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Kindlin-2 phosphorylation by Src at Y193 enhances Src activity and is involved in Migfilin recruitment to the focal adhesions

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ABSTRACT

Kindlin-2 regulates external to internal cell signaling by interaction with integrins in a process that involves the tyrosine kinase, Src. However, the underlying mechanisms remain elusive. Here we report that Src binds to and phosphorylates Kindlin-2 at Y193. Reciprocally, Kindlin-2-Y193 phosphorylation activates and maintains Src kinase activity. Kindlin-2-Y193 phosphorylation is also involved in its binding capacity with Migfilin and the recruitment of Migfilin to the focal adhesions. Functionally, we demonstrate that Kindlin-2-Y193 phosphorylation regulates Kindlin-2-mediated cell spreading and migration. These findings suggest that Src, Kindlin-2 and Migfilin together constitute a positive feedback loop that controls Src activity and regulates integrin-mediated cellular functions.

1. Introduction

Integrin-mediated cell to the extracellular matrix (ECM) adhesions provide connections between cell cytoskeleton and the ECM [1]. Besides acting as a mechanical link, cell–ECM adhesions also mediate communications between cells and microenvironment. Signaling pathways downstream of integrin, often referred to as integrin outside-in signaling, provide critical signals for cell proliferation, survival and migration [2]. Uncovering the precise mechanisms underlying integrin outside-in signaling will help to understand various cell behaviors under physiological and pathological conditions, such as cell migration, invasion, tumor growth and metastasis.

Src is an non-receptor tyrosine kinase and it was known to mediate signaling pathways downstream of various membrane receptors including integrins [3]. In Src-transformed cells, a plenty of downstream components of integrin signaling pathway were phosphorylated, such as focal adhesion kinase (FAK), p130CAS and paxillin [4,5]. These proteins were also found to associate with Src in vivo [3]. Through protein interactions Src is able to regulate integrin outside-in signaling pathway and affects cell migration [6,7], proliferation [6–8] and survival [9,10]. In addition, it was also known that Src itself can be activated by integrin signaling. Upon cell adhesion, FAK undergoes autophosphorylation at Y397, which promotes FAK-Src association. This in turn promotes Src activity through relieving Src from inhibitory intermolecular interactions [11–13].

Kindlin-2 has been recognized as an important integrin activator. Through interacting with the integrin cytoplasmic tails Kindlin-2 was known to cooperate with Talin and activates integrins [14–18], which is essential for integrin outside-in signaling pathway [16,19]. Kindlin-2 was recently found to be required for paxillin phosphorylation [19], an important event in integrin-mediated outside-in signaling [20–23]. Kindlin-2 was known to be required for the recruitment of Migfilin to the focal adhesions (FAs) [24,25], which modulates cell shape. Involving in both integrin-mediated inside-out (integrin activation) and outside-in signaling, Kindlin-2 is emerging as an important regulator of integrin functions. However, until now less is known about the mechanisms underlying Kindlin-2 regulation on integrin-mediated outside-in signaling. In addition to the regulation of the integrin-mediated signaling,

Abbreviations: WT, wild type; KD, kinase dead; CA, constitutively active; ECM, extracellular matrix

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Kindlin-2 was also reported to be involved in a variety of other signaling pathways, including Wnt/β-catenin [26], TGF-β [27] and SHH signaling pathways [28].

Migfilin, a binding partner of Kindlin-2 [24,25], has been reported to activate Src by direct interaction with Src SH3- and SH2 domains. Src is required for anoikis resistance in multiple cell types and knockdown of Migfilin promotes cell apoptosis through inhibition of Src activation [9]. It was reported that in untransformed epithelial cells, cell de-adhesion could induce degradation of Migfilin that led to reduction of Src activation. However, the mechanism regulating Migfilin-mediated Src activation at cell adhesive structures remained unclear. Most recently, Kindlin-2 was found to interact with Src to the SH3- and SH2 domains and was tyrosine phosphorylated in cells overexpressing Src [19]. Furthermore, which amino acid residue within Kindlin-2 was phosphorylated by Src and the role of phosphorylated Kindlin-2 remained completely unknown. In this report we present evidence showing that Kindlin-2 is phosphorylated by Src at Y193 and a Src-Kindlin-2-Migfilin cascade may play an important role in the regulation of Src activation and Kindlin-2-mediated cellular functions.

2. Materials and methods

2.1. Cell culture, antibodies, and other reagents

HeLa, MCF7 and 293T cells were cultured in DMEM (Invitrogen, USA) supplemented with 10% fetal calf serum (FCS) (Hyclone, USA). H1299 cells were grown in RPMI 1640 (Invitrogen, USA) supplemented with 10% FCS. Antibody specifically recognizing Y193 phosphorylated Kindlin-2 was produced by immunizing rabbits with phosphorylated peptide (KTMTPTpYDAHDGSP) (Kang Wei Shi Ji, Beijing, China). Anti-Src, anti-pSrc Y416 and pTyr-100 antibodies specifically recognizing phosphorylated tyrosine were purchased from Cell Signaling Technology, USA. Anti-Kindlin-2 monoclonal antibody was purchased from Millipore, USA. Src family kinase inhibitor PP2 was purchased from Sigma, USA.

