

GAIP-Interacting Protein, C-Terminus Is Involved in the Induction of Zinc-Finger Protein 143 in Response to Insulin-like Growth Factor-1 in Colon Cancer Cells

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Previously, we reported that the expression of zinc-finger protein 143 (ZNF143) was induced by insulin-like growth factor-1 (IGF-1) via reactive oxygen species (ROS)- and phosphatidylinositol-3-kinase (PI3-kinase)-linked pathways in colon cancer cells. Here, we investigated whether GAIP-interacting protein, C-terminus (GIPC), a binding partner of IGF-1R, is involved in ZNF143 expression through IGF-1 and IGF-1R signaling in colon cancer cells. The knockdown of GIPC in colon cancer cells reduced ZNF143 expression in response to IGF-1. IGF-1 signaling through its receptor, leading to the phosphorylation and activation of the PI3-kinase-Akt pathway and mitogen-activated protein kinases (MAPKs) was unaffected by the knockdown of GIPC, indicating the independence of the GIPC-linked pathway from PI3-kinase- and MAPK-linked signaling in IGF-1-induced ZNF143 expression. In accordance with previous results in breast cancer cells (Choi et al., 2010), the knockdown of GIPC reduced ROS production in response to IGF-1 in colon cancer cells. Furthermore, the knockdown of GIPC reduced the expression of Rad51, which is regulated by ZNF143, in response to IGF-1 in colon cancer cells. Taken together, these data suggest that GIPC is involved in IGF-1 signaling leading to ZNF143 expression through the regulation of ROS production, which may play a role for colon cancer tumorigenesis.

INTRODUCTION

Insulin-like growth factor-1 (IGF-1) is thought to regulate a variety of cellular processes, including cell survival and proliferation, by binding to the IGF-1 receptor (IGF-1R) on cell surfaces and initiating intracellular signaling cascades (LeRoith and Roberts, 2003; Miller and Yee, 2005; Ouban et al., 2003; Pollak, 2008; Rodon et al., 2008; Tao et al., 2007).

Previously, IGF-1/IGF-1R signaling was implicated in drug resistance and DNA repair mechanisms (Decraene et al., 2002; Eckstein et al., 2009; Turner et al., 1997). Trojanek and colleagues showed that IGF-1 protects against cisplatin-induced

cytotoxicity and that IGF-1/IRS1 participates in homologous recombination-directed DNA repair by regulating Rad51 localization, thereby supporting genome stability (Trojanek et al., 2003). However, the identities of the factors induced by IGF-1/IGF-1R signaling and the detailed mechanisms by which they enhance cell survival have remained unknown.

The results of yeast two-hybrid assays have shown that the PDZ domain-containing protein GAIP-interacting protein, C-terminus (GIPC) binds to the C-terminus of IGF-1R (Ligensa et al., 2001), linking it to trimeric G proteins (Booth et al., 2002). Recently, GIPC was implicated in pancreatic adenocarcinoma (Muders et al., 2006; 2009) and breast cancer (Choi et al., 2010; Lee et al., 2010). GIPC reportedly associates with various cellular proteins, including type III transforming growth factor- β receptor (Blobe et al., 2001) and human papillomavirus type 18 E6 protein (Favre-Bonvin et al., 2005). In addition, GIPC is important for the internalization of cell surface receptors (Naccache et al., 2006). Despite much research, many questions concerning the specific role of GIPC in IGF-1 signaling leading to cell proliferation and survival remain to be answered.

Zinc-finger protein 143 (ZNF143) is a human homolog of the *Xenopus* transcriptional activator Staf. ZNF143 has been implicated in DNA-damage responses and the resistance of cancer cells to cisplatin, suggesting that the protein plays a role in carcinogenesis and cancer cell survival (Ishiguchi et al., 2004; Wakasugi et al., 2007). Previously, we showed that IGF-1 induced ZNF143 expression through reactive oxygen species (ROS) and phosphatidylinositol 3-kinase (PI3-kinase)-linked signaling pathways (Paek et al., 2010). However, several questions remain to be answered, such as how IGF-1R induces ZNF143 expression.

