</td>
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<td>Thrombospondin-1</td>
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<td>Fibrinogen</td>
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<tr>
<td>BSA</td>
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%CS = surface covered by platelets expressed as percent of the total surface examined. Values presented represent means ± SD.

* Indicates that the experiments have been done once.

Fig 2. Fibulin-1-Sepharose affinity chromatography selects the integrin $\alpha_{IIb}β_3$ from platelet extracts. Platelets were surface-radioiodinated by a lactoperoxidase method and extracted in detergent buffer. The extract was preabsorbed on a column of Sepharose CL-4B and then applied to a fibulin-1-Sepharose column. Bound proteins were eluted with detergent buffer containing 25 mmol/L EDTA. Aliquots of the eluted fractions were electrophoresed in the presence of SDS in 4% to 12% polyacrylamide gradient gels under reducing (A) and nonreducing (B) conditions and the gels used to expose x-ray film. In (C), bound proteins, eluted from fibulin-1-Sepharose column with detergent buffer containing the synthetic peptide GRGDSP (lane 1) were immunoprecipitated using control mouse IgG (lane 2) or the monoclonal $\alpha_{IIb}β_3$ antibody 2G12 (lane 3). The migration positions of molecular mass standards (in kD) are indicated on the right.
ing concentrations of unlabeled receptor for 18 hours at 4°C. Bound
125IαIIbβ3 was measured by using a gamma counter.

Enzyme-linked immunoassorbent assay (ELISA) was used to examine the effect of FG on fibulin-1 binding to αIIbβ3. Microtiter wells were coated with fibulin-1 (300 nmol/L) and varying concentrations of either FG or ovalbumin in 3% BSA, 2 mmol/L CaCl2, 2 mmol/L MgCl2, TBS, pH 7.4, 0.05% Tween-20, for 18 hours at 4°C. The amount of bound fibulin-1 was indirectly measured using the fibulin-1 MoAb 3A11, goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories, Richmond, CA) and the substrate 3', 5'-tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, MD). Binding of FG to αIIbβ3 was also detected by ELISA using the FG MoAb.

Platelet adhesion to cultured fibroblast monolayers. Human lung IMR-90 fibroblasts (CCL 186) were grown to confluence in Dulbecco’s modified Eagle’s medium (DME) (Mediatech, Washington, DC) supplemented with 10% bovine calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin (GIBCO-BRL, Grand Island, NY) and 1 mmol/L sodium pyruvate. Fibroblast monolayers were washed three times with PBS, incubated for 1 hour with 1% BSA in DME, and then treated with antibodies for 2 hours at 37°C. Platelets (2 × 10^9/mL) radiolabeled with 51Cr were added to the fibroblast monolayers in the adhesion buffer in the presence or absence of 2 mg/mL of FG. Following 1 hour incubation at 37°C, 5% CO2, unattached platelets were removed by aspiration. Cell layers were rinsed with TBS-glucose buffer and the adherent platelets were lysed with 2% SDS and the 51Cr content within the lysates measured by using scintillation counting.

RESULTS

Platelets in whole blood adhere to fibulin-1 under flow conditions. The ability of fibulin-1 to promote the adhesion of platelets was evaluated by using a whole blood perfusion assay. As shown in Fig 1A, platelets under flow conditions (300 s^-1) attached and spread on surfaces coated with fibulin-1. The adhesion to fibulin-1 could be completely blocked by preincubation of the fibulin-1 coated coverslips with antifibulin-1 IgG Fab fragments (Fig 1C) but not with control IgG Fab fragments (Fig 1B). The findings indicate that the platelets in whole blood, under flow conditions, can adhere to fibulin-1.

The effect of shear rate on the ability of fibulin-1 to promote platelet adhesion was next examined. While platelets attached (21% covered surface [%CS]) to fibulin-1 under low shear conditions (300 s^-1), attachment decreased as the shear rate was increased (5% CS at 800 s^-1 and 3% CS at 1300 s^-1) (Table 1). In the parallel experiments, platelet adhesion to other adhesive proteins including FG, FN, collagen, and TSP-1 was examined under differing shear rates. Fibulin-1, FN, TSP-1, and FG behaved similarly in that the amount of platelet adhesion to each was greatest at low shear rates and decreased as the shear rate was increased. Conversely, platelet adhesion to type I collagen coatings increased with increasing shear rate as has been reported (Table 1).

