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Dok-1 Negatively Regulates Platelet Integrin αIIbβ3 Outside-in Signaling and Inhibits Thrombosis in Mice

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Summary

Adaptor proteins play a critical role in the assembly of signaling complexes after engagement of platelet receptors by agonists such as collagen, ADP and thrombin. Recently, using proteomics, the Dok (downstream of tyrosine kinase) adapter proteins were identified in human and mouse platelets. *In vitro* studies suggest that Dok-1 binds to platelet integrin β3, but the underlying effects of Dok-1 on αIIbβ3 signaling, platelet activation and thrombosis remain to be elucidated. In the present study, using Dok-1-deficient (*Dok-1*$^{-/-}$) mice, we determined the phenotypic role of Dok-1 in αIIbβ3 signaling. We found that platelets from *Dok-1*$^{-/-}$ mice displayed normal aggregation, activation of αIIbβ3 (assessed by binding of JON/A), P-selectin surface expression (assessed by anti-CD62P), and soluble fibrinogen binding. These findings indicate that Dok-1 does not affect "inside-out" platelet signaling. Compared with platelets from wild-type (WT) mice, platelets from *Dok-1*$^{-/-}$ mice exhibited increased clot retraction ($P < 0.05$ vs WT), increased PLCγ2 phosphorylation, and enhanced spreading on fibrinogen after thrombin stimulation ($P < 0.01$ vs. WT), demonstrating that Dok-1 negatively regulates αIIbβ3 "outside-in" signaling. Finally, we found that *Dok-1*$^{-/-}$ mice exhibited significantly shortened bleeding times and accelerated carotid artery thrombosis in response to photochemical injury ($P < 0.05$ vs. WT mice). We conclude that Dok-1 modulates thrombosis and hemostasis by negatively regulating αIIbβ3 outside-in signaling.

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Conflict of Interest Disclosures

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Keywords

fibrinogen; hemostasis; integrin; platelet; thrombin; thrombosis

Introduction

The integrin αIIbβ3 is a platelet surface fibrinogen receptor that plays a critical role in hemostasis and thrombosis (1). Several αIIbβ3 inhibitors have been designed and some have been FDA approved for use in humans (2); these inhibitors have significant limitations, however, because their use is associated with increased risk of major bleeding. Therefore, it is important to better understand the mechanisms by which αIIbβ3 signaling is regulated in order to develop new therapeutic approaches.

αIIbβ3 signaling is bidirectional, a process termed “inside-out” or “outside-in” signaling (3, 4). αIIbβ3 inside-out signaling, which has been studied extensively (5), occurs when platelet agonists such as ADP or thrombin induce the activation of αIIbβ3 from a low affinity to a high affinity state, and thus allow the binding of soluble fibrinogen and other αIIbβ3 ligands such as von Willebrand factor, vitronectin, and fibronectin (6). The cytoplasmic tail of β3 plays a key role in inside-out signaling through its interactions with the cytoskeletal proteins talin and kindlin-3. Talin binding to the cytoplasmic tail of β3 is known to be the final common step in αIIbβ3 activation and ligand binding (5). Kindlin-3 also binds to the β3 cytoplasmic tail, at a site distinct from talin, and is essential for platelet integrin activation (7, 8). Binding of fibrinogen to activated αIIbβ3 mediates platelet aggregation and also initiates outside-in αIIbβ3 signaling, which results in the tyrosine phosphorylation of several proteins (9, 10), including phospholipase C-gamma 2 (PLCγ2) and β3 itself (11), and triggers downstream platelet activation responses such as increased cytoskeletal reorganization (12), spreading, and clot retraction (13). In addition to its key role in mediating inside-out signaling, the cytoplasmic tail of β3 also serves an essential role in outside-in signaling by providing a site for the assembly of signaling molecules, including the Src and Syk kinases and the regulatory G-protein $G\alpha_{13}$ (9, 14-16). However, the specific regulatory proteins that control αIIbβ3 outside-in signaling are still not well understood.

Recently, using proteomics, Watson and colleagues identified a group of Dok (downstream of tyrosine kinase) adaptor proteins in both human platelets (Dok-1, Dok-2 and Dok-3) and mouse platelets (Dok-1 and Dok-2) (17-19). The Dok adaptor proteins lack intrinsic enzymatic activity, but they become phosphorylated during platelet activation (18) and have been proposed to play a role in integrin αIIbβ3 signaling by facilitating protein-protein or protein-lipid interactions (19). Previous studies have demonstrated that the phosphotyrosine-binding (PTB) domain of Dok-1 binds to the cytoplasmic tail of β3 at the same NPXY motif that provides a binding site for the talin head domain (20), and that Dok-1 may inhibit inside-out signaling by competing with endogenous talin for binding to β3 (21). Interestingly, the PTB of Dok-1 was found to bind specifically to peptide analogs of the β3 cytoplasmic tail that contain Tyr747, which is known to be required for outside-in αIIbβ3 signaling (22). However, the role of Dok-1 in regulating platelet signaling has not been

examined *in vivo*, and the importance of its interaction with the β3 cytoplasmic tail in modulating inside-out and/or outside-in signaling remains uncertain.

In this study, we sought to define the phenotypic role of Dok-1 in αIIbβ3 signaling in a murine model. Using platelets from Dok-1-deficient (*Dok-1*$^{-/-}$) mice, we demonstrate that Dok-1 negatively regulates integrin αIIbβ3 outside-in signaling, but not inside-out signaling. Moreover, we show that Dok-1 deficiency in mice shortens the tail-transection bleeding time and accelerates carotid artery thrombosis in a photochemical injury model, indicating that Dok-1 has an antithrombotic function *in vivo*.

Materials and Methods

Reagents

Human thrombin and ADP were obtained from Chrono-Log Corporation (Havertown, PA). Apyrase, PGE1, fibrinogen, and U466619 were obtained from Sigma Chemical Company (St. Louis, MO).

Plasmid constructs

The DNA sequence encoding human *DOK-1* was generated by PCR using a 5' primer (ATCGATCGGAATTCATGGACGGAGCAGTGATGGAAGG) and a 3' primer (ATCGATCGGAATTCTCAGGTAGAGCCCTCTGACTTGACCCCAG). After digestion with EcoRI, the fragment was subcloned into pCMV-tag 2B (Stratagene, La Jolla, CA) to generate FLAG-DOK-1. The nucleotide sequence was confirmed by sequencing at the DNA Core Facility of the University of Iowa. The EGFP-fused talin-H construct has been described previously (23).

Animals

The *Dok-1*$^{-/-}$ mice (129 Sv/Imj background) used in this study have been described and characterized previously (24, 25). Control aged-matched wild-type (WT) mice on the 129 Sv/Imj background were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice used were between 8 and 16 weeks of age. Mice were maintained in a specific-pathogen free facility at the University of Iowa. All procedures were approved by the University of Iowa Animal Care and Use Committee.

