Grap2 cyclin D interacting protein negatively regulates CREB-binding protein, inhibiting fibroblast-like synoviocyte growth

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Received June 17, 2020; Accepted October 19, 2020

DOI: 10.3892/mmr.2021.11916

Abstract. Rheumatoid arthritis (RA) is one of the most critical articular diseases, which is characterized by synovial hyperplasia and impaired quality of life. The clinical features of RA include chronic inflammation of the joints associated with synovial cell overgrowth. However, the mechanism regulating the outgrowth of fibroblast-like synoviocytes (FLS) is not fully understood. The present study reported that grap2 cyclin D interacting protein (GCIP), an inhibitor of DNA binding/differentiation (ID)-like helix-loop-helix protein, interacted with cAMP-response element-binding protein (CREB)-binding protein (CBP). Furthermore, GCIP repressed CREB- and NF-κB-dependent gene expression by inhibiting CBP binding to RNA polymerase II complexes. GCIP depletion via small interfering RNA enhanced FLS growth, whereas stable GCIP expression suppressed the growth of 293 cells. In addition, GCIP depletion in FLS induced the expression of cyclin D1, a CREB target gene. The present study identified a novel inhibitory mechanism in which an ID protein may functionally target the transcriptional coactivator CBP. These results suggested that GCIP downregulation may be pivotal in FLS outgrowth.

Introduction

Rheumatoid arthritis (RA) is one of the most common articular diseases, affecting 0.5 to 1% of the world population and resulting in disability due to joint destruction (1). Recently, biologic agents targeting proinflammatory cytokines have greatly improved the treatment of patients with RA. However, approximately 30% of RA patients are resistant to these therapies, suggesting that other factors are involved in RA physiopathology (2). Fibroblast-like synoviocytes (FLS) play a unique role in both inflammation and joint destruction. FLS are resistant to apoptosis and consequently overgrow, promoting the synthesis of molecules that mediate joint destruction and inflammation (3,4). *In vitro* studies have demonstrated far greater proliferation and cytokine production in synovial cells derived from RA than those derived from osteoarthritis (OA) patients (5,6). However, the mechanism that regulates synovial cell outgrowth remains incompletely understood.

Our previous studies implicated transcription factors, such as NF-κB and Jun proto-oncogene, AP1 transcription factor subunit (JUN), in the regulation of FLS proliferation, through the recruitment of the coactivator cAMP-responsive element-binding protein (CREB)-binding protein (CBP) (7,8). CBP is involved in multiple cellular processes and functions. CBP acts as a transcriptional coactivator and a histone acetyltransferase (HAT) by interacting with several transcription factors, including CREB (9,10), and a variety of nuclear hormone receptors (11). After binding various transcription factors, CBP associates with RNA helicase A (RHA)/DExH-box helicase 9, resulting in the recruitment of RNA polymerase II (Pol II) complexes (12).

A subset of helix-loop-helix (HLH) proteins called inhibitor of DNA binding/differentiation (ID) proteins function as global regulators of cell fate determination. They play pivotal roles in the coordinated regulation of gene expression during cell growth, cell cycle control, differentiation, tumorigenesis (13,14), and function by directly associating with and modulating the activity of several families of transcriptional regulators (15-17). ID proteins contain an HLH region for dimerization but lack a basic DNA binding domain. Therefore, these proteins act as transcription dominant-negative repressors by dimerizing with and sequestering ubiquitously expressed class A E-box HLH proteins (18), and in some cases, class B (tissue-specific) HLH proteins (17).

Grap2 cyclin-D interacting protein (GCIP)/cyclin D1 binding protein 1 (CCNDBP1) was originally identified by yeast two-hybrid screening. It is expressed in all human

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Key words: rheumatoid arthritis, fibroblast-like synoviocyte outgrowth, grap2 cyclin D interacting protein, CREB-binding protein

tissues and particularly highly expressed in the heart, muscles, peripheral blood leukocytes, kidneys, and brain-all associated with limited cell differentiation and/or proliferation (19). Like ID proteins, GCIP possesses an HLH domain but no basic domain. The amino acid sequence of the GCIP HLH domain shares little identity with that of the ID proteins; however, it has 78%homology with MAID, the maternal ID-like protein. GCIP and MAID also functionally inhibit E12/myogenic differentiation 1 activities (20). Transient expression of GCIP reduces the phosphorylation of RB transcriptional corepressor 1 (RB1) by cyclin-dependent protein kinases, and represses E2F transcription factor 1-mediated transcription (21). Recently, GCIP was shown to suppress hepatocyte growth, as well as cancer growth (22-24). However, the nuclear functions of GCIP are not fully understood.

