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A Conserved Lipid-binding Loop in the Kindlin FERM F1 Domain Is Required for Kindlin-mediated α IIb β 3 Integrin Coactivation^{*[S]}

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Background: Kindlins cooperate with talin to activate integrins.

Results: A polylysine motif within a loop in the F1 domain of kindlin binds membranes and is required for integrin activation.

Conclusion: The membrane-binding site in kindlin F1 is distinct from that in talin and is essential to activate integrins

Significance: Understanding the molecular basis of integrin activation requires detailed information on kindlin interactions.

The activation of heterodimeric integrin adhesion receptors from low to high affinity states occurs in response to intracellular signals that act on the short cytoplasmic tails of integrin β subunits. Binding of the talin FERM (four-point-one, ezrin, radixin, moesin) domain to the integrin β tail provides one key activation signal, but recent data indicate that the kindlin family of FERM domain proteins also play a central role. Kindlins directly bind integrin β subunit cytoplasmic domains at a site distinct from the talin-binding site, and target to focal adhesions in adherent cells. However, the mechanisms by which kindlins impact integrin activation remain largely unknown. A notable feature of kindlins is their similarity to the integrin-binding and activating talin FERM domain. Drawing on this similarity, here we report the identification of an unstructured insert in the kindlin F1 FERM domain, and provide evidence that a highly conserved polylysine motif in this loop supports binding to negatively charged phospholipid head groups. We further show that the F1 loop and its membrane-binding motif are required for kindlin-1 targeting to focal adhesions, and for the cooperation between kindlin-1 and -2 and the talin head in α IIb β 3 integrin activation, but not for kindlin binding to integrin β tails. These studies highlight the structural and functional similarities between kindlins and the talin head and indicate that as for talin, FERM domain interactions with acidic membrane phospholipids as well β -integrin tails contribute to the ability of kindlins to activate integrins.

Integrins are transmembrane $\alpha\beta$ heterodimeric cell adhesion receptors essential for the development of multicellular organisms (1, 2). Integrins support cell adhesion via their N-terminal ectodomains that bind extracellular matrix proteins,

whereas the β -integrin cytoplasmic tails are linked to the actin cytoskeleton. In addition to providing a physical link between the matrix and cytoskeletal actin, integrins transmit chemical signals into the cell, providing information on location, local environment, and adhesive state (3). Integrins lack enzymatic activity and do not directly bind F-actin, so their ability to transmit force and integrate signals across the plasma membrane involves assembly of multiprotein cytoskeletal and signaling complexes on the cytoplasmic face of integrins (3–5). However, integrin binding to extracellular ligands requires a conformational change in the integrin ectodomains, generally termed integrin activation, which results in an increase in affinity for ligand (6, 7). Integrin activation and the ensuing cell adhesion and signaling are tightly regulated by intracellular signals that target the cytoplasmic tail of the integrin β subunit (7–9).

The platelet integrin α IIb β 3 has provided an important model for the study of integrin activation and has led to the discovery of important integrin regulators. Two major FERM (4.1, Ezrin, Radixin, Moesin) domain-containing protein families have emerged as controlling integrin activation, the talins, and the kindlins (10–12). The actin-binding protein talin (270 kDa) was the first to be identified (13), and the effect of talin on integrin activation is now understood in considerable detail (9). Talin is a scaffolding protein that binds and activates the integrin, in addition to recruiting focal adhesion components such as vinculin and actin necessary for subsequent cell adhesion and spreading (13–16). There are two major closely related talin isoforms, each composed of an integrin activating N-terminal head (\approx 50 kDa) and a flexible rod domain (\approx 220 kDa) (17, 18). The talin head (residues 1–433) contains a FERM domain (residues 1–400) followed by an apparently unstructured linker (residues 401–481). The rod domain (residues 482–2541) harbors binding sites for actin and vinculin (14, 15) as well as a dimerization site and a regulatory autoinhibitory site (19–21). Unlike classical FERM domains, which are trilobed structures composed of three subdomains (F1, F2, and F3) (22), the talin FERM domain adopts a novel linear arrangement (23) and

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[S] This article contains supplemental Figs. S1 and S2.

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exhibits two other atypical features: an additional F1-like domain called F0 preceding the F1 domain, and an unstructured loop within F1 (23, 24). The talin head binds and activates integrins through an interaction of its PTB-like F3 domain with the more membrane-proximal of the two well conserved NXXY motifs in the integrin β subunit cytoplasmic tails (25–28). This interaction results in an association of the F3 with a membrane-proximal portion of the integrin β tail that is thought to destabilize inhibitory α - β tail-transmembrane interactions leading to integrin activation (29–31). Although the talin F3-integrin interaction is required to trigger integrin activation (32–35), recent studies clearly demonstrate that other regions of the talin FERM domain are also important (24, 36). Indeed, we have shown that the talin F1 loop mediates a protein-lipid interaction important for integrin activation (24) and that talin F0 also contributes to integrin activation although the mechanism is unknown (36).

The mechanisms by which kindlins impact integrin activation are less clear. Kindlins are solely composed of a FERM domain, which is unique in containing a pleckstrin homology (PH)² domain inserted into the FERM F2 domain (11, 12). The kindlin FERM is most closely related to that of talin (37) and, like talin, contains an N-terminal F0 domain (38). Vertebrates have three kindlins, kindlin-1, -2, and -3, which exhibit a tissue-specific expression pattern (39, 40). Kindlin-2 is ubiquitously expressed, whereas kindlin-1 is restricted to epithelial cells and kindlin-3 is largely specific to the hematopoietic lineage but was recently shown to be expressed in endothelial cells (39, 41). Genetic studies provide compelling evidence that kindlins are key players in integrin-mediated cell adhesion and formation of cell-matrix adhesion structures. UNC-112, the *Caenorhabditis elegans* kindlin, co-localizes with integrins and UNC-112 deficiency results in a muscle detachment phenotype resembling that seen in integrin mutants (42). In humans, kindlin-1 mutations are linked to an epithelial cell adhesion defect that causes Kindler syndrome (KS) (43, 44), whereas kindlin-3 mutations are associated with leukocyte adhesion deficiency type III characterized by recurrent bleeding and infections due to inefficient platelet aggregation and defective leukocyte recruitment to inflammatory sites (45–48). These kindlin-3 mutations result in defective activation of integrins expressed in hematopoietic cells, providing clear evidence for a role of kindlins in integrin activation. The phenotypes of kindlin-1, -2, or -3 knock-out mice, and of cells isolated from these animals strongly support an essential role for kindlins in integrin activation and signaling (44, 49, 50). We and others have demonstrated that kindlins, via their F3-PTB like domain, can bind directly to the membrane-distal NXXY motif in integrin β tails, and that this interaction is required for kindlins to enhance integrin activation (49–52). However, at least in CHO cells, we find that overexpressed kindlins are not sufficient to activate α IIB β 3 but that kindlin-mediated integrin activation requires talin, and co-expressing kindlin-1 or -2 with the talin head enhances talin-mediated

α IIB β 3 integrin activation (52). Thus kindlin-mediated integrin activation appears to require (i) binding of the kindlin F3 domain to integrin β tails, and (ii) synergy with talin, although the mechanism has yet to be defined.

The location of disease-causing mutations in human kindlin-3 (47), along with data from cell-based kindlin expression studies (38, 51), show that in addition to the F3 integrin-binding domain, other domains in kindlin are important for its effects on integrin function. Here we have drawn on the predicted structural similarities between kindlins and the talin head, and the known importance of the talin F1 loop in integrin activation (24), to investigate the role of the kindlin F1 domain. We predict that kindlin F1 has a longer insert than the one in talin F1, and show that it is unstructured, and capable of binding negatively charged phosphatidylserine lipid head groups. We have identified a well conserved polylysine motif at the start of the insert that is required for lipid binding, and we further show that this motif is required for kindlin-mediated α IIB β 3 integrin activation and for kindlin targeting to focal adhesions.

