



Integrity of kindlin-2 FERM subdomains is required for supporting integrin activation

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ABSTRACT

Kindlin family members are essential for supporting integrin activation by functionally cooperating with the talin head domain. Both the talin head and kindlin are FERM domain-containing proteins that can simultaneously interact with the integrin β cytoplasmic tails. While the talin head is well studied, the molecular basis of kindlin's interaction with integrin during integrin activation is still poorly understood. Here we defined the subdomain boundaries in kindlin-2 and evaluated their contribution to integrin activation and recognition. We found that each subdomain in kindlin-2 was required for co-activating the integrin α IIb β 3 together with the talin head (inside-out signaling) and for enhancing integrin α IIb β 3-mediated cell spreading (outside-in signaling). To evaluate the involvement of the kindlin-2 subdomains in integrin interaction, we developed a FACS-based binding assay and found that an intact FERM domain in kindlin-2 was required for the interaction. Taking all together, these findings suggest that the integrity of kindlin-2 subdomains is a prerequisite for supporting integrin recognition and for subsequent integrin activation.

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1. Introduction

Integrin activation is triggered by intracellular integrin activators [1–4]. Integrin activators can directly interact with the integrin cytoplasmic tails (CT) and control the conformational changes of integrin extracellular domains [5–7]. Among multiple integrin CT binding proteins, the talin head domain was first identified as an integrin activator [8]. Mechanistically, the talin head interacts with the membrane-proximal residues in the integrin β CT, thus unclasp the integrin α/β CT complex, and leads to integrin activation [5,9–13]. Recently, multiple lines of evidence have demonstrated that kindlin family members are also essentially required to co-activate integrin together with the talin head, however, the molecular basis of their involvement in integrin activation remains poorly understood [3,14,15].

The kindlin family contains three members in mammals, kindlin-1, -2 and -3. Kindlin-2 was first identified as a mitogen-induced-gene in human fibroblasts and also named Mig-2 [16]. Compared to a restricted expression in epithelial cells for kind-

lin-1 and in hematopoietic cells for kindlin-3, kindlin-2 is ubiquitously expressed [17–19]. Dysfunction of kindlin-1 or kindlin-3 in humans respectively results in Kindler syndrome [20] or type-III leukocyte adhesion deficiency [21,22]. Patients associated with kindlin-2 deficiency have not yet been identified but knocking out kindlin-2 in mice led to embryonic lethality [23,24], suggesting that the role of kindlin-2 might be comprehensive in vivo. Mechanistically, kindlin family members can directly bind to the membrane-distal NxxY motif in the integrin β CT and co-activate integrin together with the talin head [24–27].

Both the talin head and kindlin belong to the FERM protein family which contain three structure modules (F1, F2 and F3) as well as an N-terminal F0 subdomain [24,26–30]. Compared to talin, the kindlin FERM domain has additional insertions, a PH domain in the middle of the F2 subdomain and a large variable region (Fv) in the F1 subdomain. In this study, we delineated the kindlin-2 subdomains and found that integrity of these subdomains was required for supporting integrin recognition and activation.

2. Materials and methods

2.1. Construct preparation and transfection

The head domain (1–429) of mouse talin1 was cloned into DsRed vector. Kindlin-2 was cloned into EGFP-C vector. All the

Abbreviations: CT, cytoplasmic tail; FERM, F for 4.1 protein, E for ezrin, R for radixin and M for moesin; PH, pleckstrin homology; FACS, flow cytometry; MFI, mean fluorescence intensity.

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kindlin-2 mutants were generated using a site-directed mutagenesis kit and verified by sequencing. The transfection was done using Lipofectamine 2000.