2.2. DNA constructs, siRNAs and transfection

Expression vectors encoding Kindlin-2, Src and Migfilin were generated by inserting their cDNAs into corresponding vectors (pGEX-4T-1, pMal-c2, pEGFP-C3, and pCMV10-3×Flag). Kindlin-2 siRNA (AAGCUGGUGGAGAAACUCG) was obtained from Invitrogen, USA. Plasmids encoding HA-Src-wild type (WT) and HA-Src-KD (K298A) were kind gifts from Dr. Byung-Chul Oh (Chungbuk National University, Republic of Korea). DNA constructs were transfected into cells with Lipofactamine 2000 (Invitrogen, USA) and siRNAs were transfected with RNAimax (Invitrogen, USA) following the manufacturer's protocol.

2.3. Mutagenesis

Various mutations were introduced into DNA constructs encoding Kindlin-2 with QuikChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA) following the manufacturer's protocol. Mutations generated were confirmed by DNA sequencing.

2.4. Immunofluorescence staining

Cells were plated on collagen type I (Sigma, USA) coated cover slips and incubated at 37 °C overnight. After incubation cells were fixed with 4% paraformaldehyde in PBS. Then the fixed cells were permeabilized with 0.1% NP40. After being blocked with 5% BSA in PBS, cells were stained with indicated primary antibody at 4 °C overnight. Subsequently, the primary antibody was recognized by fluorescent conjugated secondary antibody. Then the immunofluorescence images were captured under a confocal laser-scanning microscope (Carl Zeiss LSM780, Germany).

2.5. Immunoprecipitation

Cells were collected and lysed in PBS containing 1% Triton X-100, 1 mM Na_3VO_4 and protease inhibitor cocktail (Roche, Germany) for 15 min on ice. Then the lysates were incubated with the indicated antibody (5–10 μg) overnight at 4 °C. Fifty μl protein A/G agarose beads were added and then the reaction mixtures were continued to incubate for 2 h at 4 °C. After washing with PBS containing 1% Triton X-100, 1 mM Na_3VO_4 for three times, the immunoprecipitates were analyzed by Western blot analysis.

2.6. Purification of GST fusion proteins and GST pull-down assay

GST or GST-Src constructs (Full-length, SH3 domain, SH2 domain and kinase domain) and MBP-Kindlin-2 were expressed in *Escherichia coli* BL21 (Tiangen Biotechnology, Beijing, China), and purified with Glutathione Sepharose 4B beads (Pharmacia Medtech, Piscataway, NJ, USA) or MBP-Affinity Matrix (Amylose Resin, New England Biolabs, USA) according to the protocols provided by the companies. In detail, the expressed GST or GST-fusion proteins were incubated with Glutathione Sepharose 4B beads by rotating at 4 °C for 2 h, and then the beads were washed with PBS containing 1% Triton X-100. Then the protein coated-beads were incubated with MBP-Kindlin-2 fusion protein or cell lysate in PBS containing 1% Triton X-100 and protease inhibitor cocktail (Roche, Germany) at 4 °C for 4 h. And the beads were dissolved into SDS loading buffer after centrifugation, and boiled for 5 min at 100 °C.

2.7. In vitro kinase assay

Flag-Kindlin-2 protein immunoprecipitated from 293T cells transiently expressing this protein was incubated with GST or GST-Src in kinase buffer containing 50 mM Tris–HCl (pH 7.8), 5 mM $MgCl_2$, 1.0 mM DTT, 5 mM ATP, and 150 mM NaCl at 37 °C for 30 min. Precipitates from 293T cells transfected with empty FLAG vector were incubated with GST-Src as a negative control. Then proteins were analyzed by Western blot with Src, pTyr100, pKindlin-2-Y193 and Kindlin-2 antibodies separately.

2.8. Cell spreading assay

Cover slips were coated with fibronectin (5 μg/ml, Sigma, USA) at 37 °C for 1 h and blocked with 1% heat-denatured BSA at 37 °C for 1 h. Approximately 8×10^4 cells were allowed to attach and spread for 30 min in cell adhesion buffer (RPMI 1640, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$ and 0.5% BSA). After washing with cell adhesion buffer, cells were photographed under microscope. The percentage of cells display spread morphology was determined by analyzing cells from five randomly selected fields.

2.9. Cell migration assay

Cell migration was performed by using Transwell chambers (Corning Costar Corp., Tewksbury, MA, USA) with 8.0 μm pore size. The lower surface of the chamber membranes were coated with fibronectin (5 μg/ml) at 37 °C for 1 h. Twenty-four hours post transfection, 1×10^4 H1299 cells suspended in cell adhesion buffer (RPMI 1640, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$ and 0.5% BSA) were added to the upper chamber and allowed to migrate for 5 h. Then the chamber membranes were fixed with 4% paraformaldehyde and stained with crystal violate. Five random