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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.

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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,

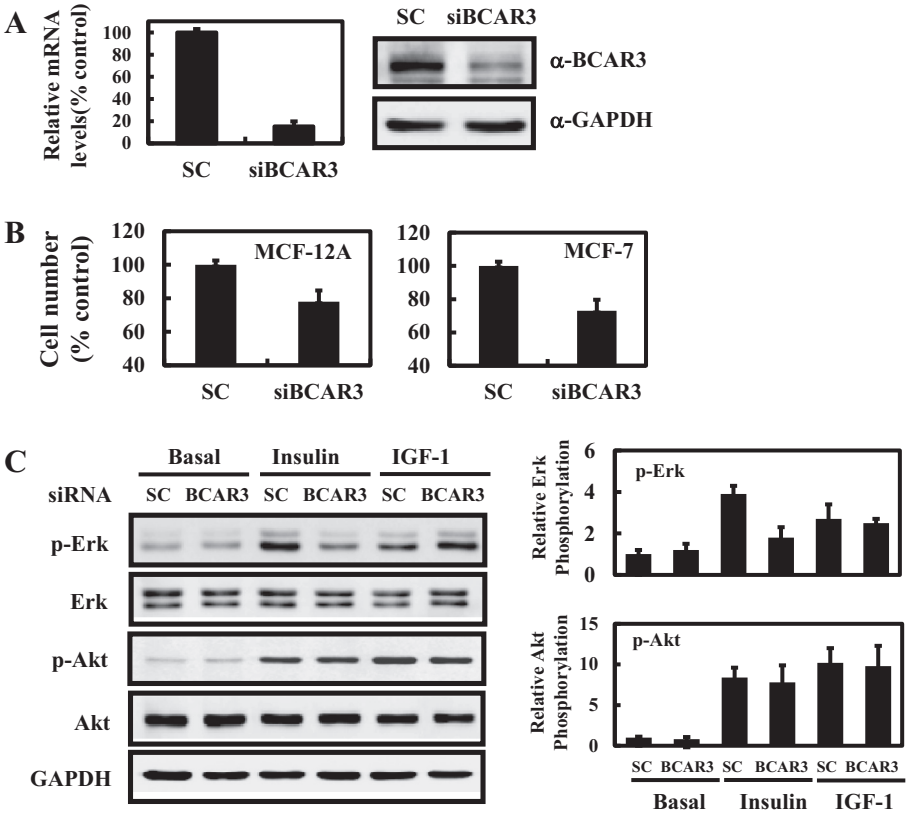

Fig. 1. BCAR3 is involved in proliferation and insulin-stimulated ERK activation in MCF-12A normal breast cells. (A) MCF-12A cells were transiently transfection with negative control or BCAR3 siRNA (100 nM) for 24 h. The expression level of BCAR3 in MCF-12A cells was analyzed by real-time PCR and immunoblotting. (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.

5′-cattgcagtggacattctgg-3′; reverse, 5′-gagaatggcagtttgggtgt-3′; β-actin, forward, 5′-ggacttcgagcaagagatgg-3′; reverse, 5′-agcactgtgt tggcgtacag-3′. PCR cycling conditions were 94 °C for 3 min and 35 cycles (94 °C for 5 s, 54 °C for 30 s and 72 °C for 20 s). Data were analyzed by a comparative cycle threshold method in Bio-Rad iQ5 software. To examine cell proliferation, transfected cells were incubated for 3 days, trypsinized, and manually counted with a hemocytometer. For immunoblotting, transfected MCF-12A cells were serum-starved for 4 h and stimulated with insulin (100 ng/ml) or IGF-1 (20 ng/ml) for 10 min. The cells were lysed and immunoblotted with antibodies against phospho-Erk, phospho-Akt, Erk, Akt and GAPDH.

2.4. Affinity purification of antibodies, preparation of GST fusion proteins, and microinjection of antibodies, siRNA, and GST-fusion proteins

We previously reported purification of anti-BCAR3 and anti-Cas antibodies, the preparation of GST fusion proteins, full length BCAR3, the SH2 domain (residues 154–253), P/S rich (residues 419–537), and microinjection of inhibitory materials [9,13]. The SH2 domains of CrkII and p85 of PI3-kinase were similarly prepared and microinjected. All microinjection materials contained rat IgG (3 mg/ml) for the detection of injected cells. Immunofluorescent staining as described below revealed that over 80% of the injected cells successfully survived.

