

The Integrin Co-activator Kindlin-3 Is Expressed and Functional in a Non-hematopoietic Cell, the Endothelial Cell^{*S}

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Integrin activation is crucial for numerous cellular responses, including cell adhesion, migration, and survival. Recent studies in mice have specifically emphasized the vital role of kindlin-3 in integrin activation. Kindlin-3 deficiency in humans also has now been documented and includes symptoms of bleeding, frequent infections, and osteopetrosis, which are consequences of an inability to activate $\beta 1$, $\beta 2$, and $\beta 3$ integrins. To date, kindlin-3 was thought to be restricted to hematopoietic cells. In this article, we demonstrate that kindlin-3 is present in human endothelial cells derived from various anatomical origins. The mRNA and protein for *KINDLIN-3* was detected in endothelial cells by reverse transcription-PCR and Western blots. When subjected to sequencing by mass spectrometry, the protein was identified as authentic kindlin-3 and unequivocally distinguished from *KINDLIN-1* and *KINDLIN-2* or any other known protein. By quantitative real time PCR, the level of kindlin-3 in endothelial cells was 20–50% of that of kindlin-2. Using knockdown approaches, we show that kindlin-3 plays a role in integrin-mediated adhesion of endothelial cells. This function depends upon the integrin and substrate and is distinct from that of kindlin-2. Formation of tube-like structures in Matrigel also was impaired by kindlin-3 knockdown. Mechanistically, the distinct functions of the kindlins can be traced to differences in their subcellular localization in integrin-containing adhesion structures. Thus, the prevailing view that individual kindlins exert their functions in a cell type-specific manner must now be modified to consider distinct functions of the different family members within the same cell type.

The contributions of integrins to the adhesive responses of cells depend upon the dynamic regulation of their activation states. Integrin activation depends upon their engagement of binding partners via their short cytoplasmic tails (1, 2). 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 (3–5). They are evolutionary conserved with an

ortholog, UNC-112, present in *Caenorhabditis elegans* (6). In mammals, there are three kindlin family members, each characterized by a C-terminal 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 (7–11). Kindlins and talin bind to the cytoplasmic tails of integrin β subunits via their F3 (PTB) subdomains within their FERM domains. However, the binding sites of talin and kindlins within the β cytoplasmic tails do not overlap (5, 12), and the two interactions appear to act cooperatively to optimize integrin activation (12, 13). Hence, cells or mice with decreased kindlin expression levels are unable to properly activate their integrins.

Kindlin-1 (UNC-112 related protein 1) is expressed predominantly in epithelial cells; and mutation in the kindlin-1 gene causes Kindler syndrome in humans (14, 15), 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 (16). Kindlin-2 (Mig-2) is expressed in most tissues and in many different cell types, and knock-out of kindlin-2 is lethal in mice and zebrafish (13, 17). Mice in which the kindlin-3 gene has been inactivated display defects in platelet (18) and leukocyte (19) responses dependent on integrin activation and the mice die by day 7 postnatally (18) from as yet undefined causes. Recently, kindlin-3 mutations have been identified in humans with a rare syndrome referred to as integrin activation deficiency disease, leukocyte adhesion deficiency III, or LADI variant (20–23). The manifestations of the kindlin-3 deficiency include episodic bleeding, susceptibility to frequent infections, and osteopetrosis, which are consequences of an inability to activate $\beta 1$, $\beta 2$, and $\beta 3$ integrins (21, 22).

To date, the original publication (4) and all reviews (24, 25) have emphasized that kindlin-3 is restricted to hematopoietic cells and the cellular defects in integrin activation deficiency disease patients support the importance of kindlin-3 in blood cell function. In the present study, using RT²-PCR, Western blotting, and mass spectrometry approaches, we demonstrate for the first time the presence of kindlin-3 in non-hematopoietic cells. Kindlin-3 is an endothelial cell protein, and this point

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^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Movies 1–3.

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² The abbreviations used are: RT, reverse transcription; CHO, Chinese hamster ovary; EGFP, enhanced green fluorescent protein; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; TNF- α , tumor necrosis factor- α ; PBS, phosphate-buffered saline; DAPI, 4',6-diamidino-2-phenylindole.

is demonstrated both in cultured endothelial cells from various anatomic origins and *in vivo*. In fact, in some endothelial cells the level of *KINDLIN-3* mRNA is ~50% of that of kindlin-2. Furthermore, kindlin-3 knockdown in endothelial cells results in impaired adhesion to integrin substrates, despite the presence of kindlin-2 in the same cells. These observations suggest different and important roles of both kindlins in integrin signaling in these vascular cells.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Rabbit polyclonal antibodies against kindlin-3 were from ProSci Incorporated, mouse monoclonal antibody against EGFP (JL-8) was from Clontech, mouse fluorescein isothiocyanate-labeled monoclonal antibody against human $\beta 3$ integrin was from BD Biosciences, mouse monoclonal antibody against GAPDH was from Affinity BioReagents, mouse monoclonal antibody against ICAM-1 was from R&D Systems, and rat monoclonal antibody against $\beta 1$ integrin was from Transduction Laboratories. Mouse monoclonal antibody against kindlin-2 was provided by Dr. Chuan-yue Wu (University of Pittsburgh School of Medicine, Pittsburgh, PA). Rabbit anti-kindlin-3 antibodies were prepared at New England Peptide, Inc. by immunizing rabbits with the GEVGEPA GTDPGLD peptide-keyhole limpet hemocyanin conjugate. Because these antibodies did not recognize mouse kindlin-3 protein, second rabbit kindlin-3 antibodies were prepared at Alpha Diagnostics International, by immunizing rabbits with ELDEDLFLQLTGGEAF peptide-keyhole limpet hemocyanin conjugate. Alexa-coupled secondary antibodies and calcein-AM were from Molecular Probes. Horseradish peroxidase-conjugated secondary antibodies and protein A/G-agarose were from Santa Cruz Biotechnology, ECL reagent was from Roche Applied Science. Human recombinant TNF- α and human recombinant vascular endothelial growth factor were from R&D Systems, human fibrinogen was from Enzyme Research Systems, human vitronectin, human laminin, and rat collagen were from Sigma. TRIzol reagent was from Invitrogen, TITANIUM One-Step RT-PCR kit was from Clontech, kindlin 2- and kindlin 3-specific and non-targeting siRNAs were from Dharmacon, and the TOPO TA Cloning Kit for subcloning from Invitrogen. RPMI, Dulbecco's modified Eagle's medium/F-12, penicillin/streptomycin, and L-glutamine were from Media Lab (Cleveland Clinic), EGM[®] Endothelial Cell Growth Medium from Lonza, and fetal calf serum from Atlanta Biologicals.

cDNA Constructs—EGFP-tagged kindlins were created by cloning full-length kindlin-1, kindlin-2, or kindlin-3 in-frame with EGFP into pEGFP-C2 vector (Clontech) as previously described (12).

Cells and Transfections—Human platelets were isolated by collecting blood into 0.38% sodium citrate. Platelets were isolated from platelet-rich plasma by gel filtration and resuspended at $1-2 \times 10^9$ platelets/ml in modified Tyrode buffer. Human lymphocytes were separated by Ficoll density gradient separation, washed twice, and resuspended at 2×10^7 cells/ml. The human megakaryoblastic leukemic cell line Meg-01, human monocytic cell line THP-1, and Chinese hamster ovary (CHO) cells were from American Type Culture Collection.

Human umbilical vein endothelial cells (HUVEC) were provided by Dr. Paul DiCorleto (Cleveland Clinic) and maintained in EGM Endothelial Cell Growth Medium. HUVEC were used between passages 2 and 4. Transient transfections of CHO cells were performed with Lipofectamine 2000. Targetect siRNA transfection kit from Targeting Systems was used for delivery of siRNAs.

Western Blotting—Human brain, human heart, and human liver protein medleys were from Clontech. 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 $2 \times$ Laemmli buffer and subjected to SDS-PAGE. Proteins were transferred to polyvinylidene difluoride, and blots were probed with antibodies of interest, using 5% bovine serum albumin as blocking agent. For assessment of the specificity of the kindlin-3 antibodies, as a negative control, kindlin-immunizing peptide was included in the blotting procedure.

Immunoprecipitation—For antibody-mediated precipitation of endogenous kindlin-3 from Meg-01 and HUVEC, 1×10^6 cells per sample 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 $12,000 \times g$ for 15 min. Aliquots of the detergent-soluble material were precleared on protein A/G-agarose for 1 h in 4 °C. Precleared lysates were incubated with 2 μ l 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 kindlin-3 antibodies from ProSci Incorporated.

