

Diminished contact-dependent reinforcement of Syk activation underlies impaired thrombus growth in mice lacking Semaphorin 4D

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We recently reported that Semaphorin 4D (Sema4D) and its receptors are expressed on the platelet surface and showed that Sema4D^(-/-) mice have a selective defect in collagen-induced platelet aggregation and an impaired vascular injury response. Here we investigated the mechanisms involved, tested the role of platelet-platelet contacts in Sema4D-mediated events, and examined the relationship between Sema4D-dependent signaling and integrin $\alpha_{IIb}\beta_3$ outside-in signaling. The results show that spleen tyrosine

kinase (Syk) activation, an early step in collagen signaling via the glycoprotein VI (GPVI)/FcR γ complex, is greatly reduced in Sema4D^(-/-) platelets and can be restored by adding soluble Sema4D. Earlier events, including FcR γ phosphorylation, occur normally; later events are impaired. In contrast, when engagement of $\alpha_{IIb}\beta_3$ was blocked, Sema4D^(-/-) and control platelets were indistinguishable in assays of Syk activation, adhesion, spreading on collagen, and activation of $\alpha_{IIb}\beta_3$. Finally, we found that, unlike the Sema4D

knockout, $\alpha_{IIb}\beta_3$ blockade inhibited FcR γ phosphorylation and that stimulating aggregation with Mn²⁺ failed to normalize Syk activation in the absence of Sema4D. Collectively, these results show that $\alpha_{IIb}\beta_3$ and Sema4D jointly promote collagen responses by amplifying Syk activation, partly by forming integrin-mediated contacts that enable the binding of Sema4D to its receptors and partly through integrin outside-in signaling. These 2 processes are interdependent, but distinguishable. (*Blood*. 2010;116(25):5707-5715)

Introduction

Semaphorins are a large family of proteins that were initially implicated in axonal guidance but have since been shown to contribute to organogenesis, vasculogenesis, angiogenesis and immune cell regulation.¹ The semaphorin family includes more than 20 proteins divided into 8 classes. We have previously shown that platelets express on their surface the class-IV semaphorin, Sema4D (CD100), as well as its 2 known receptors, CD72 and Plexin-B1.² We have also shown that platelets from Sema4D^(-/-) mice have a selectively impaired response to collagen that produces a rightward shift in the aggregation dose/response curve when platelets are activated by either collagen or the glycoprotein VI (GPVI) collagen receptor agonist, convulxin.² Notably, this is a collagen-specific defect, as platelet aggregation in response to other platelet agonists, including thromboxane A₂ (TxA₂), adenosine diphosphate (ADP) and a proteinase-activated receptor 4 (PAR4) agonist peptide, is normal. Sema4D^(-/-) mice also have impaired responses in 3 models of vascular injury performed in vivo, reduced susceptibility to atherosclerosis when crossed into a low-density lipoprotein (LDL)-receptor knockout background, and are partially protected against the sensitizing effects of dyslipidemia, suggesting that Sema4D on the surface of platelets plays a biologically relevant role.^{2,3}

Sema4D is a type I transmembrane protein that forms a disulfide-linked homodimer consisting of two 150-kDa subunits. It is present on the surface of resting platelets. However, additional copies of Sema4D are recruited to the surface upon platelet activation before being gradually cleaved and shed from the platelet surface by a disintegrin and metallopeptidase domain 17 (ADAM17).² Of the 2 best known cell surface Sema4D receptors, Plexin-B1 is expressed on neurons⁴ and endothelial cells,⁵ where it mediates cytoskeletal changes via Ras, Rho and phospholipase C γ .⁶⁻⁸ CD72 is a Sema4D receptor expressed on the surface of B cells. It has a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) that recruits Src homology phosphatase-1 (SHP-1) after B-cell antigen receptor activation, thus acting as an inhibitor of B-cell antigen receptor signaling.⁹ Engagement of CD72 with Sema4D on T cells relieves this inhibition by inducing the dissociation of the SHP-1/CD72 complex and the subsequent inactivation of SHP-1.¹⁰ Similarly, we have shown in platelets that the addition of recombinant soluble Sema4D dissociates SHP-1 from CD72.²

These observations suggest a model in which Sema4D on the platelet surface binds to receptors on adjacent platelets after integrin $\alpha_{IIb}\beta_3$ -mediated formation of stable platelet-platelet

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contacts. Subsequent signaling via *Sema4D* and/or its receptors then promotes platelet activation in a contact-dependent manner. If this hypothesis is correct, it would account for the loss of function we have observed in the *Sema4D*^(-/-) mice *in vitro* and *in vivo*.² It would also bolster the general idea that platelets communicate with each other in a contact-dependent manner once aggregation begins and provide a basis for understanding the role of *Sema4D* and its receptors in platelets. Here we have tested this model and sought the underlying mechanisms by which *Sema4D* promotes collagen responses in platelets, focusing on events downstream of the principal collagen signaling receptor, GPVI, and its immunoreceptor tyrosine-based activation motif (ITAM)-containing partner, FcR γ .

The results show that collagen-induced activation of the spleen tyrosine kinase, Syk, an early event in collagen signaling, is diminished in *Sema4D*^(-/-) platelets compared with *Sema4D*^(+/+) platelets. Addition of recombinant soluble *Sema4D* exodomain restores collagen-induced Syk phosphorylation to wild-type levels during collagen-induced platelet aggregation. In contrast, when contacts are blocked, Syk phosphorylation falls to equally low levels in *Sema4D*^(-/-) and *Sema4D*^(+/+) platelets. The defect in Syk activation in *Sema4D*^(-/-) platelets impairs phospholipase C γ 2 (PLC γ 2) activation and reduces the rise in cytosolic Ca²⁺ that otherwise accompanies platelet activation by collagen. Earlier events in the collagen receptor GPVI pathway, including phosphorylation of FcR γ , occur normally in the *Sema4D* knockout.

Collectively, these observations emphasize the importance of contact-dependent signaling in maximizing platelet responses to collagen and suggest that once integrin-dependent contacts begin to form between platelets, *Sema4D*-mediated feedback reinforces Syk activation downstream of platelet collagen receptors. Hence, integrin blockade inhibits Syk activation in part by reducing integrin outside-in signaling and in part by preventing the binding of *Sema4D* to its receptors. Although *Sema4D*-mediated signaling is dependent on $\alpha_{IIb}\beta_3$ engagement, it is distinct from outside-in signaling by the integrin, which may explain why the Syk phosphorylation defect and resulting aggregation defects in *Sema4D*^(-/-) platelets are limited to collagen and other GPVI agonists.

