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ScienceDirect
Cellular Signalling 20 (2008) 432–442
CELLULAR SIGNALLING
www.elsevier.com/locate/cellsig

Breast tumor kinase BRK requires kinesin-2 subunit KAP3A in modulation of cell migration

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Received 9 October 2007; received in revised form 5 November 2007; accepted 6 November 2007
Available online 13 November 2007

Abstract

*BR*east tumor *K*inase (BRK) also known as protein kinase 6 (PTK6) is a nonreceptor tyrosine kinase overexpressed in the majority of human breast tumors. Although some studies have implicated BRK in signalling, cell proliferation and migration, the precise intracellular role of BRK has not been fully elucidated. The RNA-binding protein Sam68, and adaptor proteins paxillin and STAT3 are the only BRK substrates that link BRK to signal transduction. To identify new BRK substrates, we screened high-density protein filter arrays by large-scale *in vitro* kinase assays using active recombinant BRK. We identified at least 4 BRK targets comprising the alpha-subunit of stimulatory guanine nucleotide binding protein (GNAS), FL139441, β-tubulin and kinesin associated protein 3A (KAP3A) and validated them as BRK substrates using a secondary assay. Further characterization revealed that KAP3A is an *in vivo* substrate of BRK and associates with BRK in breast cancer cells. We show that BRK specifically phosphorylated tyrosine residues at the C-terminus of KAP3A and induces delocalization of KAP3A from punctate nuclear localization to a diffuse nucleo-cytoplasmic pattern. Functionally, we demonstrate that KAP3A knockdown results in suppression of BRK-induced migration of breast cancer cells and show that the C-terminal deletion mutant of KAP3A acts as a dominant negative in BRK-induced cell migration. Our findings therefore reveal new substrates of BRK and define KAP3A as a physiological substrate of BRK during cell migration.

Keywords: Signal transduction; Tyrosine kinase; BRK; Breast cancer; Kinesin; KAP3A; Protein array

1. Introduction

The intracellular tyrosine kinase BRK (also called SIK and PTK6) is overexpressed in more than >60% of human breast carcinomas, but not in normal developing mammary gland or benign lesions [1–3]. In adult mice, PTK6 however is highly expressed in villus epithelial cells of the small intestine where it contributes to the maintenance of tissue homeostasis by inhibiting Akt [3]. Consequently, mice null for PTK6 displays enterocyte differentiation defects and increased Akt activity in the intestines [3]. BRK belongs to the tyrosine kinase family which includes Frk, Srm and Src42A [4]. BRK contains an SH3, an SH2, a kinase domain and a putative C-terminal regulatory tyrosine in a similar arrangement as Src family kinases, but lacks the myristoylation signal [4].

Though the cellular properties of BRK have not been fully defined there is evidence that BRK modulates signal transduction of receptor tyrosine kinases. Overexpression of BRK mRNA correlates with positive estrogen receptor status [5] and HER2 overexpression in breast cancer specimens [6] and high expression of the BRK protein was recently associated to metastases-free survival in breast cancer [7]. BRK interacts with epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 3 (HER3) and potentiates EGF-induced proliferation of mammary epithelial cells and enhances

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doi:10.1016/j.cellsig.2007.11.003

phosphorylation of HER3, inducing the coupling of EGF signalling to p85 phosphatidylinositol 3-kinase (PI3K) and Akt pathway [8,9]. EGF treatment has also been shown to the regulate the association of BRK with Akt in COS1 cells [10] and more recently BRK knockdown by short hairpin RNA in T47D breast cancer cells was shown to inhibit EGF- and heregulin-induced activation of Rac GTPase, extracellular signal-regulated kinase (ERK) 5 and p38 mitogen-activated protein kinase (MAPK) [2]. Furthermore, knockdown of Brk suppresses proliferation of T47D cells, while its overexpression enhances anchorage-independent growth of the mammary epithelial cell line HB4a and NIH3T3 murine fibroblasts [8,9].

Studies on the cellular roles of BRK in breast cancer have been limited by the restricted knowledge of the downstream effectors of BRK. Nonetheless, several BRK substrates have been reported in the literature. They include RNA-binding protein Sam68 and Sam68-like proteins SLM-1 and SLM-2 [11,12], breast tumor kinase substrate (BKS) [13], serine/threonine kinase protein kinase B/Akt [10], paxillin [14] and signal transducer and activator of transcription 3 (STAT3) [15]. Sam68 was shown to interact and colocalize with BRK in specific nuclear structures termed Sam68 nuclear bodies (SNBs) in cancer cells [16,17]. Breast cancer cell lines express tyrosine-phosphorylated Sam68 regulated by BRK activity in EGFR signalling [18]. Stimulation of breast cancer cells with either EGF or heregulin enhances phosphorylation of Sam68 [2,18]. In addition BRK has also been shown to mediate EGF-induced phosphorylation of paxillin [14]. The STAT family proteins are transcription factors that play pivotal roles in cytokine signalling [19]. Studies have revealed that STAT3 is tyrosine phosphorylated and transcriptionally activated in breast cancer cells expressing endogenous BRK, and the expression of both STAT3 and BRK is a stimulus for cellular proliferation [15]. To further illuminate the cellular roles of BRK, we set out to identify new BRK substrates by performing a large-scale kinase assay on high-density protein filter and have characterized KAP3A as a *bona fide* BRK substrate.

