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Recruitment and activation of SLK at the leading edge of migrating cells requires Src family kinase activity and the LIM-only protein 4

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ABSTRACT

The Ste20-like kinase SLK plays a pivotal role in cell migration and focal adhesion turnover and is regulated by the LIM domain-binding proteins Ldb1 and Ldb2. These adapter proteins have been demonstrated to interact with LMO4 in the organization of transcriptional complexes. Therefore, we have assessed the ability of LMO4 to also interact and regulate SLK activity. Our data show that LMO4 can directly bind to SLK and activate its kinase activity in vitro and in vivo. LMO4 can be co-precipitated with SLK following the induction of cell migration by scratch wounding and Cre-mediated deletion of LMO4 in conditional LMO4$^{fl/fl}$ fibroblasts inhibits cell migration and SLK activation. Deletion of LMO4 impairs Ldb1 and SLK recruitment to the leading edge of migrating cells. Supporting this, Src/Yes/Fyn-deficient cells (SYF) expressing very low levels of LMO4 do not recruit SLK to the leading edge. Re-expression of wildtype Myc–LMO4 in SYF cells, but not a mutant version, restores SLK localization and kinase activity. Overall, our data suggest that activation of SLK by haptotactic signals requires its recruitment to the leading edge by LMO4 in a Src-dependent manner. Furthermore, this establishes a novel cytosolic role for the transcriptional co-activator LMO4.

1. Introduction

The vast majority of cancer-related deaths are due to the dissemination of cancer cells to secondary sites. Cell migration is essential to the metastatic process by which cancer cells have acquired the ability to invade surrounding tissues and migrate from the primary tumor to distant sites within the body [1–4]. The process of cell motility involves the assembly and turnover of focal adhesions regulated by multiple intracellular signals involving focal adhesion kinase (FAK) and c-Src. Overexpression, increased activity or activating mutations in c-Src and FAK have been found in various forms of cancer including breast [5], ovary [6], head and neck [7], and pancreas [8]. In addition, increased cell motility has been reported in various forms of cancer [9–12].

The Ste20-like kinase SLK is a 220 kDa serine/threonine kinase containing three functional domains [13]. The amino terminal kinase domain shares a high degree of homology with lymphocyte oriented kinase (LOK), mammalian sterile twenty kinase (MST1), and the *Xenopus* polo-like kinase kinase (xPlkk1). The central coiled coil domain contains a Src homology 3 (SH3) binding domain as well as a caspase 3 cleavage site. The function of the coiled coil region has yet to be elucidated. The carboxy terminus of SLK consists of an AT1–46 homology (ATH) domain and is also homologous to the carboxy terminus of LOK [13,14]. The ATH domain confers an autoinhibitory function and interacts directly with the LIM domain binding transcriptional cofactor proteins Ldb1/CLIM2 and Ldb2/CLIM1/NLI. This interaction negatively regulates the kinase activity of SLK [15,16]. SLK expression was first reported to be restricted to neurogenic and myogenic lineages in the early embryo, followed by ubiquitous expression in adult tissues and cell lines [13,17]. Recent work has shown that the lack of functional SLK protein is embryonic lethal between E12.5 and E14.5 with severe defects in patterning, organogenesis and placental development [18].

In addition to development, we have found that SLK plays a critical role in cell adhesion and migration [19–23]. We have shown that SLK is recruited to the leading edge of migrating cells and co-localizes with adhesion complex components such as the microtubules, Rac1 and Paxillin. SLK was found to promote focal adhesion disassembly and cell migration through the phosphorylation of Paxillin [23]. Activation of SLK kinase activity by migratory signals is dependent on the FAK/c–Src/MAPK signaling pathway [21,23]. However, the molecular mechanisms regulating SLK complex assembly, kinase activation and its sub-cellular localization during the process of cell migration remain to be elucidated.