2.2. Flow cytometry (FACS)-based binding assay

Kindlin-2 and its mutants constructed in pEGFP vector were transiently transfected into CHO cells individually. To enrich EGFP-kindlin-2 molecules, the transfected cells were collected and their lysates were prepared to incubate with the protein-G conjugated micro-particles pre-loaded with anti-GFP antibody (Sigma). The particles then were washed with a saline buffer with 1 M NaCl followed by regular PBS. The EGFP-kindlin-2 molecules coupled on the particles were evaluated by Western blots and flow cytometry. The particles for each sample were split into two equal aliquots: one aliquot was used to incubate with biotinylated $\beta 3$ CT peptides followed by Alexa Fluor 633 conjugated streptavidin, and the other aliquot was used to incubate only with Alexa Fluor 633 conjugated streptavidin as background. After that, the particles were washed and the loading of EGFP fusions and the binding of Alexa Fluor 633 were analyzed on a BD LSR II Flow Cytometer. The peptide binding specifically to kindlin-2 on the particles was calculated with mean fluorescence intensity (MFI) of stained Alexa Fluor 633 conjugate. The MFI of stained Alexa Fluor 633 in the absence of biotinylated $\beta 3$ CT peptides was designated as MFI_a; the MFI of stained Alexa Fluor 633 in the presence of biotinylated $\beta 3$ CT peptides was designated as MFI_b. The binding index was defined as the ratio of MFI_b:MFI_a.

2.3. Integrin α IIb β 3 activation assay

Integrin activation was tested using the PAC-1 binding assay as we previously described [31]. Briefly, CHO- α IIb β 3 cells were transiently co-transfected with DsRed-talin head and EGFP-kindlin-2. The transfected cells were cultured for 24 h and then detached and stained with PAC-1 antibody followed by AlexaFluor 633-conjugated anti-mouse IgM secondary antibody. The cells double positive for DsRed and EGFP were gated on a LSR II Flow Cytometer and PAC-1 binding was evaluated by mean fluorescence intensity (MFI) of AlexaFluor 633. The MFI of PAC-1 binding to the cells only expressing DsRed and EGFP vectors was defined as 1, by which all other MFI values were normalized.

2.4. Cell spreading assay

CHO- α IIb β 3 cells were transiently transfected with EGFP vector, EGFP-kindlin-2 or its mutant constructs. 24 h after transfection, the cells were detached and used to incubate with the immobilized fibrinogen on chamber slides (Lab-Tek™ II) for 2 h at 37 °C. The non-adherent cells were washed away and the adhered cells were fixed with 4% paraformaldehyde for 10 min at 22 °C. The spreading of EGFP-positive cells was recorded by randomly selecting 8–12 fields using a fluorescence microscope and cell spreading areas were quantified using an imaging analysis system (MetaMorph).

3. Results

3.1. Define the subdomain boundaries in kindlin-2

Kindlin members share a similar subdomain arrangement [2,32,33]. Each one contains a FERM domain composed of F1, F2 and F3 subdomains which is preceded by an N-terminal F0 subdomain. A special feature in kindlin members is a PH domain insertion in the F2 subdomain. In addition, there is a variable fragment (Fv) (~100 amino-acid) in the F1 subdomain. The PH do-

main [34–36] and the F0 subdomain [37,38] in kindlin have been structurally solved. The kindlin-F0 subdomain adopts an ubiquitin-like fold, and the kindlin-PH insertion in the F2 subdomain constitutes an open phospholipid binding pocket. To further understand the molecular basis of kindlin-2 in regulating integrin function, we predicted and defined each subdomain in kindlin-2 using the I-tasser sever (<http://www.biomedcentral.com/1471-2105/9/40>), which was ranked the best in the community-wide Critical Assessment of Structure Prediction [39]. In order to obtain a more accurate prediction, we segmented kindlin-2 into three fragments, the well-defined PH domain, the N- and the C-terminal regions. The predicated structural information of these three fragments were integrated and revised based on the available kindlin structures. Not surprisingly, the determined subdomain boundaries in kindlin-2 were in close agreement with the predictions in the literature [37] (Fig. 1A). Based on this, we further generated a series of kindlin-2 deletion mutants cloned into an EGFP vector. As shown in Fig. 1B, all these kindlin-2 mutants were expressed in the transfected cells detected by an anti-EGFP antibody.

