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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 αIIbβ3 together with the talin head (inside-out signaling) and for enhancing integrin αIIbβ3-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.

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 extracelluar 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 unclasps 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 kindlin-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 EGPF-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 β3 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 LSRII 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 β3 CT peptides was designated as MFI_a; the MFI of stained Alexa Fluor 633 in the presence of biotinylated β3 CT peptides was designated as MFI_b. The binding index was defined as the ratio of MFI_b:MFI_a.

2.3. Integrin αIIbβ3 activation assay

Integrin activation was tested using the PAC-1 binding assay as we previously described [31]. Briefly, CHO-αIIbβ3 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 LSRII 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-αIIbβ3 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 domain [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 αIIbβ3 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-αIIbβ3 cells and integrin αIIbβ3 activation was evaluated by staining the cells with the PAC-1 antibody, which specifically recognizes the active αIIbβ3 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-αIIbβ3 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 αIIbβ3 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

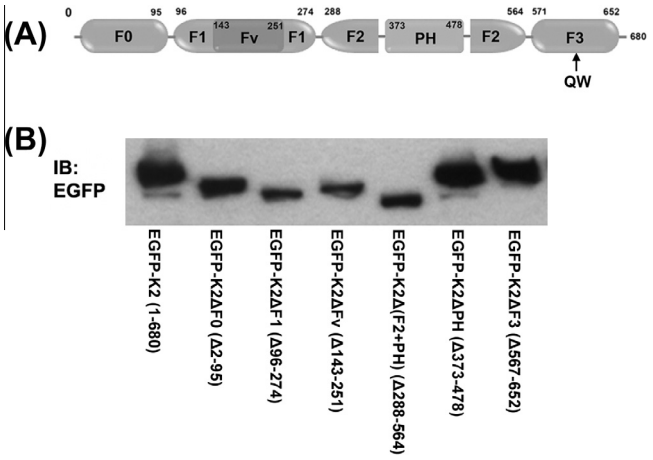

Fig. 1. Structure prediction of kindlin-2 subdomain boundaries and generation of kindlin-2 deletion mutants. (A) The boundaries of kindlin-2 subdomains. (B) EGFP-kindlin-2 mutants with defined subdomain deletions were generated by site-directed mutagenesis and their expression in transfected CHO cells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