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Site-specific Phosphorylation of Kindlin-3 Protein Regulates Its Capacity to Control Cellular Responses Mediated by Integrin $\alpha_{IIb}\beta_3$*

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Background: Kindlin-3 regulates integrin activation in hematopoietic cells.
Results: Stimulation of cells bearing integrin $\alpha_{IIb}\beta_3$ leads to enhanced kindlin-3 phosphorylation and prevention of phosphorylation-inhibited integrin-mediated cellular responses.
Conclusion: Phosphorylation is integral to the capacity of kindlin-3 to regulate integrin function.
Significance: A post-translational modification has been identified as a key event in the mechanism by which kindlin-3 regulates integrin-dependent responses.

The contributions of integrins to cellular responses depend upon their activation, which is regulated by binding of proteins to their cytoplasmic tails. Kindlins are integrin cytoplasmic tail binding partners and are essential for optimal integrin activation, and kindlin-3 fulfills this role in hematopoietic cells. Here, we used human platelets and human erythroleukemia (HEL) cells, which express integrin $\alpha_{IIb}\beta_3$, to investigate whether phosphorylation of kindlin-3 regulates integrin activation. When HEL cells were stimulated with thrombopoietin or phorbol 12-myristate 13-acetate (PMA), $\alpha_{IIb}\beta_3$ became activated as evidenced by binding of an activation-specific monoclonal antibody and soluble fibrinogen, adherence and spreading on fibrinogen, colocalization of β_3 integrin and kindlin-3 in focal adhesions, and enhanced β_3 integrin-kindlin-3 association in immunoprecipitates. Kindlin-3 knockdown impaired adhesion and spreading on fibrinogen. Stimulation of HEL cells with agonists significantly increased kindlin-3 phosphorylation as detected by mass spectrometric sequencing. Thr^{482} or Ser^{484} was identified as a phosphorylation site, which resides in a sequence not conserved in kindlin-1 or kindlin-2. These same residues were phosphorylated in kindlin-3 when platelets were stimulated with thrombin. When expressed in HEL cells, T482A/S484A kindlin-3 decreased soluble ligand binding and cell spreading on fibrinogen compared with wild-type kindlin-3. A membrane-permeable peptide containing residues 476–485 of kindlin-3 was introduced into HEL cells and platelets; adhesion and spreading of both cell types were blunted compared with a scrambled control peptide. These data identify a role of kindlin-3 phosphorylation in integrin β_3 activation and provide a basis for functional differences between kindlin-3 and the two other kindlin paralogs.

The ligand-binding function of integrin adhesion receptors, particularly on circulating blood cells, is tightly regulated. Under most conditions, the integrins exist on the cell surface in a quiescent state, incapable of binding cognate ligands with high affinity. However, when the cells are confronted with a stimulatory agonist, the integrins rapidly adopt active, ligand-competent conformations. Such integrin activation is regulated by engagement of binding partners to the short cytoplasmic tails of integrins (1–3), which elicit inside-out signals across the transmembrane domains of the integrin to the ligand binding domain within their extracellular region. Ligand binding can, in turn, elicit outside-in signaling that triggers intracellular responses. Kindlins are a family of intracellular proteins that have been implicated in bidirectional signaling across integrins. Kindlins link integrin signaling to the actin cytoskeleton and localize to integrin adhesion sites (4–6). They are evolutionarily conserved with an ortholog, UNC-112, present in *Caenorhabditis elegans* (7). In mammals, there are three kindlin family members, each characterized by a FERM domain bisected by a pleckstrin homology domain. The FERM domains of kindlins are most closely related to the FERM domain of talin, which is also involved in regulation of integrin signaling (8–12). Kindlins and talin bind to the cytoplasmic tails of integrin β subunits via their F3 (phosphotyrosine binding) subdomains within their FERM domains. However, the binding sites of talin and kindlins within the β-cytoplasmic tails do not overlap (6, 13), and the two interactions appear to act cooperatively to optimize integrin activation (13, 14). Hence, cells or mice with decreased kindlin expression levels are unable to properly activate their integrins. Kindlin-1 is expressed predominantly in epithelial cells, and mutation in the kindlin-1 gene causes Kindler syndrome in humans (15, 16), a rare disease characterized by skin blistering, poikiloderma with frequent intestinal complications. These phenotypes are recapitulated in mice in which the kindlin-1 gene has been inactivated (17). Kindlin-2 is expressed in most tissues and in many different cell types, and knock-out of kindlin-2 is lethal in mice and zebrafish during embryonic development (14, 18). Mice in which the

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kindlin-3 gene has been inactivated display defects in platelet (19) and leukocyte (20) responses dependent on integrin activation, and the mice die by day 7 postnatally (19). Kindlin-3 mutations in have been identified in humans with a rare syndrome referred to as LADIII (21–25) with manifestations that include episodic bleeding, susceptibility to frequent infections and osteopetrosis, which are consequences of an inability to activate β_1, β_2 and β_3 integrin (22, 23, 25), and variably in abnormal red cell shapes (25). Kindlin-3 is also present and functional in endothelial cells (26) and breast cancer cells (27), where it acts as a tumor promoter (27). Despite this ample evidence emphasizing the role of kindlin-3 in integrin function in variety of cells, the mechanisms underlying kindlin-mediated integrin activation are largely unknown. Recently, it has been established that the calpain I cleavage of kindlin-3 at Tyr^{373} controls the dynamics of integrin/kindlin-3 interaction and, in turn, integrin-dependent adhesion and migration of hematopoietic cells (28). The other known functional site in kindlin-3 is its integrin-binding site in its F3 domain that centers at Gln^{597}/Trp^{598}. Mice in which these two residues have been mutated to alanines are unable to stop bleeding upon tail resection or form arterial occlusions but are viable for least 6 months (29).

Here, we sought to identify possible post-translational modification(s) that regulate kindlin-3 functions in hematopoietic cells. One of the possible mechanisms regulating the ability of kindlins to activate integrins is phosphorylation. Previous work has shown that phosphorylation on the integrin β_3 CT[2] regulates kindlin-2 binding (30). In this work, we have considered how this post-translational modification might regulate the function of kindlin-3 and have utilized HEL cells and human platelets as model systems. HEL cells are suspension cells that express high levels of integrin $\alpha_{IIb}\beta_3$ (31, 32) and have served as a model of analyses of platelet responses (33, 34). When stimulated with PMA or TPO, HEL cells adhere to immobilized fibrinogen via their integrin $\alpha_{IIb}\beta_3$ (35, 36). In this study, using a variety of approaches, we confirm that kindlin-3 is crucial for integrin activation in hematopoietic cells, and this agonist-induced function of kindlin-3 depends on β_3 integrin cytoplasmic tail/kindlin-3 interaction and phosphorylation in a variable region of kindlin-3. These results establish a previously unknown role of kindlin-3 phosphorylation in the regulation of integrin function in hematopoietic cells, and they provide a molecular basis for the difference between kindlin-3 and other two kindlin family members.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Mouse monoclonal antibody (mAb) against EGFP (JL-8) was from Clontech; mouse monoclonal antibody against human β_3 integrin (RUU-PL7F12) was from BD Biosciences; mouse mAb against GAP DH was from Thermo Fisher Scientific (Waltham, MA); mouse mAb against kindlin-2 was from EMD Millipore (Billerica, MA); mouse mAb 8d4 against talin was from Sigma; mouse mAb PAC-1 against activated $\alpha_{IIb}\beta_3$ integrin was from BD Biosciences; mouse PerCP-eFluor 710-labeled mAb against CD162 (PSGL-1) was from eBioscience (San Diego); rabbit mAb against β3 integrin was from Origene (Rockville, MD); mouse PE-labeled mAb against CD62 (P-selectin) was from BD Biosciences; and rat mAb 9EG7 against activated mouse β_1 integrin was from BD Biosciences. Rabbit anti-kindlin-3 antibodies were prepared as described previously (26). Mouse mAb 2G12 against human $\alpha_{IIb}\beta_3$ integrin has been described (37). Alexa and (*R*)-PE-coupled secondary antibodies, Alexa-coupled phalloidins, and Alexa 647-coupled human fibrinogen were from Thermo Fisher Scientific. Horseradish peroxidase-conjugated secondary antibodies and A/G protein-agarose were from Santa Cruz Biotechnology (Dallas); ECL reagent was from Roche Applied Science; and human fibrinogen was from Enzyme Research Laboratories (South Bend, IN). Thrombin was from Sigma, and PMA and membrane-permeable protein kinase C inhibitor were from EMD Millipore. Kindlin 3-specific and nontargeting siRNAs from Thermo Fisher Scientific and QuikChange site-directed mutagenesis kit were from Agilent Technologies (Santa Clara, CA). Iscove's modified Dulbecco's medium, RPMI 1640 medium, DMEM/F-12, penicillin/streptomycin, and L-glutamine were from Media Lab (Cleveland Clinic); EGM® endothelial cell growth medium was from Lonza, and fetal calf serum was from Atlanta Biologicals. 9R and fluorescein-tagged peptide corresponding to residues 476–485 of kindlin-3 (FLSLQRTGSG) and a scrambled peptide (SRGLSQFGTL) were synthesized in the Biotechnology Core of the Cleveland Clinic.

cDNA Constructs—EGFP-tagged kindlin-3 was created by cloning full-length kindlin-3 in-frame with EGFP into the pEGFP-C2 vector (Clontech) as described previously (23). The PSGL-1/β_3 chimera was constructed in pcDNA3.1 vector in which the N terminus (1–91 amino acids) of human PSGL-1 was fused onto the C terminus (468–762 amino acids) of human β_3 subunit as described previously (13). All the indicated mutations of kindlin-3 and PSGL-1 chimera were introduced into the respective constructs using QuikChange site-directed mutagenesis kit (Agilent Technologies) and confirmed by gene sequencing.

Cells and Transfections—HEL cells and RAW 264.7 cells were from American Type Culture Collection (Manassas, VA). Human umbilical vein endothelial cells were provided by Dr. Paul DiCorleto (Cleveland Clinic) and maintained in EGM® endothelial cell growth medium. K562 $\alpha_{IIb}\beta_3$ cells were kindly provided by Scott Blystone (State University of New York Upstate Medical University, Syracuse). Chinese hamster ovary cell line stably expressing $\alpha_{IIb}\beta_3$ (CHO-A5) was described before (13). Transient transfections of A5 cells were performed using Lipofectamine 2000, accordingly to the manufacturer's protocol (Thermo Fisher Scientific). Transient nucleofections of cells were performed using nucleofection kit V from Lonza (Walkersville, MD), according to the manufacturer instructions and program X-005 for HEL cells, T-016 for K562 cells, and D-032 for RAW 264.7 cells with 3 μg of DNA per sample.

Ethics Statement—All experiments done with human platelets were approved by the Cleveland Clinic's Institutional

[2] The abbreviations used are: CT, cytoplasmic tail; EGFP, enhanced green fluorescent protein; HEL, human erythroleukemia; PFA, paraformaldehyde; PMA, phorbol 12-myristate 13-acetate; PSGL-1, P-selectin glycoprotein ligand-1; TIRF, total internal reflection fluorescence; TPO, thrombopoietin; MFI, mean fluorescence intensity; PE, phycoerythrin.

Review Board. Written informed consent was obtained in accordance with the Declaration of Helsinki.

Isolation of Platelets from Human Blood—Platelets were isolated as described previously (39). Briefly, blood was drawn from healthy volunteers into acid/citrate/dextrose (ACD: 65 mM citric acid, 85 mM sodium citrate, 111 mM dextrose, pH 4.61) and 2 μM prostaglandin E_1. Blood was centrifuged to obtain platelet-rich plasma, which was then further centrifuged, and the platelet pellet was suspended in Ca^{2+}- and Mg^{2+}-free Tyrode's buffer (138 mM NaCl, 12 mM $NaHCO_3$, 0.36 mM Na_2HPO_4, 2.9 mM KCl, 10 mM HEPES, 0.1% glucose, 0.1% bovine serum albumin, pH 7.1) and gel-filtered through a Sepharose CL-4B (GE Healthcare) column in Tyrode's buffer to obtain the platelet preparations. Platelet count was determined using a hemocytometer.

