Kindlin-3 in hematopoietic integrin activation: Absence in leukocyte adhesion deficiency type III

van de Vijver, E.

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CHAPTER 6

Activation of β2 integrins on human neutrophils requires recruitment of kindlin-3 to the plasma membrane: a role for the PH and PTB domain of kindlin-3

Edith van de Vijver, Paul Verkuijlen, Anton T.J. Tool, Greg Fairn, Dante Neculai, Timo K. van den Berg, Taco W. Kuijpers

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ABSTRACT

Integrin-mediated adhesion of circulating neutrophils to the blood vessel wall is essential to allow extravasation and migration to sites of infection. Activation of integrins comprises an intramolecular change from an inactive conformation towards an unclasped conformation with high-ligand binding affinity. Cytoplasmic adaptor proteins kindlin-3 and talin-1 act in concert to induce the final steps of integrin activation, downstream of complex signaling pathways. Deficiency of kindlin-3 results in Leukocyte Adhesion Deficiency type III (LAD-III, also known as LAD-I variant) syndrome, which manifests with nonpussing infections and severe bleeding.

The clinical importance of kindlin-3 has been extensively studied, but little is known about the molecular mechanism by which kindlin-3 contributes to the activation of the major integrin on human neutrophil, i.e. the β2 integrin CR3 (CD11b/CD18). Here we show that kindlin-3 is a cytoplasmic protein that is recruited from the cytoplasm to the plasma membrane upon neutrophil stimulation. Kindlin-3 mutants in human neutrophil-like NB4 cells were used to demonstrate that the PH domain of kindlin-3 interacts with the phosphoinositide $PI(3,4)P_2$ in the plasma membrane. Co-immunoprecipitations were used to demonstrate a direct interaction in phagocytes between the cytoplasmic tail of β2 integrins and kindlin-3, via its PTB domain. The binding of kindlin-3 to the plasma membrane facilitates the interaction with β2 integrins. In sum, our findings result in a model of stepwise activation of β2 integrins in human neutrophils.

INTRODUCTION

Neutrophils are key responder cells in innate immunity. During inflammation, these cells extravasate and migrate to the site of infection, to subsequently destroy pathogens by phagocytosis or extracellular killing mechanisms. Binding to endothelial cells of the vessel, tissue cells, pathogens and soluble ligands is mediated by integrins, which are transmembrane adhesion molecules on the neutrophil.[1] Integrins are composed of an α and a β subunit, and the most prominent integrins on neutrophils are complement receptor 3 (CR3, Mac-1) consisting of αM (CD11b) and β2 (CD18), and lymphocyte function-associated antigen-1 (LFA-1), consisting of αL (CD11a), and β2.[2-4]

Integrins are in a bended, inactive conformation on resting cells.[2] Integrin activation is started by stimulation of other cell surface receptors, e.g. cytokine binding to G-protein coupled receptors (GPCRs) or Toll-like receptors (TLRs) on neutrophils, which induces intracellular activation pathways known as 'inside-out' signaling.[2;4;5] Inside-out signaling results in extracellular conformational changes of the integrins and thereby increases ligand affinity. Subsequent ligand binding to the integrins induces additional conformational changes and downstream signal transduction that regulates cell spreading and alters e.g. gene expression, cell proliferation, differentiation and apoptosis ('outside-in' signaling). Integrin activation involves many proteins, e.g. the small GTPase Rap1, talin-1, filamin, α-actinin and the kindlins.[2] The kindlin family consists of the fibroblast-specific kindlin-1, the ubiquitously expressed kindlin-2 and the hematopoietic cell-specific kindlin-3, with high homology at the genetic and protein level.[6]

The importance of kindlin-3 for activation of hematopoietic integrins is illustrated by Leukocyte Adhesion Deficiency type III (LAD-III) syndrome, originally described in 1997 by our group as LAD-I/variant.[7] LAD-III is a rare autosomal recessive syndrome characterized by recurrent bacterial and fungal infections due to a neutrophil defect, in addition to a Glanzmann thrombastenia-like bleeding tendency caused by defective platelets.[7;8] Upon stimulation with several cytokines, integrin activation epitopes on hematopoietic cells are hardly detectable, and functional responses such as adhesion and aggregation are diminished or absent.[9-11] In the past four years, we and others have reported several mutations in *FERMT3*, the gene encoding kindlin-3, as the cause of this deficiency.[11-13]

Although biochemical studies do not implicate a direct interaction between kindlin and talin, both proteins seem to act in concert in the final step of integrin activation.[12;13] Talin-1 and kindlins have a highly homologous domain structure, containing a FERM domain (called after the homologous proteins 4.1, ezrin, radixin and moesin) with the subdomains F0, F1, F2 and F3.[14] The F3 subdomain bears a phosphotyrosine-binding (PTB) site specific for so-called NxxY/F motifs in the cytoplasmic tail of β integrin chains. Talin-1 binds to a NxxY/F motif in the plasma membrane proximal part of the integrin, whereas kindlins have been suggested to bind to a NxxY/F motif in the distal region of the cytoplasmic tail.[13;14] Amino acid residues surrounding these motifs have been suggested to contribute to integrin β subunit binding by talin-1 and kindlin.[15-17] In addition to its FERM-containing head domain, talin-1 has an autoinhibitory tail, which blocks the PTB-containing F3 site under resting conditions.[20-22] Kindlins have not been reported to contain any autoinhibitory domains.

As kindlins need to be localized in close proximity to the plasma membrane to contribute to activation of the transmembrane integrins, several domains have been suggested to be important for binding of kindlins to the membrane. The F2 subdomain consists of two parts, separated by a so-called pleckstrin homology (PH) domain.[14] PH domains are known for their ability to interact with phosphoinositides (PIPs) in the inner leaflet of the plasma membrane, and most PH domains have specificity for one or more PIPs.[18] As a result, the concentration of the relevant PIPs in the plasma membrane, which are subject to a dynamic process of (de) phosphorylation events, regulates the association of these proteins to the plasma membrane.