In the present study, we aimed to clarify the molecular mechanism controlling FLS growth, and identify GCIP as a CBP interacting protein. Our results demonstrate that GCIP represses CREB-dependent transcription by inhibiting interactions between CBP and Pol II, suggesting a novel inhibitory mechanism used by ID-family HLH proteins.

Materials and methods

Plasmids and antibodies. The coding sequence for full-length *GCIP* was PCR-amplified from pACT-GCIP, derived a previous yeast two-hybrid screening. A series of deletion mutants were generated by PCR. Full-length and deletion mutant versions of *GCIP* were inserted into pGEX-5X-1 (GE Healthcare) for GST pulldown assays. For transient transfection, these fragments were inserted into pcDNA3-HA. The sequences of all generated plasmids were confirmed by sequencing analysis. The RHA and CBP plasmids, PKA wild-type (wt), PKA mutant, lacking kinase activity, pGAL4-CREB and pGAL4 expression vectors, Som-Luc, pG5b-Luc, and NF-κB-Luc reporter plasmids, and the control plasmid RSV-β-gal have all been previously described (12,25,26). The following antibodies were used: anti-FLAG (M2), anti-HA (12CA5 and 3F10), anti-β-actin, and anti-cyclin D1 from Sigma-Aldrich (Merck KGaA), anti-Pol II (Progen Biotechnik GmbH), anti-CBP (Upstate Biotechnology, Inc.) and anti-His and anti-GST (GE Healthcare). Anti-GCIP rabbit polyclonal antiserum was generated against GST-GCIP (Tanpaku Seisei Kougyou). Anti-CBP rabbit polyclonal antiserum and anti-RHA antibodies have been previously described (12).

Cell culture, transient transfection and stable cell line generation. Patients with RA were receiving stable doses of methotrexate (6-10 mg/week) before joint replacement surgery. Written informed consent was obtained from all patients prior to collection of joint tissue samples. RA and OA samples were collected from Bayside Misato Medical Center (Kochi, Japan). Samples were collected from 6 patients with RA (age range, 64-78 years; mean age, 68.5 year; sex, female) between February 2012 and May 2013. Samples were collected from 6 patients with OA (age range, 67-80 years; mean age, 71.7 years; sex, female) between February 2012 and April 2012. Human FLS were obtained from patients with RA and OA by standard methods as previously described (27). Briefly, the synovial tissue was minced and digested with collagenase (Sigma-Aldrich; Merck KGaA). The adherent cells were cultured in dishes in Dulbecco's modified Eagle's medium (DMEM). 293 cells, 293T cells and FLS were cultured in Dulbecco's modified Eagle's medium (DMEM) as previously described (26,28). 239 cells were transfected with pcDNA3-HA GCIP plasmid or pcDNA3-HA plasmids (control). 293 cells stably expressing HA-GCIP or HA alone were selected and maintained in DMEM containing 400 μg/ml G418. Transient transfection assays were performed with 293 cells. Cells were lysed with cell lysis buffer (Toyo Ink Group) 24 h after transfection, and luciferase activities were measured. Reporter activity was induced by co-transfection with the PKA expression vector. -293 cells were transfected with 100 ng of CRE-Luc or pG5B-Luc reporter plasmid, 50 ng of wild-type or catalytically inactive PKA expression vector (PKAwt or PKAmut, respectively), 50 ng of RSV-β-gal control plasmid. For assay with the pG5B-Luc reporter plasmid, cells were co-transfected with 100 ng of GAL4-CREB expression vector. 293 cells transfected with NF-κB-Luc were treated with 100 ng/ml TPA or 10 ng/ml TNF-α for 24 h. Cells were lysed with a passive lysis buffer (Promega Corporation) 48 h after transfection and luciferase activities were measured and normalized to the activity of RSV-β-gal. All experiments were performed in triplicate. To ensure equal amounts of DNA, empty plasmids were added to each transfection (20).

GST pulldown assays. GST fusion proteins were expressed and purified using glutathione (GSH) Sepharose beads (GE Healthcare). ^{35}S-labeled GCIP or cell extracts were incubated with GST fusion proteins bound to the resin in 1 ml of buffer A (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% NP-40, 5% glycerol, 1 mM Na_3VO_4, 5 mM NaF, 1 μg/ml aprotinin and 1 μg/ml leupeptin) for 4 h at 4˚C. After washing with buffer A, bound proteins were resolved by SDS-PAGE and exposed to an X-ray film.

