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Prohibitin-2 is a novel regulator of p21$^{WAF1/CIP1}$ induced by depletion of γ-glutamylcyclotransferase

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ABSTRACT

Previous studies show that gamma-glutamylcyclotransferase (GGCT) is expressed at high levels in various cancer tissues and that its knockdown inhibits MCF7 cancer cell growth via upregulation of p21$^{WAF1/CIP1}$ (p21). However, the detailed underlying mechanism is unclear. Here, we used yeast two-hybrid screening and co-immunoprecipitation to identify Prohibitin-2 (PHB2) as a novel protein that interacts with GGCT. We also show that nuclear expression of PHB2 in MCF7 cells falls upon GGCT knockdown, and that overexpression of PHB2 inhibits p21 upregulation. A chromatin immunoprecipitation assay revealed that nuclear PHB2 proteins bind to the *p21* promoter, and that this interaction is abrogated by GGCT knockdown. Moreover, knockdown of PHB2 alone led to significant upregulation of p21 and mimicked the cellular events induced by GGCT depletion, including G0/G1 arrest, cellular senescence, and growth inhibition, in a p21 induction-dependent manner. Taken together, the results indicate that PHB2 plays a central role in p21 upregulation following GGCT knockdown and as such may promote deregulated proliferation of cancer cells by suppressing p21.

1. Introduction

Proteome analysis identified Chromosome 7 open reading frame 24 as highly expressed in bladder cancer tissues [1,2] and other cancers [2–4]. Later studies revealed that it was identical to gamma-glutamylcyclotransferase (GGCT) [5]. High expression of GGCT protein correlates with a poor prognosis in patients with breast cancer [3]. Depleting GGCT using an RNA interference method blocks proliferation of multiple types of cancer cells *in vitro* [2,4]; moreover, regional injection [6] and systemic administration [7] of small interfering (si)RNAs targeting GGCT show anti-tumor effects in tumor-bearing mouse models.

p21$^{WAF1/CIP1}$ (p21) is a cyclin-dependent kinase inhibitor (CDKI) [8,9], which inhibits CDK2 and is a major transcriptional target of p53; consequently, it induces cell cycle arrest at G1 phase [10]. In addition, p21 plays an important role in inducing cellular senescence [11]. In a previous study, we reported that depleting GGCT induces cellular senescence in multiple cancer cell lines by upregulating CDKIs, including p21, in a cell type-dependent manner [12]. However, the mechanism by which GGCT depletion upregulates p21 is essentially unknown.

Prohibitin (PHB) is a highly conserved protein family that has two homologs: PHB1 and PHB2 [13]. Both PHB1 and PHB2 are present in the nucleus, mitochondria, and cytosol, and each exerts a distinct function depending on its intracellular localization [14,15]. Nuclear PHB1 and 2 function independently as transcriptional repressors of target genes [15], while mitochondrial PHBs form a heterodimer that plays an important role in mitochondrial stability. In particular, PHB2 was characterized as the repressor of estrogen

Abbreviations: GGCT, gamma-glutamylcyclotransferase; p21, p21$^{WAF1/CIP1}$; CDKI, cyclin-dependent kinase inhibitor; PHB, Prohibitin; ER, Estrogen receptor; SA-β-gal, senescence-associated β-galactosidase.

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receptor activity (REA) [16]. Mitochondrial PHB2 proteins migrate to the nucleus in HeLa cells stimulated by estrogen, thereby inhibiting ERα-dependent transcription [17]. Importantly, PHBs are highly expressed by multiple types of cancer tissue, including colorectal, lung, esophagus, and urinary bladder cancers; also, serum samples from patients with colorectal cancer show higher expression of PHBs than those from non-cancer volunteers [18]. In addition, hepatocellular carcinoma tissues show markedly greater upregulation of PHB2 than adjacent non-tumor liver tissues [19].

Here, we identified a novel binding partner of GGCT protein and clarified the mechanism underlying GGCT depletion-mediated growth inhibition of cancer cells. We identified PHB2, which regulates expression of p21 and thereby plays a central role in cancer cell phenotypes upon GGCT depletion.

2. Materials and methods

2.1. Yeast two-hybrid screening

A standard yeast two-hybrid screening method was performed according to the ProNet technology manufacturer's protocol (Myriad Genetics). Briefly, cDNA encoding human GGCT amino acids 97–189 (used as bait) was subcloned into the pGBT.superB Gal4 DNA-binding domain (BD) expression vector. A breast/prostate cancer cDNA library (used as prey) was subcloned into the pGAD.PN2 Gal4 activation domain (AD) expression vector. As a negative control, AD fusion prey derived from the six selected genes was used to confirm specificity. The sequences of the two positive colonies (out of three colonies that stained blue) were identified using BLAST (NCBI).

2.2. Cells and culture

MCF7 cells were obtained from RIKEN BRC and cultured in DMEM supplemented with 10% fetal bovine serum (HyClone, South Logan, UT) and 1% penicillin and streptomycin. Cells were maintained under 5% CO_2 at 37 °C.