A recent study on the PH domain of kindlin-2 suggests that it directly binds to PIPs, in particular to $PI(3,4,5)P_3$, and that this interaction supports the activation of β1 and β3 integrins on podocytes.[19] $PI(3,4,5)P_3$ is generated by phosphorylation of constitutively present $PI(4,5)P_2$ by phosphoinositide 3-kinase (PI3K), which is activated upon cell stimulation.[20] Talin-1 contains alternative positively charged PIP-binding sites in both the F2 and F3 subdomain, which are specific for $PI(4,5)P_2$.[21] Although $PI(4,5)P_2$ is constitutively present at low levels, local upregulation by phosphatidylinositol 4-phosphate 5-kinase type I (PIPKI), downstream of GPCRs, is required to induce talin-1 recruitment and its binding to integrin β tails.[22;23]

Several studies have focused on the interaction of kindlin-1 and -2 with the β1 and β3 integrins but little is known about kindlin-3, in particular concerning the activation of the major β2 integrin on human neutrophils: CR3 (CD11b/CD18).[24] Our results demonstrate for the first time a direct interaction between kindlin-3 and the cytoplasmic tails of β2 integrins in phagocytes. Kindlin-3 is first recruited by its PH domain to the neutrophil membrane, followed by the interaction with the β2 integrin via its PTB domain. Based on our studies, we propose a model in which phosphoinositol kinases and phosphatases act in concert with small GTPases to recruit kindlin-3 and talin-1 to the inner leaflet of the plasma membrane, facilitating the activation of the β2 integrins. Our findings help to understand the molecular mechanism that results in integrin activation in human neutrophils, which fails in patients who suffer from LAD-III.

MATERIALS AND METHODS

Neutrophil isolation and NB4 cell culture

Heparinized venous blood was collected from healthy donors after obtaining informed consent. The study was approved by the Amsterdam Academic Medical Centre Institutional Medical Ethics Committee in accordance with the 1964 Declaration of Helsinki. Human neutrophils were isolated as described.[44;45] Neutrophil preparations were typically >97% pure, with the contaminating cells being mostly eosinophils. Acute promyelocytic leukemia-derived NB4 cells were cultured in RPMI medium (Gibco Life Technologies, Paisley, United Kingdom), supplemented with 20% (v/v) fetal calf serum, 300 µg/ml L-glutamine (Gibco), 100 IU/ml penicillin (Gibco) and 100 µg/ml streptavidin Gibco). A construct for the expression of GFP-tagged kindlin-3 was engineered with pRRL PPT SFFV GFP prester SIN, which was cloned into a Gateway Cassette A (Invitrogen, Carlsbad, CA, USA). The resulting plasmid was recombined with pENTR d TOPO-kindlin3 to generate pRRL PPT SFFV GFP-kindlin3. Lentivirus was produced in 293T cells and fractions of $5x10^5$ NB4 cells were transduced by centrifugation with filtered virus supernatant for 5 minutes at 1000 rpm on two subsequent days. NB4 cells were induced

to differentiate with 5 nM ATRA (Sigma-Aldrich, St. Louis, MO, USA) for 8 days, and granulocyte differentiation was validated by morphological analysis, NADPH-oxidase activity in response to zymosan and CD11b expression.[8]

Digitonin fractionation

Neutrophils were resuspended at 5x10^6/mL in Hepes buffer (20 mM Hepes [Merck Millipore, Darmstadt, Germany], 132 mM NaCl, 6.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.5 mM glucose and 0.5% (w/v) human serum albumine, pH 7.4) and were stimulated or not with 100 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich) for 25 min at 37°C and centrifuged for 5 min at 520x*g*. Cells were resuspended in ice-cold 1 mM diisopropylfluorophosphate (DFP; Fluka Chemica, Steinheim, Switzerland) in PBS, incubated for 10 min on ice and centrifuged for 5 min at 520x*g*, 4°C. Sub-cellular fractions were prepared from 10^7 cells by resuspension in digitonin buffer (250 ug/ml digitonin [Merck Millipore], 1 mM EDTA, complete protease inhibitor cocktail [PIM; Roche Diagnostics, Almere, The Netherlands] in PBS at a concentration of 10^8 cell/ml. Cells were incubated for 15 min on ice and thereafter centrifuged for 5 min at 1000x*g*, 4°C. The supernatant represented the cytosolic fraction; the pellet, containing membranes, nuclei and organelles, was incubated for another 15 min on ice in CHAPS buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 1% (w/v) CHAPS [Calbiochem, La Jolla, CA, USA], PIM and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL, USA). Both fractions were dissolved in Laemmli sample buffer (LSB; 50 mM TrisHCl, pH 6.8, 10% (v/v) glycerol, 5 mM DTT [DL-dithiothreitol, Sigma-Aldrich], 1% (v/v) 2-mercaptoethanol, 1% (w/v) sodium dodecylsulfate (SDS), 100 µg/ml bromephenol blue) and were boiled for 30 min at 95°C. All samples were stored at -20°C before subjection to Western blot.

Sonication and sucrose gradient

NB4 cells were resuspended at 10^7/ml in Hepes buffer, stimulated with 100 ng/ml PMA for 25 min at 37°C and centrifuged for 5 min at 520x*g*. Cells were resuspended in ice-cold 1 mM DFP in PBS, incubated for 10 min on ice and centrifuged for 5 min at 520x*g*, 4°C. For fractionation, 7x10^7 cells were resuspended at 10^8/ml in sonication buffer (10 mM Hepes, 170 mM sucrose, 75 mM NaCl, 1 mM $MgCl_2$, 10 µM ATP, 5 µM GTPγS, PIM and Halt) and gently sonicated with a Branson Digital Sonifier Model 250 (Branson, Danbury, CT, USA). Undisrupted cells and nuclei were pelleted at 520x*g*, and the supernatant was layered on a discontinuous sucrose gradient consisting of 5 ml of 15% (w/v) sucrose on top of 6 ml 52% (w/v) sucrose. After centrifugation (100,000x*g*, 1 hour), 700 µl of the supernatant (as the source of cytosol), 700 µl of the interface of the 15/52% sucrose layers (as the source of plasma membranes) and the pellet, resuspended in 700 µl of sonication buffer, were collected. Laemmli sample buffer was added and samples were boiled at 95°C for 30 min, and stored at -20°C.