Here, we investigated the possibility that the IGF-1R binding protein, GIPC is involved in the induction of ZNF143 in response to IGF-1 in cancer cells. We used knockdown experiments to examine whether GIPC is important in cancer cell signaling leading to ZNF143 expression in response IGF-1 in colon cancer cells.

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MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and defined fetal bovine serum (FBS) were obtained from HyClone (USA). IGF-1 was purchased from R&D Systems, Inc. (USA). Mouse monoclonal antibodies against β -actin and ZNF143, rabbit polyclonal antibodies against IGF-1R, and goat polyclonal antibodies against GIPC were obtained from Santa Cruz Biotechnology Inc. (USA). Rabbit polyclonal antibodies against phospho-Akt⁴⁷³, phospho-Akt³⁰⁸, phospho-ERK1/2, phospho-p38 kinase, phospho-JNK, Akt, and HRP-conjugated anti-mouse and anti-rabbit antibodies were purchased from Cell Signaling Technology Inc. (USA). Short hairpin (sh)RNA-lentiviral particles against human GIPC, ZNF143, and the control were acquired from Santa Cruz Biotechnology Inc.

Cell culture

The human colon carcinoma cell line HCT116 was obtained from the American Type Culture Collection (USA). HCT116 cells were maintained as monolayers in DMEM. All maintenance media were supplemented with 10% heat-inactivated FBS. All cells were grown at 37°C in a humidified 5% CO₂ atmosphere.

shRNA-mediated silencing of human GIPC and ZNF143 in HCT116 cells

To achieve the stable lentivirus-mediated expression of shRNA specific for the genes encoding GIPC and ZNF143 in HCT116 cells, cells were grown for 24 h, incubated with 5 μ g/ml polybrene for 1 h, and then infected with the lentiviral vector (approximately 1 molar ratio of infection). After 48 h, the medium was replaced and the cells were grown for 1 day. The control cell lines (HCT116 sh-control) and cell lines stably expressing ZNF143 shRNA (HCT116 sh-ZNF143) or GIPC shRNA (HCT116 sh-GIPC) were selected in 10 μ g/ml puromycin dihydrochloride for 2 weeks and maintained in growth medium containing 1 μ g/ml puromycin dihydrochloride. To avoid clonal variation, the individual clones for each stable cell line produced by infection were pooled (Choi et al., 2010; Paek et al., 2010).

Measurement of intracellular ROS by flow cytometry

Cells (10⁵ cells/ml) were grown in 6-well plate for 24 h and starved for an additional 24 h. Next, cells were treated with 100 ng/ml of IGF-1 for the indicated times, washed with warm phosphate-buffered saline (PBS), trypsinized, and quickly analyzed for green fluorescence by flow cytometry as described previously (Rhee et al., 2010; You et al., 2004). For ROS detection, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA, 10 μ M) was added 10 min before harvest, and analysis was carried out with a FACS-Calibur (Becton-Dickinson, USA) by the NCC FACS operator. The cells were sorted at approximately 500 cells/s using saline as the sheath fluid and a 488-nm argon laser beam for excitation. A two-parameter dot-plot of the side light scatter (SSC) and forward light scatter (FSC) of the population was analyzed, and the DCF fluorescence of 10,000 gated cells was measured using log amplification. The arithmetic geometric mean fluorescence channel (Geo MFC) was derived with CellQuest.

Immunoblotting

Protein samples were heated to 95°C for 5 min, separated by 8 or 10% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidene difluoride membranes for 1 h at 350 mA using a Bio-Rad transfer unit (Bio-Rad Laboratories, Inc., USA). The membranes were

blocked for 1 h in Tris-buffered saline containing 0.01% Tween 20 (TBST) and 5% nonfat dried milk, incubated for 2 h with primary antibody in TBST containing 2% bovine serum albumin (BSA), and then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies. The blots were developed using an enhanced chemiluminescence kit (West-ZOL® plus, Western Blot Detection System, Intron Biotechnology Inc., South Korea).