Preparation of washed platelets and platelet-rich plasma

Murine blood was obtained by cardiac puncture (800 μl) into 160 μl of acid citrate-dextrose solution (2.5% trisodium citrate, 2% dextrose, 1.5% citric acid (monohydrate)), then mixed with 4 ml of Pipes-saline buffer (150 mM NaCl, 20 mM PIPES, pH; 6.5). After centrifugation at 200 × g for 15 minutes, the supernatant fraction was carefully collected and 1 U/ml apyrase and 1 μM PGE1 were added. Washed platelets were prepared after centrifugation at 800 × g for 10 minutes and suspended in modified Tyrode's buffer (150 mM NaCl, 2.9 mM KCl, 0.34 mM Na_2PO_4, 12 mM $NaHCO_3$, 20 mM Hepes, 1 mM $MgCl_2$, pH; 7.35) supplemented with 5 mM Glucose and 0.5 mg/ml BSA. To prepare platelet-rich plasma, murine blood (700 μl) was obtained by cardiac puncture into 100 μl of 3.8% citrate buffer and mixed with 700 μl of saline, centrifuged at 100 × g for 10 minutes, and the

supernatant plasma collected. Platelet counts were measured using a HemaVet cell counter (Drew Scientific, Oxford, CT).

Platelet flow cytometry

30 μl of 5×10^5/ml washed platelets were stimulated with thrombin, ADP, or ADP plus U46619 for up to 15 minutes at 37°C, or left unstimulated as a control. Samples were then incubated with an activation-specific murine αIIbβ3 antibody, JON/A-PE (Emfret Analytics, Germany), and anti-CD62P-FITC (BD Pharmingen) for 10 minutes at room temperature, or soluble fibrinogen-FITC (Molecular Innovation; MFBGN-FITC) for 15 minutes at room temperature. After fixation with 1% PFA for 15 minutes on ice, the samples were analyzed by flow cytometry using a FACScan instrument (Beckton Dickinson). The data were analyzed using FlowJo software (Tree Star).

Platelet aggregation and adenosine triphosphate (ATP) secretion

Washed platelets (2.5×10^8 platelets/ml) were stirred (1,200 rpm) at 37° C for 2 minutes in a Chrono-log Whole Blood/Optical Lumi-Aggregometer (model 700–2) prior to the addition of agonists. Aggregation was induced with different agonists (thrombin, 0.1 & 0.5 U/ml; adenosine diphosphate [ADP], 5 & 10 μM) and aggregation was observed for 5 minutes. When ADP was used as the agonist, platelet aggregation was induced in the presence of 2 mM Ca^{2+} and 0.1 mg/ml fibrinogen. Aggregation was measured as percent change in light transmission, where 100% refers to transmittance through a blank sample. ATP secretion was measured with Chronolume reagent (Stock concentration, 0.2 μM luciferase/luciferin). Luminescence generated by platelet-secreted ATP was monitored using the Lumi-Aggregometer.

Human integrin αIIbβ3 activation

Activation of human integrin αIIbβ3 was assessed using the activation-specific human αIIbβ3 antibody PAC1 (BD Bioscience; #340535) and goat anti-mouse IgG secondary antibody Alexa 633 conjugate (Life Technologies; #A-21050). αIIbβ3-CHO cells were developed and cultured as described previously (23). EGFP-fused talin-H and/or FLAG-tagged DOK-1 expression constructs were transfected into αIIbβ3-CHO cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). pEGFP vector (Clontech Laboratories, Mountain View, CA) or pCMV-tag 2B (Stratagene) was transfected as empty vector to adjust the amount of plasmid DNA to be transfected. 24 hours after transfection, the cells were collected and PAC1 binding was analyzed by flow cytometry (FACS LSR II, Becton Dickinson), gating only on the EGFP positive cells.

Platelet spreading

Glass bottom microwell dishes (MatTek Corporation) were coated with fibrinogen (1 mg/ml) overnight at 4°C, and then blocked for 1 hour with 1% BSA in PBS. Murine platelets (2.5×10^7) in 500 μl of Tyrode's buffer were incubated in the presence or absence of thrombin, and platelets were allowed to adhere to the fibrinogen matrix for various periods of time. Images were obtained at intervals of 5 minutes using an Olympus IX-81 inverted microscope in differential interference contrast mode. Surface area was analyzed using ImageJ software

from NIH (Bethesda, MD). To determine the effect of Dok-1 on lamellipodia formation, detailed images of platelet spreading were taken by scanning electron microscopy. Briefly, microscope cover glasses (Fisher) were coated with fibrinogen as described above. 2.5×10^7 murine platelets in 500 μl of Tyrode's buffer were allowed to adhere to the fibrinogen matrix. After 30 minutes of incubation, non-adherent platelets were removed by gentle washing with Tyrode's buffer, and adherent platelets fixed with 2.5% glutaraldehyde in Tyrode's buffer at 4°C (overnight). The samples were treated with 1% OsO_4 in PBS for 30 minutes, and then washed with water. After dehydration, the samples were treated with hexamethyldisilizane overnight at room temperature. The samples were examined with a Hitachi S-4800 scanning electron microscope and digital images were collected with a CCD camera (Hamamatsu; ORCA-ER C4742-80-12AG) using digital image correlation.

Clot retraction

Clot retraction studies were performed as described (22). Briefly, 1 ml platelet rich plasma was placed in siliconized glass tubes containing 2 mM $CaCl_2$ and an uncoated #1 paper clip (OfficeMax). 5 μl of mouse erythrocytes were added for color contrast. Thrombin (0.5 U/ml) was added to each tube to initiate clotting, and clot retraction was allowed to proceed at 37°C. At appropriate time points, photographic images of retracting clots were recorded. After 2 hours, residual plasma volumes, which indicate the degree to which retraction has occurred, was measured and clot volume was calculated using residual plasma volumes.

Immunoblotting

Platelet lysates for immunoblotting were prepared from either washed platelets, platelets adherent to fibrinogen for 30 minutes, or platelets maintained in suspension in a BSA-coated dish for 30 minutes. Platelets were lysed with a buffer (25 mM Tris•HCl, pH 7.2, 150 mM NaCl, 5mM $MgCl_2$, 1% NP-40, and 5% glycerol) containing protease inhibitors (Roche) and a phosphatase inhibitor cocktail (Sigma). After removing the debris by centrifugation at 16,000 × g for 5 minutes, supernatant fractions were collected as platelet lysates. The protein concentration of the lysates was determined using Micro BCA Protein Assay Reagent Kit (Pierce). For some experiments, washed platelets were incubated with 1 U/ml thrombin, 0.5 mg/ml fibrinogen, and 2 mM Ca^{2+} to mimic conditions of clot retraction. Either 1 minute or 3 minutes later, the reaction samples were centrifuged at 16,000 × g for 3 minutes. After removing the supernatant fractions, the pellets were suspended with 80 μl of 2× SDS sample buffer, and then boiled at 100°C for 5 minutes. Platelet lysates were subjected to 15 % SDS-PAGE and proteins were transferred to nitrocellulose membrane (Whatman) and immunoblotted with anti-Dok-1 antibody (Santa Cruz Biotechnology; A-3), anti-talin antibody (Chemicon International, MAB1676), anti-FLAG antibody (Sigma; M2), anti-phospho-PLCγ2 (Tyr1217) antibody (Cell Signaling Technology; #3871), anti-PLCγ2 antibody (Cell Signaling Technology; #3872P), anti-phospho-Src (Tyr416) antibody (Cell Signaling Technology; #2101S), anti-Src antibody (Cell Signaling Technology; #2108S), anti-phospho-Syk (Tyr525/526) antibody (Cell Signaling Technology; #2711P) or anti-Syk (D115Q) antibody (Cell Signaling Technology; #12358P). To verify equivalent loading, membranes were stripped with Restore™ Western Blot Stripping Buffer (Thermo Scientific) and blotted with anti-β-actin antibody (Sigma; clone AC-74) or anti-PLCγ2 antibody (Cell

Signaling Technology; #3872). Immunoblots were visualized with enhanced chemiluminescence detection reagents (ECL: GE Healthcare).