Western Blotting—Cells were solubilized in a Laemmli buffer containing 62.5 mM Tris-HCl, pH 7.4, 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol. Pellets were solubilized by addition of 2× Laemmli buffer and subjected to SDS-PAGE. Proteins were transferred to PVDF, and blots were probed with antibodies of interest, using 5% BSA as a blocking agent.

Immunoprecipitation—For antibody-mediated precipitation of endogenous kindlin-3, HEL cells were stimulated with PMA or TPO, and platelets were stimulated with thrombin or PMA. Samples were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1% Nonidet P-40, 1 mM calcium chloride, containing protease and phosphatase inhibitors. Lysates were held on ice for 30 min prior to centrifugation at 12,000 × *g* for 15 min. Aliquots of the detergent-soluble material were precleared on A/G protein-agarose for 1 h at 4 °C. Precleared lysates were incubated with 2 μl of antibodies or preimmune serum and protein A/G-agarose for 16 h at 4 °C. Immunoprecipitated proteins were solubilized in Laemmli buffer and analyzed on Western blots with β_3 integrin antibodies and kindlin-3 antibodies.

Mass Spectrometry—Endogenous kindlin-3 was immunoprecipitated from HEL cells and platelets as described above. Polyacrylamide gels were stained with Coomassie Blue to visualize bands. Proteins were identified by sequencing tryptic peptides by tandem in the mass spectrometry laboratory (Cleveland Clinic). The data were analyzed using the Mascot program to analyze the collision-induced dissociation spectra collected from the LC-MS.

Immunostaining—HEL cells were stimulated with 800 nM PMA and plated on 20 μg/ml fibrinogen for the times indicated, fixed with 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100, blocked in horse serum, and stained with the indicated antibodies for ~18 h. Antigen-antibody complexes were detected by staining with Alexa-coupled secondary antibodies for 1 h. Stained cells were visualized with a ×40 or ×63 1.4 oil objective using a Leica TCS-NT laser scanning confocal microscope (Imaging Core, Cleveland Clinic). Laser intensities were adjusted to eliminate cross-talk between channels, and images were collected using Leica confocal software (version 2.5 build 1227).

Total Internal Reflection Fluorescence (TIRF)—For cell imaging by TIRF microscopy, HEL cells were nucleofected with kindlin-3 constructs, after 24 h treated with 800 nM PMA, and allowed to spread for 1 h on glass-bottom dishes (MatTek Corp., Ashland, MA) coated with 20 μg/ml fibrinogen. Cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100, blocked in horse serum, and stained with β_3 integrin antibodies for ~18 h. Antigen-antibody complexes were detected by staining with Alexa 568-coupled secondary antibodies for 1 h. Cells were imaged with a 100 × 1.49 NA TIRF objective on a Leica AM TIRF inverted microscope equipped with Dual Hamamatsu cameras (ImageEM and Orca-R2).

siRNA Interference—To knock down endogenous kindlin-3 in HEL cells, 100 nM kindlin-3, or control, nontargeting siRNAs were transfected into cells using nucleofection kit V according to the manufacturer's protocol. The level of suppression and specificity of kindlin-3 siRNA was evaluated by Western blotting with antibodies against kindlin-3 or against GAPDH as a loading control.

Platelet Aggregation—Effect of kindlin-3 and scramble peptides on platelet aggregation was examined using an aggregometer (Chrono-log Corp., Havertown, PA). Platelets (3×10^8/ml) were equilibrated at 37 °C for 5 min. Then 10 μM peptides or agonist was added, and aggregation was monitored for 10 min at 37 °C with stirring. The final volume in each cuvette was 500 μl. 1 unit of thrombin was used as a positive control, and baseline for 100% aggregation was set with Tyrode's buffer.

Isolation and Analysis of Platelet Cytoskeletons—Platelet suspensions were stimulated with thrombin for the times indicated. Aggregation was terminated by addition of an equal volume of ice-cold lysis buffer containing 2% Triton X-100, 10 mM EGTA, 100 mM Tris-HCl, pH 7.4, with protease and phosphatase inhibitors (40). Lysates were immediately centrifuged at 15,600 × *g* at 4 °C for 4 min to obtain the low speed detergent-insoluble pellet. Detergent-insoluble and -soluble fractions were solubilized by addition of an SDS-containing buffer in the presence of reducing agent (40), denatured at 95 °C, and electrophoresed on 4–20% gradient acrylamide gels in SDS. Proteins present in the fractions were detected by transferring samples to PVDF and developing the Western blots with antibodies against β_3 integrin and kindlin-3.

Flow Cytometry and Soluble Ligand Binding—To assess changes in P-selectin surface expression, platelet suspensions were stimulated with kindlin-3 peptides or 1 unit of thrombin for 30 min in the presence of 20 μl of PE-labeled P-selectin antibody and fixed with 2% PFA for 10 min. To assess fibrinogen binding to HEL cells, cells were stimulated with 800 nM PMA for 10 min, and cells were fixed with 1% PFA for 10 min in room temperature. Fixed cells were incubated with 20 μg/ml of soluble Alexa 647-labeled fibrinogen for 1 h at room temperature. To address the functional significance of kindlin-3 phosphorylation in integrin-mediated signaling, HEL cells were transfected with the indicated constructs. PAC-1 binding to the different transfectants was analyzed by gating only on the EGFP-positive cells. Mean fluorescence intensities (MFI) of PAC-1 binding were normalized based on the basal level of PAC-1 binding to cells transfected with the EGFP vector alone to obtain relative MFI values. 2×10^5 cells/sample were stimulated with 800 nM PMA for 10 min in the presence of 10 μg/ml PAC-1 in Hanks' buffered saline solution containing 0.1% BSA, 0.5 mM $CaCl_2$, $MgCl_2$. After incubation, cells were fixed with 1% PFA for 10 min in room temperature. After washing, cells were

incubated with 10 μg/ml (*R*)-PE-labeled secondary antibody for 30 min in room temperature. PAC-1 binding was normalized by $\alpha_{IIb}\beta_3$ expression level on the cell surfaces measured by 2G12 mAb, which reacts with $\alpha_{IIb}\beta_3$ independent of its activation state (37). RAW 264.7 cells were transfected, and 2×10^5 cells were incubated with 10 μg/ml 9EG7 antibody as described for PAC-1. K562 $\alpha_{IIb}\beta_3$ cells were transfected; 2×10^5 cells/sample were stimulated with 800 nM PMA for 10 min, and cells were fixed with 1% PFA for 10 min in room temperature. Fixed cells were incubated with 20 μg/ml of soluble Alexa 647-labeled fibrinogen for 1 h at room temperature. Ligand binding was analyzed using LSRFortessa flow cytometer and FlowJo software (BD Biosciences) in Cleveland Clinic Flow Core.

Adhesion and Spreading Assays—For knockdown studies, nontransfected HEL cells or HEL cells transfected with kindlin-3 or control siRNA were stimulated with 800 nM PMA for 5 min and incubated with immobilized fibrinogen in triplicate for 15, 30, or 60 min at 37 °C. After extensive washing with PBS, the adherent cells were fixed with 4% PFA and stained with Alexa 488 phalloidin. The cells were photographed at ×40, and the adherent cells were counted from 20 randomly selected fields; the cell area was measured for 300 cells. To define the β_3 CT core for kindlin-3, nontransfected HEL cells or HEL cells transfected with kindlin-3 and/or β_3 CT fused to PSGL-1 constructs were stimulated with 800 nM PMA for 5 min and incubated with immobilized fibrinogen in triplicate, for 30 min at 37 °C. After extensive washing with PBS, the adherent cells were fixed with 4% paraformaldehyde and stained with Alexa 568 phalloidin. The cells were photographed with a ×40 objective, and the adherent cells were counted from 20 randomly selected fields; the cell area was measured for 300 cells. For peptide import experiments, HEL cells were incubated with 10 μM kindlin-3 or scramble peptide for 5 min, then stimulated with 800 nM PMA for 5 min, and incubated with immobilized fibrinogen for 30 or 60 min at 37 °C. The adherent cells were fixed with 4% PFA and stained with Alexa 568 phalloidin. The cells were photographed with a ×40 objective, and the adherent cells were enumerated as described above. For peptide import studies with human platelets, isolated human platelets as described above were incubated with 10 μM kindlin-3 or scramble peptide for 5 min and plated on fibrinogen for 30 min. The adherent cells were fixed with 4% PFA and stained with Alexa 568 phalloidin. Platelets were photographed with a ×100 objective, and the adherent platelets were counted from 15 randomly selected fields.

Statistical Analysis—Two-tailed Student's *t* tests were performed where indicated in the text using SigmaPlot 11. Differences were considered to be significant at $p < 0.05$.

RESULTS

HEL Cells as a Model for Kindlin-3-mediated Regulation of Integrin $\alpha_{IIb}\beta_3$ Function—To consider whether post-translational modification(s) might regulate the integrin-activating kindlin-3 function, we first sought to establish HEL cells as a transfectable model cell system. First, we examined the levels of kindlins and talin in HEL cells as compared with K562 cells, another erythroblastoid cell line, which had been transfected to stably express $\alpha_{IIb}\beta_3$ integrin. Anti-kindlin-3 polyclonal antibodies (26) readily recognized the ~72-kDa band in whole HEL cell lysate, and this band was absent from K562 cells (Fig. 1*A*, *left-hand panel*). Kindlin-2 was absent from HEL cells and K562 cells but was readily detected in whole human umbilical vein endothelial cell lysates (Fig. 1*B*, *middle panel*). HEL cells and K562 cells expressed high amounts of talin (Fig. 1*A*, *right-hand panel*). Thus, kindlin-3 is a major kindlin in HEL cells. Levels of $\alpha_{IIb}\beta_3$ integrin in HEL cells (Fig. 1*B*) were assessed with flow cytometry, and monoclonal antibody 2G12, which recognizes the $\alpha_{IIb}\beta_3$ integrin complex (37), reacted well with the cells. HEL cells are suspension cells. Upon agonist stimulation, they increase their binding of soluble ligand (Fig. 1*C*) and require such stimulation to adhere and spread efficiently on immobilized ligands. HEL cells stimulated for 10 min with 800 nM PMA increased their binding of Alexa 647-labeled soluble fibrinogen when compared with nonstimulated HEL cells, and this interaction was completely abolished with 10 mM EDTA (Fig. 1*C*) as would be expected for an integrin-mediated binding interaction. Although the majority of HEL cells showed basal levels of fibrinogen binding in the absence of added agonist, PMA stimulation increased the extent of fibrinogen binding to the entire cell population (Fig. 1*D*). Similar increases in soluble ligand binding were observed when integrin activation was monitored with PAC-1 or when TPO was used as an agonist (data not shown).

It has been shown previously (41, 42) that HEL cells localize $\alpha_{IIb}\beta_3$ integrin to focal adhesions when spread on an integrin substrate. Knowing that kindlin-3 is a major kindlin in HEL cells, we sought to determine whether kindlin-3 colocalizes with integrin β_3 in HEL cells spreading on substrate for this integrin. It has been reported previously in several studies (36, 41–43) that HEL cell adhesion and spreading on integrin substrates, including fibronectin, vitronectin, and fibrinogen, requires stimulation with agonists such as PMA or thrombin, thereby distinguishing these cells from platelets that adhere to these substrates in the absence of exogenously added agonists. It has been suggested that PMA might increase $\alpha_{IIb}\beta_3$ integrin-dependent adhesion to immobilized ligand by increasing the local concentration of integrin, resulting in an increased avidity and changing the affinity state of $\alpha_{IIb}\beta_3$ integrin, or both (36). Under the conditions we used and consistent with these prior reports, in the absence of agonist stimulation, HEL cells displayed little adhesion, and they did not spread on fibrinogen. To determine the kindlin-3 distribution in HEL cells, cells were pretreated with 800 nM PMA for 10 min, allowed to spread on fibrinogen for 60 min, fixed, and stained with kindlin-3 and integrin-specific antibodies. At the 60-min time point, the cells were fully spread and β_3 integrin was present in focal adhesions (Fig. 2, *upper panels*). Staining for kindlin-3 revealed that kindlin-3 colocalized with β_3 integrin in these structures (Fig. 2, *upper panels*, sites of colocalization are indicated with the *arrows* in *merge panel*). To assess whether $\alpha_{IIb}\beta_3$ integrin is a major integrin present in kindlin-3-containing focal adhesions, HEL cells were stained with antibodies that specifically recognize this integrin. The staining revealed that $\alpha_{IIb}\beta_3$, detected with 2G12, was present in kindlin-3-containing focal adhesions (Fig. 2, *lower panels*, sites of colocalization are indicated with the *arrows* in *merge panel*).

