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Split focal adhesion kinase for probing protein–protein interactions

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ABSTRACT

Since protein–protein interactions (PPIs) regulate a variety of cellular processes, the detection of PPIs is crucial for elucidating the underlying molecular mechanisms as well as developing therapeutics. In this study, we propose a novel system to detect PPIs using the distinct domains of focal adhesion kinase (FAK). In this system named "split FAK", the linker and kinase domains in native FAK are tethered separately to two target proteins of interest. The interaction between the target proteins brings the linker and kinase domains into proximity, which leads to phosphorylation at Y397 of the linker domain, recruitment of another tyrosine kinase Src, and phosphorylation at Y576 of the kinase domain. PPIs are readily detected by probing phosphorylation at Y397 and Y576 of these domains. To demonstrate this system, we designed a series of split FAK chimeras with different domain structures. Consequently, dimerizer-induced interaction between FK506-binding protein 12 (FKBP) and the T2098L mutant of FKBP12-rapamycin binding domain (FRB) was clearly detected by probing phosphorylation at the specific tyrosine residues of most of the split FAK chimeras. This is a novel PPI detection system based on a mechanism-inspired design of a trans-activated split kinase.

1. Introduction

Protein–protein interactions (PPIs) play pivotal roles in regulating a variety of cellular processes. The detection of PPIs and subsequent description of PPI networks are important for elucidating the underlying mechanisms of cellular processes [1–4], as well as for developing therapeutics based on the knowledge of PPIs [5–8]. Conventionally, intracellular PPIs have been analyzed by pull-down assay, in which proteins that interact with a target protein are collected on solid phase. While this assay with subsequent mass-spectrometric analyses has been recognized as a powerful tool to map the interactome of the target protein [9–12], it inherently prefers stable multiprotein complexes, and excludes transient interactions during complex purification by multiple washing steps [13,14]. Alternatively, investigators have developed genetic methods including two-hybrid systems in yeast [14–16] and mammalian cells [17–19], fluorescence and bioluminescence resonance energy transfer-based analyses [20–23], and a variety of protein fragment complementation assays [24–26]. These methods are designed to readily detect even transient binary PPIs based on reporter gene expression and/or fluorescence/bioluminescence. However, the detection is often hampered by significant background signals which lead to false positives [27–29]. Furthermore, the reporter activity is affected by relative orientation of reporter proteins in resonance energy transfer and protein fragment complementation assays, which leads to abundant false negatives [13,30,31]. As a consequence, several different methods are needed to validate the data of PPIs [13]. Thus, there remain unmet needs to address for reliable detection of PPIs.

In this study, we propose a novel method to detect PPIs using the distinct domains of focal adhesion kinase (FAK). FAK is a tyrosine kinase composed of three structurally characterized domains; FERM, kinase and FAT (Fig. 1A) [32–35]. The activity of the kinase domain (KD) is inhibited by interacting with FERM in an inactive state (Fig. 1B) [36–38]. Integrin activation forms focal adhesion by recruitment of focal adhesion proteins including FAK and phosphatidylinositol-4-phosphate 5-kinase type-1γ (PtdIns(4)P5K1γ). The lipid kinase PtdIns(4)P5K1γ converts PtdIns(4)P located at plasma membrane to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) which interacts with the FERM domain of FAK. Upon the interaction with PtdIns(4,5)P_2, FERM is displaced from KD, which releases the inhibitory state of KD. This leads to autophosphorylation at Y397 of the linker domain (LD) between FERM and KD, and subsequently, the phosphorylated Y397 recruits another tyrosine kinase Src that phosphorylates Y576 of KD for full activation [36]. In this activation mechanism, the phosphorylation at both Y397 and Y576 depends on the proximity of LD and KD. Based on this notion, here we developed "split FAK"

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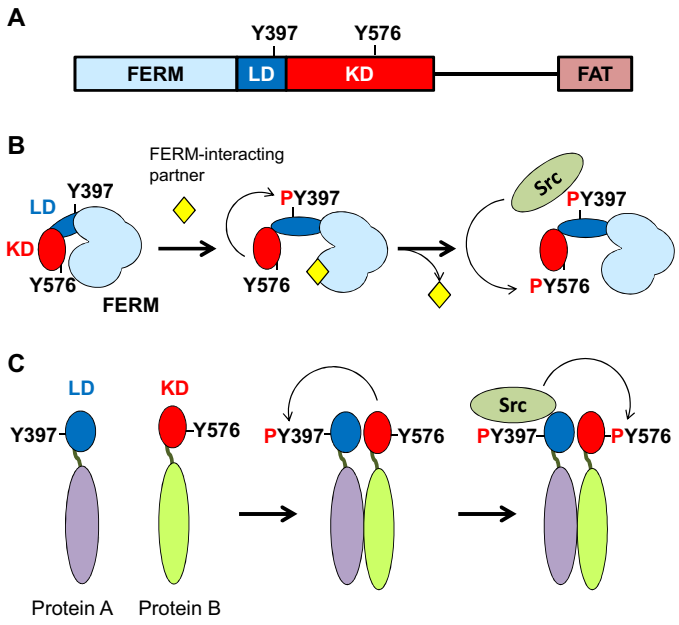