Methods

Materials

Antibodies recognizing phosphotyrosine (4G10P) and FcR γ were from Millipore, and an anti-Syk antibody (N19) was from Santa Cruz Biotechnology. The phospho-PLC γ 2 (Y1217) antibody and phospho-Syk (Y519/520) were from Cell Signaling Technology, and anti-*Sema4D* (A8) was from BD Biosciences. Fura-2-acetoxymethyl ester (Fura 2-AM) was from Invitrogen. Fibrillar Type I collagen was obtained from Chrono-log. Acid-soluble collagen was from Inamed Biomaterials (PureCol) and convulxin from Alexis Biochemicals. AYPGQV was obtained from Biopptide Co. Fibrinogen was obtained from Enzyme Research. Apyrase, aspirin, and prostaglandin E₁ (PGE₁) were obtained from Sigma-Aldrich, and Integrilin (eptifibatid, Schering-Plough) was obtained from the pharmacy of the Hospital of the University of Pennsylvania. Lec3.2.8.1 Chinese hamster ovary (CHO) cells stably transfected with h*Sema4D*(1-657) containing a C-terminal polyhistidine (His) tag¹¹ were kindly provided by Luca Tamagnone (University of Torino) and E. Yvonne Jones (University of Oxford). TALON metal affinity resin used to purify recombinant *Sema4D* was obtained from Clontech.

Mice

Sema4D^(-/-) mice were described previously^{2,12} and have been backcrossed onto a C57 BL/6 background for at least 10 generations. Comparisons were made between *Sema4D*^(+/+) and *Sema4D*^(-/-) mice obtained

from heterozygous crosses. Animal studies were carried out in compliance with University of Pennsylvania Institutional Animal Care and Use Committee approved protocols.

Isolation of human platelets

Blood obtained from healthy volunteers in accordance with the University of Pennsylvania Institutional Review Board was anticoagulated with acid-citrate-dextrose buffer (ACD; 65mM sodium citrate, 70mM citric acid, 100mM dextrose, pH 4.4) at a final ratio of 1:5 (blood:ACD) and centrifuged at 150g for 20 minutes to obtain platelet-rich plasma. Washed platelets were prepared by sedimentation at 340g for 15 minutes. Platelets were washed with HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-EDTA (ethylenediaminetetraacetic acid)-NaCl (HEN) buffer (150mM NaCl, 1mM Na₂EDTA, 10mM HEPES, pH 6.5) containing 1 μ M PGE₁ and 1 U/mL apyrase and resuspended in modified Tyrode buffer (137mM NaCl, 20mM HEPES, 5.6mM glucose, 1 g/L bovine serum albumin [BSA], 1mM MgCl₂, 2.7mM KCl, 3.3mM NaH₂PO₄, pH 7.4).

Isolation of mouse platelets

Blood was collected from the inferior vena cava of anesthetized mice (100 mg/kg pentobarbital) using a heparinized syringe (15 U/mL blood), diluted (1:1) with HEPES-Tyrode buffer and spun at 150g for 7 minutes. Platelet-rich plasma was isolated and washed with HEN buffer containing PGE₁ (1 μ M) by centrifugation at 340g for 12 minutes followed by resuspension in modified Tyrode buffer.

Cytosolic calcium concentration

Mouse platelets were isolated and washed as described above (see "Isolation of mouse platelets") and incubated with 10 μ g/mL Fura-2-AM in Tyrode buffer for 45 minutes at room temperature. Platelets were then washed once with HEN buffer and resuspended in HEPES-Tyrode buffer at a concentration of 5 \times 10⁷ platelets/mL. Changes in cytosolic Ca²⁺ were detected under stirring conditions using an Aminco-Bowman Series 2 luminescence spectrometer with excitation at 340 and 380 nm and emission at 510 nm.

Immunoprecipitation and immunoblotting

Platelets were lysed with NP-40 buffer (1% NP-40, 50mM Tris [hydroxymethyl(aminomethane)], 150mM NaCl, 1mM EDTA) containing protease (Sigma-Aldrich) and phosphatase inhibitors (Calbiochem). After centrifugation at 16 000 \times g for 15 minutes at 4°C, supernatants were incubated with antibody overnight at 4°C. Protein/antibody complexes were isolated with protein G agarose (Invitrogen) for 2 hours at 4°C. After 3 washes with lysis buffer, the beads were boiled in sample buffer (2% sodium dodecyl sulfate [SDS], 1% β -mercaptoethanol, 0.008% bromophenol blue, 80mM Tris pH 6.8, 1mM EDTA) and the proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% milk, probed as indicated, visualized by enhanced chemiluminescence (GE Healthcare) and quantified using ImageJ 1.42q software (National Institutes of Health). When antibody 4G10P was used, 3% bovine serum albumin was used in the blocking buffer instead of milk.

Flow cytometry

Platelet-rich plasma obtained from mouse whole blood was diluted with Tyrode buffer to a final concentration of 1 \times 10⁷ platelets/mL. Alexa Fluor 488-labeled fibrinogen (100 μ g/mL, Invitrogen) was added to 100 μ L of platelets with or without convulxin and incubated at 37°C for 10 minutes. Samples were fixed with 1% paraformaldehyde for 15 minutes at room temperature and washed with phosphate-buffered saline (PBS) before analysis on a FACSort flow cytometer (BD Biosciences).

Flow chamber

Studies were performed as previously described using a microfluidic flow chamber device.¹³ Each channel had a cross-sectional area of 80 μ m