Mass Spectrometry—Endogenous kindlin-3 was immunoprecipitated from HUVEC 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 by submitting all of the CID spectra collected in the LC-MS run to the data base search program Mascot.

Immunofluorescence—HUVECs were plated on 20 μ g/ml of fibrinogen, 10 μ g/ml of fibronectin, 10 μ g/ml of collagen, or 2.5 μ g/ml of vitronectin for 1 h, fixed, 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. The specificity of the kindlin-3 antibodies was examined by the use of preimmune serum or inclusion of immunizing peptide in the primary antibody labeling step. Frozen prostate cancer sections were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 diluted in 4% paraformaldehyde, blocked with 5% goat serum for 1 h at 37 °C, and stained with CD31 and kindlin-3 antibodies for ~18 h. Antigen-antibody complexes were detected by staining with Alexa-coupled secondary antibodies for 1 h. The specificity of the two kindlin-3 antibodies used in this study was examined by inclusion of the immunizing peptides in the primary antibody labeling step.

Stained cells were visualized with a $\times 40$ 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

Kindlin-3 in Endothelial Cells

and images collected using Leica Confocal Software (version 2.5 Build 1227).

RT-PCR—Total RNA was isolated from HUVEC, human aortic endothelial cells, human dermal microvascular endothelial cells, human neutrophils, and human lymphocytes with TRIzol reagent according to the manufacturer's protocol. Human smooth muscle total RNA and human skeletal muscle total RNA were purchased from Clontech. The TITANIUM One-Step RT-PCR kit was used for first strand DNA synthesis and to amplify specific cDNA fragments using following primers: *KINDLIN-2* forward, 5'-CTG GGA AAT CAA AAT GGT CAC CGT AGA GT-3'; *KINDLIN-2* reverse, 5'-GCC CTG GAT AGC TTT AAA ATA GAT GG-3'; *KINDLIN-3* forward, 5'-TCA AGG GCA GCA GGA AAG ACG A-3'; *KINDLIN-3* reverse, 5'-GGG GTA TCA GCC TCA GCC ACT CAG AC-3'; *GAPDH* forward, 5'-TGA AGG TCG GAG TCA ACG ATT TGG T-3'; *GAPDH* reverse, 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3'. cDNA fragments were cloned into pCR2.1 TOPO-TA vector, sequenced with an Applied Biosystems 3730xl DNA analyzer in our Genomics Core (Cleveland Clinic) and the resulting sequences were analyzed using BLAST.

Quantitative Real Time PCR—Total RNA (~4 μ g) was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) and random hexamers, according to the manufacturer's protocol. Real time quantitative PCR of the cDNA template was performed in an iCycler (Bio-Rad). *GAPDH*, *KINDLIN-2*, and *KINDLIN-3* primers were from SuperArray. The PCR contained 150 ng of cDNA, 10 μ M forward and reverse primers, and 12.5 μ l of 2 \times RT² Real Time SYBR Green PCR Master mix (SuperArray) in a total volume of 25 μ l. All PCR were performed in duplicate. Results were calculated as expression of the target gene relative to expression of the reference gene (*GAPDH*).

RNA Interference—To knockdown endogenous kindlins in HUVEC, siRNAs targeting kindlin-2 or kindlin-3 or control, non-targeting siRNA were transfected into cells using Targetfect siRNA transfection kit 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-2 and kindlin-3 and *GAPDH* antibody as a loading control.

Adhesion Assays—Non-transfected HUVECs or HUVECs transfected with kindlin-2, kindlin-3, or control siRNA were incubated with immobilized integrin ligands (fibrinogen, 20 μ g/ml; fibronectin, 10 μ g/ml; vitronectin, 2.5 μ g/ml), 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 and DAPI. The cells were photographed with a \times 20 objective, and the adherent cells were counted from 12 randomly selected fields.

Endothelial Cell Tube Formation—The ability of non-transfected HUVECs or HUVECs transfected with kindlin-2, kindlin-3, or control siRNA to form tubes *in vitro* was assessed on Matrigel. 12-Multiwell culture plates were coated with 500 μ l of growth factor-reduced Matrigel (BD Biosciences), containing 10 μ g/ml of bovine fibronectin (Sigma), and incubated at 37 °C for 30 min. siRNA-transfected HUVECs were seeded at a density of 2.5×10^5 cells per well on polymerized Matrigel in the

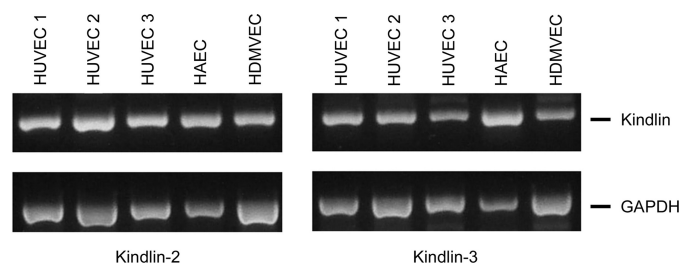


FIGURE 1. Expression of *KINDLIN-3* mRNA in endothelial cells. 200 ng of total RNA was subjected to RT-PCR, using *KINDLIN-2*- and *KINDLIN-3*-specific primers. *GAPDH* was used as a loading control.

presence of vascular endothelial growth factor (10 ng/ml). Live time-lapse photography was performed for 12 h, using 5-min intervals on a Leica DMIRB Inverted Microscope equipped with a Roper Scientific CoolSNAP HQ Cooled CCD camera, temperature controller, and CO₂ incubation chamber. Snapshots were taken using MetaMorph software and videos of tube formation were created in ImageJ.

THP-1 Cell Adhesion to HUVECs—Non-transfected or siRNA-transfected HUVECs were plated in 24-well tissue culture plates 24 h after transfection, allowed to spread and become confluent and serum-starved for 3 h. ICAM-1 expression was induced with 10 ng/ml of human recombinant TNF- α for 16 h. 5×10^5 HUVECs was resuspended in 50 μ l of Hanks' balanced salt solution with 0.1% bovine serum albumin and incubated with 2 μ l/sample of ICAM-1 monoclonal antibody for 30 min on ice. Cells were washed, resuspended in 50 μ l of Hanks' balanced salt solution, and incubated with 10 μ g/ml of Alexa 488-conjugated secondary antibody. HUVECs were washed with PBS and fixed with 2% paraformaldehyde. Antibody binding to HUVECs with or without stimulation was determined by BD Biosciences FACSscan. THP-1 cells were washed twice with PBS and resuspended in PBS at 1×10^6 cells/ml. The cells were labeled with calcein-AM at a final concentration of 1 μ M for 30 min at 37 °C. Cells were washed twice by centrifugation and resuspended in PBS containing 0.5% bovine serum albumin. Immediately before the assay, HUVECs were washed twice with PBS. Calcein-AM-labeled THP-1 cells (1×10^5 or 2×10^5 /well) were added to HUVECs and incubated for 1 h at 37 °C. To remove non-adherent cells, wells were washed twice with PBS. Relative fluorescence was read using CytoFluor II fluorescence plate reader (Applied Biosystems), using unlabeled cells as a negative control.

Statistical Analysis—Two-tailed Student's *t* tests were performed where indicated in the text using SigmaPlot 11. Significance was considered when $p < 0.05$.

RESULTS AND DISCUSSION

***KINDLIN-3* mRNA Is Present in Endothelial Cells**—Kindlin-3 deficiencies in humans, variably referred to as leukocyte adhesion deficiency III, LADI variant, or integrin activation deficiency disease (20, 22, 26, 27), and in mice (18, 19) give rise to defects in platelet and leukocyte responses dependent on integrin activation. The severity of the bleeding following minor trauma in our integrin activation deficiency disease patients led us to test whether this kindlin might also be present in endothelial cells and not entirely restricted to hematopoietic

cells. As a first step to address this possibility, we analyzed for the presence of *KINDLIN-3* mRNA in HUVEC from three different donors as well as human aortic endothelial cells, and human dermal microvascular endothelial cells. *KINDLIN-2* was used as a positive control because it is expressed abundantly in endothelial cells (12). Total RNA was isolated and 200 ng was subjected to RT-PCR using primers specific for *KINDLIN-2*

TABLE 1
The relative expression levels of *KINDLIN-2* and *KINDLIN-3* in different cell types

Total RNA from human lymphocytes, neutrophils and different preparations of HUVECs was reverse transcribed, and 150 ng of cDNA was used for PCR. All PCR were performed in duplicate. Results were calculated as expression of the target gene relative to expression of the reference gene (*GAPDH*).