Statistical analysis

All data are expressed as percentages of the control and shown as means $\pm$ S.E. Statistical comparisons between groups were made using paired *t*-tests with Prism 5.0 statistical software (GraphPad Software Inc., USA). Values of *p* < 0.05 were considered significant.

RESULTS AND DISCUSSION

GIPC is involved in ZNF143 expression in response to IGF-1 in HCT116 cells

Previously we showed that IGF-1 induced ZNF143 expression in HCT116 cells (Paek et al., 2010). GIPC, a binding protein of IGF-1R, has also shown to be involved in ROS generation in response to IGF-1 in breast cancer cells, pancreatic cancer cells and epithelial carcinoma cells (Choi et al., 2010). These studies suggested a role for GIPC in ZNF143 expression through ROS production in response to IGF-1. To test this hypothesis, we investigated whether GIPC plays a role in the induction of ZNF143 in response to IGF-1 in a human colon carcinoma cell line (HCT116 cell). First, we expressed shRNA against GIPC stably in HCT116 cells using lentiviral particles and puromycin selection (HCT116 sh-GIPC; Fig. 1A). To clarify the involvement of GIPC in ZNF143 expression in response to IGF-1, HCT116 sh-control and HCT116 sh-GIPC cells were starved and incubated with 100 ng/ml IGF-1 or buffer for 6 h. As shown in Fig. 1B, ZNF143 expression in response to IGF-1 was reduced by the knockdown of GIPC expression, demonstrating a role for GIPC in ZNF143 expression through IGF-1/IGF-1R signaling.

GIPC may be involved in IGF-1 signaling leading to ZNF143 expression independently of PI3-kinase/ERK/p38-kinase/JNK in response to IGF-1

To determine the mechanism underlying GIPC involvement in IGF-1 signaling leading to ZNF143 expression in colon cancer cells, we investigated whether GIPC affects IGF-1R expression by regulating its recycling or degradation, since GIPC was shown to bind through its PDZ domain to IGF-1R utilizing the last three amino acids of the receptor (Booth et al., 2002). Also, deletion of the C-terminus of IGF-1R was shown to be important for IGF-1R ubiquitination and internalization in cells implying a role of adaptor proteins (Sehat et al., 2007). Furthermore, previous studies showed the role of GIPC in the regulation of type III transforming growth factor-beta receptor on the cell surface (Blobe et al., 2001; Finger et al., 2008; Lee et al., 2010). Thus we first used immunoblotting to examine whether the downregulation of GIPC affects the protein expression of IGF-1R after ligand binding. As shown in Fig. 2A, IGF-1R expression was reduced after ligand binding in HCT116 sh-control cells and HCT116 sh-GIPC cells, suggesting that the knockdown of GIPC does not play an essential role in regulating IGF-1R half-life in HCT116 cells. Next, we examined whether the knockdown of GIPC might affect IGF-1/IGF-1R signaling through PI3-kinase/Akt, ERK, p38 kinase and c-Jun N-terminal kinase (JNK). IGF-1 activates various signaling pathways that enhance