Immunoprecipitation. 293T cells were transfected with HA-GCIP and FLAG-CBP expression vectors. After 48 h, the cells were lysed in 1 ml of lysis buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1% NP-40, 5% glycerol and protease inhibitors). The lysates were mixed with 1 μg of anti-HA antibody (3F10), anti-FLAG antibody (M2), or anti-CBP antiserum conjugated to protein G-Sepharose beads (GE Healthcare). After 4 h of incubation at 4˚C, the beads were washed three times with lysis buffer. Bound proteins were resolved by SDS-PAGE and analyzed by western blotting.

Immunofluorescence. Staining was performed as previously described (28). Briefly, cells were permeabilized with 0.2% Triton X-100, then incubated with rat anti-GCIP (1:100) and rabbit anti-CBP (1:100) primary antibodies followed by Alexa Fluor 594 anti-mouse and Alexa Fluor 488 anti-rabbit secondary antibodies (1:1,000; Molecular Probes). Samples were imaged on a Zeiss LSM 510 laser scanning confocal microscope.

Western blotting. The cells were lysed in 1 ml lysis buffer (20 mM HEPES, pH 7.5; 100 mM KCl; 1 mM EDTA; 1 mM DTT; 0.1% NP-40; 5% glycerol and protease inhibitors). Proteins were quantified using the Lowry Assay (Bio-Rad

Laboratories, Inc.) and 30 μg proteins were separated by 10% SDS-PAGE, and transferred onto a PVDF membrane. The membrane was incubated in 5% skim milk/TBS-T for 1 h at room temperature and then with rabbit polyclonal anti-GCIP antibody, which was generated against GST-GCIP (1:200; Tanpaku Seisei Kougyou) at 4˚C overnight. The membrane was washed three times and incubated with HRP-conjugated anti-rabbit secondary antibody (1:1,000; cat. no. A9169; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. Proteins were detected by ECL plus (GE Healthcare) and exposed to an X-ray film. Band intensity was measured using ImageJ software (version 1.53f; National Institutes of Health).

RNAi, proliferation assays and reverse transcription-quantitative PCR (RT-qPCR). GCIP siRNAs were purchased from Ambion and transfected into cells with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, 24 h before transfection, cells growing exponentially were trypsinized and transferred to a 96-well plate. Proliferation was determined by assaying viable cell numbers using the Cell Counting Kit-8 (Dojindo Molecular Technologies) according to the manufacturer's protocol and the BrdU Cell Proliferation Assay Kit (Merck Millipore). RT-qPCR was performed using the LightCycler 480 Probes Master Mix (Roche Diagnostics). Expression levels were normalized to the 18S rRNA gene levels. Two sets of primers/probes were used for PCR: *GCIP*, 5'-GAAGCCACGACTCTGACCAT-3' and 5'-GATGGCAGC ATGGACTTGT-3' (probe #86), *18S rRNA*, 5'-GCAATTATT CCCCATGAACG-3' and 5'-GGGACTTAATCAACGCAA GC-3' (probe #48).

Statistical analysis. All data are expressed as the means standard deviation (SD) and were analyzed using Excel Statistics 2012 version 1.00 (SSRI Japan Co., Ltd., Tokyo). One-way analysis of variance with a Tukey-Kramer post hoc analysis was used to compare data among multiple groups. Differences between two groups were examined using unpaired Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Study approval. The human experimental protocols in this study (approval nos. 2728 and 2729) were approved by the Ethics Review Committee of Tokyo Medical University. Written informed consent was obtained from all patients prior to the collection of joint tissue samples. Of note, all the experiments were performed in accordance with the relevant guidelines and regulations.

Results

GCIP interacts with CBP. To identify proteins that interact with CBP, we performed a yeast two-hybrid screen using the CBP C/H3 domain as bait (29) and a library of FLS-derived cDNAs as preys, and obtained clones expressing GCIP. Consistently with this result, CBP interacted with GCIP *in vitro* (Fig. 1A). To verify the interaction *in vivo*, we transiently transfected 293T cells with HA-GCIP and FLAG-CBP and performed immunoprecipitation followed by immunoblotting. FLAG-CBP coimmunoprecipitated with HA-GCIP, and HA-GCIP coimmunoprecipitated with FLAG-CBP (Fig. 1B).