KAP3A is a nonmotor subunit of the kinesin-2 heterotrimeric complex that forms a large globular domain at the base of the kinesin complex [20–22] and bridges the microtubule-based motor subunits KIF3A/3B to various cargo proteins like tumor suppressors fodrin and adenomatous polyposis coli (APC) enabling neurite building and membrane morphogenesis respectively [23,24]. The kinesin-2 complex has also been implicated in the organization of Golgi membranes and the transport Golgi-derived vesicles [25], Golgi and ER intermediate membranes [26], pigment granules [27], endosomes and lysosome derivatives [28]. KAP3A or KIF3A silencing in HeLa cells was shown to alter the steady-state kinesin-2-driven localization of endosomes and lysosomes [29]. KAP3A or KIF3A silencing was also shown to induce fragmentation of the Golgi apparatus and impair Golgi-membrane recycling and distribution of ER-Golgi recycling proteins such as the KDEL-receptor (KDEL-R) [30]. The C-terminus of KAP3A is rich in tyrosine residues that contains a consensus sequences for tyrosine phosphorylation. It has been reported that KAP3A is phosphorylated by Src tyrosine kinase reducing the affinity of KAP3A for Smg GDS [20]. It is suggested that KAP3A plays an adaptor role in linking Smg GDS or the kinesin-2 complex to both the Smg GDS-regulated small G protein and Src tyrosine kinase signalling cascades [20]. Furthermore, KAP3A is phosphorylated during mitosis, but the significance of this phosphorylation, the targeted residues or the kinases responsible for KAP3A phosphorylation *in vivo* as well as the consequences of tyrosine phosphorylation of KAP3A have not been determined [31].

In the present study, we have identified and validated KAP3A as a *bona fide* substrate of BRK both *in vitro* and *in vivo*. We show that BRK preferentially phosphorylates the C-terminal tyrosines of KAP3A and alters its localization. We also show that the C-terminus of KAP3A is a critical determinant in BRK-induced cell migration.

2. Materials and methods

2.1. High-density protein filter

The generation of the cDNA expression library and the production of high-density filters have been described previously [32]. In short, human fetal brain cDNA was directionally cloned into the pQE-30NST *E. coli* expression vector (GenBank™ accession number AF074376) for isopropyl-1-thio-β-D-galacto-pyranoside-inducible (IPTG) expression of His-tagged fusion proteins. The library is composed of full-length as well as shorter cDNA clones. The high-density protein expression filters (22×22 cm) were obtained from the Resource Center of the DHGP (www.rzpd.de). They contain over 37 000 clones of His-tagged fusion proteins arrayed in duplicates on Polyvinylidene Fluoride (PVDF) membranes.

2.2. In situ kinase assay for substrate identification

For solid-phase kinase assay, the high-density protein filter was blocked with BSA and incubated for 15 min with 50 μg of fresh GST-BRK and 100 μCi of γ^{32}P-ATP in a kinase reaction buffer containing 50 mM HEPES, 5 mM $MgCl_2$, 3 mM $MnCl_2$ and 100 μM sodium vanadate. The filter was stringently washed with TBST (25 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) at 50 °C, air-dried and exposed to X-ray film. The coordinates of the identified positives as double spots on film were used to obtain *E. coli* bacteria cultures expressing the corresponding plasmids from RZPD. For immunoblotting, the filter was probed with anti-His-tag antibody (αRGS-His) to confirm the presence of all induced His-tagged polypeptides on the filter. Validated BRK substrates are listed in Table 1.

2.3. Antibodies, reagents and plasmids

Mouse monoclonal anti-RGS-His antibody was purchased from Qiagen (Mississauga, Ont.). Anti-phosphotyrosine (anti-pTyr) clone 4G10 and anti-HA

Table 1
BRK targets identified in vitro on a high-density protein filter as substrates and BRK-interacting proteins, and their validation on a secondary screen by kinase assay and Far-Western analyses

Primary screen			Secondary screen	
Protein	Acc. no	Identified target	Kinase assay	GST-BRK binding
GNAS	NM_000516	Substrate	+	+
KAP3A	NM_014970	Substrate/interact	+	+
FLJ39441	AK096760	Substrate	+	−
β-tubulin	NM_178012	Substrate	+	+
NM23A	NM_000269	Substrate/interact	+	+
NM23B	NM_000269	Substrate/interact	+	+