3.2. Integrity of kindlin-2 subdomains is prerequisite for supporting integrin α IIb β 3 activation

Kindlin members can co-activate integrin by cooperation with talin head (inside-out signaling). To evaluate the contribution of each kindlin-2 subdomain to integrin activation, we employed a well-established integrin co-activation assay [26,27]. In this assay, talin head in a DsRed vector and kindlin-2 in an EGFP vector were co-transfected into CHO- α IIb β 3 cells and integrin α IIb β 3 activation was evaluated by staining the cells with the PAC-1 antibody, which specifically recognizes the active α IIb β 3 conformation. PAC-1 binding was calculated only on the gated cells double positive for both EGFP and DsRed (Fig. 2A). As shown in Fig. 2B, co-expression of DsRed-talin head and EGFP-kindlin-2 in CHO- α IIb β 3 cells significantly increased PAC-1 binding compared to the expression of DsRed and EGFP tags (Fig. 2B). Partial PAC-1 binding was detected on the cells expressing DsRed-talin head and EGFP without kindlin-2, verifying that kindlin-2 acts as an integrin co-activator. Next, we tested the effects of kindlin-2 subdomains on integrin α IIb β 3 activation using all the kindlin-2 mutants (as shown in Fig. 1) and found that deleting each of the subdomains in kindlin-2, except the PH domain, totally blunted their co-activation ability (Fig. 2C). Although deleting the PH domain still supported integrin

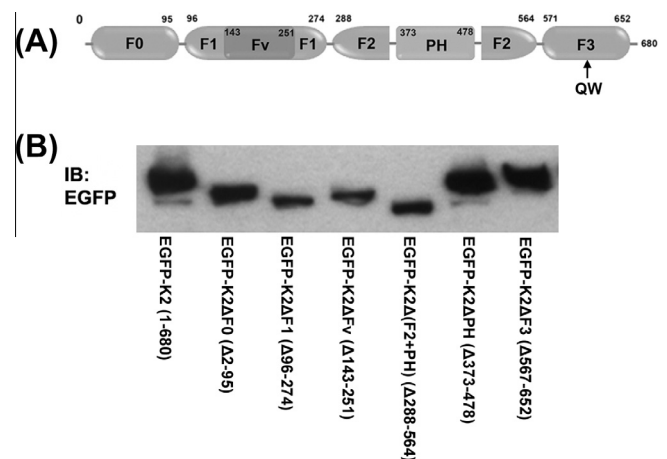


Fig. 1. Structure prediction of kindlin-2 subdomain boundaries and generation of kindlin-2 deletion mutants. (A) The boundaries of kindlin-2 subdomains. (B) EGFP-kindlin-2 mutants with defined subdomain deletions were generated by site-directed mutagenesis and their expression in transfected CHO cells were measured by Western blots using an anti-GFP antibody.

co-activation but only partial efficacy remained, which is consistent with our previous observation [26]. Taken together, these results suggest that integrity of the kindlin-2 subdomains, especially the FERM subdomain, is prerequisite for co-activating integrin together with talin head.

3.3. Integrity of the kindlin-2 subdomains is essential for supporting integrin α IIb β 3-mediated cell spreading

Kindlin-2 supports integrin-mediated cell spreading (outside-in signaling) [26,29]. To test how each of the kindlin-2 subdomains influences integrin outside-in signaling, we expressed the EGFP-kindlin-2 mutants in CHO- α IIb β 3 cells and individually evaluated their effects on α IIb β 3-mediated cell spreading. As shown in Fig. 3(A and B), compared to the EGFP control, EGFP-kindlin-2 significantly enhanced cell spreading on immobilized fibrinogen, demonstrating that kindlin-2 supports integrin α IIb β 3 outside-in