Western blot

Western blot was performed as previously described.[7] Cytoplasm or plasma membrane-containing fraction equivalents of 1x10^6 cells were loaded to each slot. For protein detection, rabbit anti-kindlin-3 antiserum ahk1836, mouse anti-p47^{phox}, mouse anti-CD18 R2E7B and

conjugates donkey anti-mouse-IgG IRDye 680 and donkey anti-rabbit-IgG IRDye 800 for Odyssey (LI-COR Biosciences, Lincoln, NE, USA) were used.[25] Detection was performed on an Odyssey Infrared Imaging system and protein levels were quantified from fluorescence intensity with Odyssey V3.0 software (both LI-COR Biosciences).

Quantification of translocation in NB4 cells

For each sample, the protein level of GFP-tagged kindlin-3 was divided by the protein level of $p47^{phox}$, to verify PMA-induced translocation and to normalize for protein sample loading. This ratio of protein translocation was set at 100% for wild-type kindlin-3. To compare translocation of kindlin-3 mutants with translocation of wild-type kindlin-3, the kindlin-3/p47 ratio of the mutants was determined relative to the wild-type kindlin-3/p47 ratio.

FRET-based liposome assay

The liposome compositions used in this study were 67.5 mol % 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 20 mol % 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), 2.5 mol % 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl) (dansyl-PE) and 10 mol % of the phosphoinositides tested. Liposomes were made by adding the required amount of stock lipids into a glass vial and drying under a stream of N_2. The phospholipids were resuspended in liposome buffer (140 mM KCl, 0.5 mM $MgCl_2$, 15 mM NaCl, and 25 mM Hepes, pH 7.5) by incubation at 37°C, vortexing and sonication. Uniform large unilamellar liposomes were produced by passing through a LiposoFast extruder with a filter of 100 nm pore size (Avestin, Ottawa, Canada). A construct for the expression of GST-PH kindlin-3 was engineered with pGEX 6P-1 (GE Healthcare). Purification of the resultant protein was performed according to manufacturer's instructions. Briefly, GST-PH expression was induced in BL21 *Escherichia coli*, and the cell lysate was loaded onto a glutathione–Sepharose column. GST-PH was eluded from the resin with 25 mM reduced glutathione and collected in 500-µl fractions. The fractions were analyzed for GST-PH by Coomassie staining of SDS–PAGE gels, the positive fractions were pooled and dialyzed in PBS at 4°C overnight to remove excess glutathione. Binding of GST-PH to liposomes was monitored via FRET by means of the intrinsic fluorescence of tryptophan residues to dansyl-PE as described previously.[26] Experiments were performed in a 5×5×30 mm quartz cuvette in a Hitachi F-2500 fluorescence spectrophotometer (Hitachi High Technologies America, Schaumburg, IL, USA) at room temperature. Excitation and emission wavelengths were 280 and 510 nm, respectively, with 5-nm slits. The total lipid concentration for the measurement was adjusted to 10 µM, GST-PH was added in increments, and the fluorescence was recorded and corrected for dilution. Data were analyzed as previously described.[26]

Co-immunoprecipitation

Culture dishes (6 cm) were coated with 5 µg/ml ICAM-1-Fc (a kind gift of dr. D Staunton, Bothell, WA, USA) in coating buffer for 1 hour at 37°C and washed 3x with PBS. For each immunoprecipitation, 40 µl of goat anti-mouse IgG Dynabeads (Life Technologies) were labeled according to the manufacturer's protocol with 10 µg/ml of mouse anti-β2 integrin antibody clone IB4 or IgG control for 45 min at 4°C and washed 3x in cold PBS. NB4 cells were

resuspended at $5x10^6$/ml in Hepes buffer and 4.4 ml of cell suspension were incubated on each dish with 100 ng/ml PMA for 25 min at 37°C. After 25 min, the supernatant of each dish was replaced by 1 ml of ice-cold CHAPS lysis buffer, and the dishes were put on ice immediately to incubate for 15 min. Lysates were harvested, $2x10^6$ cells were used for lysates and the rest was centrifuged for 8 min at 10,600x*g* at 4°C. Supernatants were incubated with labeled beads overnight at 4°C in a head-over-head rotor. The next day, supernatants were discarded, beads were washed according to protocol, and beads and whole cell lysates were boiled with 30 µl of Laemmli sample buffer for 10 min (beads) or 30 min (cell lysates), and stored at -20°C. Western blot was performed as described above.

Quantification of co-immunoprecipitations

To quantify the interaction between kindlin-3 and β2 integrins and to correct for the efficiency of immunoprecipitation, the protein level of kindlin-3 was divided by the protein level of β2 integrin in the immunoprecipitate. This ratio was set at 100% for wild-type kindlin-3. The ratio of co-immunoprecipitation of the kindlin-3 mutants to the β2 integrin was determined relative to the wild-type kindlin-3/β2 integrin ratio.

RESULTS

Kindlin-3 can be recruited to the plasma membrane via its PH domain

Kindlin-3 is required for the activation of neutrophil β2 integrins. In analogy to results reported for other kindlins and other integrins, we hypothesized that β2 integrin activation in neutrophils involves an inducible association of kindlin-3 to the integrin β chain and/or to the plasma membrane. To investigate the localization of kindlin-3 in neutrophils, we performed cell fractionation studies using the mild detergent digitonin and evaluated protein levels in the soluble cytoplasmic fraction and in the insoluble fraction, the latter harboring the plasma membranes. The phorbol ester PMA, which triggers integrin-mediated adhesion as well as NADPH oxidase activation in neutrophils, was used as a stimulus.[27;28] The NADPH oxidase component $p47^{phox}$, which associates with the plasma membrane upon PMA-induced oxidase activation, was included as a positive control in these experiments.[28] In resting cells, kindlin-3 was detected exclusively in the cytoplasm, whereas upon stimulation with PMA, a small proportion (<10%) of kindlin-3 translocated to the insoluble fraction (Figure 1A). Thus, kindlin-3 is a cytoplasmic protein that is recruited to the plasma membrane during stimulation of neutrophils to activate the integrins.