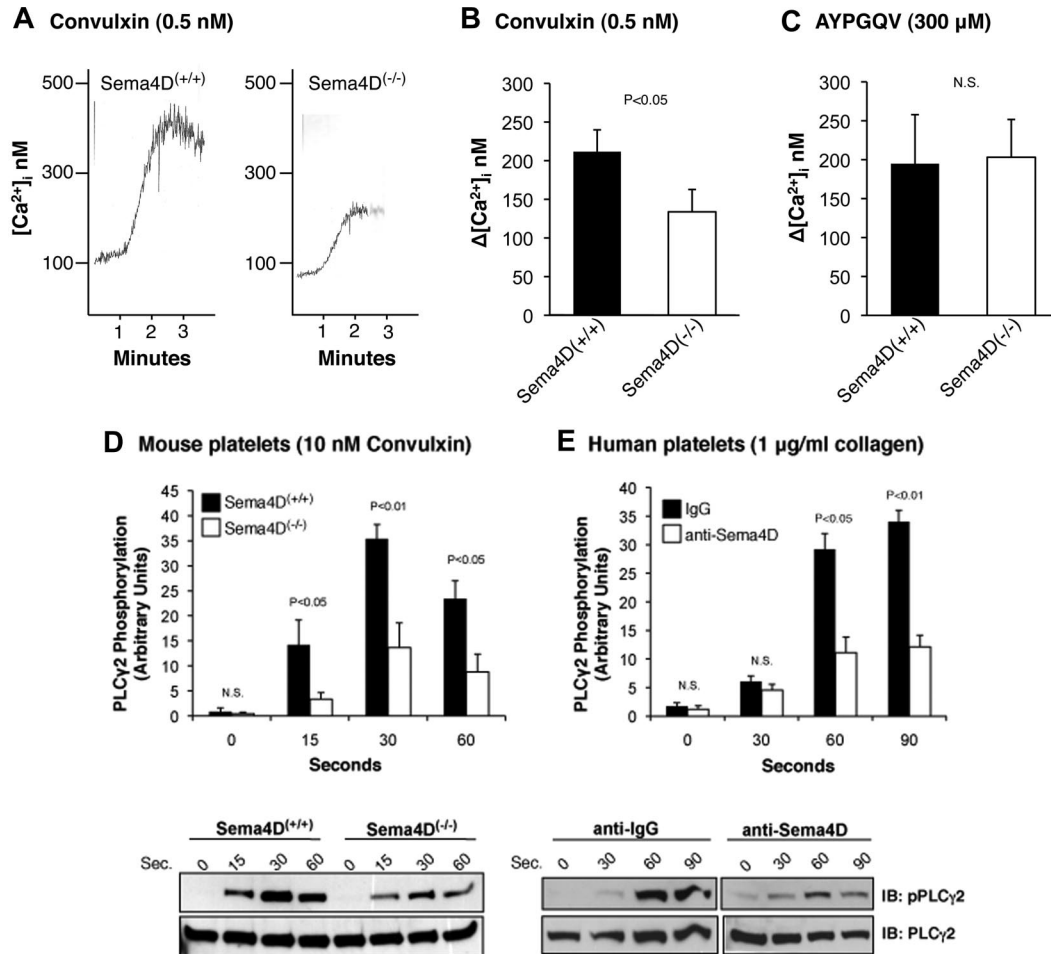


Figure 1. The Ca^{2+} response and phosphorylation of PLC γ 2 after convulxin stimulation is decreased in Sema4D $^{-/-}$ platelets. (A) Platelets from matched Sema4D $^{+/+}$ and Sema4D $^{-/-}$ mice were loaded with Fura-2 and then stimulated with 0.5nM convulxin (CVX). (B) Summary data (mean \pm SEM) from 6 studies with CVX. (C) Summary data from platelets stimulated with the PAR4 agonist peptide, AYPGQV (300 μ M, mean \pm SEM, N = 6). N.S. = not significant. (D) Platelets from matched Sema4D $^{+/+}$ and Sema4D $^{-/-}$ mice were stimulated with 10nM CVX. Phosphorylation of PLC γ 2 was measured at the times indicated. A representative immunoblot is shown and the results of 5 experiments are summarized (mean \pm SEM). (E) Human platelets preincubated for 15 minutes with either a Sema4D blocking antibody (10 μ g/mL) or an immunoglobulin control (10 μ g/mL) were stimulated with 1 μ g/mL collagen (mean \pm SEM, N = 3). N.S. = not significant.

(height) times 100 μ m (width) and was positioned perpendicular to a 100- μ m strip coated with acid soluble type I collagen (0.3 mg/mL). Whole mouse blood was anticoagulated with 93 μ M PPACK (H-(D)-Phe-Pro-Arg-chloromethylketone, Calbiochem), labeled with Alexa Fluor 488-conjugated CD41 monoclonal antibody (10 μ g/mL final concentration, BD Biosciences) and then perfused through the chamber at an average wall shear rate of 800 second^{-1} for 5 minutes. When indicated, α_{IIb} blocking antibody, Leo.H4 (EMFRET Analytics), was added at a final concentration of 25 μ g/mL before perfusion. Real-time fluorescence images of the dynamics of platelet adhesion and aggregation within the chamber were observed using a Nikon Eclipse TE2000-U inverted microscope and captured using a Hamamatsu Digital Camera C9300 coupled to SlideBook 4.2 image acquisition software (Intelligent Imaging Innovations).

Platelet spreading

Blood was collected from Sema4D $^{+/+}$ and Sema4D $^{-/-}$ mice as described above (see "Isolation of mouse platelets") and platelet-rich plasma was prepared. Glass coverslips (no. 1.5) were coated with 0.1 mg/mL acid soluble type I collagen (PureCol) overnight at 4°C and then rinsed with phosphate-buffered saline. Platelet spreading was visualized by reflection interference contrast microscopy (RICM) using an inverted microscope (Axiovert 200, Carl Zeiss) with an antilex 63 \times oil-immersion objective and appropriate polarizers. Images were recorded using a charge-coupled device camera (Retiga Exi Fast Cooled Mono 12-bit camera 32-0082B-128 QImaging) at 1 frame/s. The contact area was measured as described.¹⁴

Statistical analysis

Statistical analysis was performed using a 2-tailed Student *t* test.

Results

Syk activation and subsequent downstream events are diminished when Sema4D is blocked or genetically deleted

Because Sema4D $^{-/-}$ platelets have a selective defect in collagen-induced aggregation, we focused our studies on events downstream of the principal collagen receptor, GPVI, beginning with measurements of the increase in cytosolic Ca^{2+} that normally accompanies platelet activation by GPVI agonists. This increase in cytosolic Ca^{2+} occurs due to clustering of GPVI leading to phosphorylation of FcR γ by Src family kinases, followed by the binding of Syk to FcR γ , activation of Syk, formation of a SLP-76 (Src homology 2 domain-containing leukocyte protein of 76 kDa) and Linker for activation of T cells (LAT)-dependent signaling complex, activation of PLC γ 2 and production of 1,4,5-IP $_3$, leading to Ca^{2+} release from the dense tubular system.¹⁵

The data in Figure 1 show that the increase in Ca^{2+} initiated by convulxin, a snake venom lectin that activates GPVI, observed in Sema4D $^{-/-}$ platelets was about half that observed in platelets

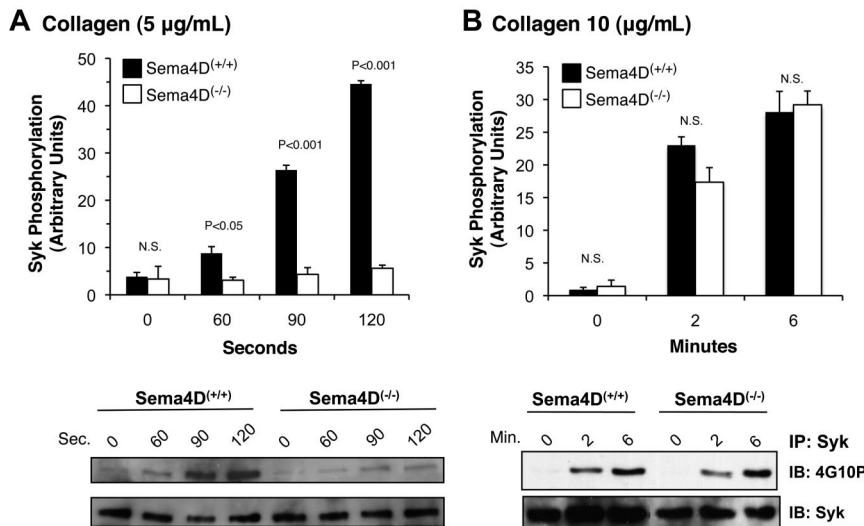


Figure 2. Decreased Syk phosphorylation in Sema4D^{-/-} platelets. Platelets from matched Sema4D^{+/+} and Sema4D^{-/-} mice were stimulated with either (A) 5 µg/mL or (B) 10 µg/mL collagen. Lysates were prepared and immunoprecipitated with anti-Syk followed by immunoblotting with the anti-phosphotyrosine antibody, 4G10P (mean ± SEM, N = 3). N.S. = not significant.