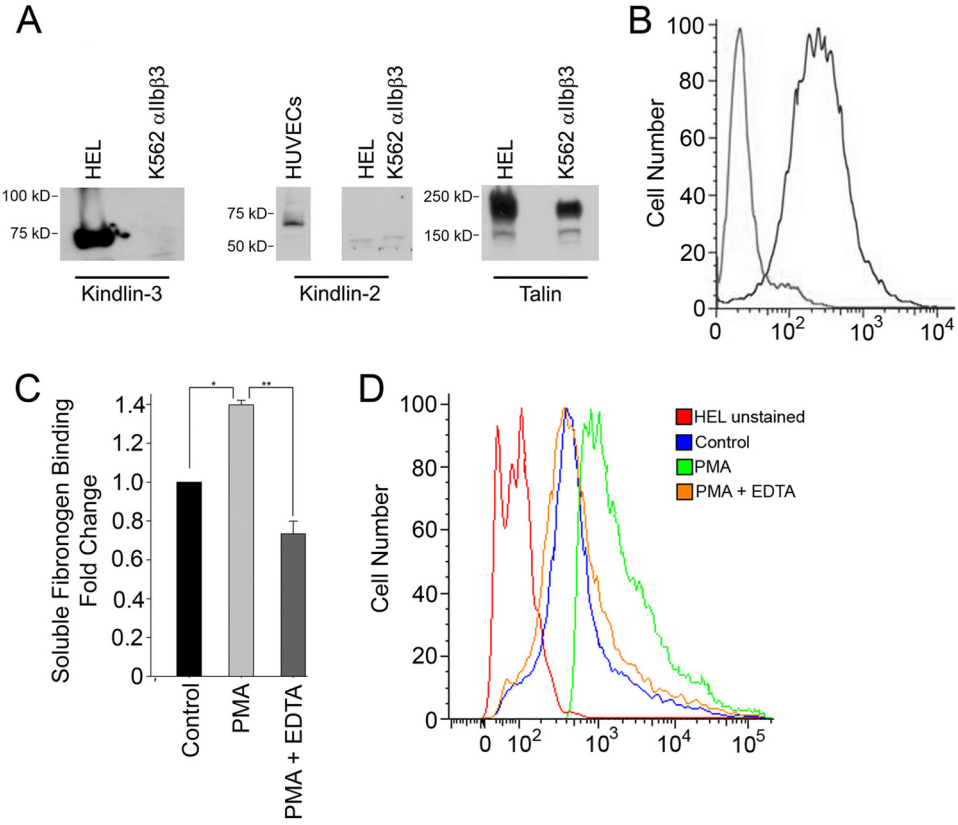


FIGURE 1. **Kindlin-3 is a major kindlin in HEL cells.** *A,* Western blots of HEL cells, K562 $\alpha_{IIb}\beta_3$ cells, and human umbilical vein endothelial cell (*HUVEC*) lysates were probed with antibodies to kindlin-3 (*left side panel*), kindlin-2 (*middle panel*), or talin (*right side panel*). *B,* $\alpha_{IIb}\beta_3$ integrin expression levels in HEL cells were determined by flow cytometry with 2G12 monoclonal antibody, and goat anti-mouse Fab conjugated to Alexa 488 was used as a secondary detection. *C,* Alexa 647-labeled soluble fibrinogen (20 μg/ml) binding to control and PMA stimulated with or without 10 mM EDTA HEL cells (*single asterisk* $p = 0.019$ and *double asterisk* $p < 0.001$). *D,* representative histogram showing the binding of Alexa 647-labeled soluble fibrinogen to control and PMA-stimulated with or without 10 mM EDTA HEL cells. The *error bars* represent means ± S.E.

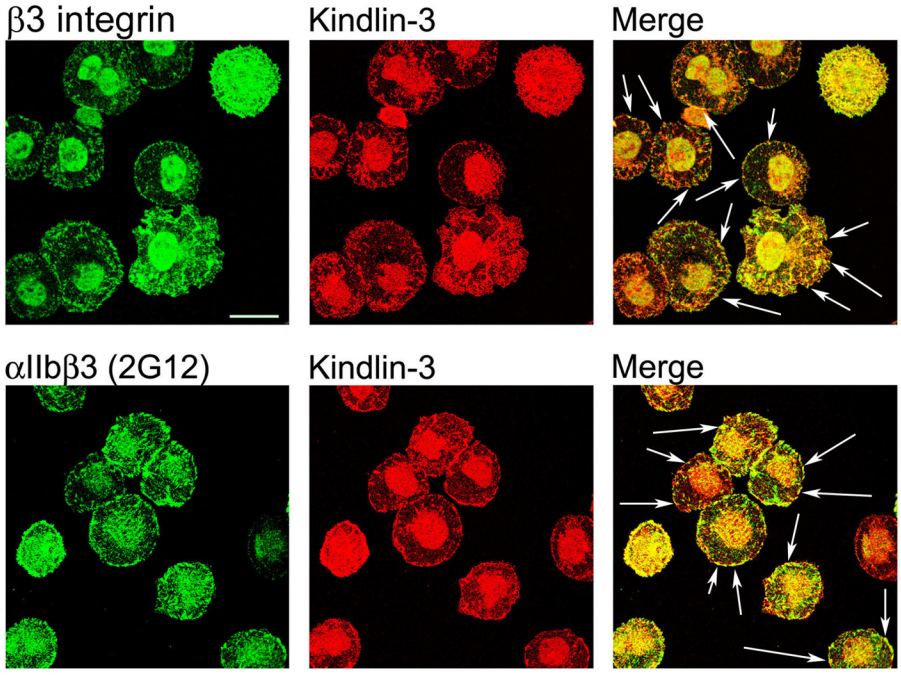


FIGURE 2. **Kindlin-3 distribution in HEL cell spreading on fibrinogen.** HEL cells were treated with 800 nM PMA and spread on fibrinogen for 60 min. Cells were fixed, permeabilized, and stained with antibodies against kindlin-3 (26), total β_3 integrin (RUU-PL7F12), and $\alpha_{IIb}\beta_3$ integrin (2G12) followed by Alexa 488 anti-mouse IgG and Alexa 568 anti-rabbit IgG. Kindlin-3 colocalized with integrins in focal adhesions (indicated with *arrows*). *Bar,* 20 μm. The images shown are representative of 12–15 independent fields with each antibody.

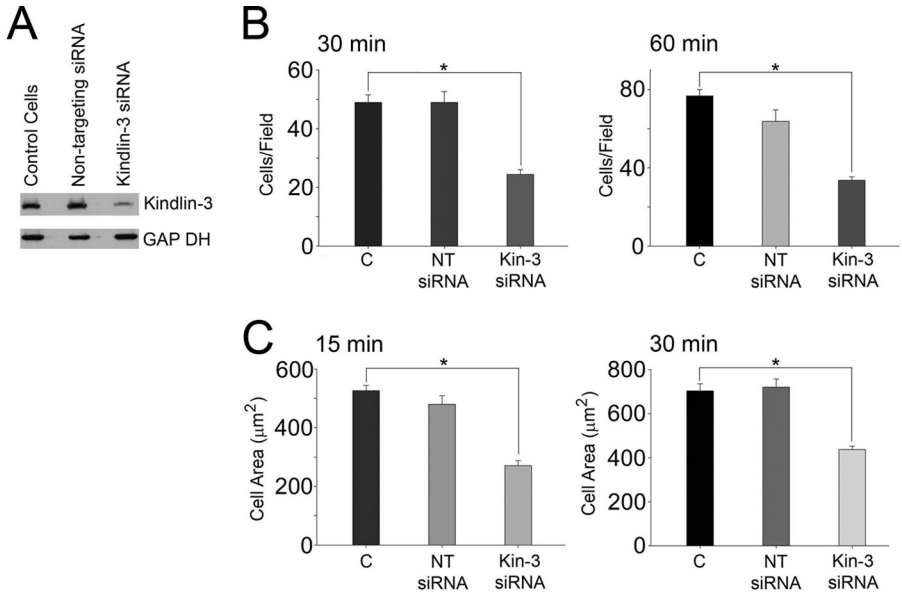


FIGURE 3. **Kindlin-3 knockdown inhibits integrin-mediated adhesion and spreading of HEL cells.** *A,* siRNA suppression of kindlin-3 expression in HEL cells. Expression of kindlin-3 in nontransfected cells, nontargeting siRNA, and kindlin-3 siRNA was analyzed by Western blotting with kindlin-3 and GAPDH antibodies. *B,* nontransfected HEL cells or HEL cell-transfected kindlin-3 or nontargeting siRNA were used in adhesion assays. The cells were treated with 800 nM PMA, allowed to adhere for 30 or 60 min, fixed, stained with Alexa 488-phalloidin, and counted (*, $p < 0.001$). The *error bars* are means ± S.E. of three independent experiments. *C,* HEL cells were treated with 800 nM PMA and allowed to adhere to fibrinogen-coated coverslips, and cell spreading was measured after 15 and 30 min. The adherent cells were fixed and stained with Alexa 488 phalloidin. The areas of cells were measured using ImageJ software, and 300 cells were quantified in each experiment (*, $p < 0.001$). The *error bars* represent means ± S.E. of three independent experiments.

Kindlin-3 Knockdown Blunts Integrin-dependent Adhesion and Spreading in HEL Cells—To determine whether kindlin-3 deficiency blunts integrin responses in HEL cells, we performed RNA interference experiments. HEL cells were transfected with siRNA specific to kindlin-3 or a nontargeting siRNA as a control. The levels of expression of kindlin-3 were analyzed on Western blots (Fig. 3*A*). Control siRNA did not have an effect on expression of kindlin-3 (Fig. 3*A*, *lane 2* of kindlin-3 blot). Kindlin-3 siRNA effectively inhibited the expression of kindlin-3 protein (Fig. 3*A*, *lane 3* of kindlin-3 blot); in three independent experiments, 60–80% knockdown was achieved. None of the siRNAs used affected β_3 integrin expression as assessed by flow cytometry with β_3 integrin-specific antibody (data not shown). To assess the function of kindlin-3 in integrin-mediated cell adhesion, nontransfected and siRNA-transfected HEL cells were stimulated with 800 nM PMA for 10 min and were plated on fibrinogen for 15, 30, or 60 min. Cells were fixed, and actin was visualized with Alexa 488 phalloidin. Cell adhesion was blunted significantly at the 30- and 60-min time points (50–60% decrease in adhesion, $p < 0.001$) in cells transfected with kindlin-3 siRNA but not with nontargeting siRNA when compared with control cells (Fig. 3*B*). Cell area was determined by measuring 300 cells at the 15- and 30-min time points. Compared with control HEL cells and cells transfected with nontargeting siRNA, knockdown of kindlin-3 inhibited β_3-mediated cell spreading by ~50% at the 15-min ($p < 0.001$) and by ~40% at the 30-min time point ($p < 0.001$) (Fig. 3*C*).