To investigate whether the PH domain of kindlin-3 plays a role in the recruitment to the plasma membrane, we generated green fluorescent protein (GFP)-tagged wild-type kindlin-3 and PH domain mutants R360C and K367A, based on amino acids in kindlin-2 that are essential for PIP binding (Figure 1B).[19;29] These proteins were stably expressed in the human myeloid NB4 cell line, and NB4 cells were differentiated with all-trans retinoic acid (ATRA) towards neutrophil-like cells prior to evaluation. We used sonication and sucrose gradients to fractionate these differentiated NB4 cells, since NB4 cells are less sensitive to digitonin (data not shown). Wild-type kindlin-3 was recruited to the plasma membrane was detected in PMA-stimulated NB4 cells, as it was in primary human neutrophils (Figure 1C).

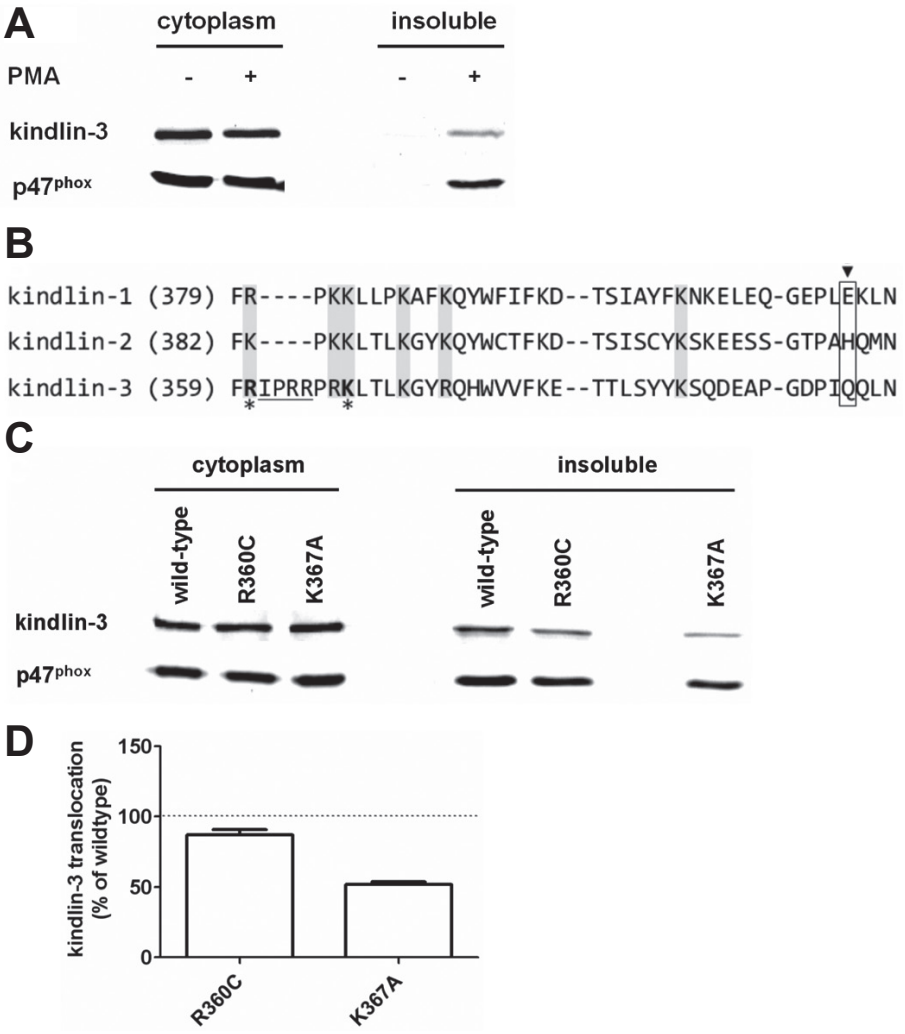

Figure 1. A. Upon cell stimulation with PMA, kindlin-3 is recruited from the cytoplasm to the insoluble fraction of plasma membranes. B. Region of the PH domains of kindlin-1, -2 and -3 involved in phosphoinositide binding. Gray residues are part of the highly positively charged K/R cluster. Rim residue H419/Q396 has been suggested to be involved in 5' phosphate binding.[29] Bold residues marked with an asterisk (*) were mutated in the current study. The underscored region represents a longer splice variant of kindlin-3; however, this variant has never been detected in any human cell type or individual sequenced by our group (data not shown). C,D. Mutation K367A in the PH domain causes a decrease in kindlin-3 recruitment, whereas R360C has little effect. Mean ± SEM, n=4.

To quantify the translocation of wild-type and mutant kindlin-3 to the plasma membrane-containing fraction, translocation of p47^{phox} was used as a control. Resting neutrophils normally do not contain any p47^{phox} at the plasmamembrane.[28] Thus, this cytoplasmic component of the NADPH oxidase enzyme complex forms evidence of the resting state of the cells prior

activation and proves the quality of separating plasma membrane and cytoplasmic fractions. Translocation of $p47^{phox}$ has been extensively reported upon subsequent activation by PMA and could thus be used for comparison of kindlin-3 translocation. Translocation of wild-type kindlin-3 was set at 100%.

We observed less than 50% of translocation with the K367A mutant compared to wild-type kindlin-3 (Figure 1D), which implies that also in kindlin-3, the PH domain is indeed responsible for the interaction with the plasma membrane and that the K367 residue is of major importance for this interaction. Mutant R360C translocated to the plasma membrane for more than 80% of what was observed with wild-type kindlin-3, indicative for a minor role of this residue in PIP binding by the kindlin-3 PH domain.