from matched Sema4D^{+/+} controls. In contrast, the Ca²⁺ response to the PAR4 (thrombin receptor) agonist peptide, AYPGQV, was the same in Sema4D^{+/+} and Sema4D^{-/-} platelets. Convulxin-induced phosphorylation of Y1217 in PLCγ2 (a surrogate marker for activation) was also impaired in platelets from the Sema4D^{-/-} mice relative to Sema4D^{+/+} controls (Figure 1D), as was collagen-induced phosphorylation of PLCγ2 in human platelets preincubated with an antibody to Sema4D relative to an immunoglobulin G (IgG) control (Figure 1E).

As already noted, PLCγ2 is activated by collagen or convulxin in platelets through a cascade of signaling proteins that includes the tyrosine kinase, Syk.¹⁶ After collagen stimulation, considerably less Syk phosphorylation was seen in Sema4D^{-/-} platelets than in matched Sema4D^{+/+} controls (Figure 2A). Increasing the collagen concentration overcame this difference (Figure 2B), just as increasing the collagen concentration overcomes the defect in platelet aggregation.² In contrast, Syk phosphorylation in response to a PAR4 agonist peptide, like the rise in cytosolic Ca²⁺, was unaffected by the absence of Sema4D (data not shown). Thus, in Sema4D^{-/-} platelets, there appears to be an agonist-selective defect in Syk activation, leading to the observed defects in PLCγ2 activation and intracellular Ca²⁺ release when platelets are activated by collagen.

Signaling events proximal to Syk activation occur normally in Sema4D^{-/-} platelets

In collagen-stimulated platelets, Syk normally becomes autophosphorylated and activated after binding to the phosphorylated ITAM domain

within FcRγ complexed with GPVI.¹⁷ FcRγ phosphorylation is mediated by the Src family kinases Fyn and Lyn, one or both of which are constitutively bound to GPVI.^{18,19} Because Syk phosphorylation was impaired in Sema4D^{-/-} platelets, we next asked whether either FcRγ phosphorylation or the binding of Syk to phosphorylated FcRγ is also affected. Two approaches were used to measure FcRγ phosphorylation, both of which gave the same results. In the first, FcRγ was immunoprecipitated with anti-FcRγ antibody, and phosphorylated FcRγ was visualized with the anti-phosphotyrosine antibody, 4G10P (Figure 3A). In the second method, platelet lysates were probed directly with 4G10P, which detects a band corresponding to phosphorylated FcRγ at approximately 12 kDa (not shown). In both cases, the extent of FcRγ phosphorylation was the same in Sema4D^{+/+} and Sema4D^{-/-} platelets. There was also no difference between Sema4D^{+/+} and Sema4D^{-/-} platelets in the amount of Syk bound to FcRγ after collagen stimulation (Figure 3B). In Sema4D^{+/+} platelets, the time course of FcRγ phosphorylation, association of Syk with FcRγ, and Syk phosphorylation were similar, all 3 events steadily increasing over the 120-second observation period. Only Syk phosphorylation in the Sema4D^{-/-} deviated from the norm. Thus, the data suggest that the defect in collagen-induced Syk activation observed in the absence of Sema4D occurs after the binding of Syk to phosphorylated FcRγ.

Stable platelet-platelet contacts are needed for optimal activation of Syk

In the immune system, Sema4D mediates contact-dependent signaling between T cells and B cells by binding *in trans* to its cell

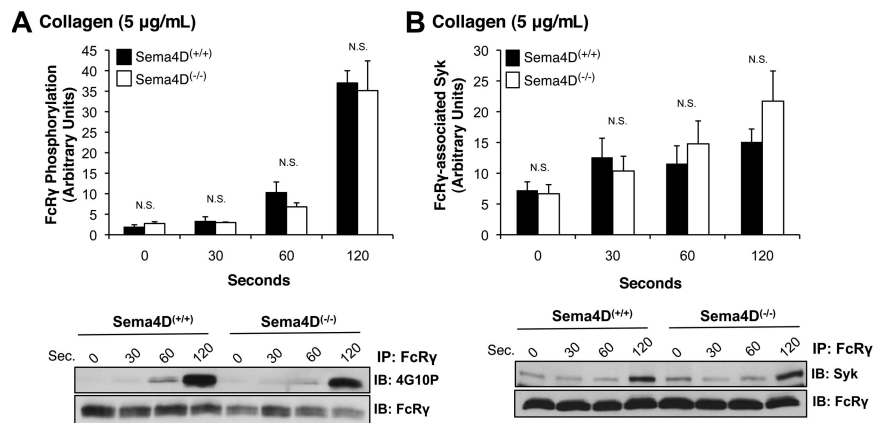
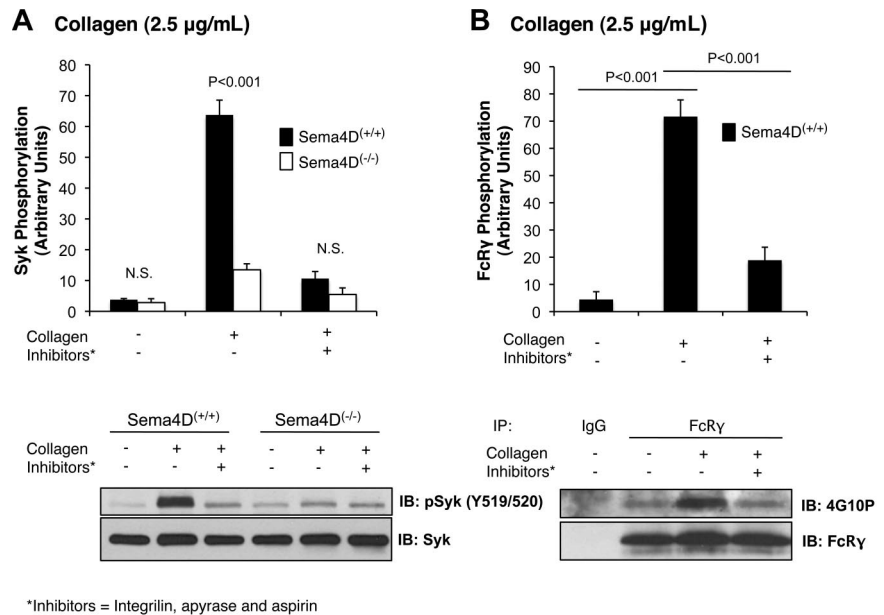


Figure 3. FcRγ phosphorylation and the formation of the FcRγ/Syk complex proceed normally in the absence of Sema4D. Platelets from Sema4D^{+/+} and Sema4D^{-/-} mice were stimulated with collagen (5 µg/mL). (A) Lysates were immunoprecipitated with anti-FcRγ, followed by immunoblotting with the anti-phosphotyrosine antibody, 4G10P (mean ± SEM, N = 3). (B) Lysates were immunoprecipitated with anti-FcRγ followed by immunoblotting with anti-Syk (mean ± SEM, N = 6). N.S. = not significant.