signaling. Surprisingly, expression of the kindlin-2 mutant with deletion of the F0, F1, Fv, or F2 + PH subdomain substantially inhibited cell spreading. However, a kindlin-2 mutant with deletion of the F3 subdomain failed to affect cell spreading. Considering that the F3 subdomain in kindlin-2 is involved in integrin interaction [25–27], we assumed that the F3-containing kindlin-2 fragments might function as dominant negatives that could possibly block integrin β 3 CT binding to the endogenous kindlin-2 in CHO- α IIb β 3 cells [26,29]. To investigate this idea, substitutions of Q614W615 to AA were introduced into EGFP-kindlin-2 and these substitutions localized in the F3 subdomain have been demonstrated to be able to block integrin interaction [25–27] (Fig. 1A). As expected, this kindlin-2 mutant failed to influence cell spreading (Fig. 3), demonstrating that the direct interaction of kindlin-2 with integrin is required for supporting β 3-integrin mediated cell spreading. Obviously, the F3 subdomain alone in kindlin-2 is incapable of

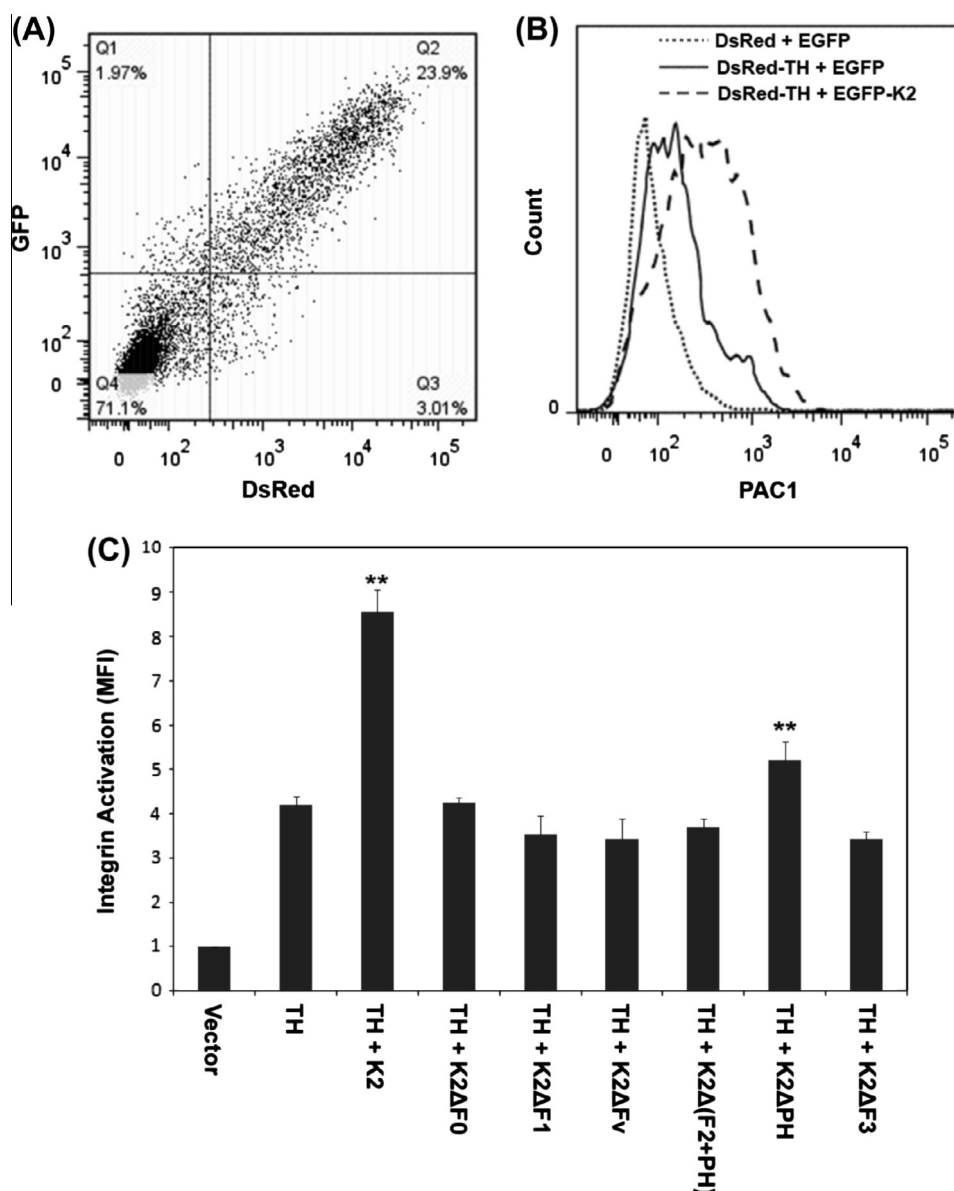


Fig. 2. Kindlin-2 subdomains contribute to co-activate integrin α IIb β 3. PAC-1 binding assay was performed on CHO- α IIb β 3 cells transiently expressing DsRed-fused talin head (TH) and EGFP-fused kindlin-2 (K2). (A) A representative FACS dot plot presents the cell population double positive for DsRed and EGFP in the right upper quadrant. (B) FACS histograms display the PAC1 binding to CHO- α IIb β 3 cells double positive for DsRed and EGFP. (C) Integrin activation was quantified and the results were presented with normalized mean fluorescence intensity (MFI). Results are means \pm S.D. ($n = 3$); ** $P < 0.01$ (versus TH).