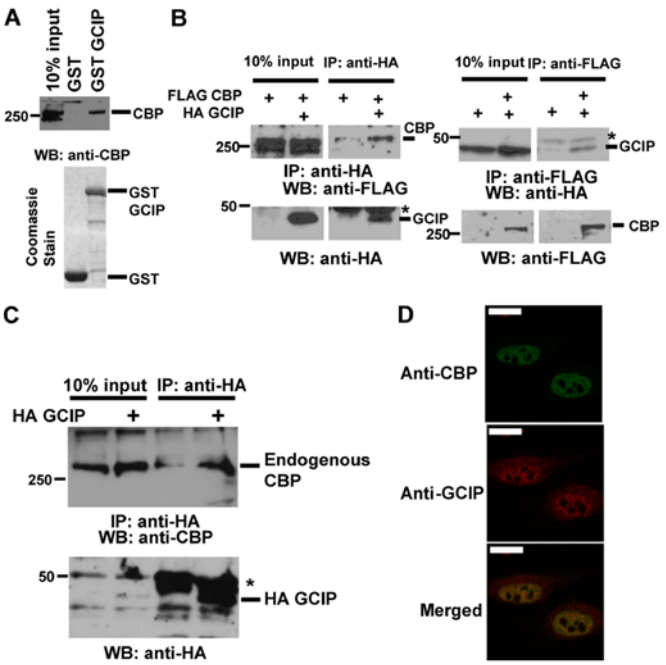

Figure 1. GCIP interacts with CBP *in vitro* and *in vivo*. (A) GST fusion proteins were incubated with nuclear extracts from 293T cells. GST and GST-GCIP were detected by Coomassie Brilliant Blue staining (lower panel). (B) Nuclear extracts from 293T cells transfected with HA-GCIP and/or FLAG-CBP expression plasmids were immunoprecipitated with anti-HA (left panel) or anti-FLAG (right panel) antibodies. * indicates the IgG heavy chain. (C) Nuclear extracts from 293T cells expressing HA-GCIP were immunoprecipitated with anti-HA antibodies and immunoblotted with anti-CBP antibodies. * indicates the IgG heavy chain. (D) 293 cells were immunoassayed with anti-CBP (green) and anti-GCIP (red) antibodies. Colocalization is represented in yellow in the overlay images. Scale bars, 20 μm. These experiments were repeated at least three times. GCIP, grap2 cyclin D interacting protein; CBP, cAMP-response element-binding protein-binding protein.

To further investigate the physiological interaction between GCIP and CBP, we transiently transfected 293T cells with HA-GCIP and performed immunoprecipitation followed by immunoblotting. As showed in Fig. 1C, endogenous CBP coimmunoprecipitated with HA-GCIP. In addition, we performed replicated the same experiment using FLS. Results show that the endogenous CBP interacted with HA-GCIP (Fig. S1A). Next, we investigated the subcellular localization of these proteins by immunofluorescence. Endogenous GCIP displayed both nuclear and cytoplasmic localization in 293 cells (Fig. 1D), while endogenous CBP was observed in the nucleus. The nuclear dots of GCIP partially overlapped with endogenous CBP. These results indicate that GCIP physically interacts with CBP in the nucleus.

Mapping the interaction domains of GCIP and CBP. GCIP contains an HLH domain in its central region. An aspartic/glutamic acid-rich domain (Acidic) and a potential leucine zipper (LZ) motif were detected in protein the C-terminal, near the HLH domain (Fig. 2A). To determine which portion of GCIP mediates its interaction with CBP, we performed GST pulldown assays using several GCIP deletion mutants and nuclear extracts from 293 cells. As showed in Fig. 2B, CBP bound to the central region of GCIP containing the HLH, Acidic and LZ domains. We then determined the