Figure 4. Phosphorylation of Syk and FcR γ is diminished when platelet-platelet contacts are prevented. Platelets from Sema4D $^{-/-}$ and matched Sema4D $^{+/+}$ mice were incubated with Integrilin (10 μ M) plus aspirin (1mM) and apyrase (2 U/mL) for 30 minutes followed by collagen (2.5 μ g/mL) for 2 minutes. (A) Immunoblot with anti-pSyk Y519/520 is shown. (B) FcR γ phosphorylation in Sema4D $^{+/+}$ platelets was detected by immunoprecipitating with anti-FcR γ followed by immunoblotting with anti-phosphotyrosine antibody, 4G10P. In both panels A and B, a representative experiment is shown and the results of 3 experiments are summarized (mean \pm SEM). N.S. = not significant.



surface receptor, CD72.¹⁰ We have proposed that Sema4D functions in a similar contact-dependent manner in platelets.² In each of the experiments presented thus far, contacts between platelets were facilitated by stirring the platelets and allowing aggregation to occur. To test our hypothesis that Sema4D-mediated events are dependent on platelet contacts, we next examined collagen-induced Syk phosphorylation when aggregation was blocked by adding the $\alpha_{IIb}\beta_3$ antagonist, Integrilin (eptifibatid). Aspirin and apyrase were also added to block the contributions of released thromboxane and adenosine diphosphate, respectively. This combination of inhibitors was sufficient to block collagen-induced aggregation completely (data not shown). It also greatly reduced Syk phosphorylation in Sema4D $^{+/+}$ platelets and eliminated the difference between Sema4D $^{+/+}$ and Sema4D $^{-/-}$ platelets in both Syk (Figure 4A) and PLC γ 2 (data not shown) phosphorylation. The level of Syk phosphorylation in Sema4D $^{+/+}$ platelets pretreated with inhibitors was similar to the level observed in Sema4D $^{-/-}$ platelets when no inhibitors were present. Similar results were obtained when contacts were limited by stimulating the platelets with collagen without stirring or when Integrilin was added without aspirin and apyrase (data not shown), suggesting the decrease in Syk phosphorylation is due to a failure to form stable platelet-platelet contacts.

In theory, preventing platelet-platelet contacts would not only block Sema4D-dependent signaling but also outside-in signaling through $\alpha_{IIb}\beta_3$, making the 2 processes difficult to distinguish if they have the same effects. However, we have observed that blocking $\alpha_{IIb}\beta_3$ either with the combination of aspirin, apyrase and Integrilin (Figure 4B), or with Integrilin alone (data not shown) also inhibits FcR γ phosphorylation (Figure 4B), while blocking Sema4D (Sema4D $^{-/-}$ platelets) does not (Figure 3A). In other words, FcR γ phosphorylation is regulated in part by the formation of contacts, but not by Sema4D-dependent signaling, while Syk phosphorylation is affected by both. This notable difference suggests that $\alpha_{IIb}\beta_3$ -dependent outside-in signaling and Sema4D-dependent signaling pathways are distinct.

Integrin engagement promotes Syk phosphorylation in response to collagen

Platelet aggregation occurs when multivalent adhesive proteins such as fibrinogen bind to activated $\alpha_{IIb}\beta_3$ on the surface of

adjacent platelets. Normally this occurs as a consequence of agonist-initiated inside-out signaling within the platelets. However, the activated conformation of integrins can also be promoted by adding Mn $^{2+}$, even in the absence of an agonist.²⁰⁻²² Here we observed that the addition of Mn $^{2+}$ and fibrinogen without an agonist to resting platelets under stirring conditions was sufficient to cause a small increase in Syk phosphorylation ($P < .05$) that was similar in Sema4D $^{+/+}$ and Sema4D $^{-/-}$ platelets (Figure 5A). Under these conditions, both types of platelets were aggregated to the same minimal extent (Figure 5B). When collagen was added after 7 minutes with Mn $^{2+}$, the extent of aggregation increased (Figure 5B). The combination of collagen and Mn $^{2+}$ also caused a large increase in Syk phosphorylation (Figure 5A), as well as PLC γ 2 phosphorylation (data not shown), but did not overcome the defect in Sema4D $^{-/-}$ platelets (Figure 5A). This suggests that achieving maximal Syk phosphorylation in collagen-stimulated platelets requires both Sema4D-dependent and integrin-dependent events. As a further test of this idea, we added recombinant Sema4D exodomain (rSema4D) as a soluble homodimer¹¹ to Sema4D $^{+/+}$ and Sema4D $^{-/-}$ platelets and measured collagen-induced Syk phosphorylation under aggregating conditions. Addition of the Sema4D exodomain to resting platelets did not affect Syk phosphorylation (data not shown); however, after collagen stimulation, it restored Syk phosphorylation in Sema4D $^{-/-}$ to levels observed in wild-type platelets (Figure 5C). It also increased collagen-induced platelet aggregation (data not shown).

Collagen-induced activation of individual platelets proceeds normally in the absence of Sema4D

To further test our model that the contribution of Sema4D to platelet activation is contact dependent, we compared the behavior of Sema4D $^{+/+}$ and Sema4D $^{-/-}$ platelets in 3 assays performed under conditions in which platelet-platelet contacts were prevented or did not occur: $\alpha_{IIb}\beta_3$ activation, spreading on collagen under static conditions, and adhesion to collagen under flow.