The PH domain of kindlin-3 can interact with PI(3,4)P_2

The PH domain of kindlin-2 interacts preferentially with the phosphoinositide PI(3,4,5)P_3, as Qu et al. recently demonstrated using binding assays with a series of membrane-immobilized PIPs.[19] To directly investigate whether the PH domain of kindlin-3 also interacts with PIPs and to determine its PIP specificity, we generated a GST-tagged construct of the PH domain of wild-type kindlin-3. Liposomes containing PI, PI(4)P, PI(4,5)P_2, PI(3,4)P_2 or PI(3,4,5)P_3 and phospholipids were incubated with the GST-tagged protein. Binding of GST-PH to the liposomes was monitored via FRET by means of the intrinsic fluorescence of tryptophan residues to dansyl-PE, as described previously.[26] The PH domain of kindlin-3 bound specifically to PI(3,4)P_2 (Figure 2). We conclude that kindlin-3 in neutrophil-like cells interacts with the plasma membrane by means of its PH domain, and in particular binds to PI(3,4)P_2.

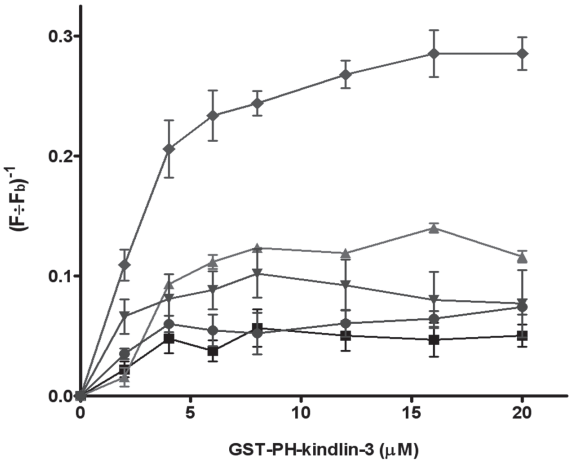

Figure 2. FRET-based analysis of the binding of the kindlin-3 PH domain to liposomes containing PI (•), PI(4)P (x), PI(4,5)P_2(▲), PI(3,4)P_2 (◆) and PI(3,4,5)P_3 (▼). Mean ± SEM, n=3.

Kindlin-3 interacts with β2 integrin

We next wanted to obtain insight into the way in which kindlin-3 interacts with β2 integrin subunits. Based on experiments with short recombinant mouse β2 cytoplasmic tails and mouse platelet lysates, Moser et al. suggested that kindlin-3 interacts directly with the β2 integrin tail.[30] To show that these interactions between kindlin-3 and the β2 integrin also take place in intact human cells, neutrophil-like differentiated NB4 cells were stimulated with PMA and allowed to adhere to ICAM-1-coated culture dishes. Cell lysates of the spread cells were immunoprecipitated with magnetic dynabeads coated either with anti-β2 integrin subunit monoclonal antibody (mAb) IB4 or with irrelevant control IgG. Adhesion to ICAM-1 occurred in a β2 integrin-mediated manner, since it was inhibited by preincubation of the cells with IB4 (data not shown).

The β2 integrin was immunoprecipitated with IB4-coated beads but not with beads coated with irrelevant control IgG (Figure 3A). A clear co-immunoprecipitation of kindlin-3 was detected exclusively when PMA-stimulated cells were used, together showing that kindlin-3 can indeed interact with β2 integrins and that this interaction specifically occurs during integrin activation.

To investigate the role of the PTB domain of kindlin-3 in the interaction with the β2 integrin, we generated two GFP-tagged mutants, Q595A and W596S, with amino acid substitutions in the PTB domain. Equivalents of W596 in kindlin-1, -2 and talin-1 are essential for the interaction with NxxY/F motifs in β integrin tails, whereas the importance of Q595 was thus far reported for talin-1 only (Figure 3B).[17] For quantification, co-immunoprecipitation of kindlin-3 was normalized to the protein level of β2 integrin in our immunoprecipitate. Co-immunoprecipitation of wild-type kindlin-3 was set at 100%. Binding of kindlin-3 mutant W596S to the β2 integrin was strongly reduced to ~20% of wild-type, indicating that the PTB domain and in particular the W596 residue is indeed essential for interaction with the integrin (Figure 3C). Q595A decreased the binding for more than 50%, implicating that this amino acid also plays a significant role in integrin binding.

As shown above, the PH domain is essential for kindlin-3 recruitment and stable localization at the plasma membrane. We hypothesized that this recruitment might be required to facilitate the interaction with the integrin, which we tested with the PH-domain mutant K367A in similar co-immunoprecipitation experiments as described above. The interaction of the K367A mutant with β2 integrin was less than 70% of the wild-type kindlin-3, which implicates that the PH domain facilitates the interaction to a large extent but is not absolutely required for the direct molecular interaction between kindlin-3 and the cytoplasmic tail of the integrins (Figure 3C).

DISCUSSION

Integrin activation on primary human neutrophils is essential for the extravasation and migration of these cells to the site of inflammation, as well as for binding to pathogens. Kindlin-3 plays an important role in hematopoietic integrin activation, as is illustrated by the severe symptoms of LAD-III patients, who lack kindlin-3.[7;8] Here we show that cytoplasmic kindlin-3 is recruited towards the plasma membrane, where its PH domain interacts with PIPs, and that this interaction facilitates subsequent binding to β2 integrin cytoplasmic tails via the PTB domain of kindlin-3.