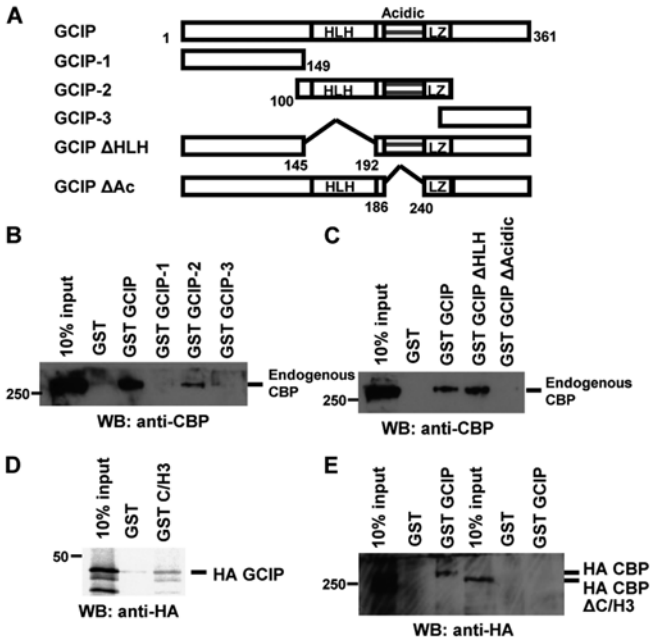

Figure 2. Determination of the binding interfaces between GCIP and CBP. (A) Schematic representation of GCIP. HLH: Helix-loop-helix domain, Acidic: Aspartic/glutamic acid-rich domain, LZ: leucine zipper. (B and C) *In vitro* binding assays were performed using 293T cell extracts and either GST or GCIP deletion mutants fused to GST. Western blot analysis was performed with anti-CBP antiserum. (D) GST or GST-C/H3 was incubated with *in vitro* translated, ^{35}S-labeled GCIP. Samples were resolved by SDS-PAGE and signals were detected by autoradiography. (E) *In vitro* binding assays were performed using extracts from 293T cells expressing HA-CBP or HA-CBP ΔC/H3 and GST or GST-GCIP. Samples were resolved by SDS-PAGE and immunoblotted with anti-HA antibodies. GCIP, grap2 cyclin D interacting protein; CBP, cAMP-response element-binding protein-binding protein.

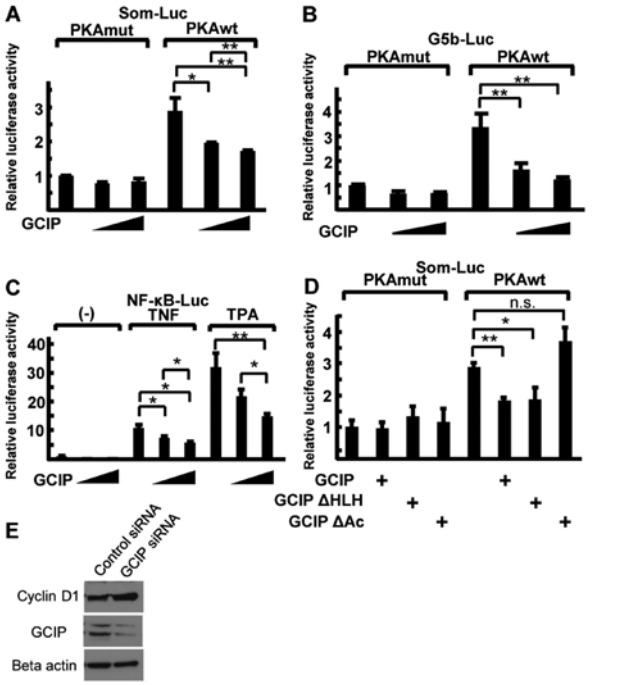

Figure 3. GCIP represses CBP-mediated transcription. (A and B) Reporter assays in 293 cells using Som-Luc (A) and GAL4-CREB, with a G5b-Luc reporter containing five GAL4 recognition sites. (B) Reporter activity was induced by the co-transfection of either WT or mutant PKA (PKAwt and PKAmut, respectively). The luciferase activity of cells transfected with empty vector and PKAmut was set to 1. (C) 293 cells were co-transfected with NF-κB-Luc and GCIP. After transfection, cells were treated with 100 ng/ml TPA or 10 ng/ml TNF-α. (D) 293 cells were co-transfected with Som-Luc and plasmids expressing various GCIP mutants. The luciferase activity of cells co-transfected with empty vector and PKAmut was set to 1. (E) Whole-cell lysates of FLS were analyzed by immunoblotting with the indicated antibodies. Data were analyzed by performing a Tukey-Kramer post hoc analysis and expressed as mean ± SD. *P<0.05, **P<0.01, n.s., not significant. These experiments were repeated at least three times. GCIP, grap2 cyclin D interacting protein; CBP, cAMP-response element-binding protein-binding protein; TPA, 12-O-tetradecanoylphorbol-13-acetate; siRNA, small interfering RNA; FLS, fibroblast-like synoviocytes.

minimal region required for CBP binding. Full-length GCIP and GCIP ΔHLH interacted with CBP, while GCIP ΔAcidic did not (Fig. 2C). To map the regions of CBP that associate with GCIP, we performed GST pulldown assays using the C/H3 domain of CBP. The C/H3 domain was sufficient to bind GCIP (Fig. 2D), and GCIP interacted with full-length CBP but not CBP ΔC/H3 (Fig. 2E). Taken together, these results demonstrate that the CBP C/H3 domain and the GCIP Acidic region are required for the CBP-GCIP interaction.