In the first assay, $\alpha_{IIb}\beta_3$ activation was detected using labeled fibrinogen after addition of convulxin to cluster GPVI. In these experiments, dilute platelets were used without stirring to minimize platelet-platelet contacts. The results show that over a range of

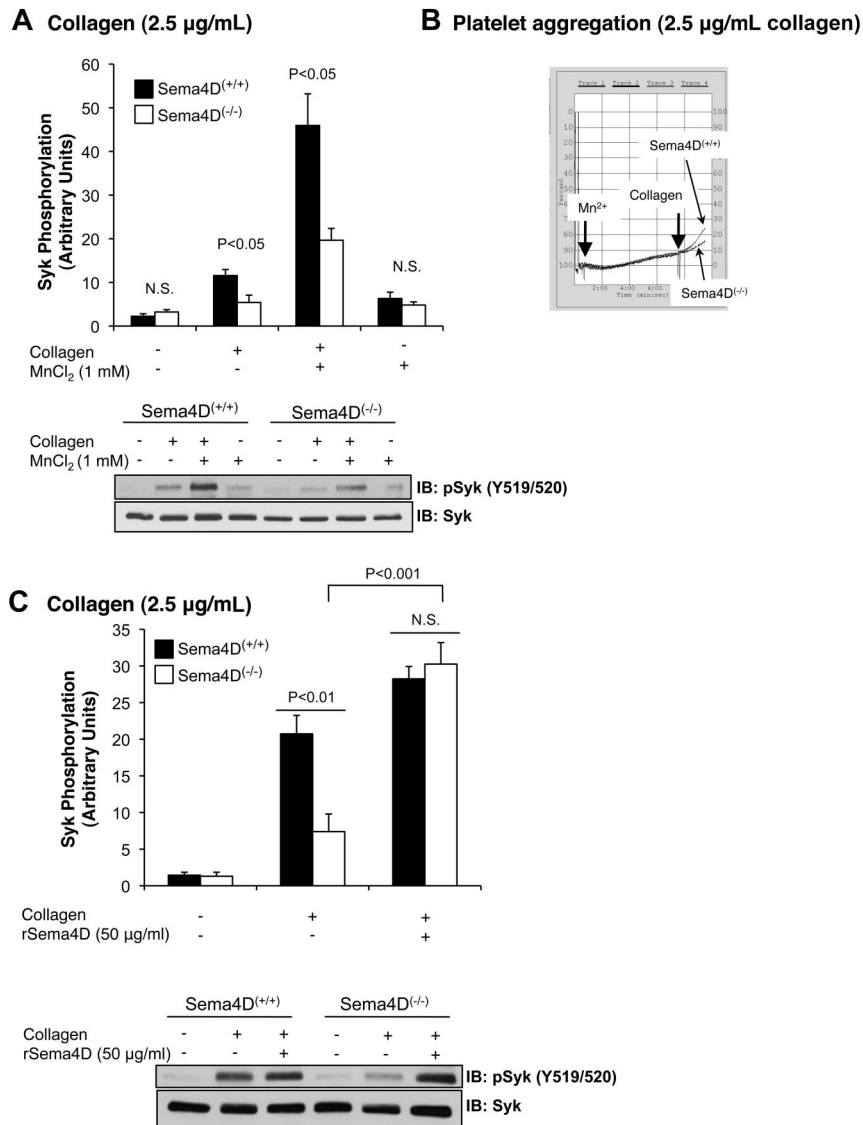


Figure 5. Promoting integrin engagement and Sema4D-mediated interactions increased Syk activation platelets. Washed platelets from Sema4D^(+/+) and Sema4D^(-/-) mice were supplemented with fibrinogen (150 $\mu\text{g/mL}$) and CaCl₂ (1mM). Where indicated, MnCl₂ (1mM) was added and the platelets were stirred for 7 minutes followed by a 2-minute incubation with or without collagen (2.5 $\mu\text{g/mL}$). (A) A representative immunoblot with anti-pSyk Y519/520 is shown, and the results of 4 experiments are summarized (mean \pm SEM). (B) Representative aggregation traces for Mn²⁺-treated samples are shown. N.S. = not significant. (C) Washed platelets from Sema4D^(+/+) and Sema4D^(-/-) mice were incubated with 50 $\mu\text{g/mL}$ rSema4D (recombinant Sema4D exodomain) for 10 minutes followed by collagen (2.5 $\mu\text{g/mL}$) for 2 minutes. A representative immunoblot with anti-pSyk Y519/520 is shown and results of 3 experiments are summarized (mean \pm SEM). N.S. = not significant.

convulxin concentrations, the extent of integrin activation (fibrinogen binding) was the same in Sema4D^(+/+) and Sema4D^(-/-) platelets (Figure 6A). In the second assay, dilute Sema4D^(+/+) and Sema4D^(-/-) platelets were allowed to adhere and spread on immobilized collagen under static conditions. The adhesion and spreading of isolated individual platelets was observed in real time using reflection interference contrast microscopy (RICM) and recorded digitally. Area measurements of these platelets showed no differences between Sema4D^(+/+) and Sema4D^(-/-) platelets in either the rate or the extent of spreading (Figure 6B).

Finally, we perfused whole blood anticoagulated with PPACK (H-(D)-Phe-Pro-Arg-chloromethylketone) over a collagen-coated surface in a microfluidics flow chamber at arterial shear rates (800 sec^{-1}).¹³ Under these conditions, an initial monolayer of platelets adhered to the collagen, after which additional platelets accumulated on top of those that arrived first, forming platelet-platelet contacts. The rate and extent of platelet accumulation was reduced by half in the absence of Sema4D, reflecting a role for Sema4D in producing optimal platelet activation on collagen (Figure 6C-E, and supplemental Videos available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). The flow studies were then repeated after blocking platelet-platelet contacts with antibody Leo.H4, which pre-

vents fibrinogen binding to mouse $\alpha_{\text{IIb}}\beta_3$. Blocking the integrin largely eliminated the formation of platelet aggregates, leaving little more than a monolayer of platelets. Under these conditions, there was no significant difference in the extent of accumulation of Sema4D^(+/+) and Sema4D^(-/-) platelets ($P = .7$ at 4 minutes, Figure 6D-E and supplemental Videos). Thus, the results of all 3 assays suggest that the loss of Sema4D affects platelet behavior only in settings in which platelet-platelet contacts are allowed to form.

Discussion

Formation of a hemostatic plug after vascular injury typically begins with the local generation of thrombin and exposure of collagen, followed by the capture of moving platelets by collagen and the subsequent accumulation of additional platelets. Once activated, platelets come in persistent contact with each other, allowing molecules on the surface of adjacent platelets to interact *in trans* and modulate thrombus growth and stability.²³ The interaction of cell surface ephrin B1 and its receptors, Eph A4 and Eph B1, is one example of contact-dependent signaling that we have described previously.²⁴ We have proposed that Sema4D and