EXPERIMENTAL PROCEDURES

Antibodies and cDNAs—Ligand-mimetic anti- α IIB β 3 PAC1 (BD Biosciences), goat anti-GFP (Rockland), goat anti-kindlin-2 (Y-15, Santa Cruz Biotechnology), and mouse monoclonal anti-vinculin (hVIN-1, Sigma) were from commercial sources. Anti-kindlin-1 antibody was a gift from Mary C. Beckerle (37), PAC1 Fab was kindly provided by S. Shattil (53), and anti- α IIB β 3 monoclonal antibody D57 (54) was a gift from M. Ginsberg. GFP-mouse talin-1 head (residues 1–433) was generated as previously described (36). The cDNAs encoding murine kindlin-1 residues 141–249 (F1 loop) and its mutants were synthesized by PCR using a mouse kindlin-1 cDNA as template, and cloned into the prokaryotic expression vector pet-151TOPO (Invitrogen). GFP and DsRed-tagged mouse kindlin-1 and human kindlin-2 constructs were described previously (38, 52). cDNAs encoding mutant mouse kindlin-1 and human kindlin-2 were generated by QuikChange mutagenesis from wild type constructs. The chimeric talin head containing the kindlin-1 F1 loop (residues 145–244) inserted between talin-1 head residues 138 and 169 was generated by overlapping PCR. A fragment of chimeric kindlin-1 containing the talin-1 F1 loop (139–168) inserted between kindlin-1 residues 139 and 245 was synthesized by Genscript and subcloned into our DsRed-kindlin-1 construct. All cDNAs were authenticated by DNA sequencing.

Analysis of Integrin Activation—The activation state of stably overexpressed α IIB β 3 integrin in CHO cells transiently expressing GFP-tagged mouse talin head and/or DsRed mouse kindlin-1 fragments was assessed in three-color FACS assays using a modification of previously described methods (52, 55). Briefly, α IIB β 3-expressing CHO cells were co-transfected with the indicated GFP and DsRed expression constructs using Lipofectamine (Invitrogen) or polyethylenimine following the manufacturer's instructions, and 24 h later, the cells were suspended and incubated with ligand-mimetic anti- α IIB β 3 monoclonal antibody PAC1 IgM (BD Biosciences) or PAC1 Fab in the presence or absence of 10 mM EDTA. α IIB β 3 integrin expression was assessed in parallel by staining with D57 antibody.

² The abbreviations used are: PH, pleckstrin homology; LMV, large multilamellar vesicles; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine.

Bound PAC1 and D57 were detected using Alexa 647 fluorophore-conjugated goat anti-mouse IgM and anti-mouse IgG (Invitrogen), respectively.

Activation of α IIb β 3 in doubly transfected (GFP-positive and red-positive) cells was quantified and the α IIb β 3 activation index was defined as $AI = (F - F_0)/(F_{\text{integrin}})$, where F is the geometric mean fluorescence intensity (MFI) of PAC1 binding, F_0 is the MFI of PAC1 binding in the presence of EDTA, and F_{integrin} is the standardized ratio of D57 binding to transfected cells. The F_{integrin} expression ratio was defined for double expressing cells as follows: $F_{\text{integrin}} = (F_{\text{trans}})/(F_{\text{untrans}})$, where F_{trans} is the geometric MFI of D57 binding to double expressing cells and F_{untrans} is the MFI of D57 binding to untransfected cells. FACS data analysis was carried out using FlowJo FACS analysis software and statistical analysis using GraphPad Prism software.

Immunofluorescence—CHO cells stably expressing α IIb β 3 integrin were transiently transfected with 3 μ g of the indicated cDNAs using polyethylenimine (linear polyethyleimine 25 kDa, Polysciences Inc.). Twenty-four hours after transfection, cells were detached and allowed to re-adhere and spread on fibrinogen-coated (10 μ g/ml) glass coverslips. After 4 h of plating, cells were fixed, permeabilized, and stained with monoclonal anti-vinculin antibody followed by anti-mouse Alexa 568 (Invitrogen) as a secondary antibody. Cells were imaged on a Nikon TE2000 microscope with a \times 100 objective and images were processed with ImageJ (NIH).

Expression of Recombinant Kindlin Polypeptides—Kindlin polypeptides were expressed in *Escherichia coli* BL21 STAR(DE3) cultured in LB media, and recombinant His-tagged kindlin polypeptides were purified by nickel-affinity chromatography following standard procedures. Due to the unstructured nature of the polypeptides and their extreme sensitivity to proteolytic degradation, the samples were boiled at 95 $^{\circ}$ C for 5 min immediately after elution and then centrifuged at 13,000 \times g for 5 min to remove precipitated material. The His-tag was removed by cleavage with AcTEV protease (Invitrogen), and after boiling for a second time, the proteins were further purified by anion-exchange chromatography. Protein concentrations were determined using extinction coefficients at 280 nm calculated from the aromatic amino acid content according to ProtParam (EXPASY) of 11,460 $\text{M}^{-1} \text{cm}^{-1}$ for kindlin residues 141–249.

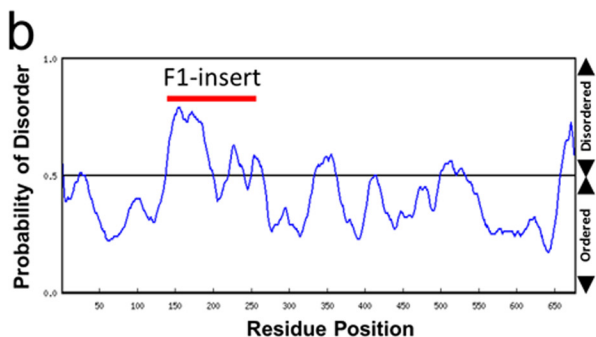
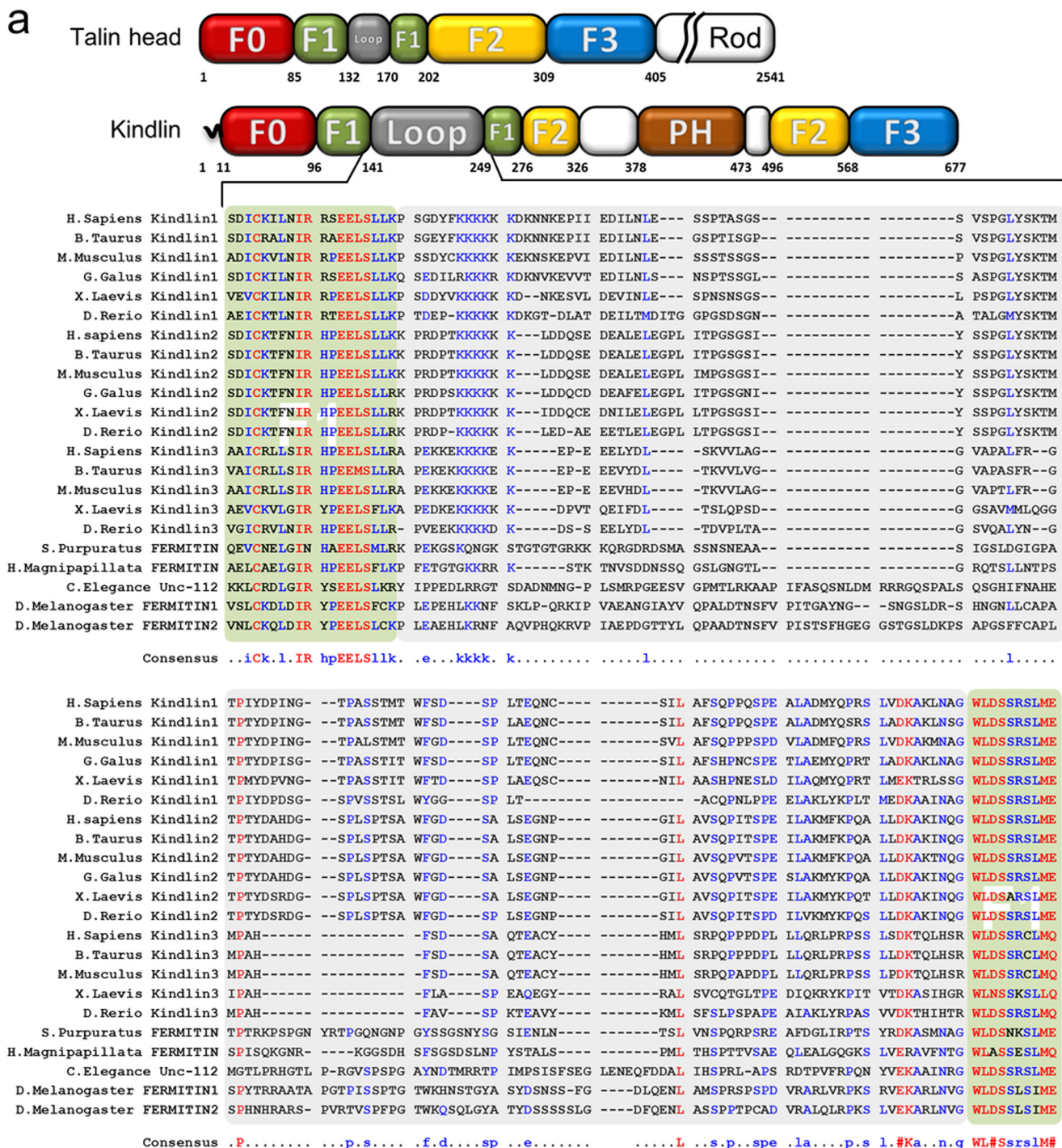
Phospholipid Cosedimentation Assay—Large multilamellar vesicles (LMV) were prepared as described previously (29, 56). Briefly, films of dried phospholipids (Sigma) were swollen at 5 mg/ml in 20 mM Hepes, pH 7.4, 0.2 mM EGTA for 3 h at 42 $^{\circ}$ C. The vesicles were then centrifuged (20,000 \times g for 20 min at 4 $^{\circ}$ C), and the pellet was resuspended in the same buffer at 5 mg/ml. Protein samples were diluted into 20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 15 mM β -mercaptoethanol. After centrifugation (20,000 \times g for 20 min at 4 $^{\circ}$ C), proteins (0.15 mg/ml) were incubated (30 min, 25 $^{\circ}$ C) in the absence or presence of phospholipid vesicles (0.5 mg/ml), 200 μ l total volume, followed by centrifugation (25,000 \times g for 20 min at 4 $^{\circ}$ C). Pellet and supernatant fractions were analyzed on a 10–20% gradient gel (Expedeon) and proteins were detected by Coomassie Blue staining.