GCIP inhibits CBP-mediated transcriptional activation. CBP activates transcription via CREB (9,10) and NF-κB (30). To examine whether GCIP plays a role in CBP-mediated transactivation, we used a somatostatin-luciferase (Som-Luc) reporter that contains an endogenous somatostatin promoter with a cAMP response element (CRE) (31). Protein kinase cAMP-activated catalytic subunit alpha (PKA) induced Som-Luc reporter activity and co-transfection with GCIP repressed Som-Luc activity in a dose-dependent manner in both 293 cells and RA-derived FLS (Figs. 3A and S1B). To rule out the possible effects of GCIP on CRE-binding proteins other than CREB (32), reporter assays were performed with G5b-Luc as an artificial state in comparison with Som-Luc as an endogenous state. Instead of Som-Luc, CREB fused to GAL4-DBD (GAL4-CREB) and pG5b-Luc, which has five copies of the GAL4 DNA-binding site upstream of Luc (12), were co-transfected with GCIP into 293 cells. The results were similar to those with Som-Luc (Fig. 3B). Next, we examined the effect of GCIP on NF-κB-mediated transactivation using NF-κB-Luc (30). The reporter was activated by phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) or TNF-α. Co-transfection with GCIP repressed NF-κB activity in a dose-dependent manner (Fig. 3C). In addition, GCIP also repressed NF-κB activity in FLS (Fig. S1C). Next we tested whether the GCIP-CBP interaction was required for GCIP-mediated transcriptional repression. Bothe GCIP and GCIP ΔHLH repressed CREB-dependent transcription, while GCIP ΔAcidic, which could not bind to CBP, had no effect (Fig. 3D). To confirm the repression activity of GCIP on CREB-mediated transcription, we further performed knockdown experiments using siRNAs. Knockdown of GCIP induced the expression of endogenous cyclin D1, one of CREB-target genes (Fig. 3E). These results indicate that GCIP represses CREB- and NF-κB-mediated transcription via interaction with CBP.

GCIP and the Pol II complex compete for CBP binding. In CREB-dependent transcription, CBP associates with phosphorylated CREB (Ser 133) (33) and recruits Pol II complexes

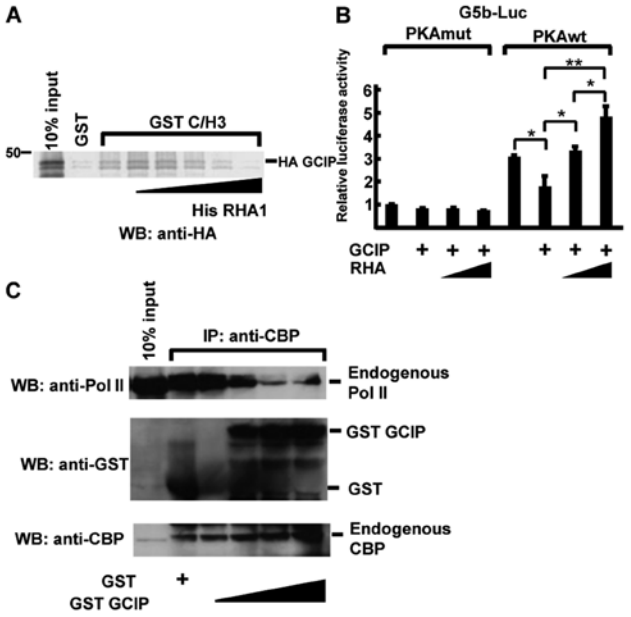

Figure 4. GCIP and the Pol II complex compete for CBP binding. (A) *In vitro* competitive binding assays. GST or GST-C/H3 was incubated with *in vitro* translated, ^{35}S-labeled GCIP and His-RHA1, and the resulting complexes were collected with GSH Sepharose beads. Samples were resolved by SDS-PAGE and signals were detected by autoradiography. (B) 293 cells were co-transfected with Som-Luc, GCIP and/or RHA. The luciferase activity of cells co-transfected with empty vector and PKAmut was set to 1. Data were analyzed by performing a Tukey-Kramer post hoc analysis and expressed as mean ± SD. *P<0.05, **P<0.01. (C) 293 cell nuclear extracts were incubated with GST or GST-GCIP and immunoprecipitated with anti-CBP antiserum. Samples were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. These experiments were repeated at least three times. GCIP, grap2 cyclin D interacting protein; CBP, cAMP-response element-binding protein-binding protein; RHA, RNA helicase A.