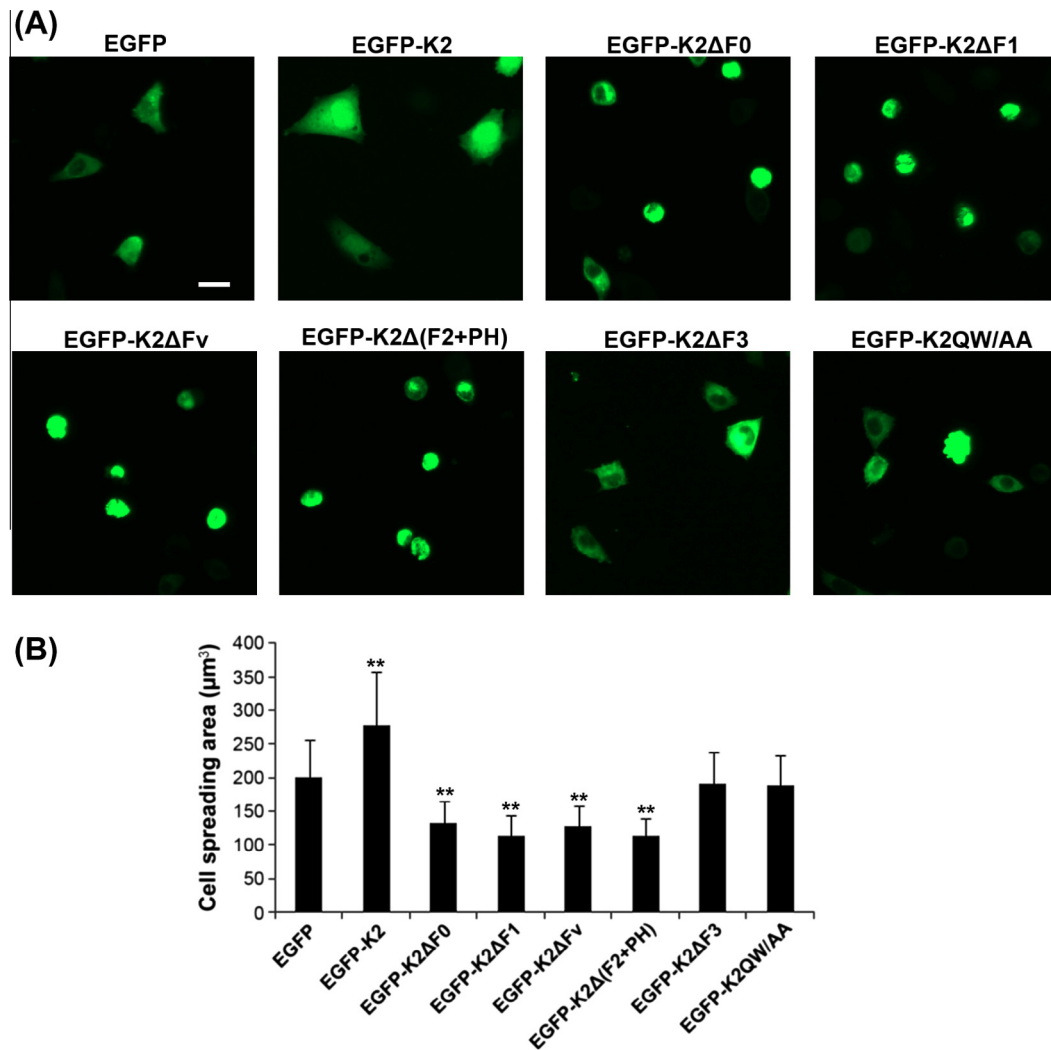


Fig. 3. Kindlin-2 enhances integrin α IIb β 3-mediated cell spreading. (A) CHO- α IIb β 3 cells transiently expressing EGFP or EGFP-fused kindlin-2 were used to adhere to immobilized fibrinogen for 2 h. The non-adherent cells were washed away and the adherent cells were fixed. The EGFP-positive cells were recorded using a fluorescence microscope. (B) Cell spreading areas were quantified using an imaging analysis software (MetaMorph); ** $P < 0.01$ (versus EGFP).

enhancing cell spreading, and integrity of the kindlin-2 subdomains is pivotal to support integrin outside-in signaling.

3.4. Integrity of kindlin-2 FERM domain is required for supporting β 3-integrin recognition

To further evaluate the involvement of kindlin-2 subdomains in interaction with the integrin β 3 CT, we developed a FACS-based binding assay, in which the protein-G coupled micro-particles were employed for FACS analysis (Fig. 4A). EGFP or EGFP-fused kindlin-2 molecules were expressed in CHO cells by transient transfection and enriched onto the Protein-G coupled particles by an anti-GFP antibody from the cell lysates. The integrin β 3 CT peptides were biotinylated and their interaction with the kindlin-2 molecules on the particles were monitored by Alexa Fluor 633 conjugated streptavidin. As shown in Fig. 4, the β 3 CT peptides significantly bound to EGFP-kindlin-2 (Fig. 4E, F and H) but not to EGFP control (Fig. 4B–D), indicative of the binding specificity. As another control, the kindlin-2 QW/AA mutant failed to mediate the β 3 CT peptide binding (Fig. 4E–H), confirming the critical involvement of the F3 subdomain in kindlin-2 in interaction with integrin. We took advantage of the expression of all the kindlin-2 deletion mutants in CHO cells (Fig. 1B) and enriched them from cell lysates and tested their binding ability to the integrin β 3 CT peptides using this

FACS-based binding assay. As shown in Fig. 4I and supplemental Fig. 1, deleting each of the subdomains in kindlin-2, except the PH domain, silenced the interaction of kindlin-2 with the β 3 CT peptides. However, kindlin-2 without the PH domain still kept the binding ability to the integrin β 3 CT the same as full length kindlin-2. These findings suggest that integrity of the FERM subdomains in kindlin-2 is obligatorily required for fully supporting integrin recognition, but the PH insertion in the FERM domain seems less likely to participate in the interaction.