Abbreviations: LMO4, LIM only protein 4; MAPK, mitogen-activated protein kinase; IP, immunoprecipitation; WCL, whole cell lysate; SYF, Src/Yes/Fyn-deficient

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The transcriptional co-regulator LIM-only protein 4 (LMO4) has been associated with poor prognosis and is overexpressed in about 60% of all human breast tumors and in over 60% of HER2-positive tumors [24,25]. The overexpression of LMO4 in murine mammary epithelium was found to induce hyperplasia and promote cell migration [24]. LMO4 is also required for ErbB2/HER2/Neu-induced cancer cell cycle progression through its regulation of cyclin D expression, downstream of PI3K [26]. While the majority of the effects of LMO4 may be caused by its transcriptional co-activator function, there is mounting evidence that LMO4 possesses cytosolic protein adapter roles in the context of migration and invasion [27,28]. Supporting this, increased cytosolic LMO4 staining in metastatic human breast tumor tissue has been presented [25,29]. Interestingly, its initial discovery was facilitated by its interaction with Ldb1 [30,31], a negative regulator of SLK activity [15]. As LMO4 interacts with Ldb1 and has been shown to also play a role in cell proliferation and motility, we investigated the possibility that SLK and LMO4 could interact. In this study, we present evidence that LMO4 binds directly to the SLK ATH domain and positively regulates SLK activity and cell migration. LMO4 is present at the leading edge of migrating cells and co-localizes with Ldb1 and adhesion proteins such as vinculin and Paxillin. Deletion of LMO4 impairs SLK recruitment to the leading edge and inhibits cell migration. Finally, c-Src deletion or inhibition impairs LMO4 expression and SLK recruitment to the leading edge. Together, these data suggest that LMO4 may contribute to the invasive potential of breast cancers through SLK and that SLK activity and localization are regulated by a potential oncogene.

2. Materials and methods

2.1. In vitro binding assays

In vitro binding assays were performed as previously described [15]. Bacteria expressing glutathione transferase (GST) fusion proteins were collected by centrifugation at 4 °C and resuspended in 500 μL RIPA lysis buffer including protease inhibitors (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 2 mM DTT, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 10 μg/mL aprotinin, 1 mM phenylmethylsulfonylfluoride and 100 μM benzamidine) and sonicated on ice. Supernatants were cleared by centrifugation; glutathione-sepharose beads (GE Healthcare, Mississauga, ON, CAN) were added and rotated for 2 h at 4 °C. Bound GST fusion proteins and beads were washed three times with 200 mM NETN buffer (20 mM Tris–HCl pH 8.0, 1 mM EDTA, 200 mM NaCl and 0.5% Nonidet P-40). In vitro translation products were obtained with the TNT quick-coupled in vitro transcription translation kit (Promega, Madison, WI) with ^{35}S-methionine (PerkinElmer, Waltham, MA) as per the manufacturer's instructions and incubated with either GST alone or GST-fusion proteins in 200 mM NETN buffer. Complexes were washed 3 times with 200 mM NETN, eluted from the beads by boiling in sample buffer and fractionated by SDS-PAGE. Proteins were visualized using Coomassie Brilliant Blue (Sigma-Aldrich, St. Louis, MO) staining. Gels were dried and subjected to autoradiography.

2.2. Cell culture, transfections and infections

Mouse embryonic fibroblast (MEF) 3T3, murine mammary carcinoma 4T1, human epithelial kidney (HEK) 293, SYF (Src/Fyn/Yes triple mutant), SYF + c-Src, and primary MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine (Invitrogen), and penicillin G (200 U/mL; Invitrogen) and streptomycin sulfate (200 μg/mL; Invitrogen) in a humidified 37 °C incubator at 5% CO_2. Primary MEFs from LMO4 floxed homozygous FVB/N female mice were established as previously described [18]. Immortalization of LMO4 floxed MEFs was achieved using a standard 3T3 spontaneous immortalization protocol.

