Cellular Signalling 21 (2009) 1415–1422

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

BRK phosphorylates PSF promoting its cytoplasmic localization and cell cycle arrest

Kiven E. Lukong, Marc-Étienne Huot, Stéphane Richard *

Terry Fox Molecular Oncology Group, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, Canada H3T 1E2
Bloomfield Center for Research on Aging, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, Canada H3T 1E2
Department of Medicine, McGill University, Montréal, Québec, Canada H3T 1E2
Department of Oncology, McGill University, Montréal, Québec, Canada H3T 1E2

ARTICLE INFO

Article history:
Received 11 March 2009
Accepted 30 April 2009
Available online 9 May 2009

Keywords:
Tyrosine kinases
BRK
Breast cancer
PSF
RNA-binding proteins
Mass spectrometry
Epidermal growth factor

ABSTRACT

*BR*east tumor *K*inase (BRK) also known as protein kinase 6 (PTK6) is a non-receptor tyrosine kinase overexpressed in the majority of human breast carcinoma. The expression of BRK is a known prognostic marker of breast carcinoma. BRK has been shown to lie downstream of epidermal growth factor (EGF) signaling and mediate effects on cell proliferation and migration. To identify BRK substrates and interacting proteins, we undertook a proteomic approach. BRK immune complexes were purified from the BT-20 breast cancer cell line and analyzed by mass spectrometry. Herein, we report the identification of PSF, the polypyrimidine tract-binding (PTB) protein-associated splicing factor, as a BRK-interacting protein and substrate. BRK and PSF co-eluted in a large protein complex that was regulated by EGF stimulation. Furthermore, BRK and PSF co-immunoprecipitated in BT-20 cells and we defined the interaction as being an SH3 domain–polyproline interaction. The C-terminal tyrosines of PSF were the site of phosphorylation by BRK. Moreover, tyrosine phosphorylation of PSF was also observed upon EGF stimulation, consistent with a role of PSF and BRK downstream of the EGF receptor. Interestingly, the tyrosine phosphorylation promoted the cytoplasmic relocalization of PSF, impaired its binding to polypyrimidine RNA, and led to cell cycle arrest. Our findings show that BRK targets the PSF RNA-binding protein during EGF stimulation.

1. Introduction

BRK (also known as protein tyrosine kinase 6 or PTK6) is a non-receptor tyrosine kinase that belongs to the BRK/FRK/SRMS tyrosine kinase family defined by a highly conserved exon structure distinct from other major soluble tyrosine kinase families [1]. The encoded 451 amino acid polypeptide of BRK is composed of a Src homology (SH) 2, SH3 and a catalytic domain [2] which display a similar architecture to and have ~30–40% sequence identity with Src kinases, the closest BRK human homologues. In addition, the catalytic activities of both BRK and Src are negatively regulated by tyrosine phosphorylation of their C-terminal tyrosines 447 and 530, respectively [3,4]. Indeed, the solution structure of BRK has revealed that it is maintained in an inactive conformation by intramolecular interactions between the C-terminal phosphotyrosine and its SH2 domain [6]. Furthermore, mutation of tyrosine 447 to phenylalanine significantly enhances the kinase activity of BRK (BRK-YF) [3]. The murine orthologue of BRK also called Src-related intestinal kinase (SIK) displays an epithelial-specific expression and shares about 80% identity with BRK [5]. BRK is localized in the nucleus and in the cytoplasm unlike Src family kinases which are mostly at the plasma membrane [1].

BRK is normally expressed in differentiating epithelial cells of the gastrointestinal tract and the skin and its overexpression is observed in many tumors [1]. BRK is overexpressed in human breast carcinomas, but not in normal developing mammary gland [1]. Approximately two-thirds of breast carcinomas contain elevated BRK expression [2,7–9]. Of 426 archival breast cancer samples from patients with long-term follow-up, BRK expression and the phosphorylation status of its substrate Sam68 correlate with significant prognostic value in the outcome of breast carcinomas [10]. BRK is also overexpressed in some metastatic melanomas [11], lymphomas [12], ovarian tumors [13], colon cancers [14], squamous cell carcinomas [15] and prostate cancers [16]. The cellular localization of BRK may be important for tumorigenesis, as the switch from nucleus to the cytoplasm is observed in high grade prostate tumors [16]. Collectively, these findings suggest that BRK may play a role in the development and/or progression of various tumors.

