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Functional roles of BCAR3 in the signaling pathways of insulin leading to DNA synthesis, membrane ruffling and GLUT4 translocation

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ABSTRACT

Breast cancer anti-estrogen resistance 3 (BCAR3) is an SH2-containing signal transducer and is implicated in tumorigenesis of breast cancer cells. In this study, we found that BCAR3 mediates the induction of ERK activation and DNA synthesis by insulin, but not by IGF-1. Specifically, the SH2 domain of BCAR3 is involved in insulin-stimulated DNA synthesis. Differential tyrosine-phosphorylated patterns of the BCAR3 immune complex were detected in insulin and IGF-1 signaling, suggesting that BCAR3 is a distinct target molecule of insulin and IGF-1 signaling. Moreover, microinjection of BCAR3 inhibitory materials inhibited membrane ruffling induced by insulin, while this did not affect insulin-mediated GLUT4 translocation. Taken together, these results demonstrated that BCAR3 plays an important role in the signaling pathways of insulin leading to cell cycle progression and cytoskeleton reorganization, but not GLUT4 translocation.

1. Introduction

Insulin and IGF-1 possess potent mitogenic and pro-migratory properties. As high concentrations of insulin and IGF-1 can elevate cancer risk and promote metastatic progression, they have been extensively studied in many malignancies [1,2]. Insulin receptor (IR) and IGF-1 receptor (IGF-1R) have similar $\alpha_2\beta_2$-heterotetrameric structures and are involved in closely related and overlapping signaling pathways. For instance, after insulin and IGF-1 bind to their cognate receptors, insulin receptor substrates are phosphorylated and facilitate delivery of signals to the PI3-kinase/Akt and Ras/ERK pathways [3]. The signals of IR and IGF-1R can ultimately increase proliferation of normal cells and cancer cells.

Breast cancer anti-estrogen resistance 3 (BCAR3) was identified as a gene responsible for the induction of anti-estrogen resistance in human breast cancer cells [4]. Overexpression of BCAR3 results in a bypass of estrogen dependence for proliferation leading to resistance to anti-estrogens [5,6]. BCAR3 belongs to the Novel SH2-containing Protein (NSP) 1–3 family and their crystal structures show similarities in molecular structure, containing an N-terminal SH2 domain, a proline/serine-rich (P/S) domain and a C-terminal GEF domain [7]. Despite similarities in overall structural features among the NSP family, there are important differences in expression patterns. For example, NSP1 and NSP3 are widely expressed in various tissues, whereas, BCAR3/NSP2 is specifically expressed in placenta, skeletal muscle, spleen and lymph node [8]. Differences in expression patterns suggest that BCAR3/NSP2 may be involved in tissue-specific functions.

The structural features of BCAR3 and other NSP family contain the SH2 and GEF domains, which suggest that the NSP family may participate in the intracellular signal transduction pathway of growth factors. It has been reported that NSP1 was tyrosine-phosphorylated with treatment of EGF and directly associated with EGF receptors through the SH2 domain of NSP1 in COS cells [8]. We previously reported that BCAR3 directly interacts with tyrosine-phosphorylated EGF receptor in normal human breast MCF-12A cells through the SH2 domain of BCAR3 [9]. The SH2 domain of NSP3/SHEP1 has also been associated with EphB1, a member of Eph receptor tyrosine kinases [10]. BCAR3 also binds to Cas and protein tyrosine phosphatase α [11,12]. This association of the NSP family with receptors and signal transducers suggests an important functional role in the signaling pathway of growth factors. But the functional roles of BCAR3 in the signaling pathways of insulin and IGF-1 have yet to be established.

In this study, we assessed the functional involvement of BCAR3 in the mitogenic signal transduction pathway of insulin and IGF-1 leading to DNA synthesis. Using single cell microinjection, we

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found that BCAR3 is an important intermediate signaling molecule in insulin-stimulated DNA synthesis and membrane ruffling, but not in IGF-1-mediated signaling. Furthermore, we found that this differential regulation is mediated through the SH2 domain of BCAR3.

2. Materials and methods

2.1. Materials

Rabbit polyclonal anti-BCAR3 antibodies were produced by Eurogentec (Belgium). Antibodies specific to Cas and Ras (Y13-259) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Super Script III was purchased from Life Technologies (Grand Islands, NY, USA). SYBR Taq reagent was obtained from Takara Bio Inc. (Shiga, Japan). Mouse anti-bromodeoxyuridine (BrdU) antibody, protein A agarose beads and glutathione-Sepharose 4B beads were from GE Healthcare Life Sciences (Uppsala, Sweden). Antibodies against phospho-ERK (Thr202/Tyr204), ERK, phospho-Akt (Ser473), Akt and GAPDH were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibody to phosphotyrosines (PY-20) were obtained from Transduction Laboratories (Lexington, KY, USA). Goat anti-mouse and anti-rabbit antibodies conjugated with fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) were from Jackson Immunoresearch Laboratories Inc. (West Groove, PA, USA). siRNA SMARTpool reagents of BCAR3, Dulbecco's modified Eagle's medium (DMEM), insulin, IGF-1, and fetal bovine serum were obtained from Thermo Fisher Scientific (Lafayette, CO, USA). Rabbit anti-GLUT4 antibody was purchased from Millipore (Temecula, CA, USA). Other reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture and cell treatment