Bleeding time

Tail-transection bleeding time was measured as described (22). Briefly, mice (6-7 weeks of age) were anesthetized with ketamine/xylazine (87.5/2.5 mg/Kg) and placed on a heating pad warmed at 37°C, and a 2 mm segment of tail was amputated with a sharp scalpel blade. The tail was immediately immersed in saline (warmed at 37°C), and the time taken for the stream of blood to stop for more than 30 seconds was defined as the bleeding time. If bleeding did not stop within 10 minutes, hemostasis was achieved by cauterizing the tail.

Carotid artery thrombosis

Experimental arterial thrombosis was induced in mice (15-16 week old mice) by photochemical injury using rose bengal (35 mg/kg) as described previously (26). Briefly, mice were anesthetized with sodium pentobarbital (70-90mg/kg intraperitoneally), and temperature was maintained at 36-38°C. During the entire procedure mice were ventilated mechanically with room air and supplemental oxygen using a Harvard rodent respirator. The right femoral vein was cannulated for the administration of rose bengal. Carotid artery blood flow was measured with a 0.5 PSB Doppler flow probe (Transonic Systems Inc) and digital recording system (Gould Ponemah Physiology Platform, version 3.33). To induce photochemical injury to the endothelial layer, the right common carotid artery was transilluminated continuously with a 1.5-mV, 540-nm green laser (Melles Griot) from a distance of 6 cm, and rose bengal (35 mg/kg) was injected via a femoral vein catheter. Blood flow was monitored continuously for 90 minutes or until stable occlusion occurred, at which time the experiment was terminated. Stable occlusion was defined as the time at which blood flow remained absent for ≥ 10 minutes.

Statistical Analysis

Differences between experimental and control groups were analyzed using the 2-tailed Student's t-test for comparisons involving single conditions, and two-way ANOVA or two-way repeated-measures ANOVA for comparisons involving multiple conditions. *P* values less than 0.05 were considered to be significant.

Results

Dok-1 deficiency does not affect inside-out signaling in murine platelets

We first confirmed by immunoblotting that Dok-1 protein is expressed in platelets from WT mice and that the expression of Dok-1 is absent in platelets from *Dok-1*$^{-/-}$ mice (Figure 1A). Dok-2 was expressed at similar levels in platelets from WT and *Dok-1*$^{-/-}$ mice (Figure 1A). Next, we performed platelet activation experiments to determine if Dok-1 modulates the inside-out activation of αIIbβ3. We performed flow cytometric analysis using an activation specific murine αIIbβ3 antibody, JON/A, and measured the surface expression of CD62P (P-selectin) to assess early platelet activation response (alpha granule release). As shown in Figure 1B, thrombin (0.05 U/ml) induced nearly identical levels of JON/A binding and CD62P surface expression in *Dok-1*$^{-/-}$ and WT platelets. The data shown in Figure 1B are

representative of platelets stimulated with thrombin for 5 minutes. When platelets were stimulated with thrombin for different time periods (between 1 and 15 minutes) no significant differences in the time course of JON/A binding or CD62P surface expression were observed between *Dok-1*$^{-/-}$ and WT platelets (data not shown). Since activation of αIIbβ3 by inside-out signaling leads to the binding of fibrinogen, we next measured fibrinogen binding to WT and *Dok-1*$^{-/-}$ platelets using flow cytometry. In agreement with the results of the integrin αIIbβ3 activation assay, WT and *Dok-1*$^{-/-}$ platelets bound similar levels of fluorescein-labeled soluble fibrinogen after stimulation with either ADP alone, ADP plus the thromboxane analog U46619, or thrombin (Figure 1C). Finally, we measured platelet aggregation responses to ADP and thrombin, and thrombin induced ATP secretion, and observed no differences between platelets from WT and *Dok-1*$^{-/-}$ mice (Supplemental Figure 1). These findings indicate that deficiency of Dok-1 does not affect early platelet activation or inside-out signaling.

Human DOK-1 *does not modulate αIIbβ3 activation*

We next examined the impact of human Dok-1 on integrin αIIbβ3 activation using CHO cells that constitutively express human αIIbβ3 (αIIbβ3-CHO cells) (23). The activation state of human αIIbβ3 was assessed using an activation-specific human αIIbβ3 antibody, PAC-1 (27). αIIbβ3-CHO cells were analyzed 24 hours after transient transfection with talin-H, FLAG-tagged DOK-1, or both talin-H and FLAG-tagged DOK-1. As shown in Figure 2, transfection with talin-H induced αIIbβ3 activation, but transfection with FLAG-tagged DOK-1 did not affect αIIbβ3 activation either in the absence (Figure 2A) or presence (Figure 2C) of talin-H. These results suggest that, like murine Dok-1, human Dok-1 does not modulate αIIbβ3 activation during inside-out signaling.

Dok-1 negatively regulates platelet spreading on fibrinogen

To determine if Dok-1 deficiency affects integrin αIIbβ3 outside-in signaling, we analyzed the spreading of fibrinogen-adherent platelets, a response that is known to be dependent on αIIbβ3 outside-in signaling (28). Because mouse platelets, in contrast to human platelets, do not spread completely on immobilized fibrinogen without cellular activation (29), we performed experiments in the presence of either 0.01 U/ml or 0.1 U/ml thrombin. With the low concentration of thrombin (0.01 U/ml), we found that *Dok-1*$^{-/-}$ platelets exhibited enhanced spreading compared with WT platelets at each time point examined ($P < 0.01$) (Figure 3A). *Dok-1*$^{-/-}$ platelets also exhibited enhanced spreading compared with WT platelets in the absence of thrombin, although lamellopodia formation was less complete (Supplemental Figure 2). Mean platelet volumes were comparable between WT (4.78 ± 0.18) and *Dok-1*$^{-/-}$ (5.01 ± 0.40) platelets, which suggests that the enhanced spreading of Dok-1$^{-/-}$ platelets was not related to differences in platelet size before spreading. When platelets adhere to fibrinogen, they initially form filopodia and then lamellipodia before adopting a fully spread form (30). Therefore, we further analyzed the spreading of WT and *Dok-1*$^{-/-}$ platelets using scanning electron microscopy. When examined 30 minutes after plating on fibrinogen in the presence of 0.01 U/ml thrombin, WT platelets exhibited prominent filopodia and lamellipodia, with less than 10% of WT platelets in a fully spread form (Figure 3B). By contrast, greater than 50% of *Dok-1*$^{-/-}$ platelets showed full spreading at the same time point. These findings suggest that Dok-1 is a negative regulator of platelet

spreading. Interestingly, in the presence of the higher concentration (0.1 U/ml) of thrombin, no differences in spreading between WT and *Dok-1*$^{-/-}$ platelets were observed (Supplemental Figure 3).