2.5. Detection of DNA synthesis, membrane ruffling and GLUT4 translocation in the microinjected cells after stimulation of insulin and IGF-1

Following a one-hour stabilization after microinjection, injected cells were stimulated with insulin (100 ng/ml) or IGF-1 (20 ng/ml) for 24 h in the presence of BrdU to detect DNA synthesis. siRNA injected cells were further incubated for 8 h before insulin or IGF-1 stimulation. For DNA synthesis, the cells were fixed and sequentially incubated with mouse anti-BrdU antibody, TRITC-conjugated anti-mouse IgG antibody and FITC-conjugated anti-rabbit IgG antibody for 1 h at 37 °C. For membrane ruffling and GLUT4 translocation, injected cells were incubated with insulin (100 ng/ml) for 5 and 15 min, respectively. The cells were subsequently incubated with rhodamine-conjugated phalloidin or anti-GLUT4 antibody. Results of microinjection experiments are represented by the mean of at least duplicates of three independently performed experiments. In each experiment, at least 200 cells were injected. Therefore, the results represent the average of 1200 injected cells.

2.6. Affinity precipitation and immunoblotting

Insulin- or IGF-1-treated HIRc cells were lysed with modified RIPA buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 4 mM Na_3VO_4, 0.5% NP-40 and protease inhibitor cocktail (Roche, Basel, Switzerland). Cell lysates were incubated with protein A-purified anti-BCAR3 antibody. For GST affinity precipitation, cell lysates were incubated for 10 μg of purified full length GST-BCAR3 and GST-SH2 BCAR3 for 90 min at 4 °C. Bound proteins were precipitated with protein A agarose beads or glutathione-Sepharose 4B beads, and immunoblotted with specific antibodies.

3. Results

3.1. BCAR3 differentially regulates the signaling pathway of insulin and IGF-1 in MCF-12A normal breast cells

Insulin and IGF-1 are important mitogens in breast cells. In order to understand the role of BCAR3 in the signaling pathway of insulin and IGF-1, we used non-tumorigenic human mammary epithelial MCF-12A cells. Expression of BCAR3 was transiently suppressed in MCF-12A cells using siRNA BCAR3 and signaling of insulin and IGF-1 was examined. As shown in Fig. 1A, the levels of BCAR3 mRNA and protein were reduced. In MCF-12A cells transfected with siBCAR3, cell proliferation was reduced up to 23% (Fig. 1B), whereas transfection with scrambled siRNA had no effect on proliferation. Similar inhibition of proliferation with siBCAR3 was observed in MCF-7 cells, suggesting that BCAR3 regulates the proliferation of both MCF-12 normal breast cells and MCF-7