	Lymphocytes	Neutrophils	HUVEC-1	HUVEC-2
<i>KINDLIN-2</i>	1	0.18	30.9	42.4
<i>KINDLIN-3</i>	1.03	1	0.018	0.058
<i>KINDLIN-2/KINDLIN-3</i>	0.0026/1	0.00069/1	1/0.22	1/0.51

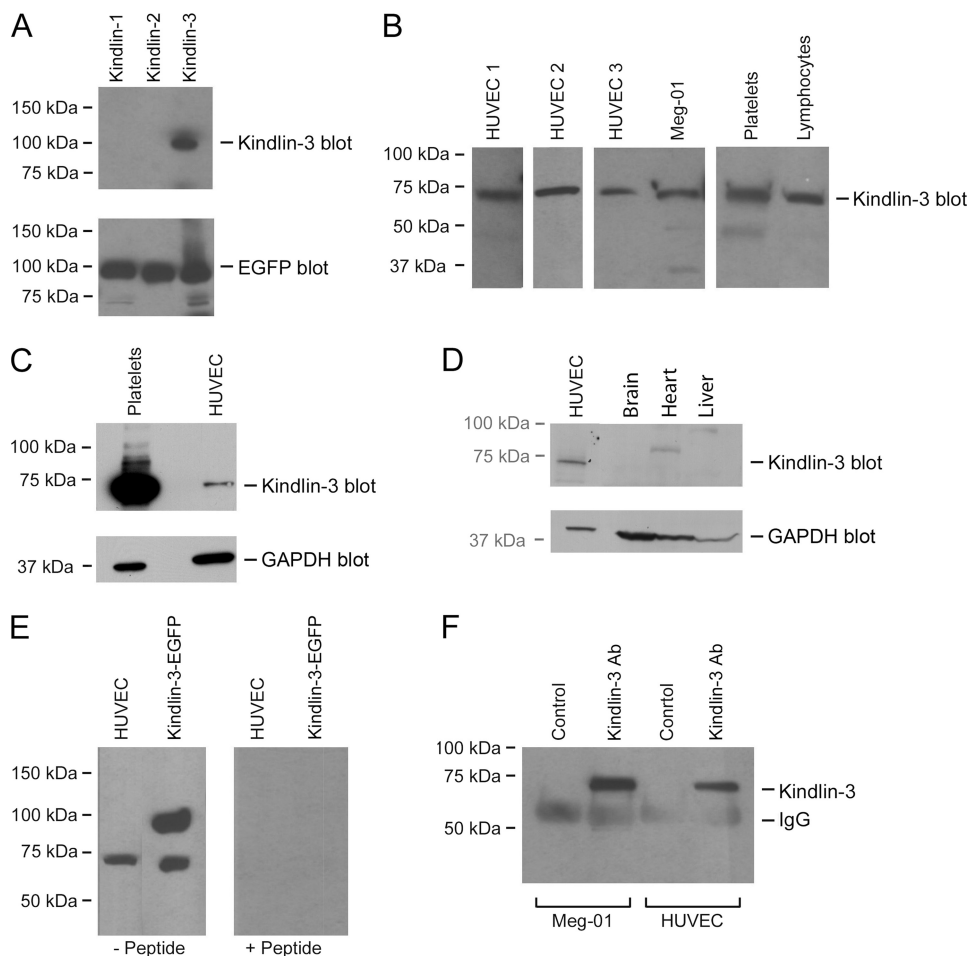


FIGURE 2. Identification of kindlin-3 protein in endothelial cells by Western blotting and immunoprecipitation. A, Western blots of CHO cells expressing EGFP-tagged kindlin-1, kindlin-2, or kindlin-3 cell lysates were probed with kindlin-3 antibodies (upper panel) or EGFP monoclonal antibody (lower panel). The specificity of the anti-kindlin-3 anti-peptide antibody is demonstrated. B, Western blots of HUVECs, Meg-01, platelet, and lymphocyte cell lysates were probed with the anti-peptide kindlin-3 antibodies. Identical results were obtained in three independent experiments. C, Western blots of HUVEC and platelet lysates were developed with the anti-peptide kindlin-3 antibodies and GAPDH antibodies. D, Western blots of HUVEC, brain, heart, and liver extracts were probed with the anti-peptide kindlin-3 antibodies and GAPDH antibodies. E, Western blots of HUVECs and CHO cells expressing EGFP-kindlin-3 cell lysates were probed with anti-peptide kindlin-3 antibodies in the absence (left-hand panel) or presence (right-hand panel) of the immunizing peptide. F, HUVECs or Meg-01 cell lysates were immunoprecipitated with kindlin-3 antibodies. Preimmune serum was used as negative control. Immunoprecipitates were analyzed on Western blots with a second kindlin-3 antibody. Identical results were obtained in three independent experiments.

and *KINDLIN-3* transcripts. The primers used to amplify *KINDLIN-3* were designed to amplify a 590-bp RT-PCR product that spans exon 14 to 15 of the *KINDLIN-3* gene, and, therefore, prevents amplification from possible contaminating genomic DNA. Expression of *KINDLIN-3* as well as *KINDLIN-2* was detected in all five RNAs analyzed (Fig. 1). The *KINDLIN-3* product was of its predicted 590 bp size. The *KINDLIN-2* product was distinct, at its predicted size of 510 bp, and was also present in all five endothelial cell isolates tested. Sequencing of the *KINDLIN-2*- and *KINDLIN-3*-derived PCR products from all the cells revealed 100% identity to human *KINDLIN-3* as well as the kindlin-2 mRNAs, respectively (data not shown). To provide insight into the relative levels of *KINDLIN-2* and *KINDLIN-3* mRNAs in HUVECs, real time quantitative PCR was performed using total RNA from different preparations of HUVEC. The analyses were also performed with total RNA from blood neutrophils and lymphocytes as hematopoietic

cells, which are known to express high levels of *KINDLIN-3* but low levels of *KINDLIN-2* (4). The data shown in Table 1 reveal that: 1) in hematopoietic cells, *KINDLIN-2* mRNA was barely detectable, whereas *KINDLIN-3* mRNA was abundant (*KINDLIN-3/KINDLIN-2* ratio was 380- and 1449-fold in lymphocytes and neutrophils, respectively); 2) *KINDLIN-2* mRNA was ~30–40-fold higher in HUVECs than in hematopoietic cells; 3) HUVECs expressed 20–50 times less *KINDLIN-3* mRNA than hematopoietic cells; and 4) HUVECs express only 2–5-fold less *KINDLIN-3* than *KINDLIN-2* mRNA (kindlin-2/kindlin-3 ratios were 1/0.22 and 1/0.51 for two different HUVEC preparations).

Kindlin-3 Protein Is Present in HUVECs—Knowing that *KINDLIN-3* is present in endothelial cells at the mRNA level, next we examined whether kindlin-3 protein is present and focused on HUVECs where isolates from several different donors were available. The polyclonal antibodies raised against a kindlin-3-specific peptide sequence readily recognized the EGFP-kindlin-3 fusion protein expressed in CHO cells (Fig. 2A, upper blot). The antibodies did not recognize EGFP-tagged kindlin-1 and kindlin-2, which were expressed at similar levels in CHO cells (Fig. 2A, lower blot). Western blot analysis of the total HUVEC lysates from three different donors revealed the presence

Kindlin-3 in Endothelial Cells

of the single ~72-kDa band reactive with the kindlin-3 anti-peptide antibody (Fig. 2B). The antibody also recognized a single 72-kDa band in hematopoietic cells, such as Meg-01 (a human megakaryocytic cell line), human platelets, and human lymphocytes, used as positive controls, further confirming the specificity of the antibody for kindlin-3. Quantitation of the kindlin-3 band intensities in platelets, lymphocytes, and HUVECs revealed that platelets and lymphocytes expressed ~40–50 times more kindlin-3 protein than HUVECs (Fig. 2C and data not shown). Ussar and colleagues (4) failed to detect kindlin-3 protein in mouse tissues, such as heart, liver, and brain, and we analyzed commercially available human brain, liver, and heart lysates for the presence of kindlin-3. Our anti-kindlin-3 antibodies readily recognized kindlin-3 band in HUVEC total lysates (Fig. 2D); however, it failed to recognize kindlin-3 in extracts of these other human tissues. We were able to detect very faint bands at a molecular weight for kindlin-3 in brain, liver, and heart extracts after prolonged blot exposure (data not shown); but the kindlin-3 expression levels were substantially lower than in HUVEC (~1% in brain, 4% in heart, and 12.6% in liver when normalized to GAPDH loading control). As further evidence that the band reactive with the antibody was authentic kindlin-3, this reaction was neutralized in HUVECs and CHO cells by expressing EGFP-kindlin-3 when the immunizing peptide was added to the blotting reagents (Fig. 2E).