4. Discussion

Kindlin family members play important roles in regulating integrin activity in cells. All three kindlin members contain a FERM domain composed of F0, F1, F2 and F3 subdomains. Interestingly, there are two additional insertions in the kindlin FERM domain, a PH domain in the F2 subdomain and a variable Fv region in the F1 subdomain. In our current study, we defined all the subdomain boundaries in kindlin-2 by combining the available structural information in the literature with the predicted structure results (Fig. 1A). To study the functional contribution of each subdomain in kindlin-2 to integrin activation, we generated all the EGFP-kindlin-2 deletion mutants in which the kindlin-2 subdomain was indi-

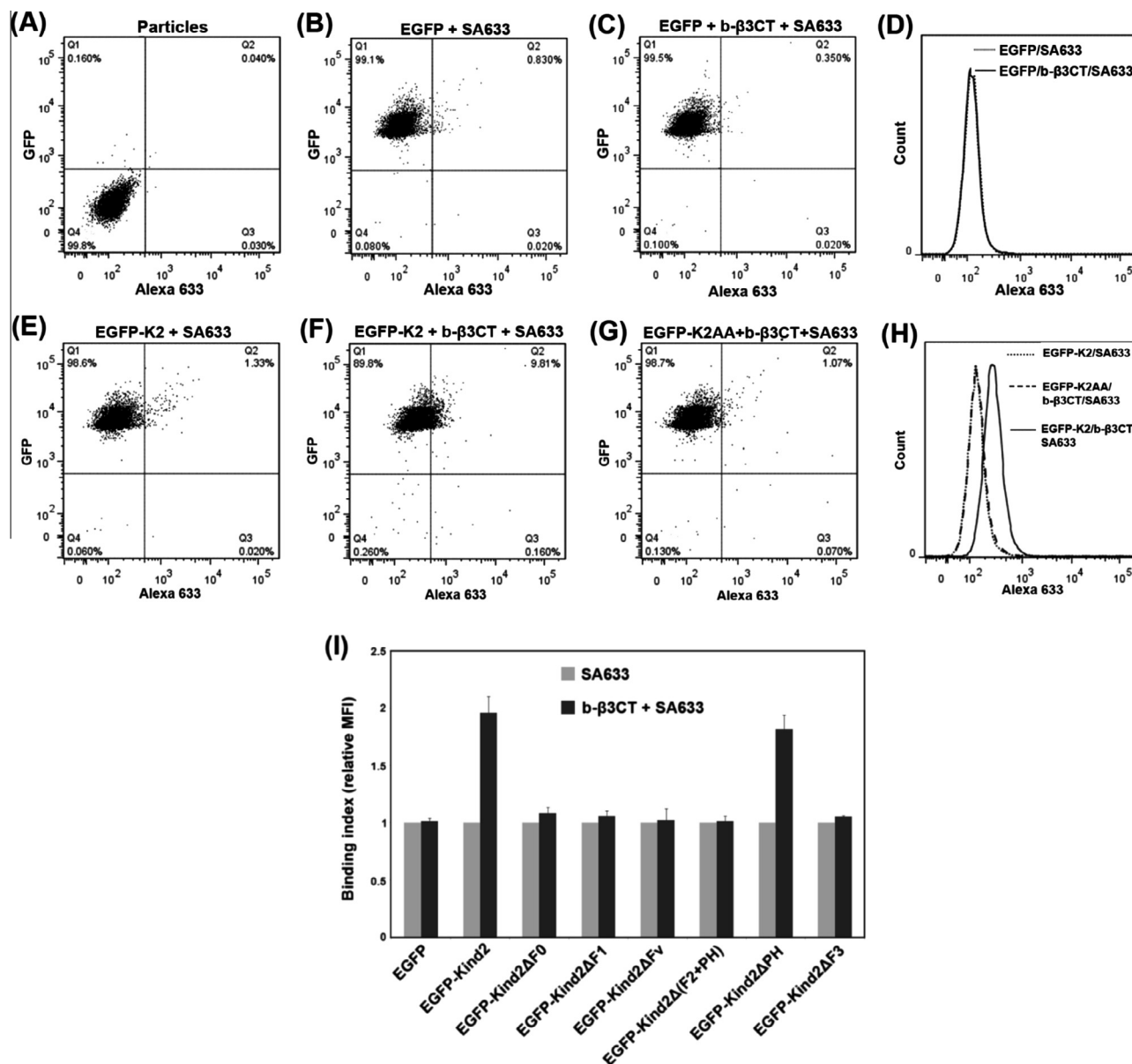


Fig. 4. The integrity of kindlin-2 FERM domain is required for fully supporting integrin recognition. Protein-G coated micro-particles (6–8 μ m) were used for flow cytometry analysis (A). These beads were first coupled with anti-GFP antibody and then incubated with the CHO cell lysates expressing EGFP (B and C), EGFP-kindlin-2 (EGFP-K2) (E and F) or EGFP-kindlin-2 QW/AA mutant (EGFP-K2AA) (G). After washing, the particles were incubated with biotinylated β 3 CT peptides (b- β 3CT) followed by Alexa Fluor 633 conjugated streptavidin (SA633) (C, F and G) or SA633 alone as controls (B and E). The bound SA-633 on the particles was displayed in histograms (D and H). (I) The binding of biotinylated β 3 CT peptide (b- β 3CT) to EGFP-fused kindlin-2 (EGFP-K2) mutants were measured and the binding index was expressed with the normalized mean fluorescence intensity (MFI). Results are means \pm S.D. ($n = 3$).

vidually deleted by site-directed mutagenesis. We presume that the structural integrity in these kindlin-2 mutants was preserved because of the following reasons: (a) only the minimal subdomain region was deleted in each mutant and the flanking linker regions were preserved (Fig. 1A); (b) all the kindlin-2 mutants were able to express in CHO cells after transfection (Fig. 1B); (c) the kindlin-2 deletion mutants were