Dok-1 negatively regulates clot retraction

To determine if Dok-1 also regulates other downstream responses to αIIbβ3 outside-in signaling, we assessed platelet-mediated clot retraction. Integrin αIIbβ3 is known to play a critical role in clot retraction by platelets, which is important in consolidating thrombus formation (22). We performed clot retraction assays using platelet-rich plasma in the presence of calcium and thrombin (0.5 U/ml). Under these conditions, the clot retraction observed with *Dok-1*$^{-/-}$ platelets was earlier and more extensive than that seen with WT platelets (Figure 4A, B).

Increased phosphorylation of PLCγ2 in Dok-1$^{-/-}$ platelets

PLCγ2 is a known regulator of platelet spreading and clot retraction. PLCγ2-deficient platelets exhibit reduced spreading on fibrinogen (31) and decreased clot retraction (32), whereas platelets expressing a gain-of-function mutant PLCγ2 (*Plcg2*$^{Ali5/+}$) have enhanced αIIbβ3 outside-in signaling, accelerated spreading on fibrinogen, and faster clot retraction. (33) Since the phenotype of *Dok-1*$^{-/-}$ mice is very similar to that of *Plcg2*$^{Ali5/+}$ mice, we hypothesized that PLCγ2 may become activated downstream of Dok-1 during integrin αIIbβ3 outside-in signaling. Therefore, to examine the mechanism of regulation of αIIbβ3 outside-in signaling, we assessed the phosphorylation state of PLCγ2 in WT and *Dok-1*$^{-/-}$ platelets. *Dok-1*$^{-/-}$ platelets exhibited enhanced transient phosphorylation of PLCγ2 at Tyr1217 after stimulation with 0.5 U/ml thrombin, 0.5 mg/ml fibrinogen, and 2 mM Ca^{2+} to mimic conditions of clot retraction ($P < 0.05$ vs. WT platelets) (Figure 5A, B).

Dok-1$^{-/-}$ mice have shortened bleeding times and accelerated arterial thrombosis

To define the hemostatic phenotype of Dok-1 deficiency in mice, we measured the tail-transection bleeding time. As shown in Figure 6A, *Dok-1*$^{-/-}$ mice exhibited significantly shortened bleeding times compared with WT mice ($P < 0.05$). To confirm that the effect of Dok-1 deficiency on bleeding time was not due to a difference in platelet count, we measured the platelet counts of 8-week-old WT and *Dok-1*$^{-/-}$ mice and found that platelet counts were comparable between WT mice (1,050,000 ± 58,000/μl) and *Dok-1*$^{-/-}$ (942,000 ± 89,000/μl). We then evaluated the susceptibility of WT and *Dok-1*$^{-/-}$ mice to arterial thrombosis by measuring the time to occlusion of the carotid artery following rose bengal-induced photochemical injury. We found that Dok1-deficient mice had significantly shortened times to occlusion compared with WT mice ($P < 0.05$; Figure 6B). Together, these findings suggest that *Dok-1*$^{-/-}$ mice have a prothrombotic phenotype *in vivo*.

Discussion

Dok-1 is expressed in myeloid cells, T and B lymphocytes, and platelets, where it has been shown to interact with the cytoplasmic tail of β3 via its PTB domain (18-20). Since β3 plays an important role in platelet activation (both inside-out and outside-in signaling), we sought to define the phenotypic role of Dok-1 in platelet function and thrombosis in *Dok-1*$^{-/-}$ mice.

Our results demonstrate clearly that Dok-1 modulates platelet function and susceptibility to arterial thrombosis via negative regulation of αIIbβ3 outside-in signaling. We found that, subsequent to fibrinogen binding to activated αIIbβ3, Dok-1 deficient platelets exhibited transiently increased phosphorylation of PLCγ2, increased spreading, and accelerated clot retraction compared with WT platelets. All of these responses are indicative of negative regulation of αIIbβ3 outside-in signaling by Dok-1. By contrast, despite prior in vitro evidence suggesting that Dok-1 may compete with talin for binding to the β3 NPXY motif (19, 20), we found no evidence that Dok-1 regulates inside-out activation of murine or human αIIbβ3.

Many platelet agonists initiate talin-dependent inside-out signaling responses that culminate in αIIbβ3 attaining an active open conformation, allowing high affinity binding of fibrinogen, which in turn initiates outside-in signaling, resulting in cytoskeletal changes, mobilization of calcium stores, and phosphorylation of multiple proteins including the β3 tail itself (22, 34). Dok-1 is recognized to bind to a motif (NPXY) on the β3 tail that is also the binding site for talin and is essential for inside-out integrin αIIbβ3 activation. Therefore, it was hypothesized that Dok-1 might regulate αIIbβ3 activation (inside-out signaling) (21). However, we found that platelets from Dok-1 deficient mice did not exhibit significant differences in αIIbβ3 activation or fibrinogen binding after stimulation with multiple platelet agonists. We also looked for an effect of Dok-1 on talin-mediated activation of human αIIbβ3 in CHO cells, but found no significant alterations of αIIbβ3 activation after co-transfection with talin-H and Dok-1. These results are discrepant with the initial findings of Campbell and colleagues, who suggested a potential role for Dok-1 in integrin activation (21). However, our findings are concordant with later findings by the same group demonstrating that Dok-1 exhibits a higher affinity for β3 when it is phosphorylated at Tyr747, a condition that does not support talin binding (35, 36). Together, these results suggest that Dok-1 either does not directly compete with talin for binding to β3 that is not phosphorylated at Tyr747 or does so inefficiently, perhaps because of the abundant levels of talin in platelets (estimated to be 3-8 % of total platelet protein) (37). In contrast, our findings suggest that Dok-1 does regulate integrin outside-in signaling, which results in the phosphorylation of Tyr747 and enhanced binding of Dok-1 to β3 (35).

Early outside-in signaling events, including those that lead to platelet spreading, appear to be independent of talin but instead involve the binding of kinases and adapter proteins to other docking motifs on the cytoplasmic tail of the β3 integrin (38). Consistent with a role for Dok-1 as a negative regulator of outside-in signaling, *Dok-1*$^{-/-}$ platelets exhibited enhanced spreading on fibrinogen, with accelerated lamellipodia formation and full spreading, and faster clot retraction than WT platelets. Recently, Du and colleagues have elegantly demonstrated that the G-protein $G\alpha_{13}$ promotes platelet spreading by binding to an EXE motif on the β3 cytoplasmic tail during early outside-in signaling (39). Our data suggest that Dok-1 negatively regulates both spreading and clot retraction, and therefore may function independently of the $G\alpha_{13}$ pathway.