Fig. 1. Activation scheme of native and split FAKs. (A) Domain structure of native FAK. The linker domain (LD) with Y397 is flanked by the FERM domain and kinase domain (KD) with Y576. The FAT domain was located at C-terminus of FAK. (B) Activation scheme of native FAK. The activity of KD is inhibited by interacting with FERM in an inactive state. Upon binding of FERM-interacting partners such as proteins and phospholipids, FERM is displaced from KD, which leads to phosphorylation at Y397 of LD, recruitment of Src, and subsequent phosphorylation at Y576 of KD. (C) Activation scheme of split FAK. LD and KD are tethered separately to two target proteins. The interaction between the target proteins brings LD and KD into proximity, which leads to phosphorylation at Y397 of LD, recruitment of Src, and subsequent phosphorylation at Y576 of KD.

in which LD and KD are tethered separately to two target proteins (Fig. 1C). The interaction between the target proteins brings LD and KD into proximity, which leads to phosphorylation at Y397 of LD, recruitment of Src, and phosphorylation at Y576 of KD. Thus, we can readily detect the existence of PPIs by detecting phosphorylation of Y397 and Y576 of these domains. To demonstrate this system, we designed a series of split FAK chimeras with different domain structures, and used FK506-binding protein 12 (FKBP) and a mutant of FKBP12-rapamycin binding (FRB) as model interacting proteins.

2. Materials and methods

2.1. Plasmid construction

A plasmid pBC-SK + FAK encoding murine FAK was obtained from Kazusa DNA Research Institute (Kisarazu, Chiba, Japan). The gene encoding the linker domain containing FAK Y397 (LD) was amplified with PCR using two primers (FAK-linker-f, 5′-CCCTCTAGACAGAAAGAAGGTGAACGG-3′; FAK-linker-r, 5′-CGCGGATCCCTCGAGCCGGGCATGGTGTATGTGTCTTC-3′) and pBC-SK + FAK as the template. The amplified fragment (LD-PCR) was digested with *Xba*I and *Bam*HI, and subcloned into pC4-RHE (ARIAD Pharmaceuticals, Cambridge, MA) to obtain pC4-LD. pC4-LD was digested with *Eco*RI and *Xho*I, and subcloned into pMK-stuffer-$(G_4S)_3$-FKBP-flag-IG (manuscript in preparation) to attach a flexible $(G_4S)_3$ linker and Flag-tagged FKBP to the C-terminus of LD, resulting in pMK-LD-$(G_4S)_3$-FKBP-flag-IG. pMK-LD-$(G_4S)_3$-FKBP-flag-IG was digested with *Eco*RI and *Bam*HI and subcloned into pFB-neo (Agilent Technologies, La Jolla, CA) to create pFB-LD-$(G_4S)_3$-FKBP-flag-IN, which is designated as the expression plasmid for “A1” chimera.

The PCR-amplified fragment (LD-PCR) described above was also digested with *Xba*I and *BamH*I, and subcloned into pC4M-F2E (ARIAD Pharmaceuticals) having an N-terminal myristoylation signal sequence to obtain pC4M-LD. pC4M-LD was digested with *Eco*RI and *Xho*I, and subcloned into pMK-stuffer-$(G_4S)_3$-FKBP-flag-IG to attach a flexible $(G_4S)_3$ linker and Flag-tagged FKBP to the C-terminus of LD, resulting in pMK-myr-LD-$(G_4S)_3$-FKBP-flag-IG. pMK-myr-LD-$(G_4S)_3$-FKBP-flag-IG was digested with *Eco*RI and *Bam*HI, and subcloned into pFB-neo to create pFB-myr-LD-$(G_4S)_3$-FKBP-flag-IN, which is designated as the expression plasmid for “A2” chimera.