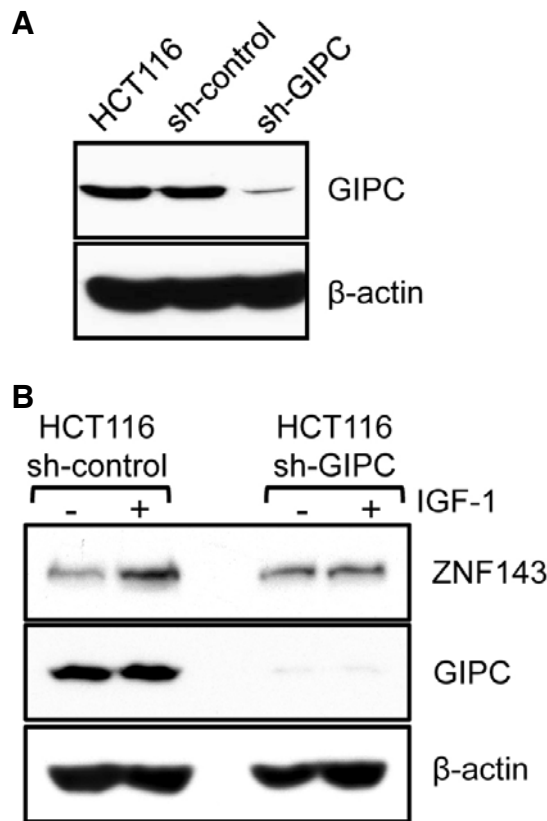


Fig. 1. Role of GIPC in ZNF143 expression in HCT116 cells. (A) HCT116, HCT116 sh-control, and HCT116 sh-GIPC cells were harvested to confirm the expression level of GIPC by immunoblotting. (B) HCT116 sh-control and HCT116 sh-GIPC cells were starved for 24 h and incubated with 100 ng/ml IGF-1 for 6 h, after which cells were washed with cold PBS and prepared for SDS-PAGE and immunoblotting. The results shown are representative of at least three independent experiments.

cell survival and proliferation, including pathways mediated by PI3-kinase/Akt (Kenchappa et al., 2004; Xu et al., 1999) and MAPKs (Chow et al., 1998; Girmata et al., 2007). Thus, we starved cells for 24 h and incubated for the indicated time periods, after which cells were harvested for immunoblotting. The phosphorylation of Akt on Thr³⁰⁸ and Ser⁴⁷³ increased in response to IGF-1 and the knockdown of GIPC expression did not alter this phenomenon in HCT116 sh-GIPC cells (Fig. 2B), implying that GIPC is not directly involved in the activation of signaling pathways downstream of the PI3-kinase/Akt pathway. This finding is in accordance with our previous results (Choi et al., 2010; Paek et al., 2010).

GIPC may participate in the induction of ZNF143 through IGF-1 by enhancing ROS production

IGF-1 increases ROS generation in treated cells and contributes to the proliferation and migration of vascular smooth muscle cells via Nox4 and Rac1 (Meng et al., 2008; Vardatsikos et al., 2009), leading to ZNF143 expression in colon cancer cells (Paek et al., 2010). To determine whether GIPC plays a role in the IGF-1-ROS-ZNF143 cascade in HCT116 cells, we have measured ROS production in response to IGF-1 and confirmed that ROS generation in response to IGF-1 in HCT116 sh-GIPC cells was much lower than that in HCT116 sh-control cells (Fig.