Pulldown Assays with Recombinant Integrin Tails—Recombinant integrin tail proteins were produced and purified as described previously (57). CHO cells stably expressing α IIb β 3 integrin were transfected with cDNAs for wild type or mutant kindlin constructs using Lipofectamine (Invitrogen) following the manufacturer's instructions, the cells were harvested 24 h later and lysed on ice with lysis buffer (50 mM NaCl, 10 mM Pipes, 150 mM sucrose, 50 mM NaF, 40 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM Na_3VO_4 , pH 6.8, 0.5% Triton X-100, 0.1% sodium deoxycholate and EDTA-free protease inhibitor tablet (Roche Applied Science)). Lysates were cleared by centrifugation, then incubated with recombinant integrin tails bound to His-bind resin (Novagen) as described previously (57); bound proteins were fractionated by SDS-PAGE using a 4–20% Tris glycine gradient gel (Bio-Rad) and detected by Western blotting or protein staining. Bands were quantified using ImageJ and data were analyzed using GraphPad Prism (GraphPad Software) and plotted as percentage of maximal binding.

RESULTS

The Kindlin F1 Domain, Like the Talin F1 Domain, Has an Unstructured Loop—Sequence analysis indicates that the domain organization of kindlins shares common features with the talin head (24, 37, 38) (Fig. 1*a*), and that talins and kindlins are FERM domain-containing proteins. Classic FERM domains contain three structurally distinct lobes termed F1, F2, and F3, but unlike classic FERM domains, the talin and kindlin FERM domains have an N-terminal duplication of the F1 domain named F0 (23, 24, 38). Moreover, the talin F1 domain has an unstructured loop (\sim 35 amino acids) emerging from between strands β 3 and β 4 of its ubiquitin-like fold, and this loop plays an important role in talin-mediated integrin activation (24). A sequence alignment of kindlin and talin predicts the presence of an even larger insertion within the kindlin F1 domain at the same position as that in talin F1 (38) (supplemental Fig. S1). Careful sequence comparison of the F1 domains from different kindlin isoforms from different species shows a clear interruption of the kindlin F1 domain sequence at the same position in all kindlins (Fig. 1*a*). This F1 domain insertion is present in all known kindlin proteins. Sequence alignment shows that the length of the kindlin F1 insert varies among isoforms, with kindlin-3 having the shortest insert compared with kindlin-1 and -2. The length of the F1 insert also varies between species; worms and insects have longer F1 inserts than higher vertebrates. In organisms with three kindlin isoforms, the F1 insert is more conserved between kindlin-1 and kindlin-2 (e.g. 60.74 and 59.04% similarity in humans and mice, respectively) than between kindlin-1 and kindlin-3 (e.g. 14.01 and 16.19% similarity in human and mice, respectively) or kindlin-2 and kindlin-3 (e.g. 14.28 and 13.33% similarity in human and mice respectively). Additionally, sequence alignments identify residues that are highly conserved across species despite the variable length and sequence of the insert. Notably, the N and C termini are more conserved than the middle of the kindlin F1 insert. The sequences of the F1 inserts in kindlins and talin share little in common (supplemental Fig. S1). Thus, unlike the talin F1 insert that contains scattered positively charged residues, the kindlin

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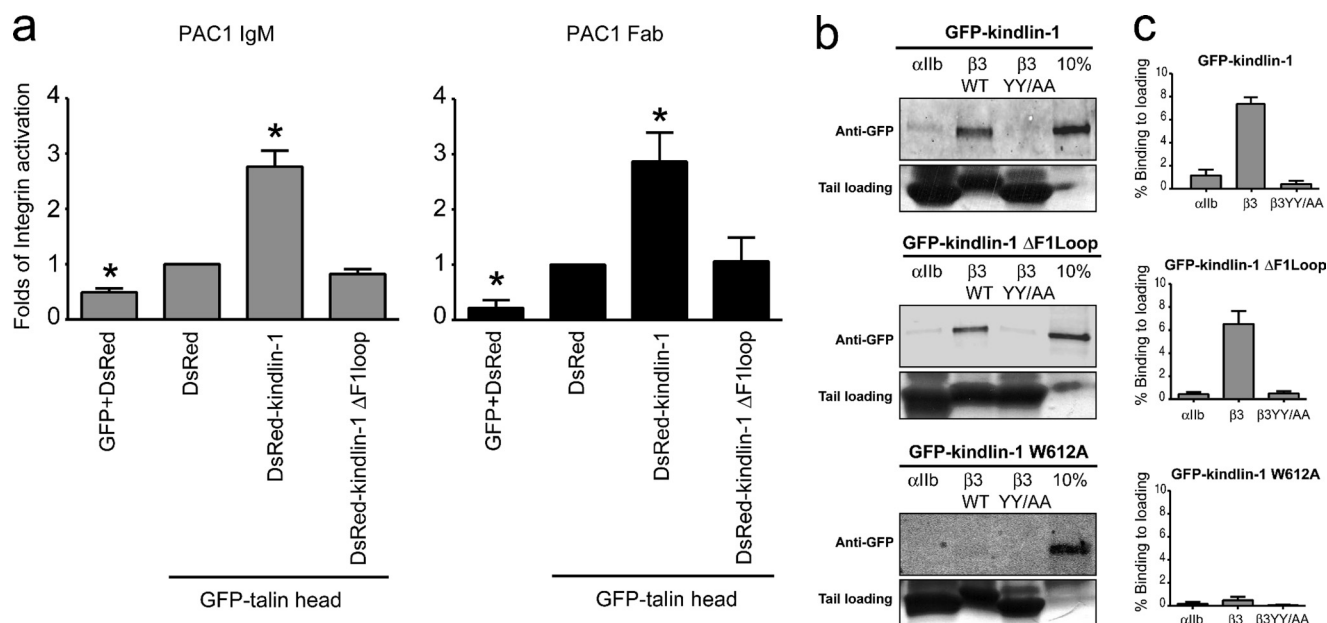