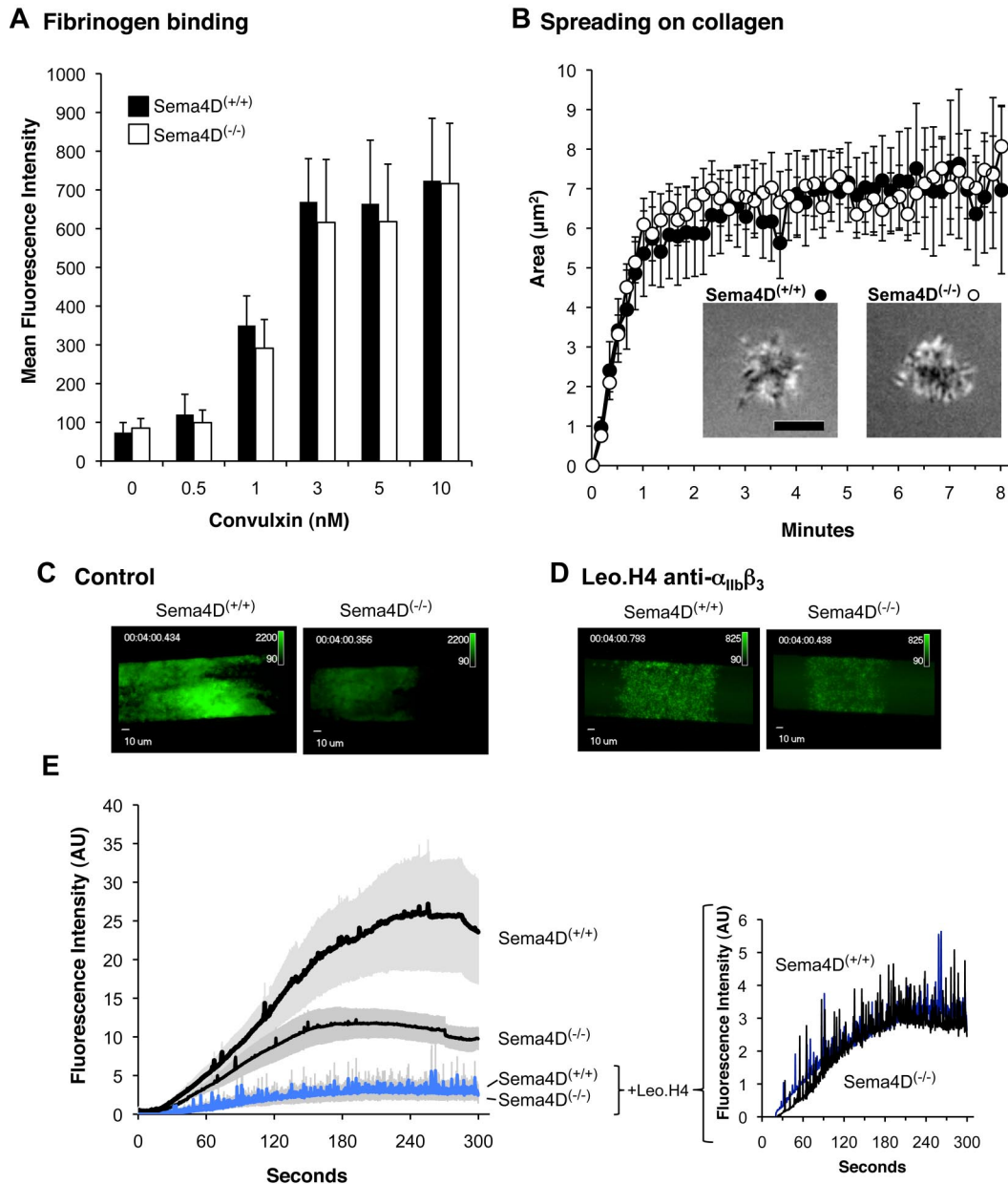


Figure 6. Fibrinogen binding, spreading on collagen and the formation of an initial platelet monolayer on a collagen-coated surface under flow proceeds normally in Sema4D^(-/-) platelets. (A) Fibrinogen binding. Platelets from Sema4D^(+/+) and Sema4D^(-/-) mice were incubated with Alexa Fluor 488–labeled fibrinogen (100 μ g/mL) and convulxin (CVX). Fibrinogen binding was measured by flow cytometry (mean \pm SEM, N = 4–8). (B) Spreading of individual platelets on collagen. Platelets from Sema4D^(+/+) and Sema4D^(-/-) mice were deposited on glass coverslips coated with acid soluble type I collagen (0.1 mg/mL). Platelet spreading was quantified by reflection interference contrast microscopy (RICM). Representative images are shown and the results of 3 experiments are summarized (mean \pm SEM). Scale bar equals 3 μ m. (C–E) Platelets in PPACK-treated whole blood obtained from Sema4D^(-/-) and matched Sema4D^(+/+) mice were labeled with Alexa Fluor 488–conjugated anti-CD41 (α_{IIb}) and perfused through a microfluidic flow chamber at 800 second⁻¹. Platelet accumulation was detected in real time. Where indicated, a second, anti-CD41 antibody (Leo.H4, unlabeled) was used to block $\alpha_{IIb}\beta_3$ and prevent platelet aggregates from forming. (C–D) Video captures after 4 minutes of platelet accumulation. Fluorescence intensities for Sema4D^(+/+) and Sema4D^(-/-) platelets have been adjusted equally for presentation. (E) Changes in fluorescence intensity over time as platelets accumulated on the collagen-coated surface (mean \pm SEM, N = 4). See supplemental videos.

its receptors provide another.² Here we investigated the mechanisms involved in Sema4D action, tested the role of contacts in Sema4D-mediated events, and examined the relationship between Sema4D-dependent signaling and integrin-mediated outside-in signaling.

The results showed that one of the early steps in GPVI signaling, phosphorylation of Syk, is greatly reduced in Sema4D^(-/-) platelets. Earlier steps, including phosphorylation of the ITAM motif in the cytoplasmic domain of FcR γ by Src family kinases, occur normally, as does Syk binding to phosphorylated FcR γ .

As a result of the Syk activation defect, downstream events were also affected, which led to decreased PLC γ 2 activation and a diminished increase in the cytosolic Ca²⁺ concentration. Raising the collagen concentration or adding soluble Sema4D exodomain dimers overcame the defect in Syk phosphorylation, just as it overcame the aggregation defect in Sema4D^(-/-) platelets.²

Notably, loss of Sema4D expression only affected platelet function when the studies were performed under conditions in which stable platelet-platelet contacts were allowed to form. When $\alpha_{IIb}\beta_3$ engagement was blocked or discouraged, individual Sema4D^(-/-) platelets