RNA-binding proteins including Sam68 (*S*rc substrate *A*ssociated in *M*itosis of *68* kDa) [3] and Sam68 *L*ike *M*ammalian proteins (SLM1 and SLM2) are known substrates of BRK [17]. Sam68, SLM1 and SLM2 are nuclear KH-domain RNA-binding proteins containing SH3- and SH2-binding motifs and interact and colocalize with BRK in specific nuclear structures termed Sam68 nuclear bodies (SNBs) in cancer cells [18,19]. Phosphorylation of Sam68 has been shown to negatively regulate its RNA-binding ability [3]. Sam68 was shown to be an effector of epidermal

* Corresponding author. Segal Cancer Centre, Lady Davis Institute, 3755 Côte Ste.-Catherine Road, Montréal, Québec, Canada H3T 1E2. Tel.: +1 514 340 8260; fax: +1 514 340 8295.
E-mail address: stephane.richard@mcgill.ca (S. Richard).

0898-6568/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2009.04.008

growth factor (EGF) stimulation and BRK contributes to Sam68 phosphorylation in EGF-treated breast cancer cells [20]. Paxillin was also identified as an interactor and direct substrate of BRK [21]. BRK phosphorylates paxillin on Y31 and Y118 enabling docking of adaptor protein CrkII and the subsequent activation of Rac1 [21]. In addition, BRK-phosphorylated paxillin enhances EGF-dependent promotion of cell migration and invasion [21].

BRK has been shown to associate with the epidermal growth factor receptor (EGFR) and to enhance the mitogenic signals of EGF by increasing the recruitment of phosphoinositol 3-kinase and the activation of Akt [22,23]. The cellular targets identified to date however do not conclusively delineate the cellular roles of BRK and all the specific pathways modulated by BRK in the pathogenesis of various cancers. To identify additional BRK-interacting proteins and substrates, we performed a proteomic analysis of BRK-interacting proteins. BT20 breast cancer cells were lysed and BRK immune complexes were purified and interacting proteins identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF). These studies led to the identification of the polypyrimidine tract-binding (PTB) protein-associated splicing factor (PSF) [24], as a BRK-interacting protein.

PSF is a 100 kDa polypeptide with 707 amino acids that functions as a multifunctional nucleic acid binding protein and exists in a complex with $p54^{nrb}$/nonO [25]. The PSF and $p54^{nrb}$/nonO complex have been shown to regulate transcription, splicing [24,26,27], transcription termination and 3′-processing [28] and DNA double-strand break repair [29]. PSF contains two canonical RNA-binding domains (RBD), a carboxyl terminal DNA-binding domain flanked by two nuclear localization signals, and an unusual amino terminus rich in proline and glutamine residues [25]. Both PSF and $p54^{nrb}$/nonO have been observed in nuclear speckles, structures that are enriched in splicing factors [30,31]. PSF was shown to coordinately repress transcription of multiple oncogenes [32].

In the present study, we report that PSF is a *bona fide in vivo* interacting protein and substrate of BRK. We show that the BRK SH3-domain interacted with N-terminal polyproline rich motifs of PSF and the PSF C-terminal tyrosines are the site of tyrosine phosphorylation by BRK. The expression of BRK and EGF stimulation promoted the cytoplasmic localization of PSF. These finding confirm PSF as a BRK target and demonstrate that tyrosine phosphorylation of PSF by BRK negatively regulated the ability of PSF to bind RNA and induced S-phase cell cycle arrest.

2. Materials and methods

2.1. Antibodies

The anti-PSF monoclonal antibody was obtained from Sigma-Aldrich (St. Louis, MO). The 9E10 anti-myc monoclonal antibody was obtained from the American Type Culture Collection (Manassas, VA). Anti-BRK polyclonal antibodies (N19 and C-17) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-actin monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO.). Anti-phosphotyrosine (anti-pTyr) clone 4G10 was from Upstate (Lake Placid, NY). Anti-PRMT1 antibody was obtained from Millipore (Billerica, MA).