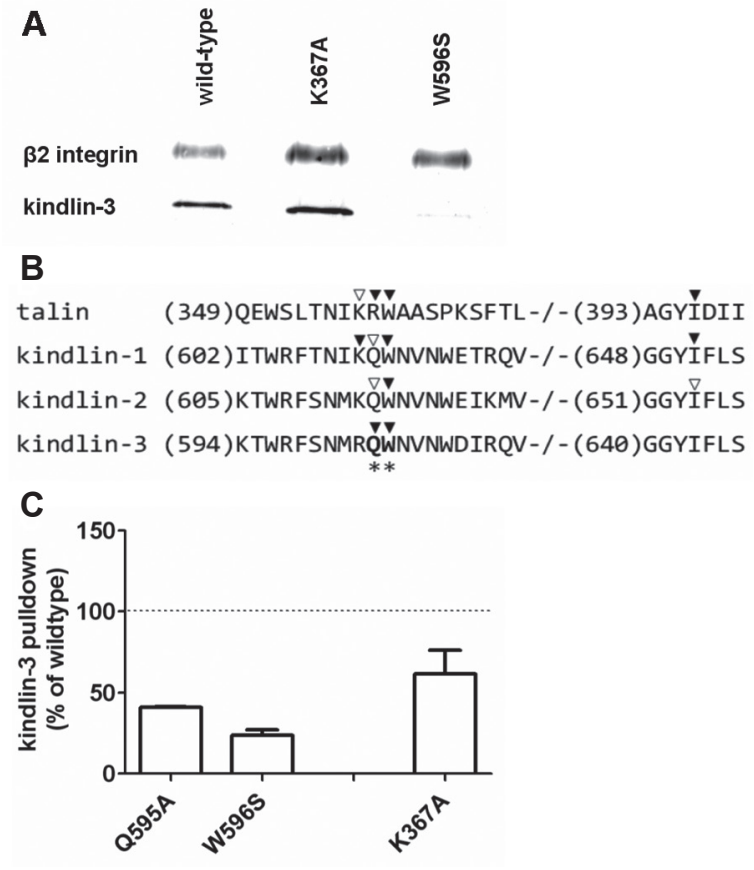

Figure 3. A. Co-immunoprecipitation of kindlin-3 with β2 integrin by means of magnetic dynabeads labeled with mAb IB4 directed against β2 integrin. B. Regions of the PTB domain of kindlin-1, -2, -3 and talin-1 involved in binding to β integrin tails. Residues known to be essential (▼) or non-essential (▽) are depicted by arrowheads, based on interaction studies with β1 and β3 (for kindlin-1, -2 and talin) and with β2 integrin in the current study.[19;37] Bold residues marked with an asterisk (*) were mutated in the current study. C. Quantification of bound protein in co-immunoprecipitations was performed on an Odyssey Infrared Imaging system and expressed relative to the amount of β2 integrin that was immunoprecipitated. Mean ± SEM, n=3 (Q595A) or n=5 (W596S, K367A).

Fractionation assays were used to reveal that kindlin-3 in human resting neutrophils is a cytoplasmic protein that is recruited to the plasma membrane during stimulation. Based on homology with other kindlins and integrins, we hypothesized that the PH domain of kindlin-3 might facilitate this interaction. Structural modeling has revealed that the PH domain of the kindlins has a core of seven β strands in two β-sheets, consisting of four β-strands (β1-β4) and three β-strands (β5-β7), respectively, which form a barrel-like structure.[29] One side of the barrel is open and highly positively charged by a cluster of six K/R residues (Figure 4).

To investigate the importance of the PH domain, we generated mutants with amino acid substitutions of residue R360 and K367, which are both part of this cluster and equivalent to

essential residues in the PH domain of kindlin-2.[19;29] We observed less than 50% of translocation with the K367A mutant compared to wild-type kindlin-3. This implies that also in kindlin-3, the PH domain is indeed responsible for the interaction with the plasma membrane and that the K367 residue is of major importance for this interaction. Interestingly, mutant R360C translocated to the plasma membrane for more than 80% of what was observed with wild-type kindlin-3, which is indicative for a minor role of this residue, and thus demonstrates that the relative contribution of specific amino acids may well vary among the different kindlins.[29]

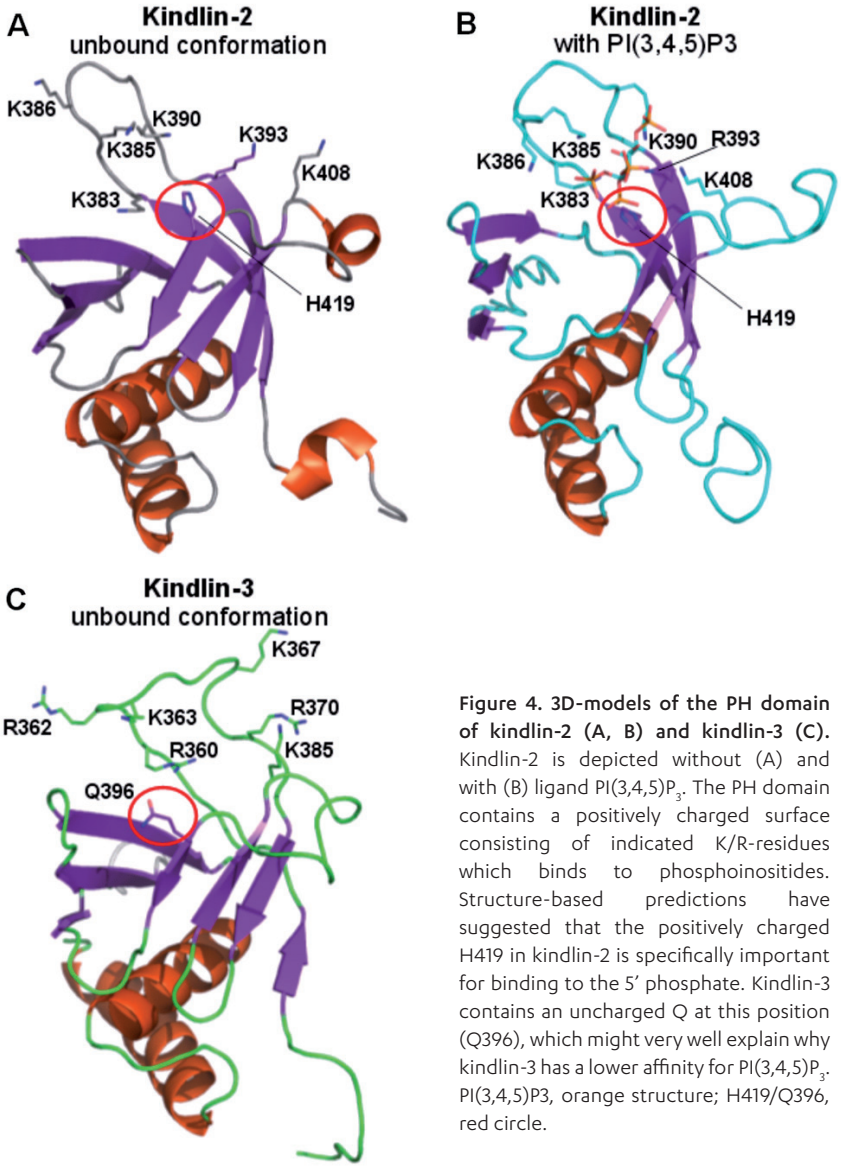

Figure 4. 3D-models of the PH domain of kindlin-2 (A, B) and kindlin-3 (C). Kindlin-2 is depicted without (A) and with (B) ligand PI(3,4,5)P_3. The PH domain contains a positively charged surface consisting of indicated K/R-residues which binds to phosphoinositides. Structure-based predictions have suggested that the positively charged H419 in kindlin-2 is specifically important for binding to the 5' phosphate. Kindlin-3 contains an uncharged Q at this position (Q396), which might very well explain why kindlin-3 has a lower affinity for PI(3,4,5)P_3. PI(3,4,5)P3, orange structure; H419/Q396, red circle.