Kindlin-binding Sites in Integrin β_3 Cytoplasmic Tail Inhibit HEL Cell Spreading on Immobilized Fibrinogen—In a prior study, the kindlin-2 binding core in integrin β_3 CT was established as Ala^{750} to Gly^{761} (44). The strategy involved expression of various β_3 integrin CT as fusion proteins linked to extracellular and transmembrane portions of PSGL-1 and with the β_3 CT replacing the PSGL-1 CT in CHO cell line stably expressing $\alpha_{IIb}\beta_3$ integrin (13). This strategy allowed the competition of chimeric proteins with endogenous β_3 CT for integrin binding partners, kindlin-2 and talin. In this study, we implemented a similar strategy in HEL cells to map the kindlin-3-binding site on β_3 CT. PSGL-1 alone and chimeric constructs were introduced into HEL cells by nucleofection. After 24 h, expression of the PSGL-1/β_3CT constructs on the cell surface as assessed by flow cytometry with anti PSGL-1 antibody differed by <10%. We then evaluated the effects of the various β_3 CT on β_3-mediated HEL cell spreading on immobilized fibrinogen. HEL cells were stimulated with 800 nM PMA for 10 min, and 2×10^5 cells were plated on immobilized fibrinogen for 30 min. Cells were fixed, and actin was visualized with Alexa 488 phalloidin. Cell area was measured for 300 cells/construct. Compared with control HEL cells and cells expressing PSGL-1 alone, expression of the wild-type β_3CT chimera inhibited β_3-mediated cell spreading by 50% ($p < 0.001$) (Fig. 4). As a specificity control, the Y747A mutation, which interferes with talin binding, resulted in a loss of inhibitory activity ($p < 0.001$), whereas Y747F mutation, which still allows talin binding, had an inhibitory effect on cell spreading. Mutations that abolished kindlin-2 binding, S752P and Y759A, also led to loss of competitive activity ($p < 0.001$) (Fig. 4). This loss was not the observed S752A substitution, which would retain kindlin binding to β_3 CT (Fig. 4). The β_3CT kindlin-2 core (Ala^{750}–Gly^{761}) (44) was also sufficient to significantly suppress cell spreading ($p < 0.001$). The β_3CT Δ748, which only expresses the talin and not the kindlin-binding site (44), also significantly inhibited HEL cell spreading ($p < 0.001$) (Fig. 4). Thus, these data establish

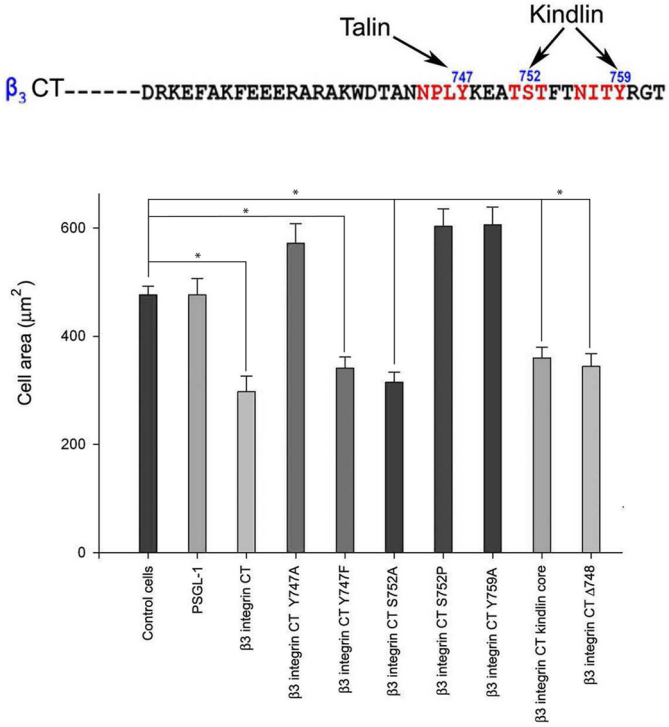


FIGURE 4. **Kindlin-binding site residues in integrin β_3 cytoplasmic tail influence HEL cell spreading.** HEL cells were stimulated with 800 nM PMA for 5 min, and cells were plated on fibrinogen for 30 min. Cells were fixed, and actin was visualized with Alexa 488 phalloidin. Compared with control HEL cells and cells expressing PSGL-1 alone, expression of the wild-type β_3CT chimera inhibited β_3-mediated cell spreading by 50%. Y747A mutation, which interferes with talin binding, resulted in a loss of inhibitory activity, whereas Y747F mutation, which still allows talin binding, had an inhibitory effect on spreading. S752P and Y759A, which block kindlin-2 binding, also led to loss of competitive activity. This loss was not observed in S752A substitution, which would retain kindlin binding to β_3 CT. The β_3 CT kindlin-2 core (Ala^{750} to Gly^{761}) was also sufficient to significantly suppress cell spreading. The β_3CT Δ748, which only expresses the talin but not the kindlin-binding site, also significantly inhibited HEL cell spreading. The areas of cells were measured using ImageJ software, and 300 cells were quantified in each experiment (*, $p < 0.001$). The *error bars* represent means ± S.E. of three independent experiments.

that both kindlin-3 and talin contributed to integrin-mediated functions in HEL cells, and kindlin-3 utilizes the same binding core on integrin β_3CT as kindlin-2 to regulate integrin function in HEL cells.

Kindlin-3 Is Phosphorylated in HEL Cells and Platelets upon Agonist Stimulation—Knowing that kindlin-3 regulates β_3 integrin functions in HEL cells upon agonist stimulation, we sought to determine whether the association of kindlin-3 with β_3 integrin is stimulus-dependent in HEL cells. and we extended this analysis to platelets. First, HEL cells or platelets were stimulated with agonists, and kindlin-3 was immunoprecipitated with anti-kindlin-3. The immunoprecipitates were then analyzed on Western blots with antibodies against β_3 integrin and kindlin-3. Anti-kindlin-3, but not preimmune serum, used as a negative control, immunoprecipitated a single ~72-kDa band (Fig. 5, *A* and *B*, *kindlin-3 blots*). Anti-β_3 integrin antibody detected a single ~100-kDa band in immunoprecipitates of cells stimulated with agonists. β_3 integrin associated with kindlin-3 in HEL cells stimulated with 800 nM PMA (Fig. 5*A*, β_3 *blot*) and in thrombin-stimulated platelets (Fig. 5*B*, β_3 *blot*), but this interaction was not detected in nonstimulated HEL cells or in resting platelets. $MnCl_2$, which activates integrin externally, failed to promote integrin-kindlin-3 association alone and did not increase PMA-induced integrin-kindlin-3 association (Fig. 5*A*).

Additional evidence for the stimulus dependence of the association of kindlin-3 with integrin was obtained from analysis of the detergent-insoluble cytoskeletal fraction of thrombin-stimulated platelets. It has been shown previously (45, 46), upon binding of fibrinogen to aggregating platelets, that a portion of the $\alpha_{IIb}\beta_3$ integrin associates with detergent-insoluble cytoskeletal fraction, which can be recovered by low speed centrifugation. Fibrinogen binding also stimulates redistribution of numerous cytoskeletal and signaling proteins, including $\alpha_{IIb}\beta_3$ integrin-binding proteins, such as c-Src and talin, to the low speed cytoskeletal fraction (40). We sought to determine whether kindlin-3 redistributes together with the β_3 subunit to detergent-insoluble fraction of thrombin-stimulated platelets. Platelets were stirred with the addition of 1 unit of thrombin for the times indicated, and detergent-insoluble and detergent-soluble fractions were separated and analyzed by Western blotting for the presence of β_3 integrin and kindlin-3 following standard protocols (40). As expected, thrombin stimulation resulted in redistribution of β_3 to the detergent-insoluble fraction (Fig. 5*C*, *lane 1* and *lanes 2–4* are the detergent-insoluble fractions from resting platelets and thrombin-stimulated platelets, respectively; *lanes 5* and *6–8* are the detergent-soluble fractions from resting and thrombin-stimulated platelets, respectively). Thrombin-induced redistribution of β_3 to the detergent-insoluble pellet was accompanied by kindlin-3 redistribution, whereas more of the kindlin-3 remained in detergent-soluble supernatant from nonstimulated platelets.

With evidence that the kindlin-3/β_3 integrin interaction was regulated, we sought to determine whether post-translational modification of kindlin-3 might play a regulatory role. HEL cells and platelets were treated with agonists to promote kindlin-3 and β_3 integrin association, and kindlin-3 was immunoprecipitated from cell lysates and resolved by SDS-PAGE. Protein bands corresponding to kindlin-3 were excised from the gel and digested with trypsin, and the tryptic peptides were subjected to tandem mass spectrometry sequencing. The stimulation of HEL cells with PMA and TPO led to increased kindlin-3 phosphorylation (Fig. 6, *A* and *B*). Thr^{482} or Ser^{484} was identified as the phosphorylation site in PMA- and TPO-stimulated HEL cells. Mass analysis of the spectra suggested a single phosphorylation site in kindlin-3 from cells stimulated with either agonist, but the identity of the site, Thr^{482} or Ser^{484}, could not be distinguished. PMA led to an ~8-fold increase in phosphorylation. TPO-induced kindlin-3 phosphorylation was dose-dependent with 600 nM TPO having a more pronounced effect than 400 nM TPO (Fig. 6*B*).

Protein kinase C (PKCs) is a family of related serine/threonine kinases that have been suggested to play indispensable roles in $\alpha_{IIb}\beta_3$ integrin activation and outside-in signaling (47–50). Because kindlin-3 is essential for integrin activation, we wished to establish whether PKC is involved in agonist-induced kindlin-3 phosphorylation. HEL cells were treated with 800 nM PMA in the absence or in the presence of membrane-permea-

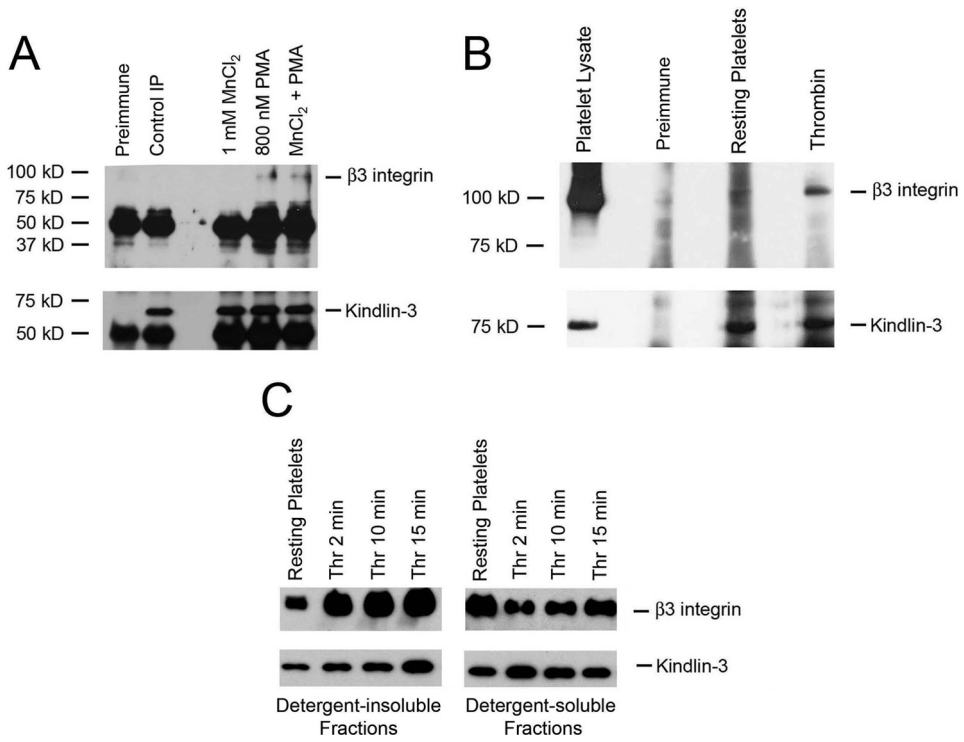


FIGURE 5. **Kindlin-3 interacts with β_3 integrin in HEL cells and platelets upon agonist stimulation.** *A,* total lysates of nonstimulated HEL cells and HEL cells treated with 1 mM $MnCl_2$ or 800 nM PMA were immunoprecipitated with control serum (preimmune) and antibodies against kindlin-3. The immunoprecipitates were subjected to Western blotting with antibodies against β_3 integrin and kindlin-3. Kindlin-3 coimmunoprecipitated β_3 integrin from HEL cells treated with 800 nM PMA but not from nonstimulated cells or $MnCl_2$-treated cells. *B,* total lysates of resting human platelets and platelets treated with thrombin were immunoprecipitated with control serum (preimmune) and antibodies against kindlin-3. The immunoprecipitates were subjected to Western blotting with antibodies against β_3 integrin and kindlin-3. Kindlin-3 coimmunoprecipitated β_3 integrin from platelets treated with thrombin but not from resting platelets. *C,* redistribution of β_3 integrin and kindlin-3 to the cytoskeletal fraction following thrombin-induced aggregation of platelets. Platelet suspensions were stirred in the presence of 1 unit of thrombin for the times indicated. Incubations were terminated by addition of Triton X-100-containing buffer. Kindlin-3 and β_3 integrin in the low speed pellets and supernatants (separated by centrifugation at 15,600 × *g* for 4 min) were detected by Western blots.