Transient DNA transfections into SYF cells were performed as per the manufacturer's instructions using Lipofectamine/PLUS reagent (Invitrogen) with a total of 4 μg of plasmid DNA per each 10 cm plate. MEF3T3 cells were transfected with 150 nM siRNA (Dharmacon RNA Technologies, Lafayette, CO) duplex for LMO4 (target sequence: 5′-GCAAGUGAGCUCGUCAUGA-3′) or Dharmacon's non-targeting duplex as control using the Lipofectamine 2000 (Invitrogen) transfection reagent. Cells were incubated for 48 h at 37 °C and 5% CO_2 and assayed for cell migration by a wound closure assay and protein expression by Western blot analysis.

For Cre-mediated deletion of LMO4, LMO4 floxed MEFs were plated at a density of 7.5×10^5 cells on 10 cm plates or 1.875×10^5 cells in 60 mm 6-well dishes in 10% FBS DMEM and incubated overnight at 37 °C and 5% CO_2. The cells were then infected with an adenovirus expressing LacZ as a control (AdLacZ) or Cre recombinase (AdCre; both a generous gift of Dr. Robin Parks, OHRI, Ottawa, ON) at an MOI of 20 in serum-free medium for 90 min at 37 °C and 5% CO_2. The infection was followed by the addition of 10% FBS DMEM and the cells were incubated at 37 °C and 5% CO_2 for 48 h prior to analysis via Western blot, cell migration or immunofluorescence.

2.3. Plasmid constructs

DNA plasmids were constructed using standard molecular cloning techniques. The GST-tagged SLK constructs were generated as described previously [15]. The murine LMO4 cDNA was PCR amplified (N-ter: 5′-ATGGTGAATCCGGGC and C-ter: 5′-TCAGCAGACCTTCTG) and sub-cloned in frame into the pCAN–Myc expression plasmid. The point mutations in pCAN–Myc–LMO4 for the quadruple LIM1 domain mutant (R33G/F34Q/L35Y/Y37Q; Myc–LMO4 QUAD (32)) were generated according to Stratagene's (Agilent, Santa Clara, CA) 'QuikChange XL site-directed mutagenesis kit' using the Myc-tagged LMO4 cDNA as the template. Clones were verified using DNA sequencing. The LMO4 deletion constructs LMO4 ΔLIM1 (aa 1–87) and LMO4 ΔLIM2 (aa 87–166), were constructed using PCR amplification with complimentary oligonucleotides (LMO4Δ1: 5′-ATGGTGAATCCGGGC and 5′-AGCACC GCTATTCCC; LMO4Δ2: 5′-TGCAGGGCCTGTGGA and 5′-TCAGCAGACC TTCTG) and sub-cloned into pCAN–Myc or pGEX 4T1.

2.4. Antibodies and immunofluorescence

The primary antibodies used in this study are as follows: FAK (BD Transduction Laboratories), FAK pY577 (Invitrogen), Ldb1 (Santa Cruz Biotechnology, Dallas, TX), Myc (9E10 mouse ascites, Sigma-Aldrich), Paxillin (BD Transduction Laboratories), Src (Cell Signaling Technology, Beverly, MA), and α-tubulin (Sigma-Aldrich). The anti-LMO4 polyclonal antibodies were custom made using full length GST–LMO4 as the immunogen (Medical and Biological Laboratories Co. Ltd., Nagoya, Japan). The anti-LMO4 monoclonal antibody has been previously described (Sum EY et al. [24], J Histochem Cytochem). The anti-SLK polyclonal has been previously described [20].

For immunofluorescence studies, cells were seeded in 10% FBS DMEM on coverslips coated with fibronectin (10 μg/mL, Invitrogen) and incubated overnight at 37 °C and 5% CO_2 or until confluent for migration studies. For coverslips intended for migration studies, a micropipette tip was used to create two parallel wounds in untreated or inhibitor-treated (1 h pre-treatment prior to wounding with 10 μM PP2 or PP3; EMD-Millipore [Calbiochem], Darmstadt, Germany) cell monolayers. Growth media were replaced with fresh 10% FBS DMEM, including any small molecule inhibitors required for the experiment, and cells were incubated for 1 h at 37 °C and 5% CO_2 prior to fixation with 4% paraformaldehyde. Following permeabilization with 0.3% Triton X-100 in STO-PBS, cells were washed and blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 20 min. Cells were