To further confirm the presence of kindlin-3 in HUVECs, additional approaches were used. First, kindlin-3 was immunoprecipitated from HUVECs and Meg-01 cell lysates with the kindlin-3 anti-peptide antibody, and immunoprecipitates were analyzed on Western blots with a different (commercially available) kindlin-3 antibody. Anti-kindlin-3, but not the preimmune serum, used as a negative control, immunoprecipitated a single ~72-kDa band

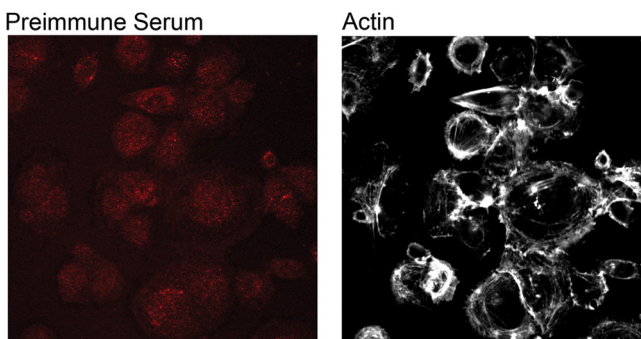
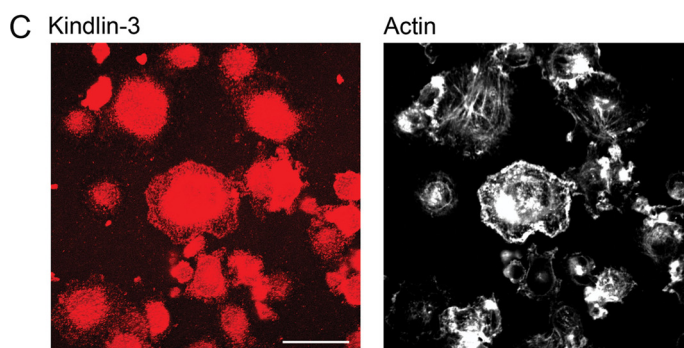
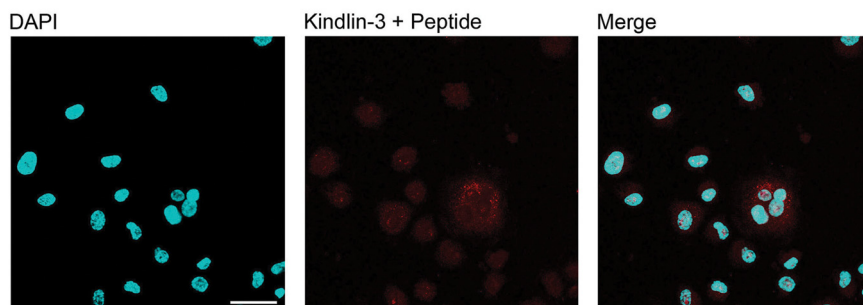
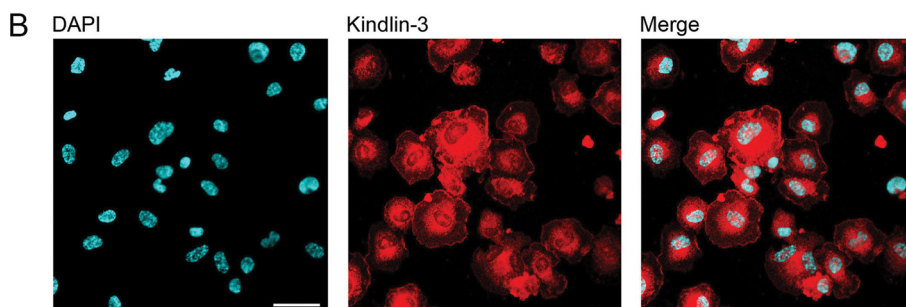
A UNC-112 related protein 2 long form [Homo sapiens]

Sequence Coverage: 41%

Matched peptides shown in **Bold Red**

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1 MAGMKTASGD YIDSSWELRV FVGEEDPEAE SVTLRVTGES HIGGVLLKIV
51 EQINRKQDWS DHAIWWEQKR QWLLQTHWTL DKYGIADAR LFFGPQHRPV
101 ILRLPNRRAL RLRASFSQPL FQAVAAICRL LSIRHPEELS LLRAPEKKEK
151 KKKEKEPEEE LYDLSKVVLA GGVAPALFRG MPAHFSDSAQ TEACYHMLSR
201 PQPPDPPLLL QRLPRPSSL DKTQLHSRWL DSSRCLMQQG IKAGDALWLR
251 FKYYSFFDL PKTDPVRLTQ LYBQARWLL LEEIDCTEEE MMVPAALQYH
301 INKLSQSGEV GEPAGTDPGL DDLVALSNL EVKLEGSAPT DVLDSLTTIP
351 ELKDLHLRIFR IPRRPRKLTL KGYRQHVWFV KETTLSEYK QDEAPGDPIQ
401 QLNLKGCEVV PDVNVSGKF CIKLLVPSPE GMSEIYLRCQ DEQQYARWMA
451 GCRLLASKGRT MADSSYTSEV QAILLFLSLQ RTGSGGPGNH PHGPDASAEG
501 LNPYGLVAPR FORKFKAKQL TPRILEAHQN VAQLSLAEAQ LRFIQAWQSL
551 PDFGISYVMV RFGKSRKDEI LGIANNRLIR IDLAVGDVVK TWRFSNMRQW
601 NVNWDIRQVA IEFDEHINVA FSCVSASCRI VHEYIGGYIF LSTRERARGE
651 ELDEDLFLQL TGGHEAF
  
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detected with the second kindlin-3 antibody (Fig. 2F). Second, the 72-kDa band immunoprecipitated by the anti-peptide antibody was subjected to identification by mass spectrometry. The kindlin-3 peptide antibody was used to immunoprecipitate the 72-kDa band from HUVEC cell lysates, and the immunoprecipitates were resolved on polyacrylamide gels and stained with Coomassie Blue to visualize bands. The 72-kDa band, which was readily detected, was excised from the gel, digested with trypsin, and the tryptic peptides were subjected to tandem mass spectrometry sequencing. Mascot program analysis of the data confirmed that the 72-kDa band was the human kindlin-3 protein (Fig. 3A). Altogether, 23 peptides showing 100% homology to human kindlin-3 were sequenced, distributed throughout the entire kindlin-3 protein, and accounted for 41% of the total kindlin-3 sequence (see Fig. 3A). Furthermore, when subjected to BLAST search against the non-redundant human protein data base, these peptides resulted in several hits with 100% identity to the human kindlin-3 but did not match well to kindlin-1, kindlin-2, or protein sequences other than kindlin-3 in the human genome (not shown). Third, immunofluorescent staining of HUVECs was performed with the anti-peptide kindlin-3 antibody. One representative of multiple fields is shown in Fig. 3B and indicates that kindlin-3 is expressed in all and not just a subpopulation of the HUVECs. Neutralization of the staining by the immunizing peptide establishes specificity. Pre-immune serum, used at the same concentration as kindlin-3 antibodies gave weak nuclear staining, as did anti-kindlin-3 serum after neutralization (Fig. 3C). However, this staining was very weak when compared with kindlin-3 peptide antibody alone, showing that the apparent nuclear staining obtained with kindlin-3 antibodies is specific. Nuclear localization of kindlin-1 and kindlin-2 has been previously observed by others (28, 29).