It is known that mobilization of intracellular calcium stores through activation of PLCγ2 positively regulates both platelet spreading on fibrinogen (31, 40) and clot retraction (32). The activation of the Src signaling cascade downstream of αIIbβ3, which also involves Syk

and SLP- 76, induces tyrosine phosphorylation of PLCγ2 and subsequent calcium mobilization (31). It is intriguing to speculate that Dok-1 may interact directly with PLCγ2, since it has been demonstrated that phosphorylated Dok-1 binds to PLCγ1, via its SH2 domain, in human myeloid cells (41). In support of this hypothesis, we found that *Dok-1*$^{-/-}$ platelets exhibit increased phosphorylation of PLCγ2 when compared to WT platelets under conditions mimicking clot retraction. The increased phosphorylation of PLCγ2 was quite transient, peaking within 3 minutes (Figure 5), which raises a question about its role in mediating the effects of Dok1 deficiency on late downstream events such as clot retraction. Alternatively, Dok-1 may negatively regulate outside-in signaling and subsequent cytoskeletal rearrangement and clot retraction by inhibiting the Src-Syk-PLCγ2 signaling axis downstream of β3. However, we did not observe any differences in the time course of Src or Syk phosphorylation between WT and Dok-1$^{-/-}$ platelets (Supplemental Figure 4). We also did not observe any differences in active RhoA, a dynamic regulator of the cytoskeleton, between WT and *Dok-1*$^{-/-}$ platelets (Supplemental Figure 5). Finally, we note that there is evidence that a second wave of talin binding to β3 may be associated with late outside-in signaling events, including clot retraction (39). It remains possible, therefore, that Dok-1 may inhibit clot retraction in part by interfering with this late talin-mediated effect on αIIbβs outside-in signaling. Clearly, more work will be needed to clarify the mechanisms of Dok-1's effects on late platelet activation, particularly clot retraction.

Integrin αIIbβ3 is critical for hemostasis, exemplified most clearly by the severe bleeding phenotype of human patients with Glanzmann thrombasthenia (42). We observed that *Dok-1*$^{-/-}$ mice had significantly shortened bleeding times and accelerated carotid artery thrombosis compared with WT mice, which supports a hemostatic role for endogenous Dok-1 *in vivo*. It cannot be determined from these experiments if the prothrombotic phenotype of *Dok-1*$^{-/-}$ mice is caused by a platelet-specific deficiency of Dok-1, since Dok-1 is also expressed in other hematopoietic cells.

In summary, our findings from *Dok-1*$^{-/-}$ mice clearly demonstrate that Dok-1 regulates αIIbβ3 outside-in signaling, and reveal a critical role for Dok-1 in platelet function and regulation of thrombosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PLCγ2	phospholipase C-gamma 2
Dok	downstream of tyrosine kinase
Dok-1$^{-/-}$	Dok-1-deficient
WT	wild-type

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What is known on this topic?

- The integrin αIIbβ3 is a platelet surface fibrinogen receptor that plays a critical role in hemostasis and thrombosis.
- In vitro studies suggest that Dok-1 is an adapter protein that binds to integrin αIIbβ3 in platelets and may regulate integrin αIIbβ3 signaling.

What this paper adds?

- Dok-1-deficient mice exhibit increased αIIbβ3 “outside-in” platelet signaling, shortened bleeding times, and accelerated carotid artery thrombosis.
- Dok-1 modulates platelet function and inhibits thrombosis by negatively regulating αIIbβ3 signaling.
- This study reveals a critical role for the Dok-1 adapter protein in hemostasis and thrombosis.

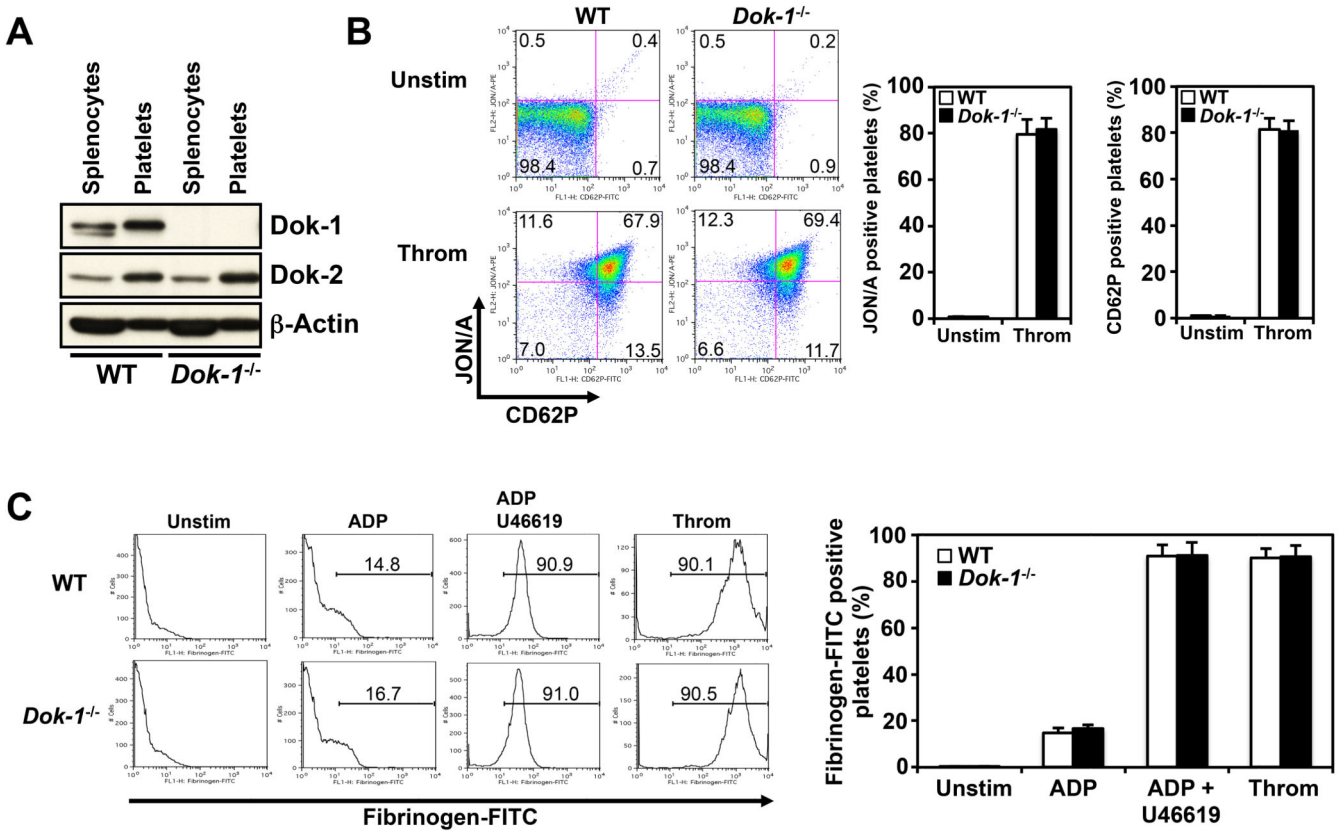

FIGURE 1. Dok-1-deficient platelets display normal inside-out activation

(A) Analysis of Dok-1 and Dok-2 expression in wild-type (WT) and *Dok-1*$^{-/-}$ platelets by immunoblotting. Splenocyte lysate was used as a positive control and β-actin as a loading control. (B) The left panel shows representative images of flow cytometric analysis of JON/A binding (y-axis) and CD62P (P-selectin) expression (x-axis) in WT or *Dok-1*$^{-/-}$ platelets that were either unstimulated or stimulated with 0.05 U/ml thrombin for 5 minutes. The right panel shows quantitative data represented as the percent of JON/A positive platelets (n = 3). (C) The left panel shows representative images of flow cytometric analysis of fibrinogen-FITC binding in WT or *Dok-1*$^{-/-}$ platelets that were either unstimulated or stimulated with stimulation with either 10 μM ADP alone, 10 μM ADP plus 3 μM U46619, or 0.1 U/ml thrombin for 15 minutes. The mean fluoresence intensity (MFI) of fibrinogen-FITC binding was 19.1 (WT) vs. 20.5 (*Dok-1*$^{-/-}$) after stimulation with ADP, 42.2 (WT) vs. 34.0 (*Dok-1*$^{-/-}$) after stimulation with ADP plus U46619, and 1114 (WT) vs. 1241 (*Dok-1*$^{-/-}$) after stimulation with thrombin. The right panel shows quantitative data represented as the percent of fibrinogen-FITC positive platelets (n = 3).