2.3. Antibodies

The following antibodies were purchased: mouse monoclonal antibodies specific for PHB2 (Santa Cruz Biotechnology, Dallas, TX), GGCT (Cosmo Bio, Tokyo, Japan), GAPDH (Wako Pure Chemical Industries, Osaka, Japan), p21 (BD Biosciences, Franklin Lakes, NJ), and the V5 epitope tag (V5) (Thermo Fisher Scientific, Waltham, MA), and a rabbit monoclonal antibody against Lamin A/C (Cell Signaling Technology, Danvers, MA). Horse anti-mouse IgG-HRP conjugates were purchased from Vector Laboratories (Burlingame, CA). The HRP-linked goat anti-rabbit IgG and mouse IgG1 isotype controls were purchased from Cell Signaling Technology.

2.4. Co-immunoprecipitation

The antibody-bead complexes were prepared by incubating 10 μg of anti-GGCT or isotype control antibody with 1.5 mg of Dynabeads Protein G (Thermo Fisher Scientific). MCF7 cells were cultured in two 10 cm dishes and then lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate-Na, and 0.1% SDS) supplemented with a protease inhibitor cocktail mix (Nacalai Tesque, Kyoto, Japan). Mixtures comprising cell lysate and each antibody-bead complex were rotated overnight at 4 °C. After washing with PBS, the complexes were eluted in SDS-sample buffer, denatured at 95 °C for 5 min, and analyzed by Western blotting as described below.

2.5. Transfection of siRNAs

Transient transfection of siRNAs was performed using Lipofectamine RNAi MAX (Invitrogen) as described previously [12]. Synthesized siRNAs were purchased from RNAi Co. LTD, Tokyo, or from Gene Design Inc., Osaka, Japan. The siRNA sequences are listed in Table S1.

2.6. Fractionation of nuclear/cytoplasmic proteins

MCF7 cells were seeded in 6-well plates and transfected with GGCT or non-targeting siRNA. At 4 days post-transfection, cellular proteins were fractionated into cytoplasmic and nuclear fractions using a LysoPure Nuclear and Cytoplasmic Extractor Kit (Wako) according to the manufacturer's protocol.

2.7. Western blot analysis

Western blot analysis was performed as described previously [12]. Briefly, cell lysates were prepared in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate-Na, and 0.1% SDS) supplemented with a protease inhibitor cocktail mix (Nacalai Tesque). The lysates or immunoprecipitation products were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Burlington, MA). After blocking with 3% fat-free dry milk in PBS/0.05% Tween-20 (PBST), or PVDF Blocking Reagent for *Can get signal* (TOYOBO, Osaka, Japan), the membranes were incubated with primary antibodies in 3% BSA/PBST or *Can get signal* Immunoreaction Enhancer Solution (TOYOBO). Proteins were visualized using the Clarity Western ECL Substrate (Bio-Rad, Hercules, CA) and detected using the ChemiDoc XRS Plus system (Bio-Rad).

2.8. Preparation of PHB2-overexpressing cells

The pENTR221-PHB2 vector was obtained from DNAFORM (clone no. 100011434, Yokohama, Japan). The pENTR221-PHB2 vector was then recombined into the pEF-DEST51 vector using a Gateway LR Clonase II Enzyme Mix (Thermo Fisher Scientific) according to the manufacturer's protocol. Next, 2×10^6 MCF7 cells were transfected with 2 μg of V5-tagged PHB2 vector or pEF-DEST51 empty vector by electroporation using Amaxa Cell Line Nucleofector Kit V and a Nucleofector™ 2b Device (Lonza, Visp, Switzerland) with a P-020 program optimized for MCF7 cells. DMEM containing 10 μg/ml blasticidin was used for clone selection.

2.9. Cell cycle analysis

Cell cycle analysis was performed as described previously [12]. Briefly, MCF7 cells were seeded in 6-well plates and transfected with GGCT, PHB2, p21, or non-targeting siRNA. The cells were then fixed in 70% ethanol at −20 °C and stained with 20 μg/ml propidium iodide containing 200 μg/ml RNase A. DNA content was analyzed using a FACSCalibur flow cytometer (BD Biosciences). At least 10,000 cells were analyzed for each sample, and data were analyzed using CellQuest Pro software (BD Biosciences).

2.10. Chromatin immunoprecipitation

At 4 days post-transfection with siRNA, 5×10^6 MCF7 cells were crosslinked with 1% formaldehyde. To generate DNA fragments, lysates were sonicated (H-amplitude, 12 pulses for 30 s each) using a Bioruptor UCD-250 (Cosmo Bio). Anti-PHB2 or isotype control antibody (final concentration, 5 μg/ml) was added to the supernatants, which were then rotated overnight at 4 °C. The reaction products were then incubated with 1.5 mg of Dynabeads Protein G

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