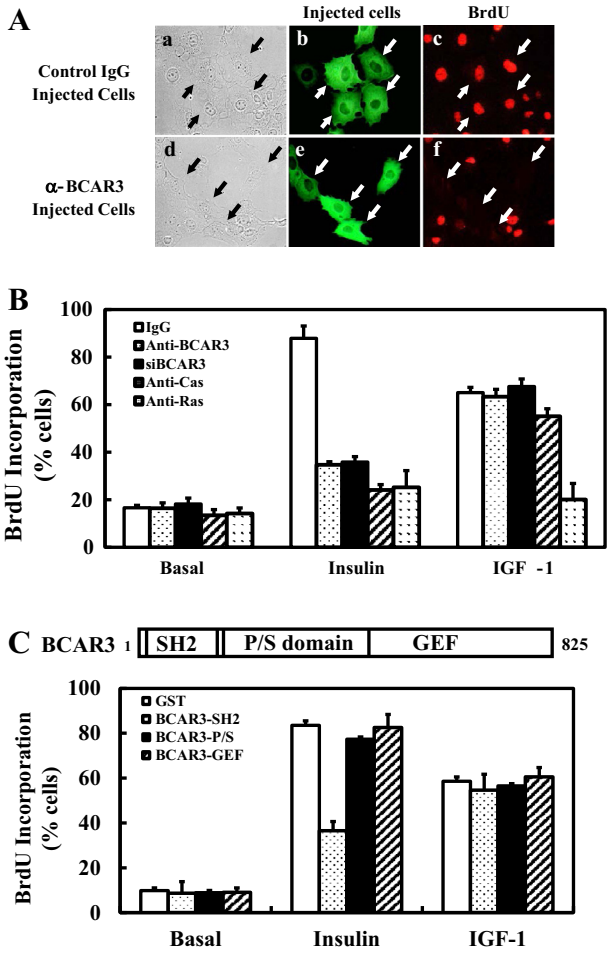

Fig. 2. Effects of microinjection of anti-BCAR3, siBCAR3 and BCAR3-SH2 on DNA synthesis induced by insulin or IGF-1. (A) HIRc cells were grown on acid-washed coverslips, serum-starved, and microinjected with control rabbit IgG (a–c) or affinity purified rabbit anti-BCAR3 antibodies (d–f) and stimulated insulin (100 ng/ml) for 24 h. The injected cells were identified by cytoplasmic immunostaining (b, e) and DNA synthesis was detected by nuclear anti-BrdU immunostaining (c, f). Arrows indicate the injected cells. (B) Serum-starved HIRc cells were microinjected with the indicated inhibitory materials and stimulated with insulin (100 ng/ml) or IGF-1 (20 ng/ml) for 24 h. (C) Functional schematic structure of BCAR3 is shown on the upper panel. Serum-starved HIRc cells were microinjected with GST protein containing the SH2 domain, P/S domain or GEF domain of BCAR3. The injected cells were stimulated with insulin or IGF-1 for 24 h. The inhibitory effect on the DNA synthesis was determined by anti-BrdU antibody. Results are obtained from three independent experiments in which at least 1200 cells were injected. The data are expressed as the percent of total injected cells. *Bars*, the mean result ± S.E.

tumor cells. Next, insulin- and IGF-1-mediated activation of ERK and Akt proteins were assessed in MCF-12A cells. Insulin-mediated ERK activation was suppressed by siRNA BCAR3, while IGF-1-mediated ERK activation was not affected (Fig. 1C). In addition, Akt activation by insulin and IGF-1 was also unaffected by siRNA BCAR3. These results suggest differential involvement of BCAR3 in the signaling pathways of insulin and IGF-1.

3.2. BCAR3 differentially regulates DNA synthesis induced by insulin and IGF-1

To further confirm the differential regulation by BCAR3 in insulin-mediated signaling, we utilized the microinjection technique to examine the functional role of BCAR3 in the signaling pathway of insulin and IGF-1 leading to DNA synthesis. Due to a low response of DNA synthesis to insulin or IGF-1, MCF-12A cells or other breast cells were not applicable to the single cell microinjection technique, particularly for insulin and IGF-1 actions [9]. Instead, we used Rat-1 cells constitutively overexpressing the human insulin receptor (HIRc) to study the role of BCAR3 in mitogenic actions of insulin and IGF-1. Previously these cells have been used to examine insulin and IGF-1 actions [13,15].

We microinjected the inhibitory materials, anti-BCAR3 antibodies and siRNA BCAR3, into cell cycle-arrested HIRc cells. Subsequently, DNA synthesis stimulated with insulin or IGF-1 was monitored in the injected cells according to our previously reported methods [9,13]. Fig. 2 shows the epi-fluorescence image of microinjected cells and the results of approximately 1200 microinjected cells after stimulation with insulin or IGF-1. Microinjected BCAR3 antibodies inhibited DNA synthesis induced by insulin (Fig 2B). DNA synthesis stimulated by insulin was 84% and 37% in the control rabbit IgG-injected cells and BCAR3 antibody-injected cells, respectively, while DNA synthesis stimulated by IGF-1 was not affected. This result suggests that the endogenous inhibition of BCAR3 by antibody microinjection led to decreased sensitivity for DNA synthesis specifically stimulated by insulin, but not by IGF-1. Similarly, microinjection of siRNA BCAR3 inhibited DNA synthesis in cells stimulated by insulin, but not in cells stimulated by IGF-1 (Fig. 2B). Microinjection of anti-Cas antibody differentially inhibited insulin-stimulated DNA synthesis. As a control, microinjection of anti-Ras antibody inhibited DNA synthesis stimulated by both