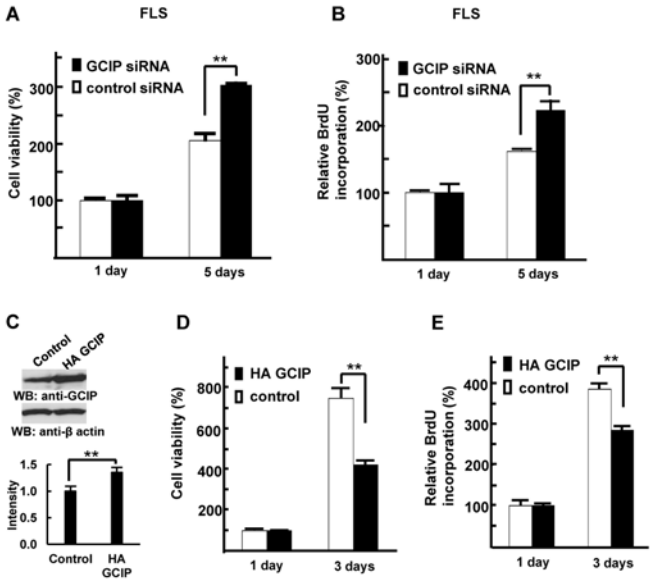

Figure 5. Effects of GCIP on cell proliferation. (A and B) FLS were transiently transfected with GCIP or control siRNAs. After transfection, cell viability was determined by (A) Cell Counting Kit-8 or (B) BrdU incorporation assays. (C) Whole-cell lysates of 293 cells stably transfected with either HA-GCIP plasmid (HA GCIP) or a control vector (control) were analyzed by western blotting with anti-GCIP antibody and anti-β-actin antibody. Band intensity was measured using ImageJ software. Statistical analysis was performed using the unpaired Student's t-test. Data are presented as the mean ± SD. **P<0.01. Cells expressing HA or HA-GCIP were plated, and cell viability was determined at the indicated times using the (D) Cell Counting Kit-8 or (E) BrdU incorporation assays. Data were analyzed by performing a Tukey-Kramer post hoc analysis and expressed as mean ± SD. **P<0.01. These experiments were repeated three times. GCIP, grap2 cyclin D interacting protein; siRNA, small interfering RNA; FLS, fibroblast-like synoviocytes.

by binding RHA, which interacts with the C/H3 domain (12). RHA interacts with CBP via its N-terminal region (amino acids 1-262), termed RHA1 (12). We hypothesized that GCIP and RHA competitively bind CBP, resulting in the repression of CREB-dependent transcription. To examine this, we first performed an *in vitro* competitive binding assay to address the possibility of direct competition between GCIP and RHA for CBP binding. As shown in Fig. 4A, His-RHA1 directly competed with GCIP for association with GST-C/H3 in a dose-dependent manner. Next, reporter assays were performed with G5b-Luc in 293 cells co-transfected with RHA and GCIP. In PKA-stimulated cells, GCIP repressed reporter activity (Fig. 4B), which was recovered in a dose-dependent manner in the context of RHA co-transfection (Fig. 4B). To further examine whether GCIP inhibits Pol II recruitment to CBP, we performed immunoprecipitation assays (Fig. 4C). Pol II coimmunoprecipitated with endogenous CBP and the addition of GST-GCIP decreased the formation of complexes between CBP and Pol II (Fig. 4C). These results demonstrate that GCIP inhibits Pol II complex recruitment to CBP, resulting in the repression of CREB-dependent transcription.