ble myristoylated PKC inhibitor (51), and kindlin-3 was immunoprecipitated from cell lysates and resolved by SDS-PAGE as described above. Mass spectrometry analysis revealed that the PKC inhibitor blocked PMA-induced kindlin-3 phosphorylation in the dose-dependent manner (Fig. 6*C*), suggesting a direct or indirect role of PKC in kindlin-3 phosphorylation.

Identical phosphorylation sites, Thr^{482} or Ser^{484}, were identified in kindlin-3 from thrombin- or PMA-stimulated platelets (Fig. 6*D*). Interestingly, platelets achieved maximal kindlin-3 phosphorylation in the presence of EDTA, which prevents platelet aggregation, but it still allows integrin activation, suggesting that kindlin-3 phosphorylation in platelets may be a dynamic process (Fig. 6*D*). These phosphosites reside in a hyper-variable region of kindlin-3 region, *i.e.* it is not present in either kindlin-1 or kindlin-2 (Fig. 7*A*). To determine whether these phosphoresidues are conserved among the species, we aligned the human kindlin-3 peptide sequence in question with kindlin-3 from several other species (Fig. 7*B*). The phosphorylation site(s) were found to be evolutionarily conserved among the species, with at least one residue present in all sequences analyzed (Fig. 6*B*).

T482A/S484A Kindlin-3 Mutant Impairs Integrin-mediated Cell Responses—To address the functional significance of kindlin-3 phosphorylation in integrin-mediated signaling, site-directed mutagenesis was performed in which Thr^{482} and Ser^{484} were replaced with alanines, and the effects of these mutations on kindlin-3 localization, integrin-induced spreading, and soluble ligand binding were explored in HEL cells. EGFP alone or EGFP-tagged kindlin-3 constructs were introduced into HEL cells by nucleofection. Wild-type kindlin-3 was used as a positive control, and Q597A/W598A kindlin-3, which disables the primary integrin-binding site in the F3 subdomain of kindlin-3 (29), was a negative control. By Western blotting, we confirmed that kindlin-3 mutants were of the same size as wild-type kindlin-3 (data not shown). First, we investigated localization of the kindlin-3 mutant in PMA-treated HEL cells spread on fibrinogen. Because the expression level of EGFP-tagged kindlin-3 in HEL cells is so high as to obscure subcellular localization of its fluorescence, we implemented TIRF microscopy that provides a means to selectively excite fluorophores near the adherent cell surface while minimizing fluorescence from intracellular regions. Because TIRF illuminates only fluorophores very close (*e.g.* within 100 nm) to the coverslip-sample interface, this serves to increase the signal-to-noise ratio (52). We have previously used TIRF to visualize EGFP-tagged kindlin-3 at contact sites of the adherent cells with the substrate in rescue experiments in transformed lymphocytes from the human subjects lacking kindlin-3 expression (23). HEL cells were allowed to spread on fibrinogen for 60 min, fixed, and stained with kindlin-3 and integrin-specific antibodies. At the 60-min time point, the cells were fully spread, and β_3 integrin was present in focal adhesions (Fig. 8, *A* and *B*, *middle panels*). Wild-type kindlin-3

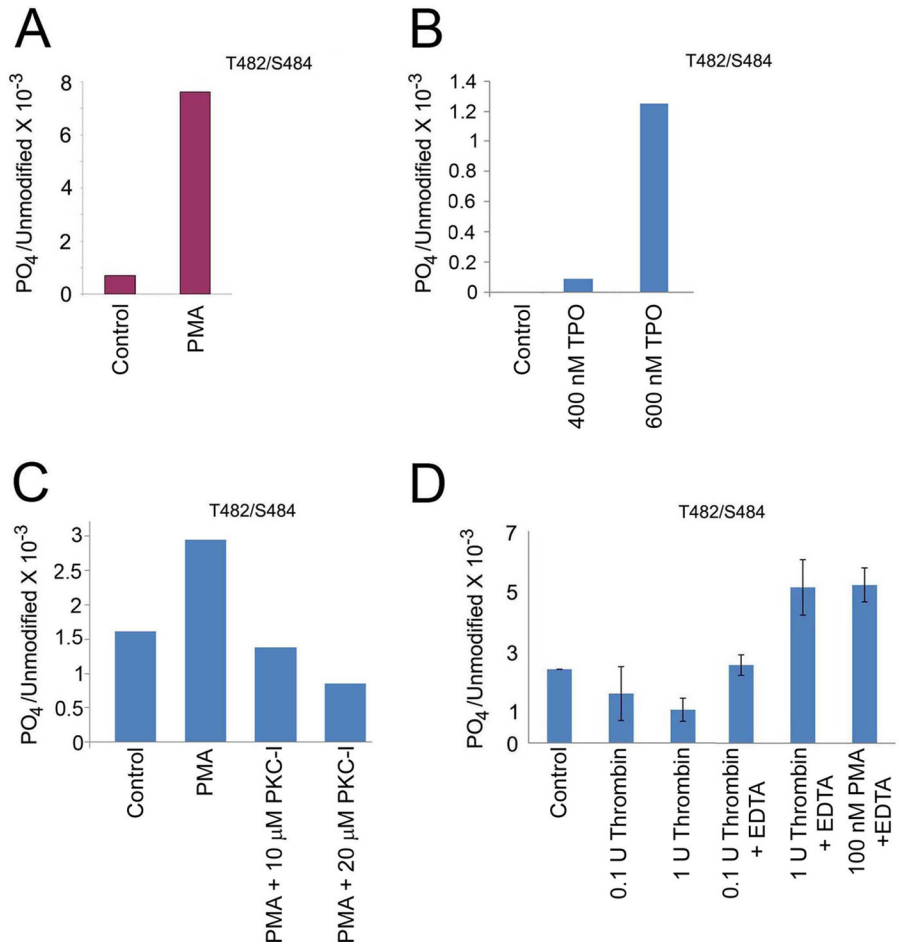


FIGURE 6. **Kindlin-3 is phosphorylated in HEL cells and platelets upon agonist stimulation.** *A,* HEL cells were treated with PMA, and endogenous kindlin-3 was immunoprecipitated with kindlin-3 antibodies. The 72-kDa kindlin-3 band was excised from the gel and subjected to mass spectrometry sequencing. Thr^{482} and/or Ser^{484} were identified as a phosphosite in three independent experiments. *B,* HEL cells were treated with TPO, and endogenous kindlin-3 was immunoprecipitated with kindlin-3 antibodies. The 72-kDa kindlin-3 band was excised from the gel and subjected to mass spectrometry sequencing. Thr^{482} and/or Ser^{484} were identified as a phosphosite in two independent experiments. *C,* HEL cells were treated with PMA in the absence or presence of membrane-permeable PKC inhibitor, and endogenous kindlin-3 was immunoprecipitated with kindlin-3 antibodies. The 72-kDa kindlin-3 band was excised from the gel and subjected to mass spectrometry sequencing. Thr^{482} and/or Ser^{484} were identified as a phosphosite in two independent experiments. *D,* human platelets were treated with thrombin or PMA, and endogenous kindlin-3 was immunoprecipitated with kindlin-3 antibodies. The 72-kDa band was excised from the gels and subjected to mass spectrometry sequencing. Mass analysis of the spectra suggested a single phosphorylation site in kindlin-3 from both cell types, but the identity of the site, Thr^{482} or Ser^{484}, could not be distinguished. These phosphosites reside in a hyper-variable region of kindlin-3 region, *i.e.* it is not present in either kindlin-1 or kindlin-2.

(Fig. 8*A*, *left-hand panels*), identified with EGFP fluorescence, colocalized with β_3 integrin in these structures (Fig. 8*A*, *right-hand panels*, sites of colocalization are indicated with *arrows*). T482A/S484A kindlin-3 mutant (Fig. 8*A*, *left-hand panel*) also colocalized with β3 integrin in focal adhesions (Fig. 8*A*, *left-hand panels,* sites of colocalization indicated with *arrows*); however, the majority of T482A/S484A kindlin-3 mutant transfected cells were less spread than nontransfected cells in the same experiment. Overexpression of Q597A/W598A kindlin-3, a mutant that binds poorly to β_3 integrin (29), impaired cell spreading on fibrinogen as well; however, we were able to identify some partially spread cells expressing Q597A/W598A mutant. In these cells, the Q597A/W598A kindlin-3 mutant (Fig. 8*B*, *left-hand upper panel*) did not colocalize with β_3 integrin in focal adhesions close to the cell surface (Fig. 8*B*, *right-hand upper panel*, transfected cells indicated with *arrows*). EGFP alone (Fig. 8*B*, *left-hand lower panel*) also did not localize to β_3-containing focal adhesions (Fig. 8*B*, *right-hand lower panel,* transfected cells indicated with *arrows*). Knowing that T482A/S484A kindlin-3 mutant is still present in β_3-containing focal adhesions, we sought to determine whether the kindlin-3 mutant still associates with β_3 when compared with wild-type kindlin-3. EGFP alone, EGFP-tagged wild-type kindlin-3, and EGFP-tagged T482A/S484A kindlin-3 mutant were expressed in CHO-A5 cells (expressing human $\alpha_{IIb}\beta_3$ integrin), and the capacity of anti-EGFP to pull down β_3 integrin was evaluated by SDS-PAGE and Western blotting using anti-β_3 integrin antibody (Fig. 8*C*, *upper blot*). Expression levels of EGFP constructs were evaluated with EGFP antibody. Wild-type and mutant kindlin-3 expressed at similar levels, and EGFP alone expressed at much higher level (Fig. 8*C*, *lower blot*). Despite the high level of expression, EGFP alone did not bring down β3 integrin; however, the kindlin-3 constructs coimmunoprecipitated β3 equally well (Fig. 8*C*). These results are consistent with the TIRF data and support the notion that kindlin-3 phosphorylation does not affect integrin binding directly.