Fibulin-1-Sepharose affinity chromatography selects the integrin αIIbβ3 from platelet extracts. To identify the platelet receptor that mediates platelet adhesion to fibulin-1 we performed ligand-affinity chromatography on extracts of surface-radiolabeled platelets using a column of fibulin-1 coupled to Sepharose. As shown in Fig 2, EDTA elution of the fibulin-1 Sepharose column released two radiolabeled polypeptides that when run on SDS-PAGE gave M, of 120,000 and 110,000 when reduced (Fig 2A), and 150,000 and 95,000 when nonreduced (Fig 2B). Subsequent elution of the column with urea-containing buffer did not release additional radiolabeled proteins (data not shown) indicating that the bulk of the material that bound to the column was released with EDTA buffer.

The electrophoretic properties, including M, values and response to reduction, of the fibulin-1-Sepharose binding proteins were reminiscent of the biophysical features of the
platelet integrin αmβ3. Immuno precipitation analyses were performed to confirm this possibility. As shown in Fig 2C, monoclonal αmβ3 antibody precipitated both polypeptides from fractions containing the proteins eluted from the fibulin-1-Sepharosecolumn. Antibody to the integrin αv subunit did not precipitate either of the fibulin-1-Sepharose binding proteins (data not shown). The results indicate that the proteins selected from detergent extracts of platelets by fibulin-1-Sepharose affinity chromatography are the subunits of the integrin αmβ3.

Integrin αmβ3 antibodies block adhesion of platelets in whole blood to fibulin-1 under flow conditions. To establish that integrin αmβ3 was mediating platelet adhesion to fibulin-1 under the conditions of the perfusion assay we evaluated the effect of a monoclonal integrin αmβ3 antibody known to block integrin αmβ3 ligand binding activity. The results showed that the integrin αmβ3 antibody completely inhibited platelet adhesion to the fibulin-1(Fig 3A) whereas control mouse IgG had no effect. The integrin αmβ3 antibody was also able to completely block platelet adhesion to surfaces coated with FG, the predominant integrin αmβ3 ligand (Fig 3B). The results indicate that integrin αmβ3 mediates platelet adhesion to fibulin-1.

Purified integrin αmβ3 does not bind directly to fibulin-1 in solid phase binding assays. To determine whether integrin αmβ3 was capable of binding directly to fibulin-1 we performed solid phase binding assays using purified components. As shown in Fig 4, 125I-integrin αmβ3 did not bind to microtiter wells coated with fibulin-1 but was able to bind wells coated with FG (Fig 4A). Similarly, fibulin-1 did not bind to wells coated with integrin αmβ3 whereas FG did bind to immobilized integrin αmβ3 (Fig 4B). The results indicate that integrin αmβ3 is not able to interact directly with fibulin-1.

FG acts to bridge the interaction between integrin αmβ3 and fibulin-1 in platelet adhesion assays. The failure of integrin αmβ3 to bind to fibulin-1 in solid phase assay seemed contradictory to the results from platelet adhesion and affinity chromatography that implicated integrin αmβ3. A possible explanation for these apparently disparate results is that some protein found in blood or in platelet extracts was acting to bridge the interaction between integrin αmβ3 and fibulin-1. To test this possibility we first examined the role of FN, a known ligand for both integrin αmβ3 and fibulin-1.12 Platelet adhesion assays were performed using platelets in either whole blood, reconstituted blood or reconstituted blood depleted of FN. As shown in Table 2, platelet adhesion to fibulin-1 was not effected by the lack of FN in the reconstituted blood.

We next evaluated the role of FG, another known ligand for both integrin αmβ3 and fibulin-1,13,31,32 as a potential mediator of platelet binding to fibulin-1. Because it was impractical to completely deplete blood of FG static adhesion assays were performed instead of the perfusion type assay. As shown in Fig 5A and B, washed 51Cr-labeled platelets in plasma-free buffer were found to adhere poorly to fibulin-1 coated surfaces.51Cr-labeled platelet adhesion to fibulin-1 was increased when the fibulin-1-coated surfaces were first preincubated with FG (Fig 5B). In addition, when FG was coincubated with 51Cr-labeled platelets, the level of adhesion was increased further (Fig 5B) and approached levels obtained with FN and FG coated surfaces (Fig 5A). In contrast, coincubation of the washed 51Cr-labeled platelets with FN did not increase the level of platelet adhesion to fibulin-1 (Fig 5A). Coincubation of washed 51Cr-labeled platelets with FG did not increase the low level of platelet adhesion to ovalbumin coated surfaces. The results indicate
that platelet adhesion to fibulin-1 coated surfaces is promoted by FG but not FN.