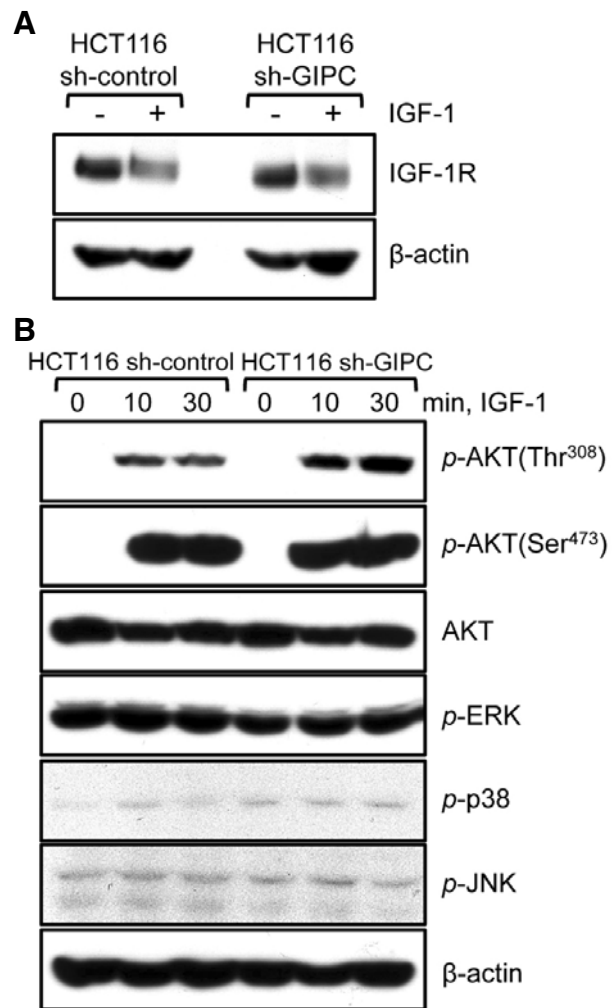


Fig. 2. The effect of GIPC knockdown in Akt, ERK, p38 and JNK activation in response to IGF-1 in HCT116 sh-control and HCT116 sh-GIPC cells. (A, B) Cells were grown in DMEM containing 10% FBS for 24 h and starved for additional 24 h. Then, cells were incubated with IGF-1 for 6 h (A) or for the indicated time periods (B) and harvested for immunoblotting. The results shown are representative of at least three independent experiments.

3, Supplementary Fig. S1). These data suggest that GIPC is involved in IGF-1/IGF-1R-stimulated ZNF143 expression by regulating ROS generation.

GIPC may participate in Rad51 expression in response to IGF-1 by regulating ZNF143 expression

Rad51 is a ZNF143 target gene important for DNA repair (Trojanek et al., 2003; Wakasugi et al., 2007). We showed that the IGF-1-ZNF143 cascade induces Rad51 expression by comparing the induction of Rad51 in response to IGF-1 in HCT116 sh-control and HCT116 sh-ZNF143 cells (Paek et al., 2010). To confirm the role of GIPC in IGF-1 signaling leading to ZNF143 expression, we examined the expression of Rad51 in response to IGF-1 in HCT116 sh-control, HCT116 sh-ZNF143 and HCT116 sh-GIPC cells. As knocking down ZNF143 reduced Rad51 expression in response to IGF-1, knocking down GIPC also attenuated Rad51 expression (Fig. 4), suggesting a role for GIPC in ZNF143 expression in IGF-1-stimulated