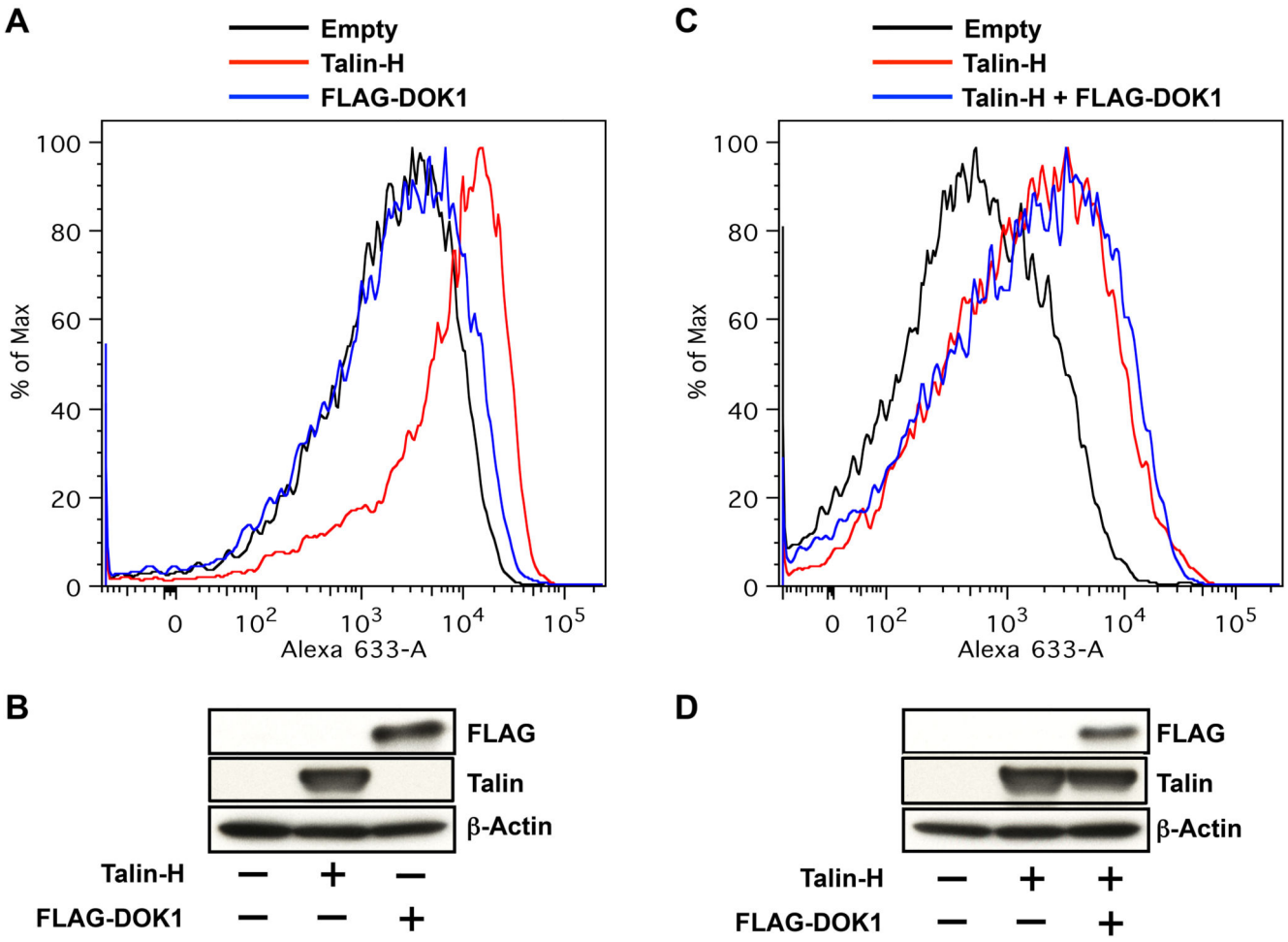

FIGURE 2. Dok-1 does not affect the activation of human αIIbβ3 in αIIbβ3-CHO cells

(A and C) EGFP-fused talin-H and/or FLAG-tagged DOK1 were transiently transfected into αIIbβ3-CHO cells. αIIbβ3 activation was assessed by PAC1 binding using flow cytometry. (B and D) Expression of FLAG-tagged Dok-1 and Talin were detected by immunoblotting. The data are representative of three experiments.