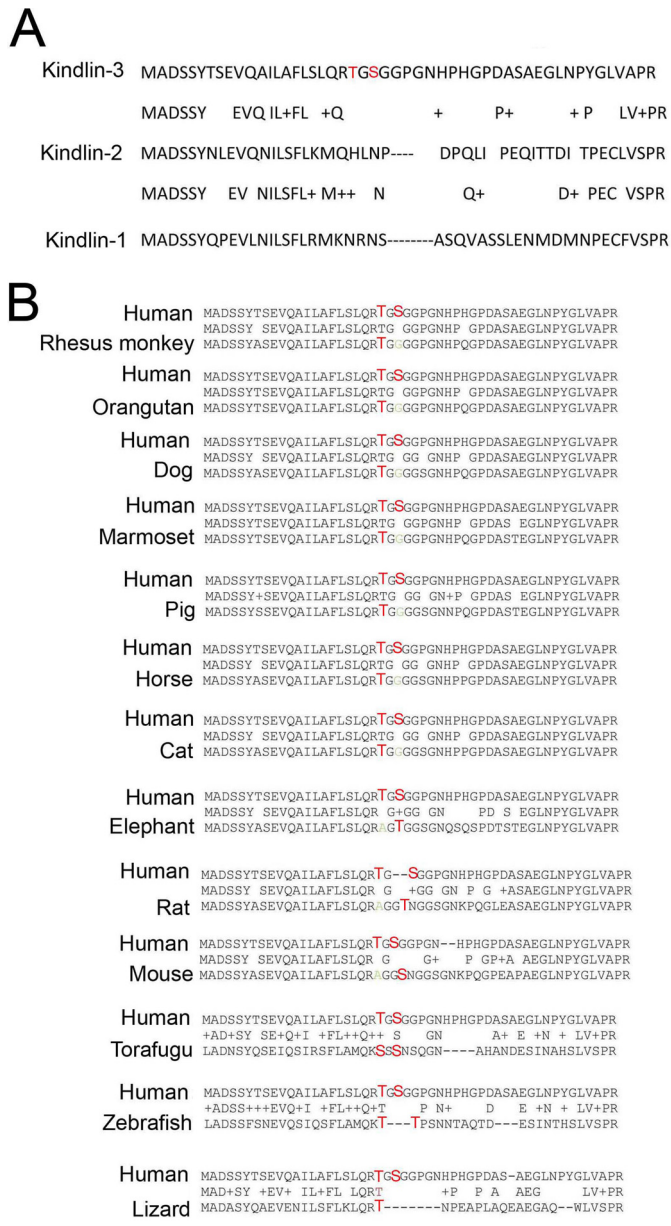


FIGURE 7. **Kindlin-3 phosphosites are evolutionarily conserved.** *A,* identified kindlin-3 phosphosites reside in a hypervariable region of kindlin-3 compared with kindlin-2 and kindlin-1. *B,* comparison of the human kindlin-3 sequence with kindlin-3 from several other species. The phosphorylation site(s) were found to be evolutionarily conserved among the species (conserved residues depicted in *red*), including very distant species of *T. rubripes* (torafugu) and *D. rerio* (zebrafish).

To assess the function of kindlin-3 phosphomutant in integrin-mediated cell spreading, transfected HEL cells were stimulated with 800 nM PMA for 10 min and plated onto fibrinogen for 30 min. Cells were fixed, and actin was visualized with Alexa 568 phalloidin. Cell area was determined by measuring 300 cells/construct. Compared with control HEL cells and cells transfected with wild-type kindlin-3, the phosphomutant inhibited β_3-mediated cell spreading by 40%, which was similar to the effect of Q597A/W598A mutant kindlin-3, suggesting the importance of phosphoresidues for integrin-induced cell spreading (Fig. 9*A*). Because the T482A/S484A mutant still localized in β_3-containing focal adhesions (Fig. 8*A*) and bound to β_3 integrin (Fig. 8*C*), these data suggest that Thr^{482} and/or Ser^{484} phosphorylation might facilitate kindlin-3 binding to a still unidentified protein that is involved in integrin-induced outside-in signaling and cell spreading. Next, we assessed the effect of kindlin-3 phosphomutant overexpression on the activation of endogenous $\alpha_{IIb}\beta_3$ integrin in HEL cells. The cells were nucleofected with EGFP alone or with EGFP-tagged kindlin-3 constructs. After 24 h, cells were stimulated with 800 nM PMA for 10 min and subsequently incubated with PAC-1. The populations of cells expressing EGFP-tagged proteins were gated; 60–80% of the cells were EGFP-positive cells, and binding of PAC-1 antibody to these populations was measured. Expression of wild-type kindlin-3 significantly increased activation of $\alpha_{IIb}\beta_3$ integrin (Fig. 9*B*). HEL cells express endogenous kindlin-3 (Fig. 1*A*). However, it has been shown previously (53) that in another hematopoietic cell line, CMK megakaryocytic cells, which express high levels of endogenous kindlin-3, transient expression of kindlin-3 can augment $\alpha_{IIb}\beta_3$ integrin activation in the presence but not in the absence of agonist, as measured by PAC-1 binding (53). Thus, despite the high levels of endogenous kindlin-3, overexpression of WT kindlin-3 increased agonist-induced integrin activation. In contrast, T482A/S484A kindlin-3 mutant failed to stimulate integrin activation in HEL cells as compared with EGFP-expressing cells (Fig. 9*B*). Next, we extended our studies to other cell models, K562 cells expressing exogenous $\alpha_{IIb}\beta_3$ integrin and RAW 264.7 mouse macrophage cell line, which expresses high levels of endogenous $\alpha_5\beta_1$ integrin. The K562-$\alpha_{IIb}\beta_3$ cell line we use has undetectable levels of kindlin-2 and kindlin-3 (see Fig. 1*A*); thus, kindlin-dependent responses are entirely dependent on expressed proteins. (We are aware that the K562-$\alpha_{IIb}\beta_3$ cells we used differ from those reported by others (28), who found substantial amounts of kindlin-3 in their K562 cells and attribute this to subline variability.) K562-$\alpha_{IIb}\beta_3$ cells are suspension cells, and they activate integrins upon agonist stimulation. K562 cells were nucleofected with EGFP constructs, and binding of soluble Alexa 647-labeled fibrinogen was measured. When EGFP-tagged kindlin-3 constructs were expressed in K562 cells, wild-type kindlin-3 increased binding of soluble fibrinogen significantly (Fig. 9*C*), whereas T482A/S484A kindlin-3 was as ineffective in increasing integrin activation as the Q597A/W598A kindlin-3 (Fig. 9*C*). RAW 264.7 mouse macrophage cell line, expressing endogenous kindlin-3, was previously shown to activate $\alpha_5\beta_1$ integrin when wild-type but not Q597A/W598A kindlin-3 was expressed (19). In our assay, we used 9EG7 monoclonal antibody that recognizes activated mouse β_1 integrin (54). Wild-type kindlin-3 expression in RAW 264.7 cells yielded a significant increase of 9EG7 binding when compared with EGFP control (Fig. 9*D*). Expression of T482A/S484A kindlin-3 did not trigger integrin activation, and 9EG7 binding was comparable with EGFP, and Q597A/W598A kindlin-3 was used as a negative control (Fig. 9*D*). Taken together, these data indicate that kindlin-3 is capable of inducing integrin activation in various cell models, and Thr^{482}and/or Ser^{484} are required for this activity.

Kindlin-3-derived Peptide Inhibits Adhesion and Spreading of HEL Cells and Platelets—A peptide corresponding to the kindlin-3 peptide Phe^{476}–Gly^{485}, designated WT-K3 peptide,

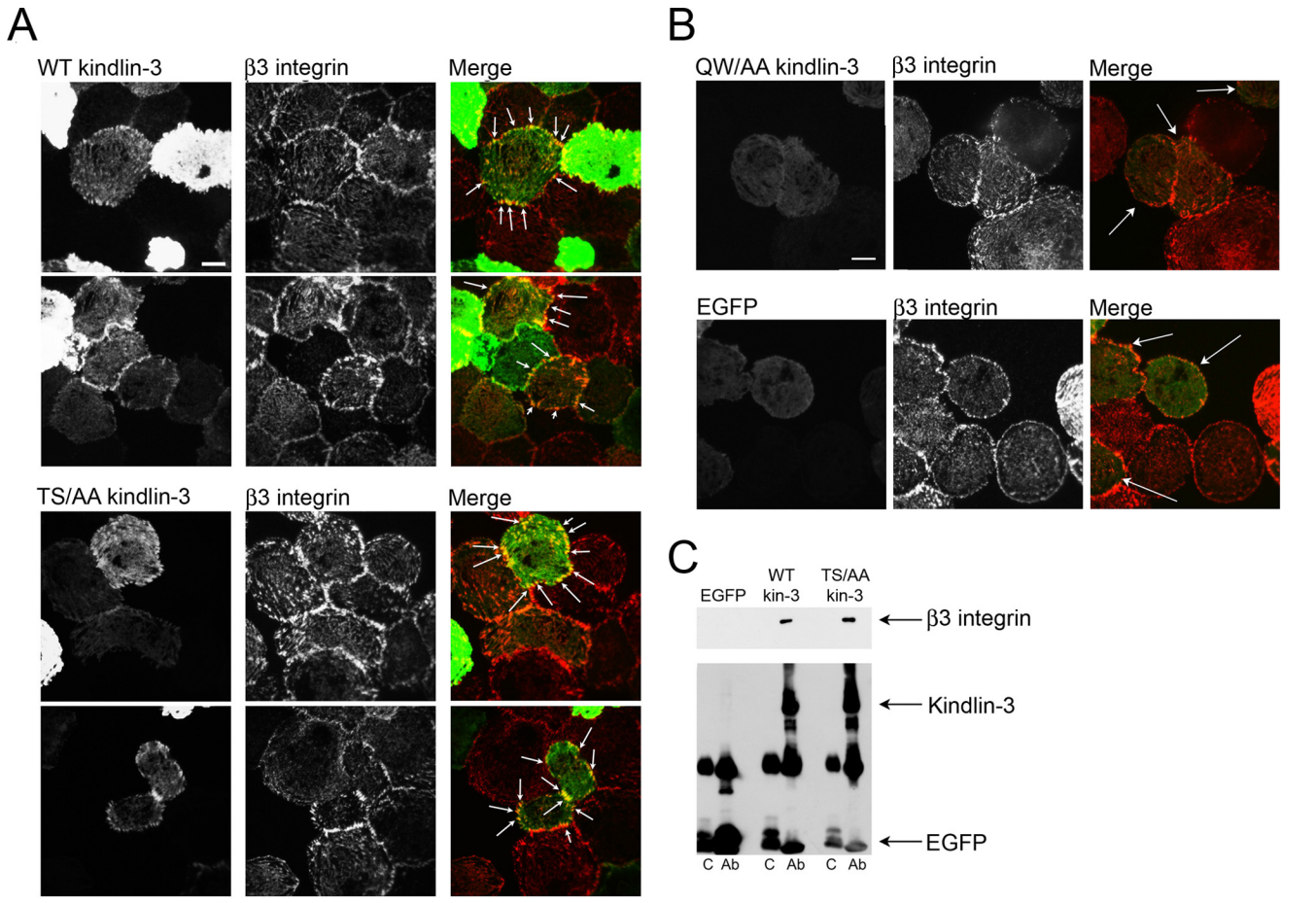


FIGURE 8. **Kindlin-3 phosphomutant (S482A/T484A ($S^{482}T^{484}$/AA) colocalizes with β_3 integrin in focal adhesions as assessed by TIRF and binds to β_3 integrin.** *A* and *B,* Hel cells were transiently transfected with plasmids encoding EGFP alone and EGFP-kindlin-3 constructs. Transfected HEL cells were treated with PMA and allowed to adhere to fibrinogen for 1 h. The adherent cells were fixed and stained with β_3 integrin antibody followed with Alexa 568-conjugated anti-mouse secondary antibody. Kindlin-3 distribution was visualized with EGFP fluorescence. *A,* wild-type and phosphomutant kindlin-3 colocalized with integrins in focal adhesions (indicated with *arrows*). *B,* Q597A/W598A (*QW/AA*) kindlin-3 (mutation of the primary integrin-binding site) and EGFP alone were absent from β3-containing focal adhesions (transfected cells indicated with *arrows*). *Bar,* 20 μm. *C,* lysates of CHO-A5 cells transfected with EGFP-tagged wild-type kindlin-3, S482A/T484A kindlin-3, or EGFP alone were used for coimmunoprecipitation assays. After incubating with A/G-agarose and EGFP antibody (*Ab*), β_3 integrin bound to kindlin-3 constructs was evaluated by SDS-PAGE and Western blotting using anti-β_3 integrin antibody. Kindlin-3 and EGFP levels in immunoprecipitates are also shown as detected with an EGFP antibody. *C,* control.