To confirm that the FG-promoted platelet adhesion to fibulin-1 was mediated by the integrin αIIbβ3, we evaluated the ability of monoclonal integrin αIIbβ3 antibodies to inhibit the adhesion. As shown in Fig 6A, two different integrin αIIbβ3 MoAb, that have been shown to block receptor binding to FG, were able to completely block FG-promoted 51Cr-labeled platelet adhesion to fibulin-1. In addition, these antibodies blocked platelet adhesion to FG coated surfaces (Fig 6B). The FG-promoted adhesion to fibulin-1 coated surfaces could also be blocked by monoclonal or polyclonal antibodies to fibulin-1 (Fig 6A) whereas platelet adhesion to FG (Fig 6B) or FN (not shown) coated surfaces was unaffected by these antibodies. The results indicate that the FG-promoted platelet adhesion to fibulin-1 coated surfaces is mediated by the integrin αIIbβ3, presumably by binding to FG that is bound to fibulin-1.

FG promotes binding of integrin αIIbβ3 to fibulin-1 in solid phase binding assays. Given that FG was able to promote platelet adhesion to fibulin-1 via integrin αIIbβ3, we next sought to show by using purified components that FG could promote the binding of integrin αIIbβ3 to fibulin-1. When FG was included in the buffer along with integrin αIIbβ3, a four-
ROLE OF FIBULIN-1 IN PLATELET ADHESION

Fig 7. FG promotes the binding of integrin $\alpha_{\text{IIb}}\beta_3$ to fibulin-1. In (A), microtiter wells coated with fibulin-1 were incubated with $^{125}$I-integrin $\alpha_{\text{IIb}}\beta_3$ (5 nmol/L) in the absence (Control) or presence of either (FN, 0.33 mg/mL) (FG, 3 mg/mL) or ovalbumin (OVB). In (B), microtiter wells coated with integrin $\alpha_{\text{IIb}}\beta_3$ were incubated with fibulin-1 (300 nmol/L) in the presence of increasing concentrations of either FG or ovalbumin (0.013 to 30 $\mu$mol/L) and the amount of fibulin-1 bound to $\alpha_{\text{IIb}}\beta_3$ was detected by ELISA. The curve represents the best-fit of the data to a single class of sites.

Fold increase in $\alpha_{\text{IIb}}\beta_3$ binding to fibulin-1 coated wells was observed (Fig 7A). Inclusion of FN or ovalbumin had little or no effect on integrin $\alpha_{\text{IIb}}\beta_3$ binding to fibulin-1 coated wells (Fig 7A). Similarly, incubation of fibulin-1 with FG resulted in the binding of fibulin-1 to wells coated with $\alpha_{\text{IIb}}\beta_3$ that increased with increasing dose of FG (Fig 7B). These findings are in support of the results from platelet adhesion assays indicating that fibulin-1 promotes platelet adhesion via a FG bridge to the integrin $\alpha_{\text{IIb}}\beta_3$.

Divalent cations are required for FG-promoted platelet adhesion to fibulin-1. We next evaluated the requirement for divalent cations in the process of FG-promoted platelet adhesion to fibulin-1. As shown in Fig 8, in the absence of divalent cations, FG did not promote platelet adhesion to fibulin-1 coated surfaces. The inclusion of either 2 mmol/L Ca$^{2+}$, 2 mmol/L Mg$^{2+}$, 2 mmol/L Mn$^{2+}$, Ca$^{2+}$ and Mg$^{2+}$ at 2 mmol/L each or Mg$^{2+}$ and Mn$^{2+}$ at 2 mmol/L each, resulted in a four to eightfold increase in platelet adhesion to fibulin-1. The adhesion was greatest when both Mg$^{2+}$ and Mn$^{2+}$ were present. This later observation is consistent with the cation requirements necessary to achieve optimal binding of the integrin $\alpha_{\text{IIb}}\beta_3$ to synthetic peptides containing the sequence RGD.17