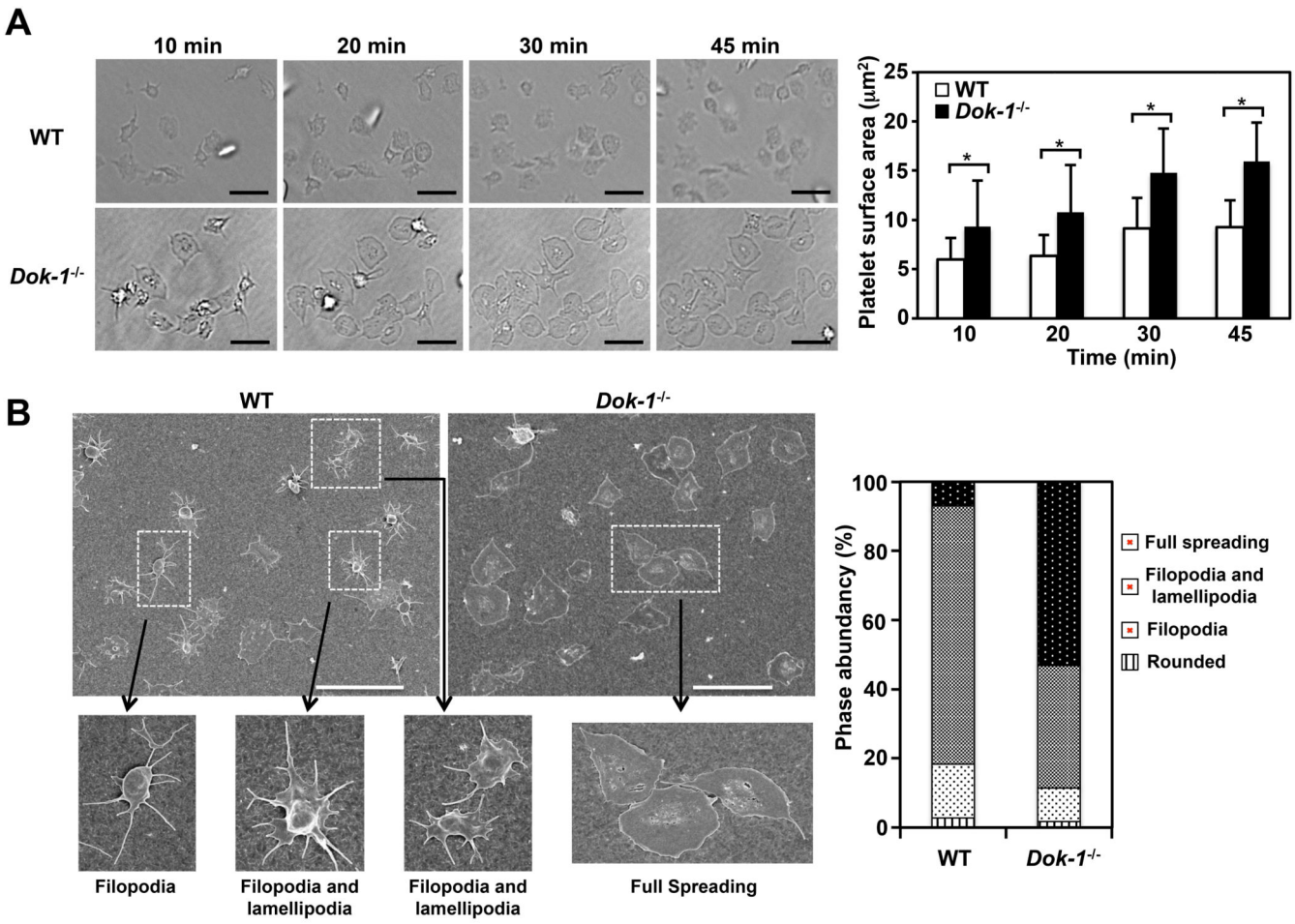

FIGURE 3. Dok-1 deficient platelets exhibit enhanced spreading

(A) Platelets from WT or *Dok-1*$^{-/-}$ mice were plated on fibrinogen-coated glass microwell dishes and stimulated with thrombin (0.01 U/ml). The left panel shows microphotographs taken at the indicated time points. Bar: 10 μm. The right panel shows quantification of platelet surface area. Values are expressed as mean± SD, with n = 26 platelets/genotype (**P* < 0.05). (B) Platelets from WT or *Dok-1*$^{-/-}$ mice were plated on fibrinogen-coated cover glass dishes and stimulated with 0.01 U/ml thrombin. After 30 minutes, non-adherent platelets were removed by washing, and adherent platelets were fixed and imaged by scanning electron microscopy. Bar: 10 nm. The left panel shows representative images, and the right panel shows percent phase abundancy. N = 200 platelets/genotype.

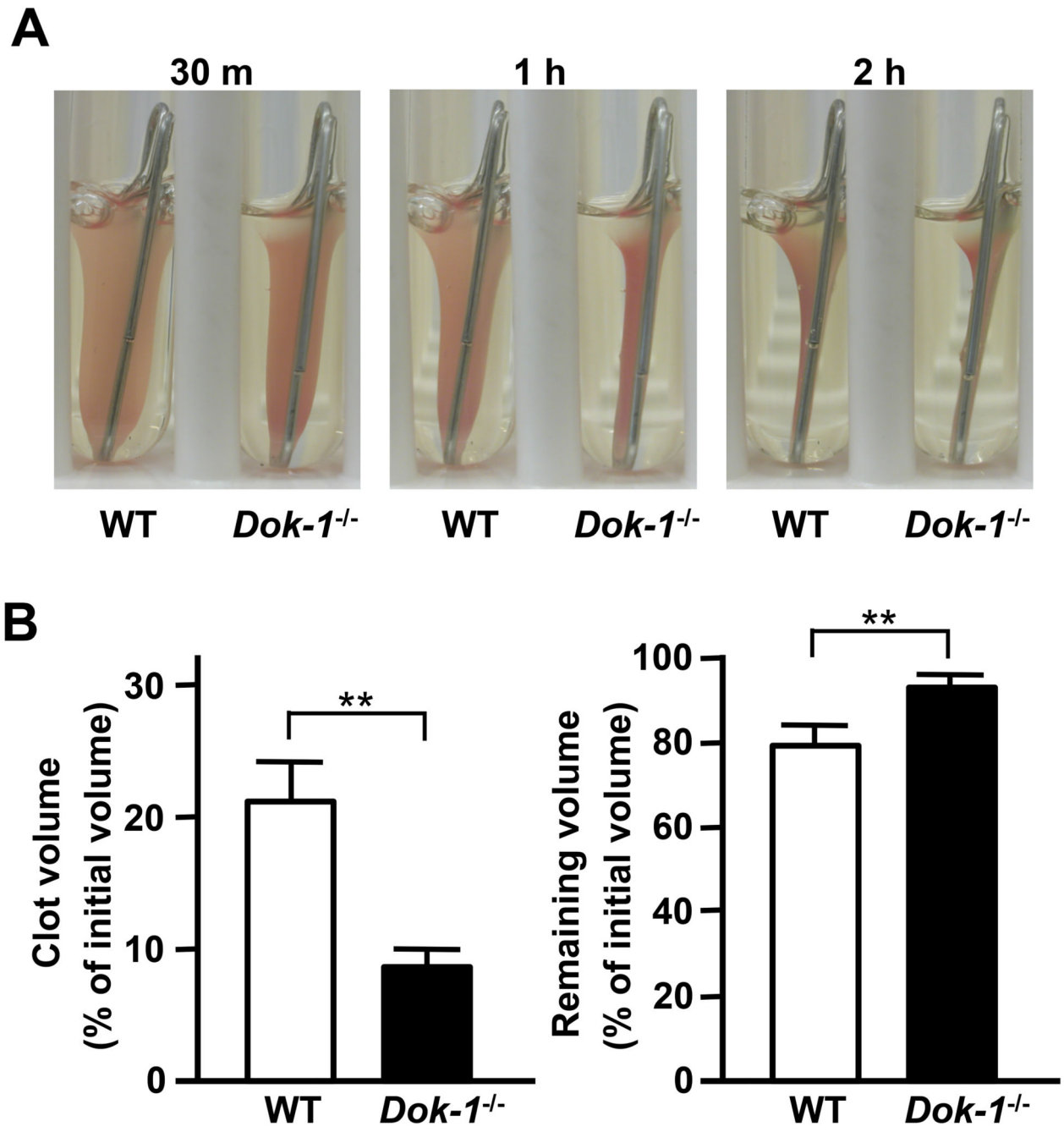

FIGURE 4. Dok-1-deficiency accelerates clot retraction

(A) Representative images of clot retraction at 30 minutes, 1 hour and 2 hours after stimulation with 0.5 U/ml thrombin. (B) Residual clot volume at 2 hours. Values are mean ± SD, with n=4/group (** $P < 0.01$).