Kindlin-3 Is Present in Endothelial Cells *in Vivo*—The results described above show the presence of kindlin-3 in cultured endothelial cells. To determine whether kindlin-3 is expressed in endothelial cells *in vivo*, first we assessed its presence in a prostate tumor as a highly vascularized tissue. Frozen sections of prostate tumor were used for immunostaining (Fig. 4A). Double staining of prostate cancer frozen sections with antibodies against the endothelial cell marker CD31 and kindlin-3 revealed the presence of well developed blood vessels that stained positively for both CD31 and kindlin-3 (Fig. 4A, *right-hand panel*, double-positive cells marked with *arrows*). Interestingly, kindlin-3 was present in CD31-negative cells as well, suggesting that kindlin-3 might be present in other non-hematopoietic cells (Fig. 4A, *left-hand panel*). Verifying the specificity of immunofluorescence staining, inclusion of the immunizing peptide during the primary antibody reaction with the sections (Fig. 4A, *left-hand panel*) resulted in complete loss of kindlin-3 staining. Although the above analyses focused on

kindlin-3 in human endothelial cells, we developed a second anti-peptide antibody to a kindlin-3 sequence conserved in the human and mouse protein and found that the antibody reacted with kindlin-3 in mouse platelets (Fig. 4B, *left-hand panel*). Further evidence for specificity of the antibody was demonstrated by neutralization of its reactivity when immunizing peptide was included in primary antibody step (Fig. 4B, *right-hand panel*). By immunostaining, this antibody reacted with CD31-positive cells in normal mouse skeletal muscle tissue (Fig. 4C, *left-hand panel*), suggesting the presence of kindlin-3 in endothelial cells present in normal as well as pathological tissue samples. This reactivity also was neutralized by the peptide immunogen (Fig. 4C, *right-hand panel*).

Kindlin-3 and Kindlin-2 Localize Differently during Endothelial Cell Spreading on Integrin Substrates—Our previous study had established that kindlin-2 associates dynamically with $\beta 3$ integrin during spreading (12), with $\beta 3$ integrin and kindlin-2 colocalizing in lamellipodium, and in focal adhesions in fully spread cells. Here, we sought to determine kindlin-3 localization in HUVECs and whether kindlin-2 and kindlin-3 localization differed during HUVEC adhesion on various $\beta 1$ and $\beta 3$ integrin ligands. To ensure that our anti-kindlin-3 antibody reacted with fixed cells in a specific manner, we stained HUVECs spread on fibrinogen (Fig. 3B) and confirmed that staining was abolished by preincubation of the antibody with the immunizing peptide. To determine kindlin-3 distribution in HUVECs, cells were allowed to spread on vitronectin, fibrinogen, fibronectin, and collagen for 1 or 2 h, fixed, and stained with kindlin-2, kindlin-3, and integrin-specific antibodies. In cells spread on $\beta 3$ or $\beta 1$ integrin substrates, integrins localized to lamellipodia at the edges of spreading cells and in focal adhesions (Fig. 5, *A and B*). Staining for kindlin-3 revealed that it was highly concentrated at the regions of extending membranes where it colocalized with $\beta 3$ and $\beta 1$ integrins (Fig. 5, *A and B*, *large arrows*). Kindlin-3 was noticeably absent from $\beta 3$ -containing and $\beta 1$ -containing focal adhesions (Fig. 5, *A and B*, *small arrows*). In contrast, whereas kindlin-2 also was present with integrins at the membrane extensions, it was found in focal adhesions (Fig. 5, *A and B*, *double arrows*) where kindlin-3 was absent. To assess more clearly the differences in kindlin-2 and kindlin-3 distribution on the integrin substrate, the merge of kindlin-2 and kindlin-3 staining was created (Fig. 5C). The areas of kindlin-2 staining (focal adhesions) are marked with *boxes* and kindlin-3 is absent from these areas. Ussar *et al.* (4) had previously reported that kindlin-2 and kindlin-3 showed different localization pattern cells when the proteins were over-expressed as EGFP fusion proteins in cells. Our data demonstrates such differences at the level of the endogenous proteins expressed and in non-hematopoietic cells not previously known to express the two kindlins.

FIGURE 3. Detection of kindlin-3 in endothelial cells by mass spectrometry and immunofluorescence. *A*, endogenous kindlin-3 was immunoprecipitated from HUVEC with peptide antibodies. The 72-kDa band was excised from the gel and subjected to mass spectrometry sequencing. Peptide coverage is shown, matched peptides shown in *red*. Peptide coverage was 41%. *B*, HUVECs spread on fibrinogen for 30 min were fixed, permeabilized, and stained with peptide antibodies to detect kindlin-3, followed by Alexa 568 anti-rabbit IgG and DAPI to visualize all the cells (*upper panels*). The specificity of the anti-kindlin-3 peptide antibodies was examined by inclusion of immunizing peptide in the primary antibody labeling step (*lower panels*). *C*, HUVECs spread on vitronectin for 1 h were fixed, permeabilized, and stained with peptide antibodies to detect kindlin-3, followed by Alexa 568 anti-rabbit IgG and phalloidin-Alexa 633 to visualize all the cells (*upper panels*). Preimmune serum from the same rabbit was used for staining to establish background staining (*lower panels*). Bars = 40 μ m.

Kindlin-3 in Endothelial Cells

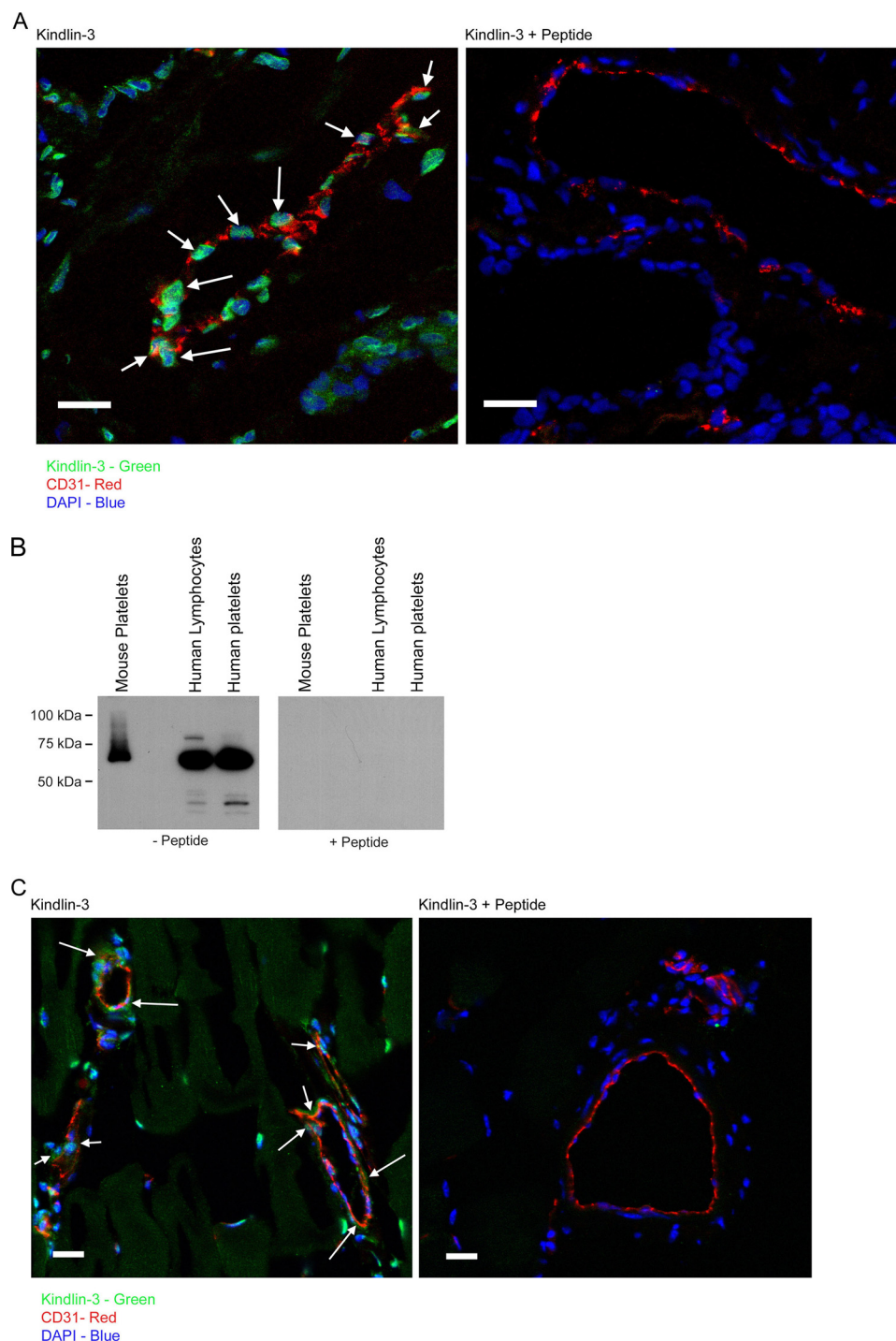


FIGURE 4. Kindlin-3 is present in endothelial cells *in vivo*. *A*, frozen tissue sections of human prostate cancer were stained for kindlin-3 (green) and CD31 (red) and mounted in DAPI containing medium. Endothelial cells positive for CD31 and kindlin-3 are marked with arrows. The kindlin-3 immunizing peptide used to raise the antibodies was included as specificity controls. *B*, Western blots of mouse and human platelet and human lymphocyte cell lysates were probed with the anti-peptide kindlin-3 antibodies in the absence (left-hand panel) or presence (right-hand panel) of the immunizing peptide. *C*, frozen tissue sections of mouse skeletal muscle were stained for kindlin-3 (green) and CD31 (red) and mounted in DAPI containing medium. Endothelial cells positive for CD31 and kindlin-3 are marked with arrows. The kindlin-3 immunizing peptide used to raise the antibodies was included as specificity controls. Bars = 20 μ m.