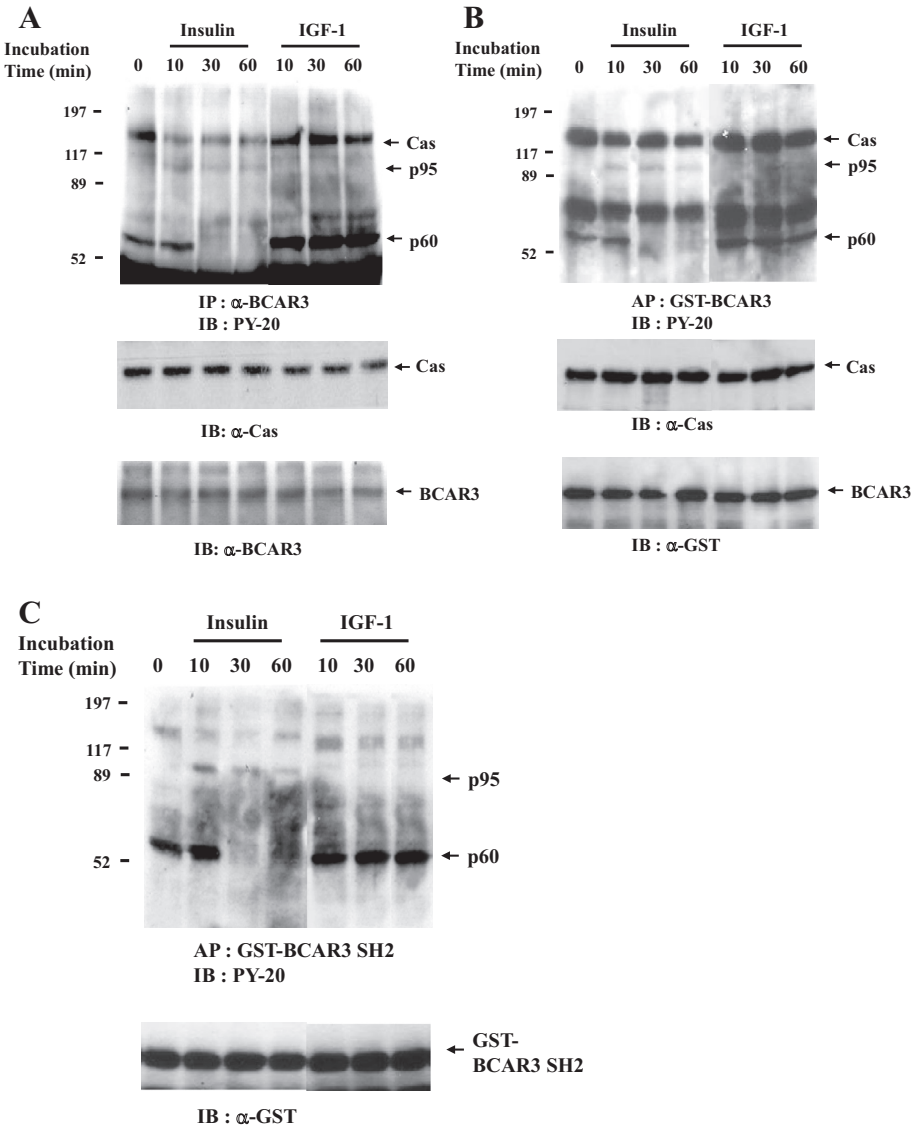

Fig. 3. Identification of tyrosine-phosphorylated protein associated with BCAR3. HIRc cells were serum-starved and stimulated with insulin or IGF-1 for the indicated times. The cell lysates were affinity-precipitated with anti-BCAR3 antibody (panel A), or full length GST-BCAR3 (panel B), or GST-BCAR3 SH2 domain (panel C). The precipitates were immunoblotted with antibodies against phosphotyrosine (PY-20), Cas, BCAR3 and GST.

insulin and IGF-1, suggesting the specific role of BCAR3 and Cas in the insulin signaling pathway.