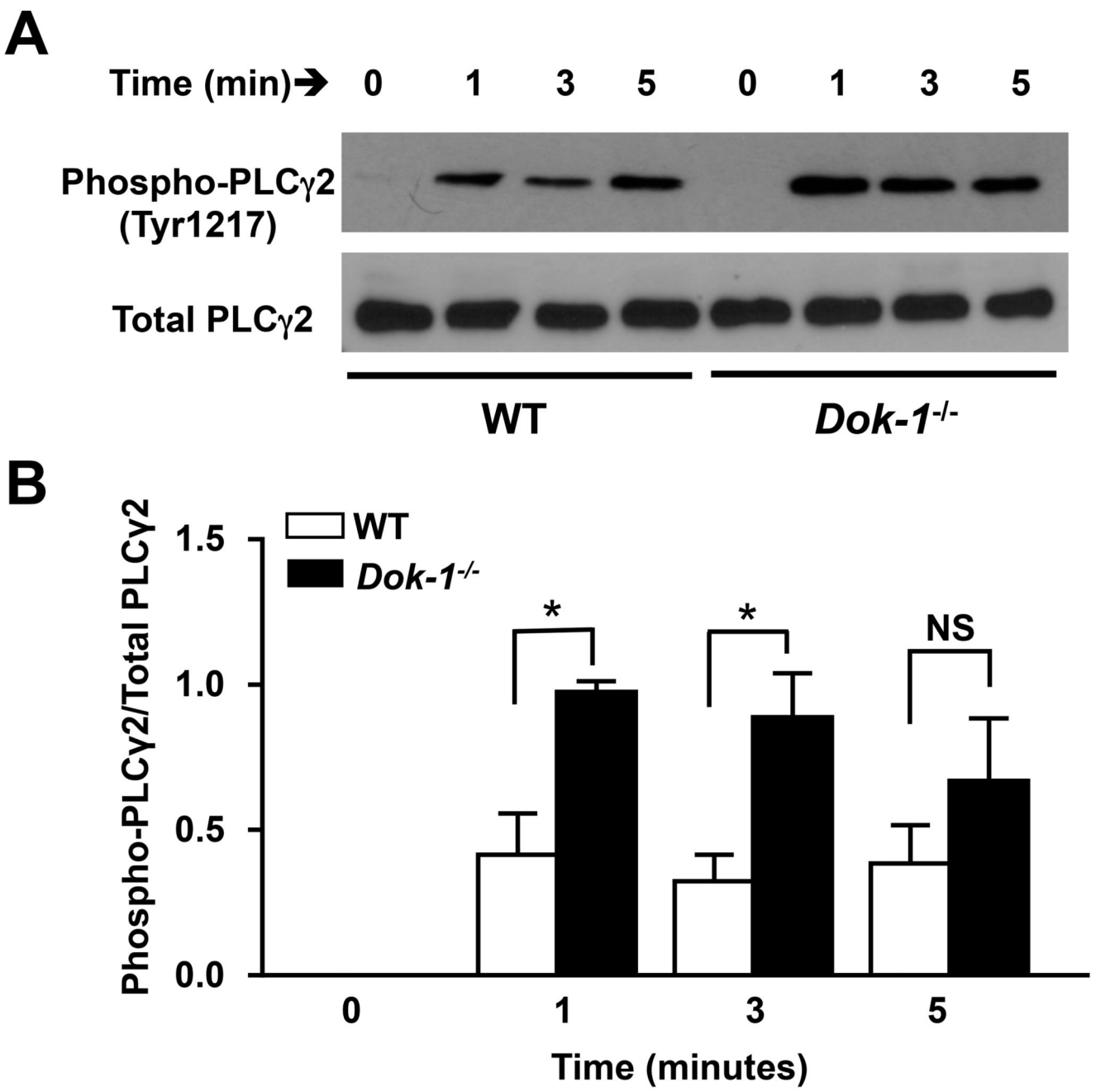

FIGURE 5. Increased phosphorylation of PLCγ2 in *Dok-1*$^{-/-}$ platelets

(A) Representative images of immunoblots of phosphorylated PLCγ2 (Tyr1217) and total PLCγ2 in WT or *Dok-1*$^{-/-}$ platelets treated with 0.5 U/ml thrombin, 0.5 mg/ml fibrinogen, and 2 mM Ca^{2+} (to mimic conditions of clot retraction) for the indicated periods of time. (B) Densitometric analysis of immunoblots using ImageJ. The data are expressed as a ratio of phospho PLCγ2 (Tyr1217)/total PLCγ2. Values are mean ± SD, with n=5/group (*$P < 0.05$).

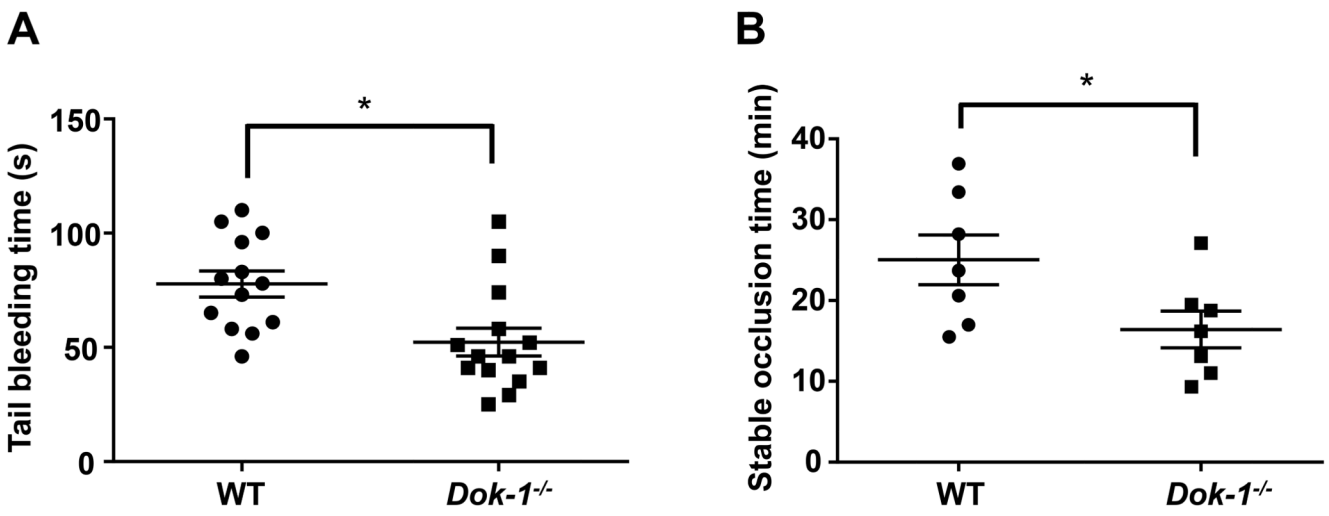

Figure 6. Dok-1$^{-/-}$ mice exhibit shortened bleeding times and accelerated carotid artery thrombosis

(A) The tail-transection bleeding time was determined as the time taken for initial cessation of bleeding after transection in WT or *Dok-1-/-* mice. Each symbol represents a single mouse. Horizontal bars show the mean of each group (*$P < 0.05$). (B) Time to occlusive thrombus in the photochemical-induced carotid artery injury model. Each symbol represents a single mouse. Horizontal bars show the mean of each group (*$P < 0.05$).