FIGURE 2. The kindlin-1 F1 loop is required for α IIb β 3 integrin activation. *a*, CHO cells stably expressing α IIb β 3 integrin were co-transfected with GFP or GFP-tagged talin head (residues 1–433) and DsRed or DsRed-tagged kindlin-1 cDNAs as indicated. Activation indices of α IIb β 3 integrin from co-expressing cells with similar fluorescence of GFP and DsRed tags were calculated using either PAC1 IgM or PAC1 FAB as indicated and normalized for integrin expression (see “Experimental Procedures”). The results represent the mean \pm S.E. ($n \geq 3$). Results that are significantly different from DsRed + GFP-talin head (t test $p < 0.05$) are indicated (*). *b*, kindlin-1 F1 loop is not required for kindlin-1 binding to β 3-integrin tail. Pull-down assays using wild type and mutant recombinant α IIb or β 3 tail proteins were performed with CHO cell lysates. Binding of overexpressed kindlin constructs was assessed by Western blotting. Loading of each tail protein was judged by protein staining. Lysate represents 10% of the starting material in the binding assay. *c*, binding of different kindlin constructs was quantified by densitometry and normalized to the lysate control ($n \geq 3$).

F1 insert has a conserved cluster of positively charged amino acids at its N terminus.

NMR spectroscopy shows that the talin-1 F1 insert forms a largely unstructured loop emerging from the F1 domain (24). Similarly, a probability prediction of disorder for mouse kindlin-1 highlights an unstructured 108-amino acid stretch (141–249) within the F1 domain in agreement with the talin-kindlin domain structure comparison (Fig. 1*b*). This prediction is also true for all the other kindlin proteins (supplemental Fig. S2) and is supported by the ^1H - ^{15}N heteronuclear single quantum coherence spectrum of the isolated mouse kindlin-1 insert (141–249), which indicates a substantially disordered conformation (see supplemental Fig. S3 of Ref. 38). Therefore, both kindlin and talin have an unstructured loop inserted at the same position of their respective F1 domains. In kindlins, despite their variable lengths, these loops still share some conserved amino acids.

Kindlin-1 F1 Loop Is Required for α IIb β 3 Integrin Activation—Kindlin and talin are regulators of α IIb β 3 integrin activation. We and others have shown that, when co-expressed in CHO cells, kindlins potentiate talin head-mediated α IIb β 3 activation (44, 50–52). Furthermore, the talin F1 loop is important for optimal α IIb β 3 activation by the talin head (24). Given the sim-

ilarities between the kindlin and talin FERM domains, we sought to test whether the kindlin F1 loop also plays a role in kindlin-mediated α IIb β 3 integrin activation. For this purpose, we compared the ability of overexpressed DsRed-tagged wild-type kindlin-1 and kindlin-1 lacking the F1 loop (Δ 145–244) to co-activate α IIb β 3 in the presence of GFP-talin head. We measured the binding of the ligand-mimetic anti- α IIb β 3 antibody PAC1 in a well established three-color flow cytometry-based assay to assess integrin activation in doubly expressing CHO cells co-transfected with GFP or GFP-talin head along with DsRed or DsRed-tagged kindlin constructs (52, 55). EDTA-treated cells were used to control for PAC1 binding specificity and α IIb β 3 total cell surface expression level was assessed by staining with the activation-independent anti- α IIb β 3 monoclonal antibody D57.

As shown in Fig. 2*a*, consistent with previous reports, expression of the GFP-talin head triggered α IIb β 3 activation and co-expression of wild type DsRed-kindlin-1 with the GFP-talin head further increased α IIb β 3 integrin activation. However, when the DsRed-kindlin-1 Δ F1 loop (Δ 145–244) was co-expressed with the GFP-talin head, it did not increase talin head-mediated α IIb β 3 integrin activation (Fig. 2*a*). This strongly suggests that the F1 loop of kindlin has an important role in

FIGURE 1. *a*, the domain structure of talin head and kindlin. Schematic representation of talin head and kindlin domain structure. The individual domains: F1, F2, and F3, that make up a canonical FERM domain are shown in green, yellow, and blue, respectively; the F0 domain is shown in red. The kindlin PH domain is indicated by a brown box. The unstructured F1-loop region is shown in gray and other unstructured regions are in white. The position of the long C-terminal talin rod is indicated. The horizontal scale in both schematics is the same. Sequence alignment of kindlin FERM domains showing the F1 sequence (green background) bordering the F1 insert (gray background). Highly conserved (>90% identity) residues are colored in red, residues with >50% identity are colored in blue, and other residues appear in black in the kindlin sequence. # is any NDQE. Kindlin sequences from NCBI data base were processed by Multalin server. *b*, the probability of disorder for mouse kindlin-1. Mouse kindlin-1 full-length sequence (NCBI data base NP_932146.2) was submitted to the RONN (Regional Order Neural Network) server to predict natively disordered regions based on the amino acid sequence. The 117-amino acid F1 insert (shown by the red line) comprises residues 141–249 and contains >80 of these inherently disordered residues. Prediction false positive rate was set at 5%.

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integrin activation. Furthermore, as activation was measured in tightly gated cell populations with fixed GFP and DsRed fluorescence and corrected for their surface-expressed α IIB β 3 integrin levels, differences in expression of wild type and mutant kindlin or effects on talin head or integrin expression levels cannot account for this result.

PAC1 is an IgM and we therefore repeated our assays using the PAC1 Fab fragment, which has previously been described as an effective reporter of the integrin activation state (53). As shown in Fig. 2*a*, we obtained very similar results with PAC1 and PAC1 Fab. Thus, the kindlin-1 F1 loop is required for kindlin-1 to mediate its synergistic effect with the talin head in activating α IIB β 3 integrin. We conclude that the kindlin F1 loop, like the talin F1 loop, is involved in the integrin activation process.

Kindlin-1 F1 Loop Is Not Required for Kindlin-1 Binding to β 3 Integrin— α IIB β 3 integrin activation by kindlin requires binding of the PTB-like kindlin F3 domain to the kindlin-binding site within the β 3 cytoplasmic tail (44, 52, 58). To test whether the lack of α IIB β 3 integrin co-activation by the kindlin-1 Δ F1 loop could result from an inability to bind the β 3 tail, we attempted to pulldown full-length kindlin-1 wild type and mutant recombinant proteins from CHO cell lysates using different integrin tails immobilized on beads. The results, presented in Fig. 2*b*, show that both wild type kindlin-1 and the kindlin-1 Δ F1 loop bind the β 3 cytoplasmic tail. Binding was specific as neither the α IIB tail nor a β 3 tail bearing a mutation in the kindlin-binding site (Y747A, Y759A) were able to pulldown wild type kindlin-1 or the kindlin-1 Δ F1 loop. As a further specificity control for the β 3 tails we assessed binding of kindlin-1 (W612A), which contains a point mutation in its F3 domain that is known to impair binding to β 3 tail (44). This protein was well expressed but did not bind any of the tested integrin tails (Fig. 2*b*). As shown in Fig. 2*c*, quantitative analysis shows no impairment in the ability of the kindlin-1 Δ F1 loop mutant to bind specifically to β 3 tails compared with wild type kindlin-1. Taken together, our results suggest that the requirement of the kindlin F1 loop for α IIB β 3 integrin co-activation is independent of the ability of kindlin-1 to bind to β 3 tails.