Kindlin-3 Knockdown Blunts Integrin-dependent Adhesion in HUVECs—To determine whether kindlin-3 plays a role in integrin function in endothelial cells, we performed RNA interference experiments. In our previous study, we have shown that

kindlin-2 knockdown impairs α v β 3-mediated adhesion and migration in HUVECs (12), and therefore kindlin-2 siRNA was used as a control in the subsequent experiments. Because kindlin-2 and kindlin-3 mRNAs show partial homology, it was important to establish that kindlin-3 or kindlin-2 knockdown does not show an off-target effect on expression of each other. HUVECs were transfected with siRNAs specific to kindlin-2 or kindlin-3, or a non-targeting siRNA as a control. The levels of expression of kindlin-2 and kindlin-3 were analyzed on Western blots (Fig. 6A). Control siRNA did not have an effect on expression of either kindlin (Fig. 6A, lane 2 of *kindlin-2* and *kindlin-3* blots). In three experiments, the levels of expression of kindlin-2 and kindlin-3 in cells treated with the control siRNA were 97.3 ± 9.3 and $94.3 \pm 11.49\%$, respectively, compared with the untreated cells. Kindlin-2 and kindlin-3 siRNAs effectively inhibited the expression of their respective target proteins, but did not exhibit off-target effects (Fig. 6A, lanes 3 and 4 of *kindlin-2* and *kindlin-3* blots), confirming the specificity of each siRNA. Kindlin-2 siRNA decreased kindlin-3 protein expression by $69.3 \pm 17.3\%$, whereas kindlin-3 expression remained at $99.2 \pm 10.6\%$ (values relative to non-transfected cells in three independent experiments). Kindlin-3 siRNA decreased kindlin-3 expression by $61.1 \pm 16.3\%$ but did not affect kindlin-2 expression. None of the siRNAs used affected the expression of β 1 and β 3 integrins as assessed by immunofluorescence and Western blotting (data not shown).

To assess the function of kindlin-3 in integrin-mediated cell adhesion, non-transfected and siRNA-transfected HUVECs were plated on integrin β 1 and β 3 ligands. Consistent with our previous results (12), kindlin-2 knockdown had an inhibitory effect on cell adhesion to the α v β 3 ligand, vitronectin (Fig. 6B), but not to the α 5 β 1 ligand, fibronectin (Fig. 6C). Kindlin-3 knockdown did not have an effect on cell adhesion to vitronectin (Fig. 6A); however, cell adhesion to fibronectin was

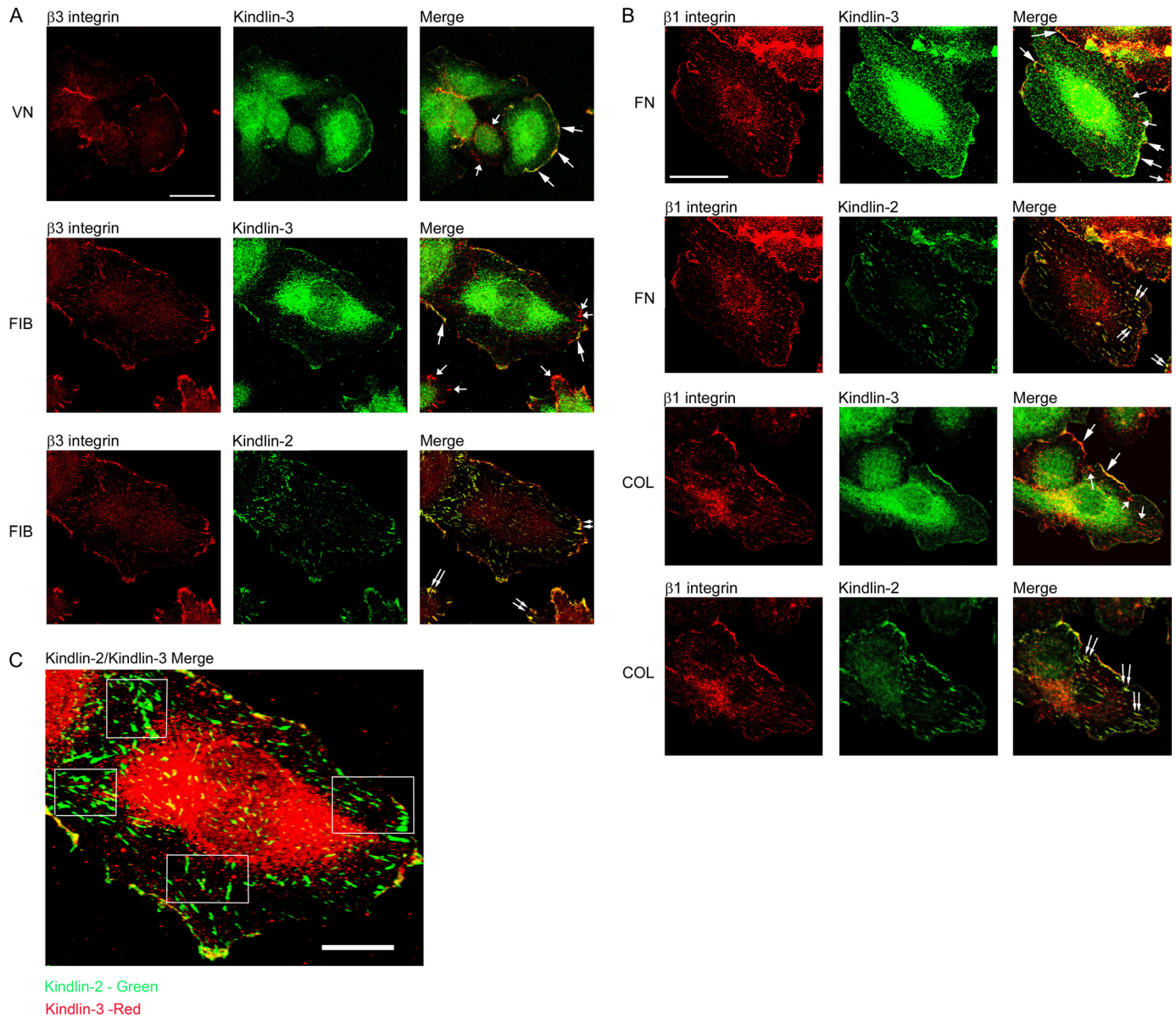


FIGURE 5. Kindlin-3 distribution in HUVECs spread on integrin substrates. HUVECs spread on vitronectin (VN) and fibrinogen (FIB) (A) for 1 h or fibronectin (FN) and collagen (COL) (B) for 2 or 1 h, respectively, were fixed, permeabilized, and stained with antibodies to detect kindlin-2, kindlin-3, $\beta 3$ integrin, and $\beta 1$ integrin followed by Alexa 633 anti-mouse IgG, Alexa 568 anti-rabbit IgG, and Alexa 488 anti-rat IgG. Kindlin-3 colocalized with integrins at the sites of membrane extensions (large arrows) but not in the focal adhesions (small arrows). Kindlin-2 colocalized with integrins at sites of membrane extension and in focal adhesions (double arrows). C, merge of kindlin-2 and kindlin-3 staining from A is shown. Areas rich in kindlin-2 and devoid of kindlin-3 are marked with boxes. Bars = 20 μ m. The images shown are representative of 12–15 independent scans for each integrin ligand.

blunted significantly (52% decrease in adhesion, $p < 0.001$) in cells transfected with kindlin-3 siRNA (Fig. 6C). Thus, in the absence of an integrin activating stimulus, the two kindlins exhibited selective effects on the functions of different integrins.