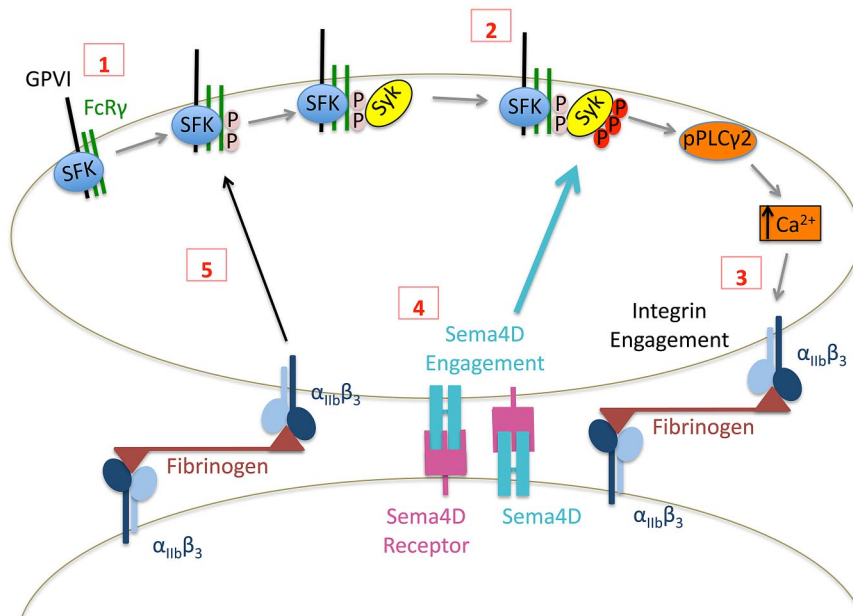


Figure 7. Proposed contact-dependent role of Sema4D in GPVI signaling. (1) Based on work by others, clustering of GPVI leads to Src family kinase (SFK)-mediated phosphorylation of FcR γ resulting in the recruitment and (2) subsequent phosphorylation of Syk. Subsequent signaling through phospholipase C γ leads to integrin activation and (3) the formation of stable, integrin-dependent contacts between platelets. (4) The data presented here suggest that this allows Sema4D to engage *in trans* with its receptors, amplifying Syk activation. It also allows outside-in signaling by the integrin to promote Syk phosphorylation, in part by increasing FcR γ phosphorylation (5).

were indistinguishable from wild type in terms of the (much diminished) extent of Syk activation, the formation of a platelet monolayer under flow on a collagen-coated surface, and spreading on collagen under static conditions. Activation of $\alpha_{IIb}\beta_3$ in single platelet suspensions was also normal in Sema4D $^{-/-}$ platelets. Thus, Sema4D appears to promote collagen-induced platelet activation by increasing Syk activation in a contact-dependent manner after Syk binds to phosphorylated FcR γ . How this might occur is discussed below (see “A model for Sema4D in platelets”).

Integrin outside-in signaling versus Sema4D-mediated signaling

The relationship between outside-in signaling and Sema4D-dependent signaling is complex. The binding of Sema4D and other cell surface ligands to their receptors on adjacent cells can only occur when integrin engagement stabilizes platelet-platelet contacts. Previous studies have shown that outside-in signaling through $\alpha_{IIb}\beta_3$ also promotes Syk phosphorylation.^{25,26} Therefore, it might seem difficult to distinguish outside-in signaling from Sema4D-mediated signaling. However, we found that stimulating integrin engagement and outside-in signaling by adding Mn $^{2+}$ in combination with collagen increased Syk phosphorylation without eliminating the difference between Sema4D $^{+/+}$ and Sema4D $^{-/-}$ platelets, while adding soluble Sema4D boosted collagen-stimulated in Sema4D $^{-/-}$ platelets to the same level as in Sema4D $^{+/+}$ platelets. We also found that inhibiting integrin engagement impaired FcR γ phosphorylation, which was not affected in the Sema4D knockout. Thus, Sema4D signaling and integrin outside-in signaling are intertwined, but distinguishable. In other words, while Sema4D signaling in platelets depends on prior integrin engagement, Sema4D makes its own contribution at a distinct point in the collagen signaling pathway once contacts have formed. This may be part of the explanation for why the aggregation defect in Sema4D $^{-/-}$ platelets is limited to collagen and other GPVI agonists.

A model for Sema4D in platelets

Taken together, these observations suggest a model in which Sema4D functions in a contact-dependent signaling pathway that reinforces Syk phosphorylation downstream of GPVI as illustrated in

Figure 7. Although adhesion and the initial responses of platelets to collagen can occur normally in the absence of Sema4D, once platelets begin to form stable integrin-mediated contacts with other platelets, Sema4D can bind to its receptors. Along with integrin outside-in-mediated signaling, this amplifies collagen responses by increasing Syk activation and PLC γ 2 activity. *In vitro*, the defect in Syk activation in the absence of Sema4D can be overcome by increasing the collagen concentration. This may be due to increased GPVI clustering and enhanced initial signaling that bypasses the need for the Sema4D-mediated boost in signaling. The decrease in platelet accumulation after laser or rose Bengal injury in Sema4D $^{-/-}$ cremaster muscle arterioles and the prolonged time to occlusion in carotid arteries exposed to FeCl $_3$ that we have described previously all argue for the relevance *in vivo* of platelet Sema4D.^{2,3}

In theory, Sema4D could contribute to Syk phosphorylation by either promoting phosphorylation or inhibiting dephosphorylation. CD72 10 and Plexin-B1 4 are the best characterized receptors for Sema4D and we have previously reported the expression of both these receptors in human platelets.² We have proposed that either or both of these receptors are involved in Sema4D signaling in platelets as they are in other cell types, but definitive evidence is still lacking. We have also shown previously that CD72 is associated with SHP-1 in resting human platelets and that addition of either soluble Sema4D or a platelet agonist causes dissociation of the CD72/SHP-1 complex,² similar to events in B cells.^{9,10} However, neither the CD72 knockout nor the plexin-B1 knockout alone appear to phenocopy the effects of the Sema4D knockout (H.J. and L.F.B., unpublished observations, December 2007). Therefore, it remains to be established whether Sema4D engages either of these receptors or some other receptor *in trans* when aggregation begins. It is also formally possible that signaling mediated by Sema4D occurs in a retrograde fashion when it engages receptors on nearby platelets. Such signaling is known to occur when ephrins engage with Eph kinases in platelets, and has also been shown for another semaphorin family member, Sema6D, during cardiac development.²⁷ Evidence in T cells has suggested the possibility of signaling downstream of Sema4D.^{28,29}

In conclusion, we have shown that Sema4D contributes toward collagen signaling in platelets, and we propose that Sema4D plays a key role in the contact-dependent reinforcement of Syk activation

downstream of GPVI. We have also provided evidence that contacts between platelets are important for Sema4D-mediated events, as is the case when T cells expressing Sema4D come in contact with B cells expressing CD72.³⁰ Much still remains to be learned about the role of Sema4D in platelets. The absence of gross bleeding when Sema4D is genetically deleted in mice suggests that Sema4D is not essential for normal hemostasis. However, the observation that the loss of Sema4D expression impairs platelet responses to vascular injury,² reduces platelet hyperactivity associated with dyslipidemia, and confers protection against the development of atherosclerosis³ suggests that Sema4D plays a larger role in response to pathological injury. Future studies will aim to further understand how Sema4D functions to promote collagen signaling in platelets.

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Authorship

Contribution: K.M.W. designed the study, performed experiments, analyzed the data, and wrote the paper; L.Z. designed the study, performed experiments, analyzed the data, and wrote the paper; H.J. performed experiments and analyzed the data; K.P.F. performed experiments and analyzed the data; T.J.S. designed the study; D.L. performed experiments and analyzed the data; A.N.T. performed experiments; K.B.N. provided reagents/analytical tools; S.M. provided reagents/analytical tools; A.K. provided reagents/analytical tools; H.K. provided reagents/analytical tools; D.A.H. provided reagents/analytical tools; S.L.D. provided reagents/analytical tools; and L.F.B. designed the study, analyzed data, and wrote the paper.

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