and containing the candidate phosphosites and a scrambled peptide, designated Scr-K3, were synthesized with a nine arginine (9R) membrane-penetrating tag at their C terminus to support intracellular delivery (54, 55). To visualize the uptake of WT-K3 and Scr-K3 peptides by cells, each peptide was labeled with fluorescein (Fig. 10*A*). The capacity of these peptides to penetrate intact HEL cells was evaluated by flow cytometry. Trypan blue quenching was performed to distinguish between extracellular (quenched) and intracellular pools of peptides. WT-K3 and Scr-K3 peptides (10 μM) were incubated with HEL cells for 10 min, and then cells were left untreated or treated with 0.2 mg/ml trypan blue for 5 min. After fixation, samples were analyzed by flow cytometry. The fluorescence signal was detected for all cells analyzed, and it was reduced after quenching by ~50% (Fig. 10*A*, *blue lines*) when compared with unquenched cells (Fig. 10*A*, *green lines*). These results indicated efficient and comparable uptake of 9R WT-K3 and Scr-K3 peptides into the HEL cells. Their uptake was monitored by flow cytometry, and we first examined the effects of the peptides on integrin-mediated adhesion and spreading in HEL cells plated on immobilized fibrinogen. HEL cells were incubated with 10 μM peptides in the presence of 800 nM PMA, and cells were plated on fibrinogen. After 30 min, the cells were fixed, and actin was visualized with Alexa 568 phalloidin. Cell adhesion was dramatically blunted at 30 min in cells treated with WT-K3 peptide (Fig. 10*B*), whereas Scr-K3 peptide did not have an effect on adhesion when compared with control cells (Fig. 10*B*). Cell area was determined by measuring 300 cells/peptide at 30 min and WT-K3 peptide inhibited cell spreading by ~50% when compared with cells treated with Scr-K3 peptide and control cells (Fig. 10*C*).

Next, we sought to determine whether the WT-K3 peptide affected platelet responses. Evidence for the functional activity of WT K3 peptide was first sought in platelet aggregation studies. Surprisingly, addition of 10 μM WT-K3 peptide added to resting platelets induced significant platelet aggregation, comparable with that induced by thrombin, whereas Scr-K3 peptide did not have any effect (Fig. 10*D*). These differential effects of the WT-K3 peptide and Scr-K3 peptide on platelet aggregation were reproducible with three different donors. However, the

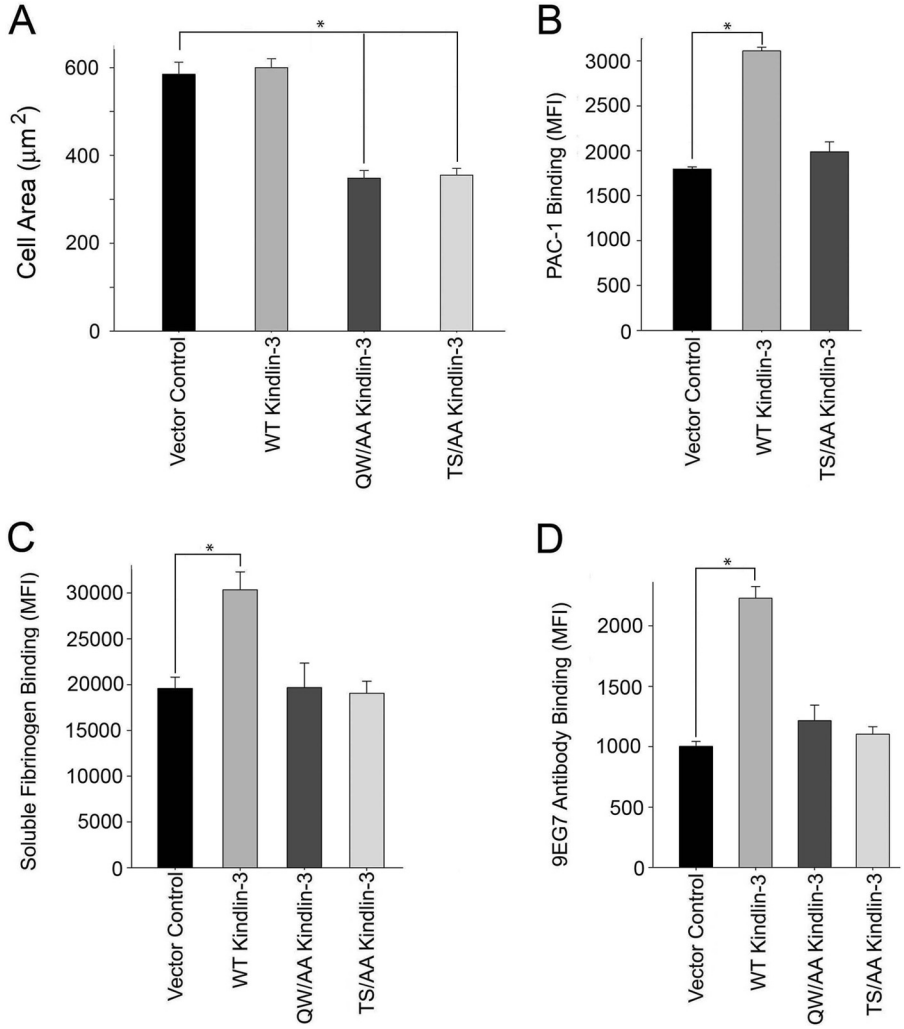
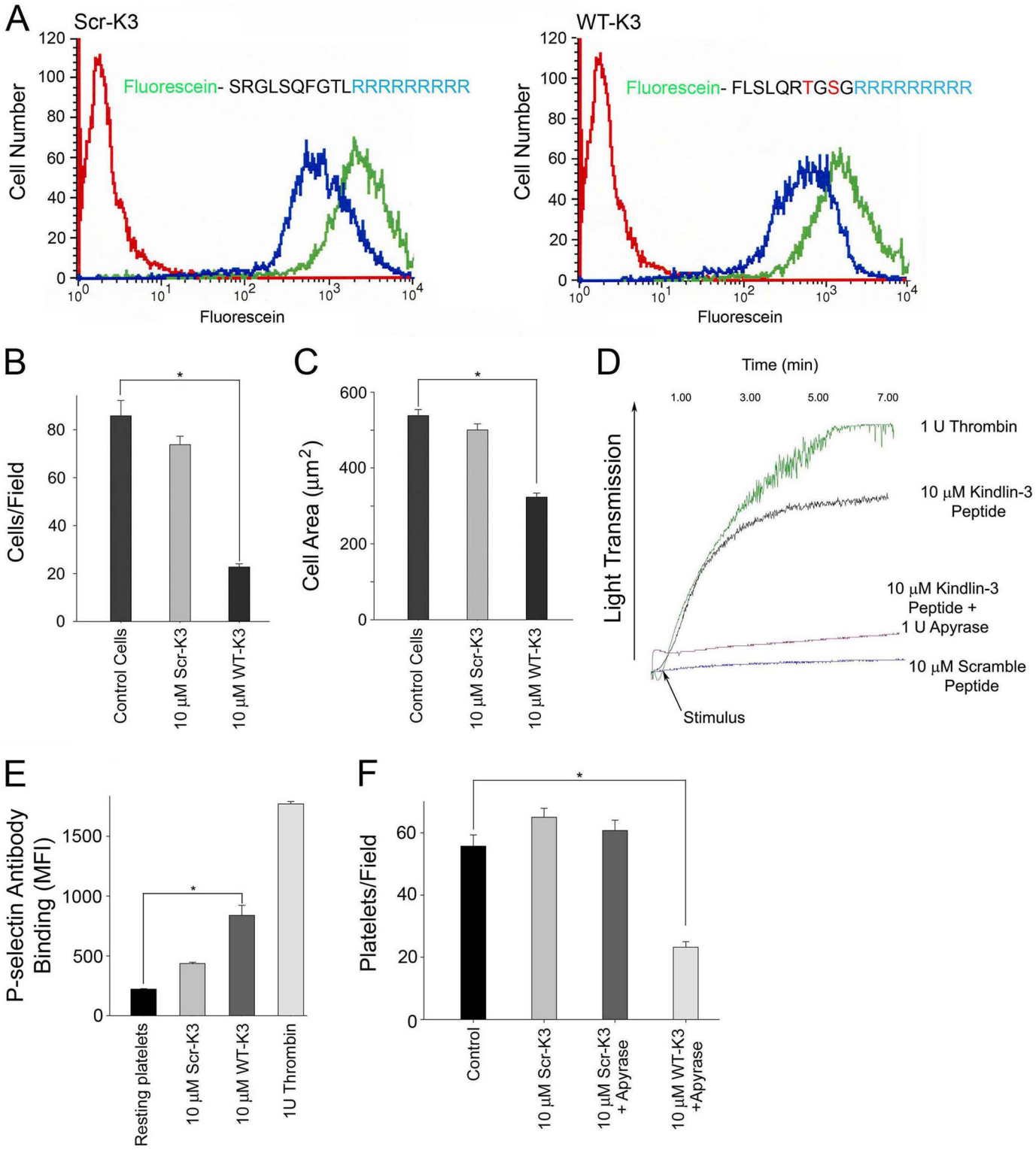


FIGURE 9. **Kindlin-3 phosphomutant (S482A/T484A ($S^{482}T^{484}$/AA)) blunts integrin-induced cell spreading and soluble ligand binding.** *A,* HEL cells were transiently transfected with plasmids encoding EGFP alone and EGFP-kindlin-3 constructs. EGFP expression levels in HEL cells were determined by flow cytometry; transfection efficiency was 70–80%. Transfected HEL cells were treated with PMA and allowed to adhere to fibrinogen coated coverslips, and cell spreading was measured after 30 min. The adherent cells were fixed and stained with Alexa 568 phalloidin. The areas of cells were measured using ImageJ software, and 300 cells were quantified in each experiment. The *error bars* represent means ± S.D. of three independent experiments (*, $p < 0.001$). *B,* plasmids encoding EGFP alone or EGFP-kindlin-3 constructs were transiently transfected to HEL cells. HEL cells were stimulated with 800 nM PMA, and the transfected cells were stained with PAC-1 to assess $\alpha_{IIb}\beta_3$ activation. Flow cytometry was used to measure the MFI of PAC-1 binding. Integrin expression of transfected cells was measured in parallel with 2G12 antibody that binds $\alpha_{IIb}\beta_3$ integrin in an activation-independent manner. The *error bars* represent means ± S.D. of three independent experiments (*, $p < 0.001$). *C,* plasmids encoding EGFP alone or EGFP-kindlin-3 constructs were transiently transfected to K562 $\alpha_{IIb}\beta_3$ cells. EGFP expression levels were determined by flow cytometry with a transfection efficiency of 70–80%. The transfected cells were stimulated with 800 nM PMA and incubated with 20 μg/ml human fibrinogen, and the flow cytometry was used to measure the MFI of fibrinogen binding. The *error bars* represent means ± S.E. of three independent experiments (*, $p < 0.001$). *D,* mouse RAW 264.7 cells were transiently transfected with plasmids encoding EGFP alone or EGFP-kindlin-3 constructs. The transfected cells were stained with 9EG7 monoclonal antibody to assess β_1 integrin activation. Flow cytometry was used to measure the MFI of 9EG7 binding. The *error bars* represent means ± S.E. of three independent experiments (*, $p < 0.001$).

observed response was blocked by the ADP scavenger, apyrase, indicating that the effects arose from released ADP, either by induction of secretion or by lysis of some platelets (Fig. 10*D*). To assess whether WT-K3 peptide induced α-granule secretion in platelets, P-selectin surface expression upon treatment with membrane-permeable peptide platelet suspensions was measured with fluorescently labeled P-selectin antibody. Treatment with 1 unit of human thrombin served as a positive control and stimulated an ~8-fold increase in P-selectin surface expression when compared with unstimulated platelets (Fig. 10*E*). Scr-K3 (10 μM) peptide induced a slight but not statistically significant increase in P-selectin surface expression. However, 10 μM WT-K3 peptide increased P-selectin surface expression ~4-fold when compared with resting platelets. Accordingly, in testing the effects of the peptides in platelet adhesion assays, we also included 5 μM apyrase to degrade released ADP. Platelets were pretreated with 10 μM WT-K3 or Scr-K3 peptide in the absence or presence of apyrase, were plated on fibrinogen for 30 min, and then fixed and stained with Alexa 568 phalloidin. There was no effect on platelet adhesion upon treatment with Scr-K3 peptide when compared with control platelets (Fig. 10*F*). Treatment with apyrase had no effect on adhesion of platelets incubated with the scrambled peptide (Fig. 10*F*); adhesion of platelets treated with WT-K3 peptide in the presence of apyrase was significantly blunted when compared with control or Scr-K3 peptide-treated platelets (Fig. 10*F*).