The gene encoding FERM and LD containing FAK Y397 was amplified with PCR using two primers (Linker-P3, 5′-CATGGCTTCTAGAATGGCAGCTGCTTATCTTGAC-3′; HA-D2-P4, 5′-TCCACCGCTCGAGCCGGGCATGGTGTATGTGTCTTCCTCATC-3′) and pBC-SK + FAK as the template. The amplified fragment was subcloned into the fragment of A1 which was amplified with PCR using two primers (linker-P1, 5′-TCTAGAAGCCATGGTGGCG-3′; HA-D2-P2, 5′-GGCTCGAGCGGTGGAG-3′) by utilizing the In-Fusion HD Cloning Kit (Clontech, Mountain View, CA) to obtain pFB-FERM-LD-$(G_4S)_3$-FKBP-flag-IN, which is designated as the expression plasmid for “A3” chimera.

The gene encoding FERM and LD containing FAK Y397 was amplified with PCR using two primers (HA-D2-P3, 5′-GCGCTGCTCCGTACGATGGCAGCTGCTTATCTTGACCCAAAC-3′; HA-D2-P4, 5′-TCCACCGCTCGAGCCGGGCATGGTGTATGTGTCTTCCTCATC-3′) and pBC-SK + FAK as the template. The amplified fragment was subcloned into the fragment of pFB-HA-EpoRD2TM-stuffer-$(G_4S)_3$-FKBP-flag-IN (manuscript in preparation) which was amplified with PCR using two primers (HA-D2-P1, 5′-CGTACGGAGCAGCGCGAG-3′; HA-D2-P2, 5′-GGCTCGAGCGGTGGAG-3′) by utilizing the In-Fusion HD Cloning Kit to obtain pFB-HA-EpoRD2TM-FERM-LD-$(G_4S)_3$-FKBP-flag-IN, which is designated as the expression plasmid for “A4” chimera.

The gene encoding the kinase domain (KD) of FAK was amplified with PCR using two primers (FAK-kinase-f, 5′-CCCTCTAGAAGCACCAGGGATTATGAGATTC-3′; FAK-kinase-r, 5′-CGCGGATCCCTCGAGCCCTGCACCTTCTCCTCCTC-3′) and pBC-SK + FAK as the template. The amplified fragment (KD-PCR) was digested with *Xba*I and *Bam*HI, and subcloned into pC4-RHE to obtain pC4-KD. pC4-KD was digested with *Eco*RI and *Xho*I, and subcloned into pMK-stuffer-$(G_4S)_3$-FRB-myc-IG (manuscript in preparation) to attach a flexible $(G_4S)_3$ linker and myc-tagged FRB_{T2098L} to the C-terminus of KD, resulting in pMK-KD-$(G_4S)_3$-FRB-myc-IG. pMK-KD-$(G_4S)_3$-FRB-myc-IG was digested with *Eco*RI and *Bam*HI, and subcloned into pMK-ΔL-stuffer-ΔTM-Kit-Flag-IP (manuscript in preparation) to create pMK-KD-$(G_4S)_3$-FRB-myc-IP, which is designated as the expression plasmid for “B1” chimera.

The PCR-amplified fragment (KD-PCR) described above was also digested with *Xba*I and *Bam*HI, and subcloned into pC4M-F2E to obtain pC4M-KD. PC4M-KD was digested with *Eco*RI and *Xho*I, and subcloned into pMK-stuffer-$(G_4S)_3$-FRB-myc-IG (manuscript in preparation) to attach a flexible $(G_4S)_3$ linker and myc-tagged FRB_{T2098L} to the C-terminus of the kinase domain, resulting in pMK-myr-KD-$(G_4S)_3$-FRB-myc-IG. pMK-myr-KD-$(G_4S)_3$-FRB-myc-IG was digested with *Eco*RI and *Bam*HI, and subcloned into pMK-ΔL-stuffer-ΔTM-Kit-Flag-IP to create pMK-myr-KD-$(G_4S)_3$-FRB-myc-IP, which is designated as the expression plasmid for “B2” chimera.

The gene encoding KD and FAT together with their intervening region was amplified with PCR using two primers (kinase-P3, 5′-CATGGCTTCTAGATCGACCAGGGATTATGAGATTCAGAGAG-3′; V5-D2-P4, 5′-CCGCCTCCACCGCTCGAGCCGTGTGGCCGTGTCTGCC-3′) and pBC-SK + FAK as the template. The amplified fragment was subcloned into the fragment of B1 which was amplified with PCR using two primers (Linker-P1, 5′-TCTAGAAGCCATGGTGGCG-3′;