Effect of Kindlin-3 Knockdown on Complex Endothelial Cell Responses—To begin to consider how kindlin-3 might influence HUVEC responses beyond cell adhesion, we analyzed the effects of kindlin-3 knockdown on: 1) endothelial cell-monocyte interaction, and 2) formation of tube-like structures in Matrigel. For the effects on leukocyte interaction, HUVECs were transfected with non-targeting, kindlin-2 or kindlin-3 siRNA, plated in a 24-well tissue culture vessel, and treated with or without 10 ng/ml of TNF- α . Calcein-AM-labeled THP-1

monocytoid cells, a cell line that has been used extensively to study the interaction of cells of the monocytoid lineage with endothelial cells (30–32) were then cocultured with HUVEC for 1 h, and adherence of THP-1 cells was determined by measuring fluorescence intensity. There was minimal adhesion of THP-1 cells to HUVEC that had not been treated with TNF- α . The fluorescence intensity increased only ~50% over the control (non-fluorescent THP-1 cells) and examination of the wells under a microscope revealed that only a few THP-1 cells adhered to the HUVEC monolayer. TNF- α treatment promoted an ~15 increase in the adherence of THP-1 monocytes to the HUVEC, and this was associated with an ~10-fold increase of ICAM expression as determined by fluorescence-activated cell sorter. However, there was no significant differ-

Kindlin-3 in Endothelial Cells

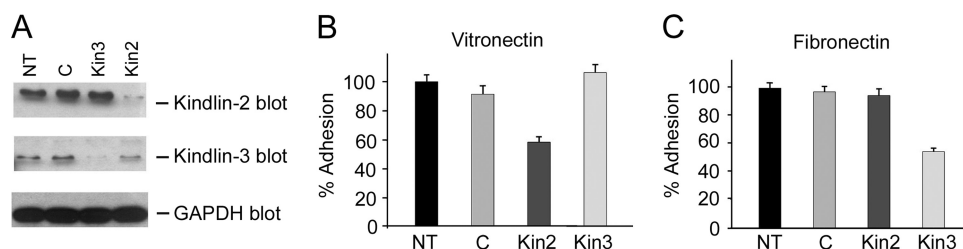


FIGURE 6. Kindlin-3 knockdown inhibits integrin-mediated adhesion in HUVECs. A, siRNA suppression of kindlin-3 expression in HUVECs. Expression of kindlin-2 and kindlin-3 in non-transfected cells (NT), kindlin-2 (Kin2), kindlin-3 (Kin3), and non-targeting siRNA (C) transfectants were analyzed by Western blotting with kindlin-2, kindlin-3, and GAPDH antibodies. B and C, non-transfected or HUVECs transfected with kindlin-2 (Kin2), kindlin-3 (Kin3), or non-targeting siRNA (C) were used in adhesion assays. The cells were fixed, stained with DAPI and Alexa 568-phalloidin, and counted. The error bars are mean \pm S.E. of three independent experiments.

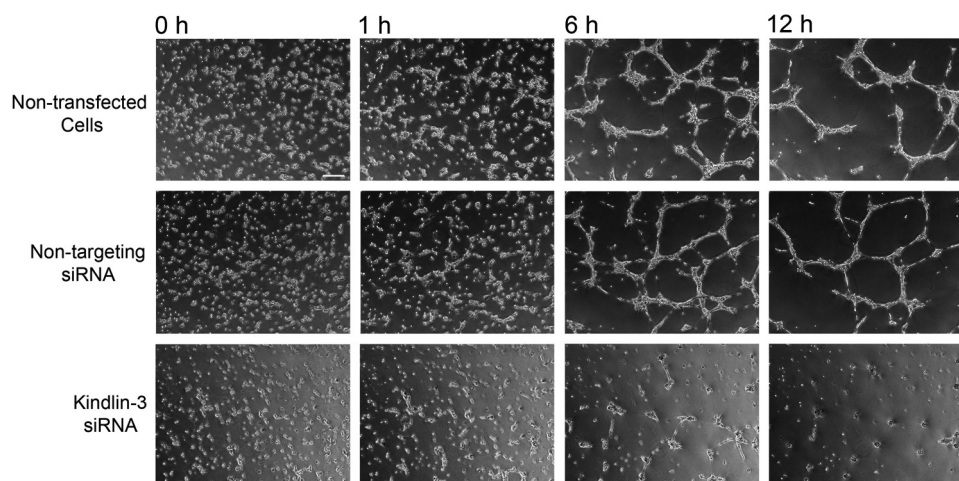


FIGURE 7. Kindlin-3 knockdown inhibits formation of tube-like structures *in vitro*. Non-transfected, non-targeting siRNA-transfected or kindlin-3 siRNA-transfected HUVECs were seeded on growth factor-reduced Matrigel containing 10 μ g/ml of fibronectin and 10 ng/ml of vascular endothelial growth factor. The figure shows representative fields of three separate experiments, each performed in triplicate. Bar = 200 μ m.

ence in the adhesion of THP-1 cells to non-targeting siRNA or kindlin-2 or kindlin-3 siRNA-transfected HUVECs. Adhesion of THP-1 cells to kindlin-2 siRNA-transfected cells was 99% and kindlin-3 siRNA-transfected cells was 110% when compared with adhesion of THP-1 cells to HUVECs transfected with non-targeting siRNA. Based on the work of Moser *et al.* (19), these data suggest that the role of kindlin-3 on leukocyte-endothelial cell interactions depends primarily on leukocyte rather than endothelial cell kindlin-3.

To begin to consider whether kindlin-3 might be involved in angiogenesis, we studied the ability of kindlin-3 siRNA-transfected HUVECs to form tube-like structures in Matrigel, a widely used *in vitro* model of the angiogenic response. When the cells were seeded on growth factor-reduced Matrigel, in non-transfected cells, and non-targeting siRNA-transfected cells, formation of an interconnected network of endothelial cells took place between 1 and 6 h after seeding (Fig. 7 and see [supplemental Movie 1](#) for non-transfected cells and [supplemental Movie 2](#) for non-targeting siRNA-transfected cells) and this was observed in three independent experiments. As shown in Fig. 7, *bottom panels*, and [supplemental Movie 3](#), kindlin-3 siRNA-transfected cells showed a significantly impaired ability to form tubes on Matrigel compared with non-transfected and non-targeting siRNA-transfected HUVECs. A

significant inhibition of tube formation in the kindlin-3 siRNA-transfected HUVECs was observed at all time points studied (Fig. 7 and [supplemental Movies 1–3](#)). The difference was most visible at 6–12 h (frames 72–144 in [supplemental Movies 1–3](#)) when a tubular network of interconnecting branches was well developed in non-transfected and non-targeting siRNA-transfected HUVECs samples, whereas very few contacts between cells with short protrusions only and few branches were observed in the kindlin-3 siRNA-transfected samples. These data indicate that kindlin-3 markedly influences the development of tube-like structures by endothelial cells on Matrigel.

In summary, using RT-PCR, Western blotting, and mass spectrometry, we demonstrate the presence of kindlin-3 in non-hematopoietic cells. Kindlin-3 is an endothelial cell protein. *KINDLIN-3* mRNA is present in HUVEC as well as human aortic endothelial cells and human dermal microvascular endothelial cells. Moreover, kindlin-3 protein is present in HUVEC isolated from multiple donors, as assessed with Western blotting and

immunoprecipitation. Mass spectrometric sequencing of the band immunoprecipitated with kindlin-3 antibodies confirmed it to be authentic kindlin-3. Furthermore, kindlin-3 knockdown in endothelial cells results in impaired adhesion to integrin substrates, despite the presence of kindlin-2 in the same cells. This observation suggests important but different roles of both kindlins in integrin signaling in these vascular cells. This interpretation is supported by differences in the distribution of kindlin-2 and kindlin-3 as endothelial cells spread on integrin substrates. Although the literature suggests similar residues with integrin β subunits are involved in kindlin-2 and kindlin-3 binding, our data indicate that their recognition of integrins cannot be identical. In HUVEC, knockdown approaches implicate kindlin-2 in regulation of integrin α v β 3 function, engagement of vitronectin (33), whereas kindlin-3 knockdown suppresses recognition of the α 5 β 1 ligand fibronectin (34). These ligand and integrin selective effects of knockdown of either kindlin further suggest that they cannot fully compensate for one another. Our results also lay the groundwork for examining the effects of kindlin-3 in more complex endothelial cell responses, such as angiogenesis. Our initial results showing a suppression of formation of tube-like structures in Matrigel by HUVECs in which kindlin-3 was knocked down support this possibility and will now need to be pursued in *in vivo* analyses.