A Polylysine Motif in Kindlin-1 F1 Loop Binds to Lipid Membrane—We have previously shown that the F1 loop in the talin FERM domain, which is also important for integrin activation, binds to acidic membrane phospholipids, and that this induces helix formation with positively charged residues aligned along one surface (24). We therefore sought to assess whether the kindlin-1 F1 loop also interacts with membranes using an *in vitro* co-sedimentation assay (24). We expressed and purified the kindlin-1 F1 loop (amino acids 141–249) and incubated it with LMV containing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), or a mixture of both (4:1 PC:PS). After centrifugation, protein contents from pelleted vesicles and supernatant were analyzed by SDS-PAGE. As shown in Fig. 3*a*, the kindlin-1 F1 loop does not bind to POPC or PC:PS mixed LMV and remains in the supernatant (S) fraction. However, the loop cosediments with negatively charged LMV composed of POPS as shown by the disappearance of the protein from the supernatant lane (S) and its appearance in the

pellet lane (P). This result shows preferential binding of the kindlin F1 loop to POPS over other lipid mixtures.

In the case of the talin F1 loop, membrane binding was mediated by an α -helix whose formation was stabilized upon membrane binding (24). Analysis of the sequence and NMR spectrum of the kindlin-1 F1 loop (38) did not reveal α -helices. However, the kindlin-1 F1 loop does have a high ratio of positively charged Arg and Lys residues. Notably these residues are enriched at the N-terminal end of the kindlin F1 loop including a stretch of 6 lysines at the start of the loop (residues 147–152). Sequence alignment shows that this short stretch of lysine residues is highly conserved in all mammalian kindlins (kindlin-1, -2, and -3), and is also present in kindlins of birds, fish, reptiles, and amphibians (Fig. 3*c*). Furthermore, whereas it is often shorter and less well conserved, stretches of lysines or arginines are still evident at the start of the F1 loop in invertebrate kindlins (insects, nematodes, sea urchin, hemichordata, and Hydra) (Fig. 3*c*). This high degree of conservation suggests functional significance and polylysine sequences are known to confer membrane binding activity on proteins (59, 60). To test whether the stretch of lysines was important for the kindlin-1 F1 loop binding to lipids, cosedimentation assays were performed with purified F1 loop constructs lacking the N-terminal polylysine motif (Δ lysines) or containing charge reversal point mutations targeting the same motif (KKKKKK/AEAAEA). As shown in Fig. 3*b*, deletion or disruption of the polylysine motif markedly inhibited F1-loop binding to POPS containing LMVs. Thus, our data demonstrate that the kindlin-1 F1 loop can bind negatively charged phosphatidylserine lipid head groups and that this interaction is mediated through a conserved polylysine motif.

The Lipid-binding Polylysine Motif in Kindlin-1 F1 Loop Is Required for α IIB β 3 Coactivation—To test whether binding of the kindlin-1 F1 loop to acidic membrane phospholipids contributes to the synergy between kindlin-1 and the talin head in α IIB β 3 integrin activation, we assessed the impact of overexpressing DsRed-kindlin-1 F1 loop mutants containing either charge reversal mutations (KKKKKK/AEAAEA) or a deletion of the polylysine motif (Δ lysines). α IIB β 3-expressing CHO cells were cotransfected with GFP empty vector or GFP-talin head along with DNAs encoding DsRed, wild type DsRed-kindlin-1, or DsRed-kindlin-F1 loop mutants. Integrin activation and total integrin expression of doubly expressing cells were measured with PAC1 and D57 antibodies, respectively. Activation indices were then calculated and standardized to the GFP + DsRed control as described under “Experimental Procedures.”

As expected, GFP-talin head-activated α IIB β 3 integrin, and DsRed-kindlin-1 enhanced the talin-mediated activation. In contrast, the DsRed-kindlin-1 Δ F1 loop construct could not cooperate with the GFP-talin head to activate α IIB β 3 integrin (Fig. 4*a*). Interestingly, neither the DsRed-kindlin-1 charge reversal mutant (KKKKKK/AEAAEA) nor DsRed-kindlin-1 Δ lysines were capable of increasing α IIB β 3 integrin activation above the level reached by the GFP-talin head plus DsRed control. The results show that, as for talin, membrane binding via the F1 loop is important for kindlin-1-mediated integrin activation.

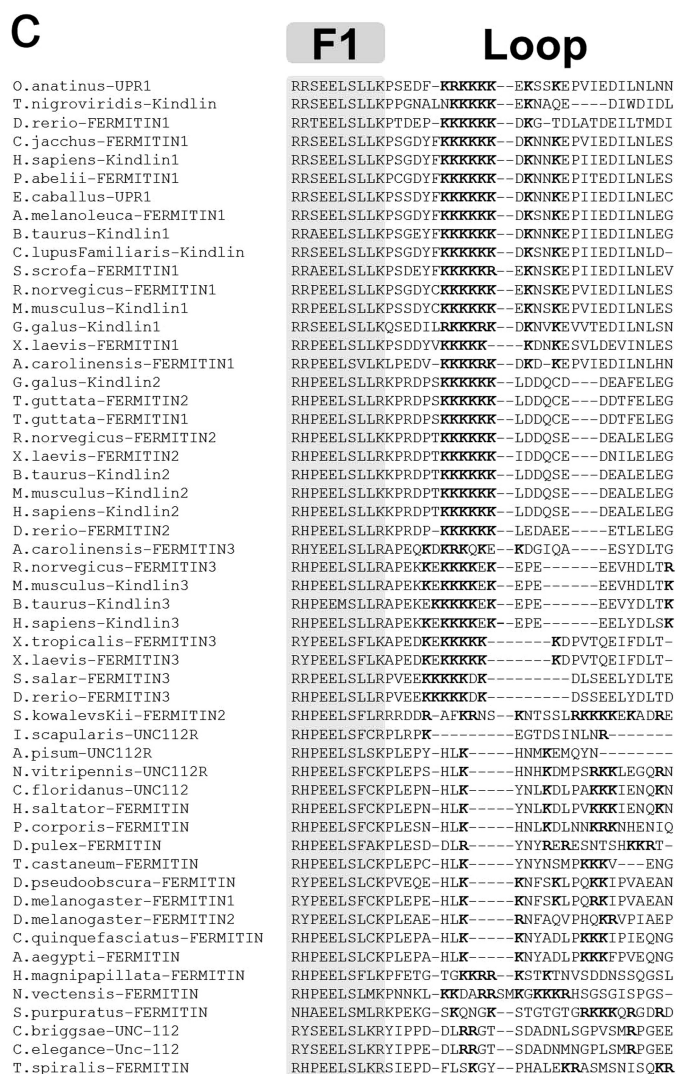
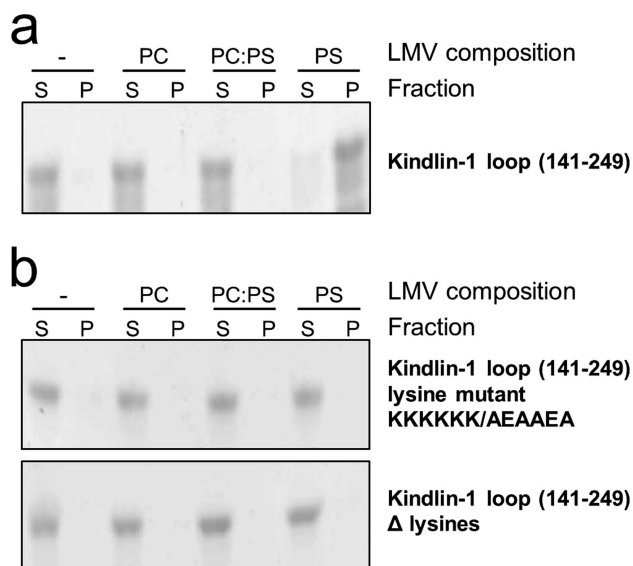