BCAR3 protein is composed of three functional domains, the SH2 domain, the P/S domain, and the GEF domain. We further examined which of these domains is implicated in the regulation of DNA synthesis (Fig. 2C). Insulin-stimulated DNA synthesis was inhibited in the SH2 domain protein-injected cells, but not in P/S or GEF domain protein-injected cells. IGF-1-stimulated DNA synthesis was not inhibited by microinjection of the different BCAR3 protein domains. These results suggest that the BCAR3 protein is an endogenous signal transducer of insulin-mediated cell cycle progression and is differentially involved in the signaling pathway of insulin and IGF-1.

3.3. Identification of tyrosine-phosphorylated protein associated with BCAR3

Having demonstrated that BCAR3 and specifically the BCAR3 SH2 domain play a differential regulatory role in insulin and IGF-1 signaling, we next examined possible tyrosine-phosphorylated proteins interacting with BCAR3. To do this, we performed affinity precipitation using BCAR3 antibodies or GST fusion proteins containing full length or the SH2 domain of BCAR3 in HIRc cells (Fig. 3). We found that full-length BCAR3 directly interacted with phosphorylated Cas, and proteins migrating at 95 (p95) and 60 (p60) kDa, while the SH2 domain of BCAR3 only interacted with p95 and p60, and not with Cas. Tyrosine phosphorylation of p95 and p60 was time-dependent and undetectable after 30 min. Also this disappearance was only specific to stimulation by insulin, and not IGF-1. The identity of p95 and p60 is currently unknown. Consistent with prior studies [11,12], Cas interacted with full length BCAR3 through the carboxyl terminal, but not with its SH2 domain. These results suggested that some tyrosine phosphorylated proteins differentially interacted with the SH2 domain of BCAR3 and may regulate signaling of insulin and IGF-1.

3.4. A functional role of BCAR3 in membrane ruffling and GLUT4 translocation stimulated by insulin

We next examined whether BCAR3 is involved in regulation of membrane ruffling and GLUT4 translocation induced by insulin. Membrane ruffling was monitored by staining with actin-binding phalloidin (Fig. 4A). Microinjection of anti-BCAR3 antibody and GST-BCAR3-SH2 inhibited insulin-stimulated membrane ruffling. Control injection of the same concentration of Rabbit IgG and GST protein had no effect on membrane ruffling. In agreement with [16], Crk-SH2 and p85-SH2 protein domains inhibited insulin-induced membrane ruffling as well (Fig. 4A). For GLUT4 translocation analysis, microinjection was performed into differentiated 3T3-L1 adipocytes followed by immunostaining with anti-GLUT4 antibody as previously reported [14] (Fig. 4B). Interestingly, insulin-stimulated GLUT4 translocation was not inhibited by microinjection BCAR3 antibody and GST-BCAR3-SH2. These results suggest that the BCAR3 protein plays an important role in insulin-stimulated cytoskeletal reorganization, but not in GLUT4 translocation.

4. Discussion

We examined the functional role of BCAR3 in the signaling pathway of insulin and IGF-1 leading to mitogenesis. We found that BCAR3 directly regulates the activation of DNA synthesis by insulin signaling, but not by IGF-1 signaling. Furthermore, we demonstrated that the SH2 domain of BCAR3 was involved in the differential regulation of mitogenic signaling of insulin and IGF-1. In addition, insulin stimulation of ERK activation was blocked