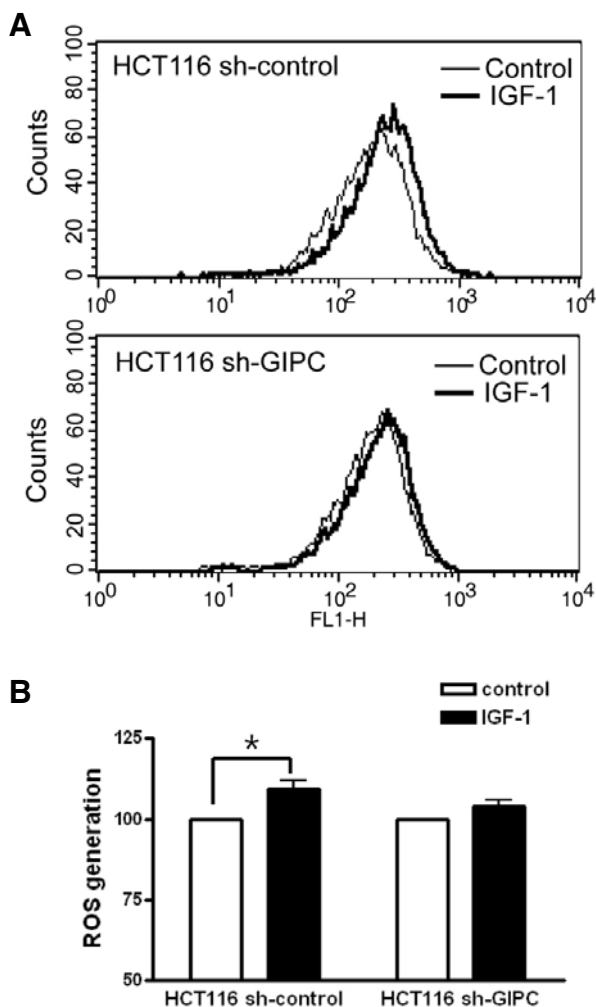


Fig. 3. Role of GIPC in ROS generation in response to IGF-1 in HCT116 sh-control and HCT116 sh-GIPC cells. (A, B) HCT116 sh-control and HCT116 sh-GIPC cells were grown in DMEM containing 10% FBS for 24 h and starved for additional 24 h. Then, cells were incubated with 100 ng/ml IGF-1 or buffer for 30 min, washed and trypsinized. Cells were incubated with 10 μ M DCFH₂DA 10 min before harvest. DCF fluorescence, reflecting the relative level of ROS (arbitrary units), was measured using a FACS caliber flow cytometer. The representative result was shown in (A) and statistical analysis of at least three independent experiments was shown in (B). Data are expressed as means $\pm$ S. E. of at least three independent experiments. Statistical significance was assessed using paired Student's *t* tests (*, *p* < 0.01).

HCT116 cells through ROS production. Also, AG1024, an IGF-1R inhibitor affected ZNF143 expression in HCT116 sh-control cells but not in HCT116 sh-GIPC cells, supporting a role of GIPC in IGF-1/IGF-1R signaling to ZNF143 expression (Supplementary Fig. S2).

In this study, we confirmed that ZNF143, a transcription factor which is implicated in drug resistance, can be induced by IGF-1 as part of the GIPC-ROS cascade. Although our data are interesting, many questions still remain to be answered, including how GIPC can regulate ROS production and signaling pathways leading to ZNF143 expression in IGF-1-stimulated cells. In terms of ZNF143 in cancer cell DNA repair, we are investi-

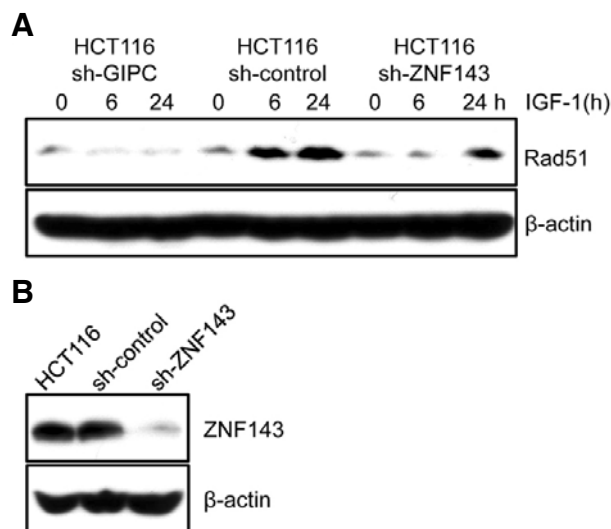


Fig. 4. Role of GIPC and ZNF143 in Rad51 expression in response to IGF-1 in HCT116 cells. (A) HCT116 sh-control, HCT116 sh-GIPC and HCT116 sh-ZNF143 cells were starved and incubated with 100 ng/ml IGF-1 for 0, 6, and 24 h. Then, cells were harvested and prepared for SDS-PAGE and immunoblotting. The results shown are representative of at least three independent experiments. (B) HCT116, HCT116 sh-control, and HCT116 sh-ZNF143 cells were harvested to confirm the expression level of ZNF143 by immunoblotting. The results shown are representative of at least three independent experiments.

gating how ZNF143 and its target genes regulate the DNA-damage response in anticancer drug-treated cells.

In summary, we found that ZNF143 expression can be upregulated by IGF-1 via a mechanism involving ROS and PI3-kinase (Paek et al., 2010). We also confirmed that the binding protein of IGF-1R, GIPC plays a role in IGF-1 signaling leading to ZNF143 expression and that ROS production in response to IGF-1 may be a major pathway in which GIPC is involved. ZNF143 expression was also involved in the expression of Rad51, which is important for DNA repair, in response to IGF-1 in HCT116 cells, and this was also affected by GIPC expression. These data suggest that ZNF143 induction by IGF-1/IGF-1R is involved in cancer cell survival through a variety of cellular processes, including drug resistance and DNA repair. We are currently investigating how ZNF143 and its target genes regulate the characteristics of cancer cells and how GIPC is involved in the regulation of IGF-1R in cancer cells.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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