functional in cells as dominant negatives (Fig. 3). Here we believed that the well defined subdomain boundaries in kindlin-2 would ensure a more accurate measurement. Substantially, we observed that all the kindlin-2 subdomains essentially contributed to co-activate integrin α IIb β 3 (Fig. 2), which further solidifies the previous observation [26,27,34,38]. Moreover, we found that all the kindlin-2 subdomains were required for supporting integrin α IIb β 3-mediated cell spreading (Fig. 3). Based on these results, we conclude that integrity of the

kindlin-2 subdomains is prerequisite for kindlin-2 to support integrin bidirectional signaling.

To test the involvement the kindlin-2 subdomains in the interaction with integrin, we developed a FACS-based binding assay as described in the experimental procedures. We believe that this assay has several advantages: (a) kindlin-2 molecules expressed in mammalian cell (CHO) can be directly enriched on the particles; (b) the loading of EGFP-kindlin-2 molecules on the particles can be easily monitored by flow cytometry analysis; (c) critical washing condition can be used to avoid indirect interaction; (d) binding of integrin β 3 CT peptide to kindlin-2 can be quantified by flow cytometry analysis. Using this method, we found that all the kindlin-2 subdomains except the PH domain were essentially required for integrin interaction (Fig. 4). However, how these FERM subdomains in kindlin-2 cooperate with each other during the interac-

tion needs further investigation. Hypothetically, the F3 subdomain itself may present a binding site for integrin since the QW/AA mutations located in the F3 subdomain can abolish integrin interaction, but meanwhile the other FERM subdomains may provide additional structural support to stabilize the binding pocket. Although the PH domain does not participate in integrin interaction, the membrane-attaching ability must be a key mechanism to explain its functional contribution to integrin [34,40].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.03.086>.

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