FIGURE 3. The kindlin F1 loop interacts with negatively charged membrane phospholipids via a positively charged sequence at its N terminus. *a* and *b*, wild type kindlin F1 loop (residues 141–249; 0.15 mg/ml) (*a*) or kindlin F1 loop containing mutations in the polylysine motif (*b*) was mixed with vesicles (0.5 mg/ml) consisting of PC, PS, or a 4:1 ratio of PC:PS and then centrifuged. Protein present in the vesicle-containing pellet (*P*) or the vesicle-free

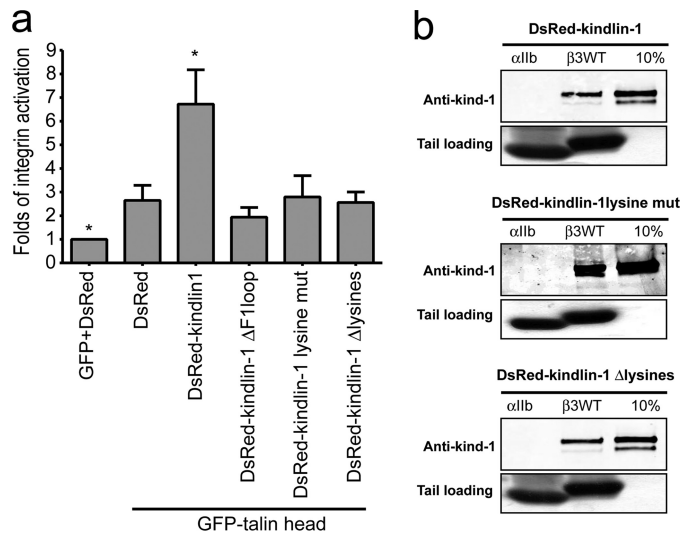


FIGURE 4. The lipid-binding polylysine motif is required for kindlin-1-mediated α IIb β 3 integrin activation but not integrin β tail binding. *a*, CHO cells stably expressing α IIb β 3 integrin were co-transfected with GFP or GFP-tagged talin head (residues 1–433) and DsRed or DsRed-tagged kindlin-1 cDNAs as indicated. Activation indices of α IIb β 3 integrin from co-expressing cells with similar fluorescence of GFP and DsRed tags were calculated and normalized for integrin expression (see “Experimental Procedures”). The results represent the mean \pm S.E. ($n \geq 3$). Results that are significantly different from DsRed + GFP talin head ($p < 0.05$) are indicated (*). *b*, pull-down assays using wild type and mutant recombinant α IIb or β 3 tail proteins were performed with CHO cell lysates. Binding of overexpressed GFP-kindlin-1 wild-type or F1 loop mutant recombinant proteins was assessed by Western blotting. Loading of each tail protein was judged by protein staining. Lysate represents 10% of the starting material in the binding assay.

We also checked whether, in a pull-down assay, these same mutations affected the ability of full-length kindlin-1 to bind to the β 3 tail. As shown in Fig. 4*b*, kindlin-1 F1 loop mutants (Δ lysines and KKKKKK/AEAAEA lysine mutant) retain their specific binding to β 3 immobilized tails *in vitro* as does the wild type kindlin protein. This data clearly demonstrate that the interaction between the kindlin-1 F1 loop and acidic phospholipids is required for the synergy with the talin head in α IIb β 3 integrin activation, and that this is independent of kindlin binding to the β 3 tail.

The Polylysine Motif in the Kindlin F1 Loop Is Required for Kindlin Targeting to Focal Adhesions—In adherent cells, kindlin-1 localizes to focal adhesions where integrins anchor the cytoskeleton to the extracellular matrix. Kindlin F3 interaction with the β tail is crucial for targeting kindlin into these structures (37). To investigate whether the kindlin F1 loop polylysine motif also plays a role in this process, we compared the localization of overexpressed GFP-tagged kindlin-1 wild type and mutants in adherent α IIb β 3-expressing CHO cells. GFP-kindlin-1-transfected cells were harvested, then allowed to reattach and spread onto coverslips coated with the α IIb β 3 integrin ligand, fibrinogen. Four hours after plating, cells were fixed, stained for the focal adhesion marker vinculin, and analyzed by

supernatant (*S*) was assessed by SDS-PAGE and protein staining. *c*, sequence alignment of kindlins showing the N-terminal part of the kindlin F1 loop bordered by F1 sequence (gray). Positively charged amino acids, arginine (*R*) and lysine (*K*) are in bold. Kindlin sequences from NCBI data base were aligned using the Multalin server.

Lipid-binding Loop in the Kindlin FERM F1 Domain

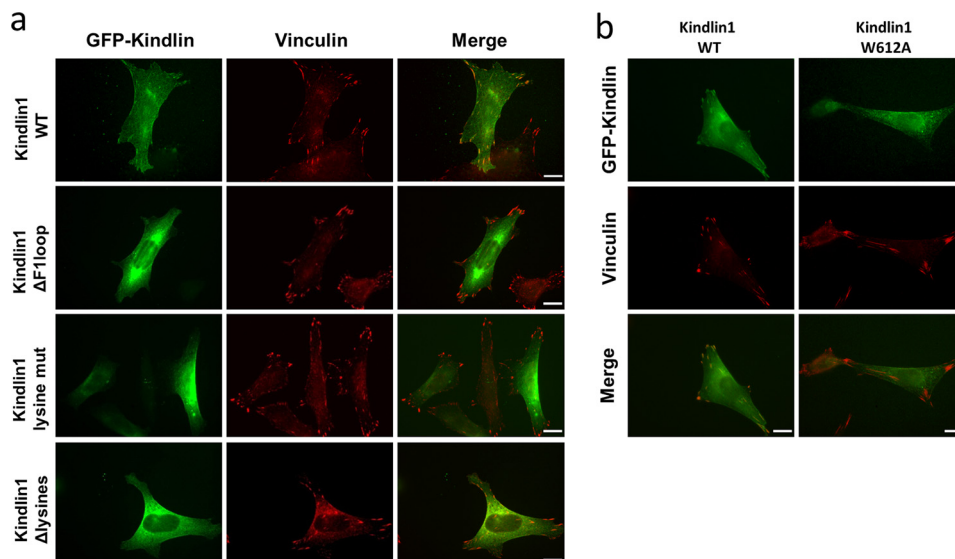


FIGURE 5. The kindlin F1 loop poly-lysine motif is required for FA targeting of kindlin-1 in adherent cells. Images of CHO cells stably expressing α IIb β 3 integrin transiently transfected with (a) GFP-tagged kindlin-1 wild type and F1 loop mutants or (b) GFP-tagged kindlin-1 wild type and W612A mutant expression constructs after plating on fibrinogen coverslips. Kindlin-1 wild type but not kindlin-1 F1 loop mutants nor kindlin-1 W612A co-localizes with vinculin at focal adhesions. White bar = 10 μ m.

epifluorescence microscopy. We have previously shown that adhesion, spreading and focal adhesion formation by α IIb β 3-expressing CHO cells on fibrinogen are dependent on α IIb β 3 (61). Representative images (Fig. 5a) show that, as previously reported (37), GFP-kindlin-1 is clearly targeted to vinculin-containing focal adhesions. However, none of the GFP-kindlin-1 F1 loop mutants (kindlin-1 Δ loop, Δ lysines, and KKK-KKK/AEAAEA lysine mutant) were found in focal adhesions. Instead they displayed diffuse cytoplasmic staining without any particular localization. We also assessed targeting of the integrin-binding defective kindlin-1 mutant W612A (44). As expected, GFP-kindlin-1(W612A) failed to target to focal adhesions confirming the specificity of our assay (Fig. 5b). Expression of the mutant kindlins did not significantly alter the presence of focal adhesions as judged by vinculin localization (Fig. 5, a and b). We conclude that the F1 loop lipid-binding motif is required for targeting kindlins to focal adhesions.