Although recruitment to the plasma membrane was reduced for more than 50% with mutant K367A, residual recruitment could still be detected. For kindlin-2, it was reported that the F0 domain – the N-terminal part of the FERM domain – contains an HKHWK cluster, which creates an additional positively charged surface that can bind to negatively charged phospholipids.[31] A similar positively charged polylysine loop in the F1 domain of kindlin-1 has been shown to interact with membrane phospholipids.[32] Based on structural homology, equivalent clusters in the F0 and F1 subdomain of kindlin-3 may be responsible for the residual recruitment we detected.

Using a FRET-based liposome assay, we revealed that the PH domain of kindlin-3 bound specifically to $PI(3,4)P_2$. $PI(3,4)P_2$ is generated upon dephosphorylation of $PI(3,4,5)P_3$ by phosphatases such as SHIP, which is activated upon neutrophil stimulation.[20;33] As mentioned above, $PI(3,4,5)P_3$ is generated from $PI(4,5)P_2$, to which talin-1 can bind, and these findings may imply that talin-1 interacts with the membrane prior to kindlin-3. In line herewith, recent findings on LFA-1 suggest that talin-1 is required for initial integrin activation and kindlin-3 for a later step to induce maximal ligand-binding affinity.[34]

Kindlin-2 was previously reported to bind preferentially to $PI(3,4,5)P_3$, and it was suggested that the recognition mode would be conserved among the kindlins.[24] We show here that the affinity for specific PIPs varies among the kindlins. Structure-based predictions have suggested that the positively charged H419 in kindlin-2 is specifically important for binding to 5' phosphate in phosphoinositides.[29] Kindlin-3 contains an uncharged Q at this position (Q396), which might very well explain why kindlin-3 has a lower affinity for $PI(3,4,5)P_3$ (Figure 4).

Co-immunoprecipitation studies were used to demonstrate for the first time in intact human neutrophil-like cells that kindlin-3 interacts with β2 integrins, which had previously been suggested from findings with mouse platelets and recombinant integrin tails.[30] The interaction is induced only during integrin activation. Mutants with amino acid substitutions in the PTB domain of kindlin-3 were used to reveal that this site facilitates the intermolecular interaction. Binding of kindlin-3 mutant W596S to the β2 integrin was strongly reduced to ~20%, while the Q595A mutation also decreased binding for more than 50%. Decreased co-immunoprecipitation of PH domain mutant K367A, to less than 70%, suggests that interaction with the plasma membrane facilitates integrin binding. Binding to PIPs via the F0 and F1 domain of kindlin-3, as suggested above, may also contribute to recruitment to the membrane.

Although the PTB domain of talin-1 and kindlins is highly homologous and the importance of these molecules for integrin activation is shared, the relative contribution to integrin binding of certain conserved amino acids is highly variable (Figure 3B).[17] In talin, three conserved amino acids, i.e. R358, W359 and I396, are important for binding to the cytoplasmic tails of β1 and β3 integrins, whereas K357 is less important in this interaction.[15;17;35] For the interaction of kindlin-1 with β1 integrins, the equivalent amino acids K610, W612 and I651 in kindlin-1 are important, but Q611 is not.[17] For the interaction of kindlin-2 with the β1 integrins, equivalent W615 is important, but Q614 and I654 are not.[17] For interaction of β3 integrin with kindlin-1 and kindlin-2, solely the importance of W612 and W615, in each kindlin, respectively, has been confirmed. Here we show that for optimal interaction of kindlin-3 with the β2 integrins, the amino acid residues Q595 and W596 are both essential. In addition, functional studies of Moser et al. have confirmed that the

equivalent amino acid residue Q597 in murine kindlin-3 is important for β3 integrin activation in mice.[36] Together these findings reveal that variation in the specific binding characteristics exists among talin-1 and the different kindlins.

Our data lead to a model in which lipid-modifying effects, as shown in Figure 5, contribute to activation of b2 integrins on neutrophils, e.g. downstream of GPCRs. In resting neutrophils, the major b2 integrin CR3 (αMβ2 or CD11b/CD18) is in an inactive conformation, and talin-1 and kindlin-3 are not bound to the membrane (Figure 5A, see 1). Cell stimulation induces phosphatidylinositol 4-phosphate 5-kinase type I (PIPKI) to generate a steep rise in local PI(4,5)P_2 levels.[37] The F3 region of talin-1 can bind to areas with high levels of PI(4,5)P_2, which abrogates its autoinhibition and allows binding to the membrane-proximal NPLF domain of αMβ2.[21;22;40] In parallel, phosphoinositide 3-kinase (PI3K) phosphorylates PI(4,5)P_2 to PI(3,4,5)P_3.[20] PI(3,4,5)P_3 is subsequently dephosphorylated into PI(3,4)P_2 by phosphatases such as SH2-containing 5' inositol phosphatase-1 (SHIP), which induces recruitment of kindlin-3 (Figure 5A, see 2).[33]

Kindlin-3 binds to PI(3,4)P_2 via positively charged surfaces in its PH domain. Since PI(3,4)P_2 is generated as a product of (de)phosphorylation of talin-binding PI(4,5)P_2, we suggest that kindlin-3 recruitment and integrin binding takes place after initial talin-integrin binding, which would also fit with the order of events in current models on LFA-1 activation.[34] Additional positively charged surfaces in the F0F1 domain of kindlin-3 may induce binding to other PIPs, including PI(4,5)P_2, as was suggested for kindlin-1 and -2.[31;32] At the plasma membrane, the PTB domain of kindlin-3 can bind the membrane-distal NPKF domain of αMβ2 and thereby contribute to integrin activation (Figure 5A, see 3)).