2.2. Mass spectrometry

BT20 breast cancer cells (5×10^7) were lysed in a buffer containing 1% Triton X-100 (Roche Applied Science), 20 mM Tris, pH 7.4, 150 mM NaCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.01% phenylmethanesulfonyl fluoride. Endogenous BRK was immunopurified from the cell lysate using anti-BRK antibodies coupled to protein A-Sepharose (Sigma). After extensive washings with lysis buffer and PBS, the bound proteins were eluted with 500 μl of PBS containing 6.0 M urea. Eluted proteins were dialyzed against water overnight, lyophilized, resolved by SDS-PAGE and revealed by Coomassie Blue R-250 staining. Selected protein bands of approximately 100, 60 and 36 kDa were excised and sent to the University of Calgary Mass Spectrometry Proteomics Facility for in-gel tryptic digestion and MALDI-TOF analysis.

2.3. Expression constructs

An expression plasmid encoding a GFP-PSF fusion protein was kindly provided by Dr. Herbert H. Samuels (Departments of Pharmacology and Medicine, New York University School of Medicine, New York, USA) [33]. GFP-PSF mutants ΔRRM2, NT ΔN1, ΔN2 were kindly provided by James G. Patton (Departments of Biological Sciences and Pediatrics, Vanderbilt University, Nashville, Tennessee) [31]. Details on the construction of the myc-tagged and GFP-tagged BRK plasmids have been published [20,34].

2.4. Cell culture

The following cell lines were obtained from the American Type Culture Collection (Manassas, VA): HeLa, MDA-231, MDA-468, BT20, MCF-7, T47D and U2OS. The cells were maintained in DMEM (Life Technologies-BRL, Grand Island, NY) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (all from ICN, Costa Mesa, CA), and 10% bovine calf serum (Hyclone, Logan, UT). Cells were maintained at 37 °C in 5% CO_2.

2.5. Protein expression and immunoprecipitations

HeLa or HEK293 cells were transfected with LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. For immunoprecipitations, cell lysates were incubated on ice with the primary antibody for 1 h. Then 30 μl of 50% protein A-Sepharose slurry was added and incubated at 4 °C for 30 min with constant end-over-end mixing. The beads were washed twice with lysis buffer and once with PBS. Protein samples were analyzed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblotting was performed using the anti-PSF, anti-GFP and anti-BRK antibodies. Immunoreactive proteins were visualized using either goat anti-mouse or goat anti-rabbit antibodies conjugated to horseradish peroxidase (ICN Pharmaceuticals, Costa Mesa, CA) and chemiluminescence (ECL) detection kit (DuPont, Wilmington, DE). For RNA interference against PSF, human osteosarcoma U2OS cells were transfected with siGENOME SMARTpool from Dharmacon (Lafayette, CO) using Lipofectamine™ RNAi MAX (Invitrogen) according to the manufacturer's instructions with a final concentration of 20 nM siRNA. For EGF treatment, cells were starved overnight in media containing 0.5% bovine calf serum and stimulated at various time intervals with EGF (100 ng/ml).

2.6. Immunofluorescence

HeLa cells were cultured directly on glass coverslips in a 6-well dish. Transfection of HeLa cells for immunofluorescence was achieved using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol using 2 μg of DNA. For EGF treatment, HeLa cells were starved overnight on coverslips, stimulated at various time intervals with EGF (100 ng/ml). In both cases, the cells were fixed with 1% paraformaldehyde in PBS, pH 7.4, for 5 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature (RT). If the cells were to be visualized only for GFP, then the coverslips were mounted onto glass slides with glycerol containing 4′,6-diamidino-2-phenylindole (DAPI) to stain the nuclei. If the cells required antibody staining, the permeabilized cells were first blocked with 10% calf serum (Hyclone) in PBS for 30 min, then incubated with the primary antibody (1:200) for 1 h in PBS at RT. The cells were washed with 0.1%