Kindlin-2 Requires the Lipid-binding Polylysine Motif for α IIb β 3 Coactivation—As shown in Fig. 3c, the polylysine motif is the most conserved part of the kindlin F1 loop. Its N-terminal position within the loop and its length (KKKKKK) are preserved across species and between the three different kindlin isoforms. To investigate whether the F1 loop is also important for kindlin-2-mediated integrin activation, we assessed the effect of deleting the polylysine motif in the kindlin-2 F1 loop on α IIb β 3 integrin activation. The α IIb β 3 integrin activation indices (Fig. 6a) indicate that, whereas wild type kindlin-2 cooperates with the talin head to activate α IIb β 3, the kindlin-2 F1 Δ lysine mutant is unable to do so in the presence of the coexpressed GFP-talin head. Importantly, the Δ lysine mutation had no effect on kindlin-2 binding to integrin β tails (Fig. 6, b and c), which is required for integrin activation (51, 52). Therefore, our data clearly demonstrate that the lipid-interacting lysine motif in the F1 loop is required for both kindlin-1 and -2-mediated α IIb β 3 integrin activation, although the F1 loop plays no direct role in binding of kindlins to β -integrin tails.

The Membrane-binding F1-loops of Kindlin-1 and Talin Are Not Functionally Equivalent—The preceding data show that like talin, the kindlin F1 loop contains an unstructured loop capable of binding lipid head groups and important for integrin activation. However, the talin and kindlin loops bind membranes in a different manner; talin via a region with helical propensity near the middle of the loop and kindlin via a polybasic stretch at the N terminus of the loop. To determine whether the kindlin loop could substitute for the talin loop we generated a chimeric talin head in which the talin F1 loop (amino acids 139–168) was replaced with the kindlin-1 F1 loop (amino acids 145–244). Testing the resultant construct in integrin activation assays showed that the loop-swapped mutant activates α IIb β 3 integrin significantly less well than the wild type talin head (Fig. 7a). We also generated and tested the reciprocal chimera in which we inserted the talin F1 loop into kindlin-1. Again the resulting protein was impaired in its ability to activate integrins (Fig. 7b). These data show that whereas the F1 loops of talin and kindlin have important related functions they are not functionally interchangeable.

DISCUSSION

Kindlins directly bind integrin β subunit cytoplasmic domains, target focal adhesions in adherent cells, and play an important role in integrin activation (37, 44, 49–51, 58, 62). However, the mechanisms by which kindlins impact integrin activation remain largely unknown. A notable feature of kindlins is their similarity to the integrin-binding and activating talin head domain. Drawing on this similarity, we report here the identification of an unstructured insert in the kindlin F1 FERM domain, and provide evidence that a polylysine motif in this loop supports kindlin binding to negatively charged phospholipid head groups. We further show that the F1 loop and its membrane binding activity are required for kindlin-1 targeting to focal adhesions, and for the cooperation between kindlin-1 or kindlin-2 and the talin head in α IIb β 3 integrin activation, but

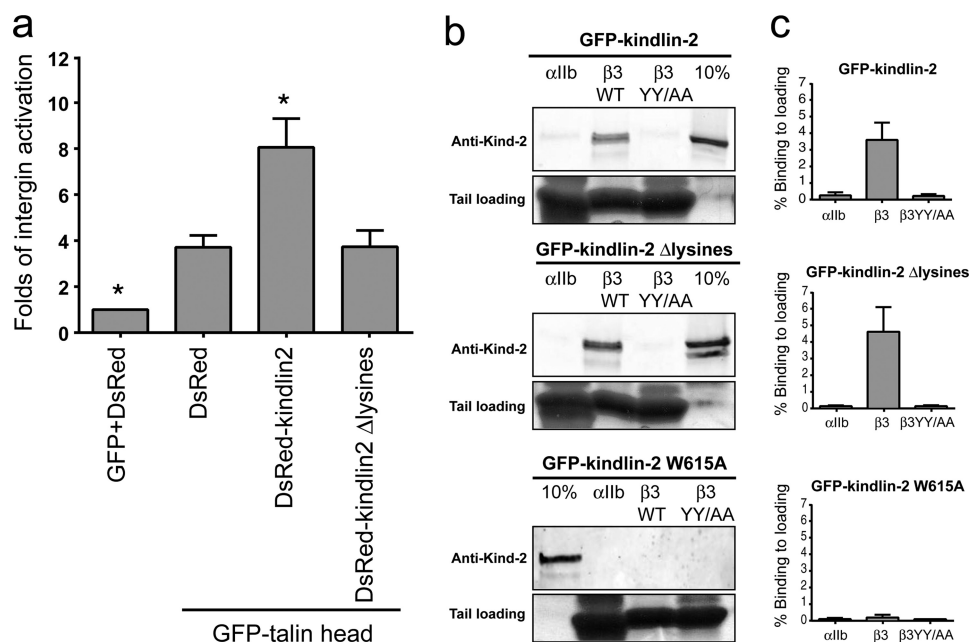


FIGURE 6. Kindlin-2-mediated α IIB β 3 integrin activation requires F1 loop lipid-binding polylysine motif. *a*, similar to Fig. 4*a*. CHO cells stably expressing α IIB β 3 integrin were co-transfected with GFP or GFP-tagged talin head (residues 1–433) and DsRed or DsRed-tagged kindlin-2 cDNAs as indicated. Activation indices of α IIB β 3 integrin from coexpressing cells with similar fluorescence of GFP and DsRed tags were calculated and normalized for integrin expression (see “Experimental Procedures”). The results represent the mean \pm S.E. ($n \geq 3$). Results that are significantly different from DsRed + GFP-talin head (t test $p < 0.05$) are indicated (*). *b*, similar to Fig. 4*b*. Pull-down assays using wild type and mutant recombinant α IIB or β 3 tail proteins were performed with CHO cell lysates overexpressing GFP-kindlin-2 wild type or F1 loop mutants proteins. Binding of kindlin constructs was assessed by Western blotting. Loading of each tail protein was judged by protein staining. Lysate represents 10% of the starting material in the binding assay.

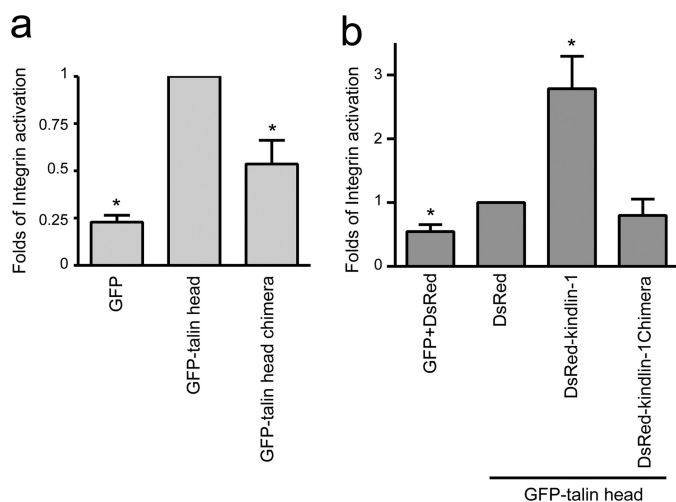


FIGURE 7. Swapping the F1 loops between talin head and kindlin-1 impairs their ability to activate α IIB β 3 integrin. *a*, GFP empty vector, GFP-talin head (residues 1–433) or chimeric GFP-talin head (kindlin-1 loop) cDNAs were transfected into CHO cells stably expressing α IIB β 3 integrin. Activation indices of α IIB β 3 integrin from expressing cells with similar GFP fluorescence were calculated and normalized for integrin expression (see “Experimental Procedures”). The results represent the mean \pm S.E. ($n \geq 3$). Results that are significantly different from the GFP-talin head (t test $p < 0.05$) are indicated (*). *b*, similar to Fig. 4*a*, CHO cells stably expressing the α IIB β 3 integrin were co-transfected with GFP or GFP-talin head (residues 1–433) and DsRed, DsRed-kindlin-1, or chimeric DsRed-kindlin-1 (talin loop) cDNAs as indicated. Activation indices of α IIB β 3 integrin from coexpressing cells with similar fluorescence of GFP and DsRed tags were calculated and normalized for integrin expression (see “Experimental Procedures”). The results represent the mean \pm S.E. ($n \geq 3$). Results that are significantly different from DsRed + GFP-talin head (t test $p < 0.05$) are indicated (*).

not for kindlin binding to integrin β tails. These studies highlight the structural and functional similarities between the talin head and kindlins (23, 24) and, together with reports revealing a

role for the kindlin F0 and PH domains in α IIB β 3 activation (38, 51, 63), indicate that multiple lipid interactions contribute to the ability of kindlins to activate integrins.