Rat-1 fibroblasts overexpressing wild-type human insulin receptors (HIRc) were maintained as described previously [13]. Immortalized normal human breast MCF-12A cells were purchased from the ATCC (Mannassa, VA, USA) and cultured as suggested by the ATCC. Cell cycle of MCF-12A cells was arrested with DMEM containing 0.5% FBS for 24 h followed by stimulation with insulin (100 ng/ml) and IGF-1 (20 ng/ml) for the indicated time. 3T3-L1 cells were cultured and differentiated as described previously [14].

2.3. siRNA transfection, real-time PCR analysis, cell counting and immunoblotting

The MCF-12A cells were transfected with control and BCAR3 siRNA with media including serum for 24 h. After transfection, total RNAs from the transient transfected MCF-12A cells were extracted using easy-BLUE kit (iNtRON biotechnology, Seongnam, Korea). Real-time fluorescence quantitative PCR was used to measure BCAR3 mRNA with Bio-Rad iCycler (Bio-Radm Hercules, CA, USA). cDNA was synthesized using Super Script III and amplified using SYBR Premix EX Taq premix reagent. The primer sequences used for real-time PCR were as follows: BCAR3, forward,

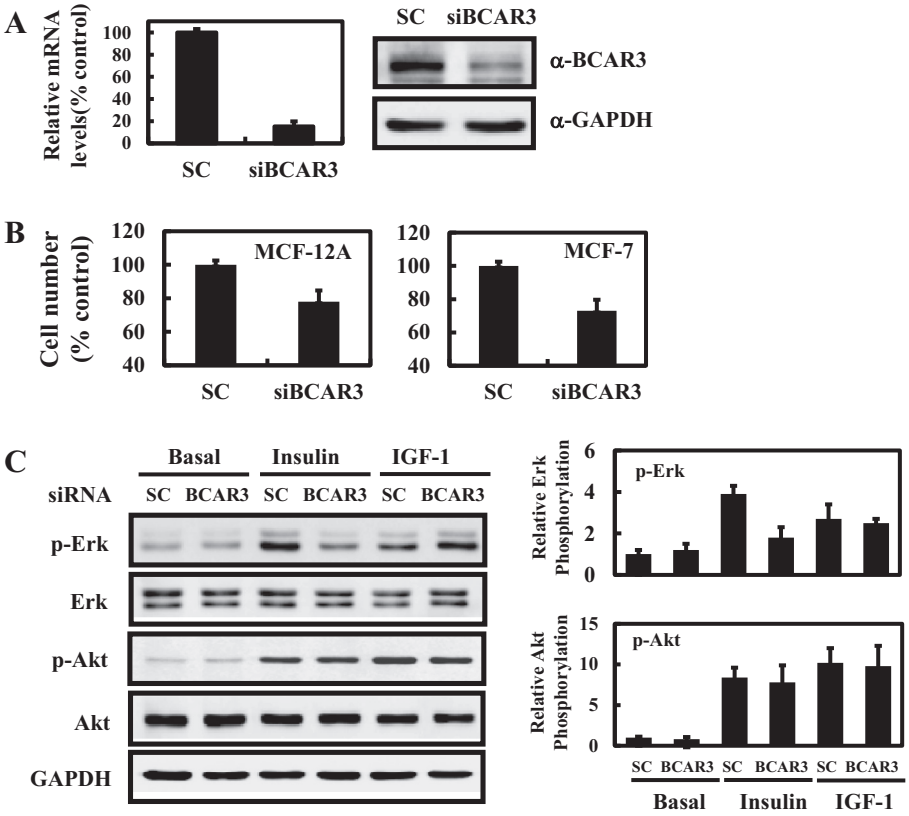

Fig. 1. BCAR3 is involved in proliferation and insulin-stimulated ERK activation in MCF-12A normal breast cells. (A) MCF-12A cells were transiently transfection with negative control or BCAR3 siRNA (100 nM) for 24 h. The expression level of BCAR3 in MCF-12A cells was analyzed by real-time PCR and immunoblotting. (B) The siBCAR3-transfected MCF-12A cells and MCF-7 cells were cultured for 3 and 5 days, respectively, and were manually counted with a hemocytometer. Relative cell growth in triplicate and standard error is shown. Results are expressed as the percentage of control scrambled siRNA-transfected cells. (C) MCF-12A cells transiently transfected with negative control or BCAR3 siRNA (100 nM) for 24 h were starved with serum-free media for 4 h and stimulated with insulin (100 ng/ml) or IGF-1(10 ng/ml) for 5 min. Levels of activated Akt and Erk were determined by immunoblotting. Relative phosphorylation of Erk and Akt compared to the control siRNA-transfected basal MCF-12A cells is depicted.