Apart from the lipid-modifying effects, neutrophil stimulation leads to the recruitment of structural proteins via small GTPases of the Ras superfamily such as Rap1, Rac1 and Cdc42.[2] Activation of Rap1 results in generation of a Rap1 interacting adaptor molecule (RIAM)/talin/integrin complex that interacts with the cytoskeleton.[2] In addition, the Rho-family GTPases Rac1 or Cdc42 can activate PI3K as a positive-feedback loop in enforcing cell polarity. For LFA-1 on lymphocytes, activation of PKC leads to the formation of a ternary complex of RACK1, kindlin-3 and the integrin.[41] Phosphorylation of S and T residues in the cytoplasmic β2 integrin tail has been implicated to contribute to regulation of the binding of several of these adaptor proteins, including filamin, 14-3-3, talin-1 or kindlin-3, during integrin activation.[4;42;43]

In conclusion, in the current study, we elucidate that the PH domain of kindlin-3 is required for its recruitment to the plasma membrane, which facilitates subsequent binding of kindlin-3 to β2 integrin cytoplasmic tails via its PTB domain. Our data add to the understanding of kindlin-integrin interactions in neutrophils, of which little was known regarding kindlin-3 and the β2 integrins.

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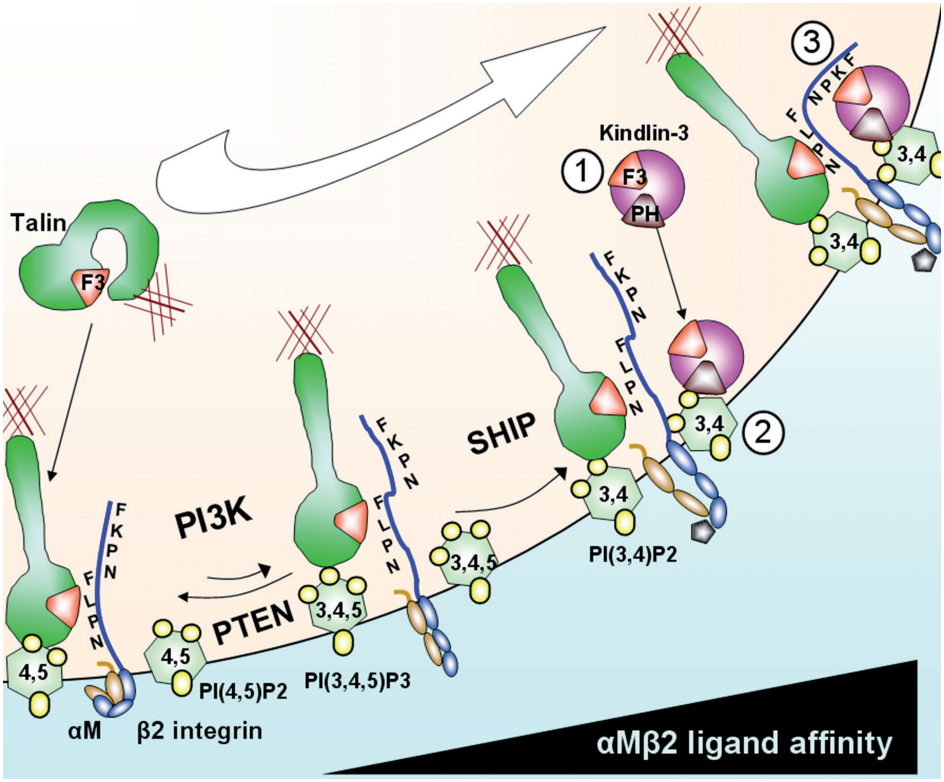

Figure 5. Model for b2 integrin activation. A. In resting neutrophils, the major integrin αMβ2 is in an inactive conformation, and talin-1 and kindlin-3 are not bound to the membrane. Cell stimulation, e.g. via GPCRs, induces phosphatidylinositol 4-phosphate 5-kinase type I (PIPKI) to generate a steep rise in local PI(4,5)P_2 levels.[37] The F3 region of talin-1 can bind to areas with high levels of PI(4,5)P_2, which abrogates its autoinhibition and allows binding to the membrane-proximal NPLF domain of αMβ2.[21;22;40] In parallel, phosphoinositide 3-kinase (PI3K) phosphorylates PI(4,5)P_2 to PI(3,4,5)P_3.[20] PI(3,4,5)P_3 is subsequently dephosphorylated by phosphatases such as SH2-containing 5' inositol phosphatase-1 (SHIP) into PI(3,4)P_2, which induces recruitment of kindlin-3 **(1)**. Kindlin-3 binds to PI(3,4)P_2 via positively charged surfaces in its PH domain **(2)**. Since PI(3,4)P_2 is generated as a product of (de)phosphorylation of talin-binding PI(4,5)P_2, we suggest that kindlin-3 recruitment and integrin binding takes place after initial talin-integrin binding, which would fit with the order of events in current models for LFA-1 activation.[34] Additional positively charged surfaces in the FOF1 domain of kindlin-3 may induce binding to other PIPs, including PI(4,5)P_2, as was suggested for kindlin-1 and -2.[31;32] At the plasma membrane, the PTB domain of kindlin-3 can bind the membrane-distal NPKF domain of αMβ2 and thereby contribute to integrin activation **(3)**. B. Regions of the cytoplasmic tail of β1, β2 and β3 integrin that interact with kindlins and talin. Structural studies by means of NMR implicate that the upstream W contributes to talin-1 binding, whereas interaction studies revealed the TT/TST/TTT region as an additional binding site for kindlin-1, -2 and -3.[19;37;38] Phosphorylation of this region regulates binding of filamin and 14-3-3 proteins and RhoA recruitment.[38]

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