In describing kindlin-3 knock-out mice, Moser *et al.* (18) observed that the phenotype of global kindlin-3 inactivation was more severe than in bone marrow chimeras (irradiated wild-type mice transplanted with kindlin-3 null fetal liver), suggesting an important role of kindlin-3 in non-bone marrow-derived cells. The present results suggest that the role and function of kindlin-3 should be examined in other non-hematopoietic cell types as well. The extent of knockdown of kindlin-2 and kindlin-3 we obtained with siRNA was only partial but yet had detectable functional effects on endothelial cell adhesion and tube formation. These observations lead to the prediction that humans with reduced levels of kindlins could also exhibit abnormal functions of endothelial cells as well as other non-hematopoietic cells.

REFERENCES

1. Qin, J., Vinogradova, O., and Plow, E. F. (2004) *PLoS Biol.* **2**, 726–729
2. Ma, Y. Q., Qin, J., and Plow, E. F. (2007) *J. Thromb. Haemost.* **5**, 1345–1352
3. Tu, Y., Wu, S., Shi, X., Chen, K., and Wu, C. (2003) *Cell* **113**, 37–47
4. Ussar, S., Wang, H. V., Linder, S., Fässler, R., and Moser, M. (2006) *Exp. Cell Res.* **312**, 3142–3151
5. Shi, X., Ma, Y. Q., Tu, Y., Chen, K., Wu, S., Fukuda, K., Qin, J., Plow, E. F., and Wu, C. (2007) *J. Biol. Chem.* **282**, 20455–20466
6. Rogalski, T. M., Mullen, G. P., Gilbert, M. M., Williams, B. D., and Moerman, D. G. (2000) *J. Cell Biol.* **150**, 253–264
7. Calderwood, D. A., Zent, R., Grant, R., Rees, D. J., Hynes, R. O., and Ginsberg, M. H. (1999) *J. Biol. Chem.* **274**, 28071–28074
8. Vinogradova, O., Velyvis, A., Velyviene, A., Hu, B., Haas, T., Plow, E., and Qin, J. (2002) *Cell* **110**, 587–597
9. Garcia-Alvarez, B., de Pereda, J. M., Calderwood, D. A., Ulmer, T. S., Critchley, D., Campbell, I. D., Ginsberg, M. H., and Liddington, R. C. (2003) *Mol. Cell* **11**, 49–58
10. Tadokoro, S., Shattil, S. J., Eto, K., Tai, V., Liddington, R. C., de Pereda, J. M., Ginsberg, M. H., and Calderwood, D. A. (2003) *Science* **302**, 103–106
11. Nieswandt, B., Moser, M., Pleines, I., Varga-Szabo, D., Monkley, S., Critchley, D., and Fässler, R. (2007) *J. Exp. Med.* **204**, 3113–3118
12. Ma, Y. Q., Qin, J., Wu, C., and Plow, E. F. (2008) *J. Cell Biol.* **181**, 439–446
13. Montanez, E., Ussar, S., Schifferer, M., Bösl, M., Zent, R., Moser, M., and Fässler, R. (2008) *Genes Dev.* **22**, 1325–1330
14. Jobard, F., Bouadjar, B., Caux, F., Hadj-Rabia, S., Has, C., Matsuda, F., Weissenbach, J., Lathrop, M., Prud'homme, J. F., and Fischer, J. (2003) *Hum. Mol. Genet.* **12**, 925–935
15. Siegel, D. H., Ashton, G. H., Penagos, H. G., Lee, J. V., Feiler, H. S., Wilhelmson, K. C., South, A. P., Smith, F. J., Prescott, A. R., Wessagowit, V., Oyama, N., Akiyama, M., Al, Aboud, D., Al, Aboud, K., Al, Githami, A., Al, Hawsawi, K., Al, Ismaily, A., Al-Suwaid, R., Atherton, D. J., Caputo, R., Fine, J. D., Frieden, I. J., Fuchs, E., Haber, R. M., Harada, T., Kitajima, Y., Mallory, S. B., Ogawa, H., Sahin, S., Shimizu, H., Suga, Y., Tadani, G., Tsuchiya, K., Wiebe, C. B., Wojnarowska, F., Zaghoul, A. B., Hamada, T., Mallipeddi, R., Eady, R. A., McLean, W. H., McGrath, J. A., and Epstein, E. H. (2003) *Am. J. Hum. Genet.* **73**, 174–187
16. Ussar, S., Moser, M., Widmaier, M., Rognoni, E., Harrer, C., Genzel-Boroviczeny, O., and Fässler, R. (2008) *PLoS Genet.* **4**, e1000289
17. Dowling, J. J., Gibbs, E., Russell, M., Goldman, D., Minarcik, J., Golden, J. A., and Feldman, E. L. (2008) *Circ. Res.* **102**, 423–431
18. Moser, M., Nieswandt, B., Ussar, S., Pozgajova, M., and Fässler, R. (2008) *Nat. Med.* **14**, 325–330
19. Moser, M., Bauer, M., Schmid, S., Ruppert, R., Schmidt, S., Sixt, M., Wang, H. V., Sperandio, M., and Fässler, R. (2009) *Nat. Med.* **15**, 300–305
20. Kuijpers, T. W., van, d. V., Weterman, M. A., de, B. M., Tool, A. T., van den Berg, T. K., Moser, M., Jakobs, M. E., Seeger, K., Sanal, O., Unal, S., Cetin, M., Roos, D., Verhoeven, A. J., and Baas, F. (2009) *Blood* **113**, 4740–4746
21. Svensson, L., Howarth, K., McDowall, A., Patzak, I., Evans, R., Ussar, S., Moser, M., Metin, A., Fried, M., Tomlinson, I., and Hogg, N. (2009) *Nat. Med.* **15**, 306–312
22. Malinin, N. L., Zhang, L., Choi, J., Ciocea, A., Razorenova, O., Ma, Y. Q., Podrez, E. A., Tosi, M., Lennon, D. P., Caplan, A. I., Shurin, S. B., Plow, E. F., and Byzova, T. V. (2009) *Nat. Med.* **15**, 313–318
23. Ehlers, R., Ustinov, V., Chen, Z., Zhang, X., Rao, R., Lusinskas, F. W., Lopez, J., Plow, E., and Simon, D. I. (2003) *J. Exp. Med.* **198**, 1077–1088
24. Larjava, H., Plow, E. F., and Wu, C. (2008) *EMBO Rep.* **12**, 1203–1208
25. Moser, M., Legate, K. R., Zent, R., and Fässler, R. (2009) *Science* **324**, 895–899
26. Alon, R., and Etzioni, A. (2003) *Trends Immunol.* **24**, 561–566
27. McDowall, A., Inwald, D., Leitinger, B., Jones, A., Liesner, R., Klein, N., and Hogg, N. (2003) *J. Clin. Invest.* **111**, 51–60
28. Kato, K., Shiozawa, T., Mitsushita, J., Toda, A., Horiuchi, A., Nikaido, T., Fujii, S., and Konishi, I. (2004) *Hum. Pathol.* **35**, 55–60
29. Lai-Cheong, J. E., Ussar, S., Arita, K., Hart, I. R., and McGrath, J. A. (2008) *J. Invest. Dermatol.* **128**, 2156–2165
30. Narizhneva, N. V., Razorenova, O. V., Podrez, E. A., Chen, J., Chandrasekharan, U. M., DiCorleto, P. E., Plow, E. F., Topol, E. J., and Byzova, T. V. (2005) *FASEB J.* **19**, 1158–1160
31. Postea, O., Krotz, F., Henger, A., Keller, C., and Weiss, N. (2006) *Arterioscler. Thromb. Vasc. Biol.* **26**, 508–513
32. Mori, D., Ishii, H., Kojima, C., Nitta, N., Nakajima, K., and Yoshida, M. (2007) *J. Atheroscler. Thromb.* **14**, 213–218
33. Cheresh, D. A., and Spiro, R. C. (1987) *J. Biol. Chem.* **262**, 17703–17711
34. Argraves, W. S., Suzuki, S., Arai, H., Thompson, K., Pierschbacher, M. D., and Ruoslahti, E. (1987) *J. Cell Biol.* **105**, 1183–1190