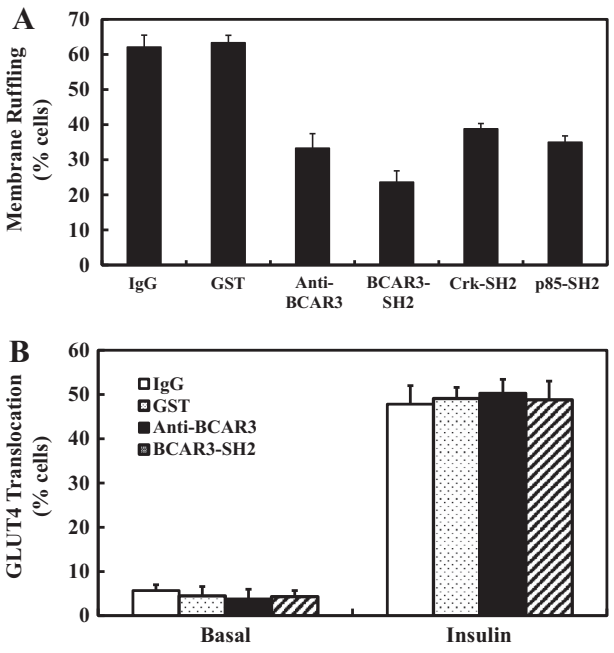

Fig. 4. BCAR3 regulates insulin-stimulated membrane ruffling, but not GLUT4 translocation. (A) Serum-starved HIRc cells were microinjected with control materials or BCAR3 inhibitory materials and stimulated insulin (100 ng/ml) for 10 min. Membrane ruffling was monitored with phalloidin staining. (B) Fully differentiated 3T3-L1 adipocytes were serum-starved and microinjected with the indicated inhibitory materials and stimulated with insulin (100 ng/ml) or for 15 min. GLUT4 translocation was determined with anti-GLUT4 antibody. Results are obtained from three independent experiments in which at least 1200 cells were injected. The data are expressed as the percent of total injected cells. *Bars*, the mean result ± S.E.

through cellular knock-out by siBCAR3, but not by stimulation with IGF-1. We also found that BCAR3 mediated insulin-stimulated membrane ruffling, but not GLUT4 translocation. Taken together, our results suggest that BCAR3 is an important signaling molecule mediating the mitogenic effect of insulin, but not of IGF-1.

IR and IGF-1R are highly homologous and their signaling pathways frequently overlap. Despite this close association, the physiologic outcomes of insulin and IGF-1 are very distinct. Therefore, signaling pathways unique to insulin have long captured the interest of investigators. Several signaling molecules have been reported to be specifically involved in IR signaling, but not IGF-1R signaling. Among them, pp120, MAD2, Cbl and APS have been shown to specifically interact with the activated IR, but not IGF-1R, and to regulate IR internalization and GLUT4 translocation [17–20]. In addition, FAK has been reported to become dephosphorylated by insulin, while it became phosphorylated by IGF-1 [21]. These data suggest that the existence of specific differential signaling of insulin in addition to those that overlap with IGF-1 signaling. Our results suggest that BCAR3 is involved in the differential signaling pathway unique to insulin.

We also observed the direct association of BCAR3 with tyrosine-phosphorylated proteins such as Cas, p95 and p60. More importantly, this association was differentially regulated between insulin and IGF-1. The association of BCAR3 with p95 and p60 may play an important role in not only mitogenic signaling of insulin but also of ERK activation by insulin. Currently, the molecular identity of p95 and p60 is yet to be uncovered. The association of BCAR3 with Cas has been reported and this association is important in cell motility and metastasis [22,23]. BCAR3 and Cas co-localize at the cell membrane, and synergistically increase cell proliferation and transformation with Src oncogenes in breast cancer cells [11]. Although many studies show that BCAR3 regulates cell motility [22,23],

the functional role of BCAR3 in the signaling pathways of growth factors such as insulin and IGF-1 has not completely been elucidated.

We and others reported that BCAR3 itself had a mitogenic activity in normal human breast cells and cancer cells [5,6,9]. There is also growing evidence that insulin and IR play a key role in cancer both *in vitro* and *in vivo* [24]. Further, studies show that elevated levels of insulin and IR are present in human breast tumors and are involved in tumor growth [25–27]. For these reasons, insulin mutant analogs and diabetic drugs have been actively applied in chemotherapeutics. Our results on the role of BCAR3 in insulin-specific mitogenic signals suggest that further investigation on pathways related to BCAR3 can yield potential therapeutics of breast cancer and may elucidate mechanisms of antiestrogen resistance.

In summary, we have shown that BCAR3 regulates insulin-induced ERK activation, DNA synthesis and membrane ruffling. Our results demonstrate, for the first time, that BCAR3 is a key mediator in cell cycle progression induced by the signaling pathway of insulin, but not of IGF-1. Specifically, the SH2 domain of BCAR3 is involved in insulin-stimulated DNA synthesis. Different tyrosine-phosphorylated patterns in the BCAR3 immune complex were detected in insulin and IGF-1 signaling, suggesting that BCAR3 has distinct target molecules in insulin and IGF-1 signaling. Taken together, these results demonstrate that BCAR3 protein plays a differential role in the DNA synthesis during insulin and IGF-1 signaling. Furthermore, BCAR3 is involved in the cytoskeleton reorganization stimulated by insulin, but not in GLUT4 translocation.

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