We, and others, have shown that kindlins bind directly to the membrane-distal NXXY motif in integrin β -tails via their F3 domain and that this is required for kindlins to enhance talin-mediated integrin activation (32, 44, 49–52). However, additional sites in kindlin are also involved as deletion of N-terminal kindlin domains ablates the ability of kindlin to cooperate with talin during α IIB β 3 activation (38, 51, 63). N-terminal domains in the talin head also contribute to talin-mediated integrin activation (36) and we recently showed the importance of a membrane-binding loop within the talin F1 domain for integrin activation (24).

Alignments of kindlin and talin sequences led us to identify an insert interrupting the kindlin F1 domain at the same position as the loop in talin F1 domain (Fig. 1*b* and supplemental Fig. S2). The kindlin F1 insert sequence is much longer than the talin F1 loop and there is little or no sequence similarity between the talin and kindlin loops. However, like the talin F1 loop, the kindlin-1 insert is disordered (Fig. 1*b* and supplemental Fig. S2) as confirmed by the poor signal dispersion of its ^1H - ^{15}N heteronuclear single quantum coherence spectrum (38). Moreover, the unstructured kindlin-1 F1 loop, like the talin F1 loop, is well conserved between species and among isoforms indicating a potentially important role in kindlin function. We found that kindlin-1 and -2 lacking the F1 loop are unable to cooperate with talin head in α IIB β 3 integrin activation. This effect is not due to an inability to bind integrins as kindlin-1 and -2 lacking the F1 loop retained their ability to bind to purified recombinant β 3 cytoplasmic tails. Thus, as in

Lipid-binding Loop in the Kindlin FERM F1 Domain

talin, an extended disordered loop in the kindlin F1 domain is important for integrin activation.

In addition to protein-protein interactions, classical FERM domains mediate protein-lipid interaction through a positively charged groove at the F1-F3 interface (22). The extended talin FERM domain, despite its atypical structure, also binds to lipid membranes, but by means of lysine and arginine residues in the F1 loop, F2 and F3 domains that are distributed along one face of the FERM domain (23, 24, 29, 31). Disruption of any one of these membrane-targeting sites markedly impaired talin-mediated integrin activation (24, 29, 31). In this study, we showed that the kindlin-1 F1 loop also binds to phosphatidylserine containing vesicles and, more interestingly, that this binding is mediated through a highly conserved polylysine motif present at the start of the predicted F1 loop. Our data suggest that binding is due to the interaction with negatively charged lipid head groups, as is the case for talin (24). Deletion or mutation of the polylysine motif prevents loop binding to membranes, and in the context of the intact protein blocks the ability of kindlin-1 or kindlin-2 to potentiate talin head-mediated integrin activation suggesting that membrane targeting of kindlins is an important step in the activation process.

Like talin, kindlin has several membrane binding sites. A PH domain-mediated kindlin-2-lipid interaction was previously implicated in integrin activation and while our work was in revision additional papers confirmed the importance of membrane binding via the PH and F0 domains for integrin activation (63, 64). Our data extend this to show that additional membrane-targeting sites are also required, and specifically that a polylysine motif in the F1 loop is needed. Thus, as is the case in talin, the effect of kindlin on integrin activation may require multiple interactions with the membrane. The important role for the polylysine motif is conserved in mammalian kindlin-1 and kindlin-2. The polylysine motif is also present in kindlin-3, but as kindlin-3 does not target to focal adhesions or trigger α IIB β 3 activation in CHO cells, or most other non-hematopoietic cells (58), we were unable to test its functional significance. Nonetheless, the high degree of sequence conservation of the polylysine motif suggests that it will play a similar essential function in all vertebrate kindlins. The polylysine motif is less rigorously conserved in invertebrates but stretches of lysines or arginines are still evident at the start of the F1 loop in invertebrate kindlins, suggesting that membrane binding by the F1 loop will be conserved across most kindlins.

The role of both the talin and kindlin F1 loops in membrane targeting and integrin activation shows the structural and functional similarities between these proteins. However, the sequences of the talin and kindlin loops and their modes of membrane binding are different. The central part of the talin F1 loop has propensity to form an α -helix with positively charged amino acids arranged on one face. This helical structure is stabilized by interaction with negatively charged lipid head groups (24). The F1 loop in kindlin is longer than that of talin and NMR analysis revealed no helical propensity in the kindlin-1 F1 loop, instead membrane binding is mediated by a stretch of lysines at the N terminus of the loop. The location of the membrane-binding site close to the start of the loop suggests that in kindlin, the loop may not function in exactly the same way as the talin

loop. Indeed when we swap the F1 loops between kindlin-1 and talin we find that both kindlin and talin are impaired in their abilities to activate α IIB β 3 integrins, such that each loop swapped chimera behaves similarly to constructs lacking the loop (Fig. 2*a*) (24). Thus despite similarities there are important differences between kindlin and talin. This is reinforced by observations that although the F1-loop of kindlins is required for their ability to activate integrins, this is not the case for talin as F3 alone, or talin head lacking the loop, can activate α IIB β 3 albeit less well than the intact talin head (24, 36).

Our data clearly demonstrate that the kindlin F1 lipid-binding polylysine motif is not only required for α IIB β 3 activation but also for kindlin localization to focal adhesions in adherent α IIB β 3-expressing CHO cells (Figs. 4–6). Deletion or point mutation of the KKKKKK motif prevents kindlin-1 targeting to focal adhesion, yet, neither of these mutations substantially affect the ability of kindlin to bind the β 3 tail distal NITY motif. We note that in all our assays the CHO cells express endogenous kindlin-2 and it is therefore possible that some of the impaired targeting in F1 loop mutants reflects an inability to effectively compete with endogenous protein. Nonetheless, this still reinforces our conclusion that the F1 loop plays important roles in kindlin targeting and activation.

In light of previously published data, our study reveals that the kindlin F1 loop mediates a protein-lipid interaction previously unknown for kindlin. This interaction between a polylysine motif within the loop and acidic phospholipid-rich lipid membranes has relevance for kindlin functions in the cell. We hypothesize that interactions between the kindlin-F1 loop, F0, and the PH domains may provide stronger membrane tethering and/or it may orientate kindlin at the membrane and stabilize its interaction with the β 3 tail thus facilitating α IIB β 3 integrin activation. Disabling any of these protein-lipid interactions may lead to unstable or inefficient membrane-kindlin- β 3 tail interactions, which fail to sustain talin head-mediated activation. Interestingly, kindlin targeting to focal adhesions requires the kindlin F1 loop and the polylysine motif but apparently does not require a PH domain-lipid membrane interaction (65). Therefore, different lipid-interacting portions of kindlin may serve distinct functions, providing through their lipid-binding selectivity, another layer of regulation of this protein family.

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