DISCUSSION

Kindlins play an indispensable role in integrin activation, but the mechanisms by which they have an impact on integrin activation remain unresolved. Despite their sequence similarity, kindlin-3 fails to activate $\alpha_{IIb}\beta_3$ in CHO cells where kindlin-1 and kindlin-2 exhibit coactivator function. Recently, Kasirer-Friede *et al.* (56) were able to show an effect of kindlin-3 on integrin activation in CHO cells coexpressing $\alpha_{IIb}\beta_3$ integrin, PAR1, adhesion and degranulation adaptor protein, mouse talin, and kindlin-3. Association of the adaptor protein with $\alpha_{IIb}\beta_3$ integrin and talin enabled agonist-dependent, kindlin-3-induced modest $\alpha_{IIb}\beta_3$ integrin activation (56). In this study, we have developed HEL cells as a transfectable model and have used human platelets to show that a post-translational modifi-



cation of kindlin-3 might regulate its agonist-induced activity toward integrins. HEL cells are well established hematopoietic cell model to study integrin $\alpha_{IIb}\beta_3$ function and signaling (33, 34). The work from different laboratories established agonist-dependent $\alpha_{IIb}\beta_3$ integrin activation in HEL cells, measured by soluble ligand binding and increased adhesion and spreading on integrin substrates, with $\alpha_{IIb}\beta_3$ integrin being a prominent component of focal adhesions (35, 36). Although HEL cells have been used broadly and are supported by our studies as an excellent model for $\alpha_{IIb}\beta_3$-dependent functions, it is noted that, unlike platelets that adhere to fibrinogen in the absence of agonist stimulation, HEL cells require agonist stimulation to adhere to substratum (this study and see Refs. 36, 41–43). This difference may depend on the high density of $\alpha_{IIb}\beta_3$ on platelets (57) and the release of ADP from dense granules, which activates the integrin (58).

Here, we show that kindlin-3 is highly expressed in HEL cells (Fig. 1), kindlin-3 colocalizes with β_3 integrin in focal adhesions during the cell spreading on fibrinogen (Fig. 2), kindlin-3 knockdown inhibits adhesion and spreading (Fig. 3), and both kindlin-3 and talin contribute to agonist-induced integrin function in HEL cells (Fig. 4). Of note, using PSGL-1 chimeras, kindlin-3 was found to utilize the same residues in the integrin β3 subunit as kindlin-2 uses in CHO cells (44). Point mutations and kindlin core spanning residues 749–762 of β_3 integrin CT (44) were equally effective in blunting cell spreading. The β_3 CT (Δ748), which only expresses the talin but not the kindlin-binding site, also significantly inhibited cell spreading. These data are consistent with the contribution of both talin and kindlin-3 to integrin-mediated functions in HEL cells.

In vitro studies using purified kindlins and integrin cytoplasmic tails have showed their direct interaction by various approaches (30, 44, 59, 60). However, in both HEL cells and platelets, the agonist stimulated was needed to demonstrate substantial interaction by coimmunoprecipitation approaches. Agonist stimulation was found to be required for the association of kindlin-3 with β_2-containing integrins in T cells (28, 61). Taken together, these data suggest agonist stimulation is a common mechanism in promoting association of kindlin-3 with integrins.

Phosphorylation of talin and β_3 CT itself has been proposed to serve as a key regulator of integrin-mediated functions (30, 62, 63), with β_3 phosphorylation decreasing integrin/talin and integrin/kindlin interactions and phosphorylation of talin controlling talin turnover and cell migration. In our study using mass spectrometry sequencing, we identified agonist-induced kindlin-3 phosphorylation in HEL cells and platelets. PMA in HEL cells and thrombin in platelets promoted kindlin-3-β_3 integrin association and at the same time promoted kindlin-3 phosphorylation on Thr^{482} and/or Ser^{484} (Fig. 5).

The identified phosphosite resides in a highly variable region of kindlin-3, and at least one of the residues, Thr^{482} or Ser^{484}, is evolutionarily conserved in all kindlin-3 orthologs analyzed (Fig. 7), including very distant species of *Takifugu rubripes* (torafugu) and *Danio rerio* (zebrafish), consistent with the importance of these phosphosites in kindlin-3 function. T482A/S484A kindlin-3 mutant impeded soluble ligand binding and cell spreading in HEL cells to a level comparable with Q597A/W598A kindlin-3 mutant, which is unable to bind integrin with high affinity (Fig. 9). Comparison of T482A/S484A mutant and wild-type kindlin-3 in other cell types, namely K562 $\alpha_{IIb}\beta_3$ cells and mouse macrophage cell line RAW 267.4, allowed us to extend our findings to another model cell type expressing $\alpha_{IIb}\beta_3$ integrin and to the regulation of β_1 integrin activation. The K562 $\alpha_{IIb}\beta_3$ cells that we used express talin (Fig. 1) but can only activate their integrin upon PMA stimulation when kindlin-3 is exogenously expressed. In this kindlin-3 null background, T482A/S484A kindlin-3 failed to activate integrin (Fig. 9), despite the expression levels comparable with wild-type kindlin-3, emphasizing further the pivotal role of phosphosites. RAW 267.4 cells had been used previously to demonstrate a role for kindlin-3 in β_1 integrin activation (19). In our experiments, we used 9EG7 monoclonal antibody, which recognizes only activated β_1 integrin and showed that T482A/S484A mutant-transfected RAW cells showed virtually no increase in 9EG7 antibody binding, comparable with Q597A/W598A kindlin-3 and vector control, whereas wild-type kindlin-3 was effective in β_1 integrin activation. These data indicate the importance of Thr^{482} and Ser^{484} also in β_1 integrin activation, suggesting a general mechanism for phosphorylation of kindlin-3-induced integrin activation. Supporting this conclusion, recent phosphoproteomic analyses identified Thr^{482} phosphorylation in T cells upon T cell receptor stimulation with anti-CD3 antibody (64) and Ser^{484} as a target of phosphorylation upon platelet activation by thrombin (65). In rat kindlin-3, Thr but not an Ser is present, whereas in mouse kindlin-3, Ser but not Thr is present. Thus, phosphorylation may be dependent on the species and cell type.

Mutation of Thr^{482} and Ser^{484} affects both inside-out (integrin activation) and outside-in (cell spreading) signaling, but it does not affect the binding of kindlin-3 to the β3 integrin. One possible explanation is that phosphorylation of Thr^{482} or Ser^{484}

FIGURE 10. **Kindlin-3 peptide blunts adhesion and spreading in HEL cells and platelets.** *A,* ability of peptides to enter HEL cells was evaluated by flow cytometry with trypan blue used to distinguish between extracellular and intracellular pools of peptides. Peptides (10 μM) were incubated with HEL cells for 10 min, and the fluorescence signal was reduced after quenching by ~50% (*blue lines*) compared with the unquenched signal (*green lines*). *B,* HEL cells were treated with 10 μM Scr-K3 peptide or WT-K3 peptide for 5 min. The cells were treated with 800 nM PMA, allowed to adhere to immobilized fibrinogen for 30 min, stained with and Alexa 568-phalloidin, and counted (*, $p < 0.001$). The *error bars* are means ± S.E. of three independent experiments. *C,* HEL cells were treated with 10 μM peptides followed by 800 nM PMA and allowed to adhere to fibrinogen-coated coverslips, and cell spreading was measured after 30 min. The adherent cells were fixed and stained with Alexa 568 phalloidin. The cell areas were measured using ImageJ software, and 300 cells were quantified in each experiment (*, $p < 0.001$). The *error bars* represent means ± S.E. of three independent experiments. *D,* representative traces showing the aggregation kinetics of human platelets treated with 10 μM kindlin-3 peptides in the absence or presence of apyrase. 1 unit of human thrombin was used as a positive control. Platelets were aggregated for 7 min in 37 °C. *E,* surface expression levels of P-selectin were evaluated in human platelets treated with kindlin-3 peptides. Human platelets were left untreated (*resting platelets*) or treated with 10 μM kindlin-3 peptides. 1 unit of thrombin was used as a positive control. Flow cytometry was used to measure the MFI of P-selectin antibody binding (*, $p < 0.001$). *F,* human platelets were pretreated with 10 μM peptides in the absence or presence of apyrase and plated on fibrinogen for 30 min, then fixed and stained with Alexa 568 phalloidin and counted (*, $p < 0.001$). The *error bars* are means ± S.E. of two independent experiments.

in this sequence may create a binding site(s) for yet unidentified kindlin-3 partners. The T482A/S484A mutant acted as dominant negative in adhesion and spreading assays, suggesting that it may compete with endogenous kindlin-3 for integrin binding and at the same time prevent an endogenous protein from binding to kindlin-3. In peptide-based functional assays, where cell-permeable peptides were introduced into HEL cells and platelets, the kindlin-3-derived peptide was very effective in inhibiting cell spreading and adhesion (Fig. 10), consistent with this hypothetical kindlin-3 binding partner. Interestingly, kindlin-3 peptide caused α-granule secretion, as assessed by an increase in surface expression of P-selectin and subsequent platelet aggregation, and this effect was abolished with apyrase treatment, an ADP scavenger (Fig. 10, *D* and *E*). The kindlin-3 peptide significantly increased platelet adhesion to immobilized fibrinogen, and this effect was abolished completely with apyrase treatment, with very few platelets adhering, when compared with control or scramble peptide. This observation suggests that this region of kindlin-3 may be involved in linking kindlin-3 to the cytoskeleton of HEL cells and platelets, and this interaction may regulate granule secretion in activated platelets.

We noted that thrombin induced phosphorylation of kindlin-3, and the extent of this phosphorylation was increased in the presence of EDTA (Fig. 6*D*), which would inhibit platelet aggregation. This effect is somewhat surprising, but several explanations can be considered. Most likely, the phosphorylation of kindlin-3 may be followed by rapid dephosphorylation. PKC-induced phosphorylation events, as we have shown kindlin-3 to be a PKC phosphorylation target (Fig. 6*C*), are often followed by rapid activation of phosphatases (38). Time course experiments will need to be undertaken to test this possibility. Another possibility is that binding of fibrinogen released from platelets to $\alpha_{IIb}\beta_3$ that occurs in the presence of divalent ions, but is prevented by EDTA, may blunt the phosphorylation of kindlin-3. There may also be decreased recovery of kindlin-3 from aggregated platelets, perhaps due to incorporation into the insoluble cytoskeleton, and we present some evidence consistent with this possibility in Fig. 5*C,* although we do not yet know whether it is phosphorylated kindlin-3 that selectively associates with the cytoskeleton.

In summary, we identified a novel mechanism of regulating kindlin-3 function and integrin activation in hematopoietic cells. Our combined data provide evidence that kindlin-3 phosphorylation is crucial for effective integrin activation in different cell models and platelets and provide an explanation for functional differences between kindlin-3 and the two other kindlin family members.

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