FG promotes platelet adhesion to fibulin-1 present in fibroblast cell layers. We next sought to determine whether fibulin-1 incorporated within an extracellular matrix could mediate platelet adhesion in a FG dependent manner. Given that fibroblasts produce an elaborate fibrillar fibulin-1 extracellular matrix and that previous studies have shown that platelets adhere to cultured fibroblasts we examined the adhesion of $^{31}$Cr-labeled platelets to fibroblast monolayers in the presence or absence of FG and fibulin-1 antibodies. As shown in Fig 9A, platelet adhesion to fibroblast monolayers in the absence of FG was unaffected by anti-fibulin-1 IgG. However, fibulin-1 antibodies did inhibit platelet adhesion to fibroblasts monolayers by 30% ($P = .04$) when FG was included with the platelets (Fig 9B). In contrast, platelet adhesion to fibroblasts in both the presence and absence of FG was inhibited by FN antibodies. The degree of inhibition of platelet adhesion in the presence of FG achieved using FN antibodies was of a similar magnitude to that achieved using fibulin-1 antibodies. The results indicate that fibulin-1 incorporated into an extracellular matrix can promote platelet adhesion in the presence of FG.

DISCUSSION

The results presented in this manuscript document for the first time that fibulin-1 coated surfaces can promote the adhesion of platelets. This adhesion phenomenon was initially discovered using assays in which whole blood was perfused over surfaces coated with fibulin-1 or under static conditions when PRP was added to microtiter wells coated with fibulin-1. However, when platelets free of plasma were added to microtiter wells coated with fibulin-1 no adhesion...
proteins, FN and FG, were obvious candidates for such a plasma mediator based on the fact that both could bind fibulin-1 as well as bind to platelets. When FG was mixed with platelet extracts free of plasma, adhesion to fibulin-1 coated surfaces occurred whereas FN did not promote such adhesion. Consistent with these findings were other results that showed that antibodies to the FG-binding integrin, \( \alpha_{IIb} \beta_3 \), blocked platelet adhesion to fibulin-1 in both whole blood perfusion assays as well as in microtiter well assays performed in the presence of FG. Furthermore, when platelet extracts, containing FG, were applied to an affinity matrix of fibulin-1-Sepharose, the integrin \( \alpha_{IIb} \beta_3 \) was found to have specifically bound. Finally, we showed that the binding of purified integrin \( \alpha_{IIb} \beta_3 \) to fibulin-1 was dependent on the presence of FG. Based on these findings we conclude that platelet adhesion to fibulin-1 involves indirect interaction with the platelet integrin \( \alpha_{IIb} \beta_3 \) via a bridge of FG.

Platelet adhesion to fibulin-1 via a bridge of FG may be physiologically relevant in the processes of thrombosis and hemostasis. It is known that fibulin-1 is a component of subendothelial and extravascular ECM and that it can bind FG. It is possible that exposure of subendothelial fibulin-1 following vascular injury leads to FG binding to fibulin-1 given that the KD for the FG-fibulin interaction is \(-3 \mu\text{mol/L}\) and the plasma concentration of fibrinogen is 6 to 13 \( \mu\text{mol/L} \). FG immobilized on surfaces has been shown to promote adhesion of activated as well as unstimulated platelets in a process mediated by the integrin \( \alpha_{IIb} \beta_3 \). Therefore, the binding of blood borne FG to matrix-incorporated fibulin-1 could lead to integrin \( \alpha_{IIb} \beta_3 \)-mediated platelet adhesion to the fibulin-1-bound FG. In this way, fibulin-1 can be considered as a thrombogenic component of connective tissue ECM. Supportive of such an in vivo role for fibulin-1 are the results presented herein that showed fibulin-1, expressed by cultured fibroblasts and incorporated into the fibroblast monolayer ECM, can mediate FG-dependent platelet adhesion.

Collagens have been previously implicated as prominent thrombogenic agents present in ECMs and evidence indicates that platelet adhesion to collagens type I, III, and IV is dependent on vWF whereas adhesion to denatured type I and III collagens is dependent on FN. In addition, platelet adhesion to FN has been shown to be dependent on vWF derived from either plasma or platelet a-granules. These interactions serve as precedence for the occurrence in vivo of platelet interactions with exposed ECM proteins via a plasma protein bridge. A current hypothesis for platelet response to vascular injury put forth by Z. M. Ruggeri is that circulating platelets bind predominantly to vWF and fibrinogen/fibrin immobilized at sites of vascular injury. Fibulin-1 must now be regarded as a candidate for a mediator of FG immobilization at such sites in vivo and therefore serve as a participant in the earliest events of thrombus formation.

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