GCIP inhibits FLS growth. To investigate the role of GCIP in FLS growth, we depleted GCIP from RA patients' FLS. Control or *GCIP* siRNAs were transiently transfected into FLS, and their viability and proliferation were measured. GCIP depletion resulted in increased FLS viability compared with control cells (Fig. 5A). We next measured BrdU incorporation in these cells. As shown in Fig. 5B, treatment with *GCIP* siRNA enhanced FLS proliferation compared with control siRNA. Next, we generated 293 cell lines stably expressing either HA-GCIP or a control vector, as we were unable to generate FLS with stable GCIP expression. First, we confirmed overexpression of GCIP (Fig. 5C). As shown in Fig. 5D and E, the growth rate of GCIP-expressing 293 cells was lower than that of control cells. These results indicate that GCIP negatively regulates cell proliferation, suggesting that GCIP downregulation in the FLS of patients with RA could result in FLS overgrowth.

Downregulation of GCIP in FLS derived from patients with RA. Based on the rapid proliferation of RA-derived FLS, we hypothesized that the expression of GCIP might be low in RA-derived FLS compared to osteoarthritis (OA)-derived FLS. Therefore, we next investigated *GCIP* expression in FLS. As shown in Fig. 6A, RA-derived FLS displayed reduced *GCIP* transcript levels compared with those observed in patients with OA. The GCIP protein level was also significantly decreased in RA-derived FLS (Fig. 6B).

Discussion

Four members of the ID protein family are present in mammalian cells (13). Outside their conserved HLH

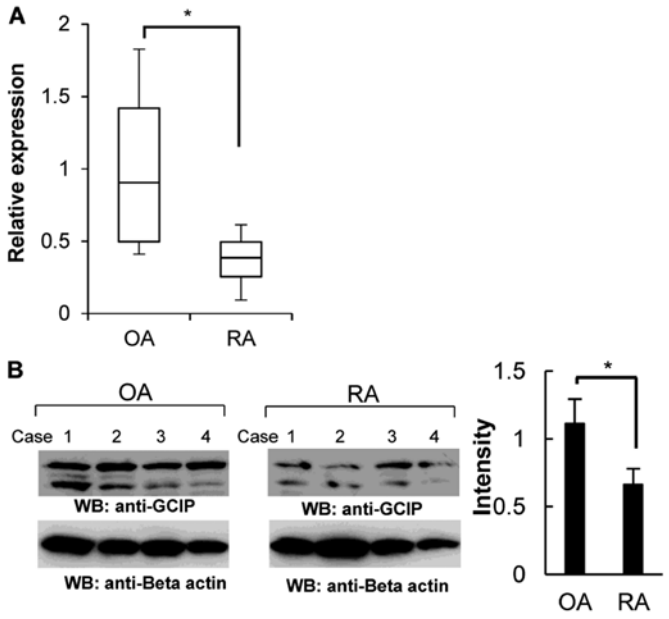

Figure 6. Downregulation of GCIP in the RSCs of patients with RA. (A) Total RNA was isolated from FLS of patients with RA and OA, and subjected to reverse transcription-quantitative PCR analysis. Individual measurements were normalized to 18S rRNA, and the average OA value was set to 1. OA, n=6; RA, n=6. Statistical analysis was performed using Student's t-test. Data represent the mean ± SD. *P<0.05. (B) Western blot analysis of GCIP in FLS from patients with RA and OA. The case denotes the code number of each patient. Expression of β-actin was used as an internal control. Band intensity was measured using ImageJ software. Statistical analysis was performed using Student's t-test. Data represent the mean ± SD. *P<0.05. GCIP, grap2 cyclin D interacting protein; RA, rheumatoid arthritis; OA, osteoarthritis; FLS, fibroblast-like synoviocytes.

domains, the ID proteins display extensive sequence and function divergence (14,17). ID proteins act as act as dominant-negative regulators by dimerizing with different partners (14,17), and mainly bind to basic HLH (bHLH) proteins, as well as a few non-bHLH proteins such as RB1 (34,35) and some transcription regulators, including the paired box (13), ADD1/SREBP-1c (36), MIDA1 (37), and ETS-domain transcription factors (38). However, to date, the association between ID proteins and mediator proteins have not been described. GCIP also possesses an HLH domain without a basic domain and is related to the ID protein family (19). In this study, we found that GCIP interacts with the coactivator CBP and represses CREB- and NF-κB-dependent transcription through this interaction. To the best of our knowledge, this study is the first to describe an interaction between a transcriptional coactivator and an ID-like protein.

Previous studies demonstrate that CBP activates CREB- and NF-κB-dependent transcription and functions in cell growth of FLS (5,14). RHA is a cofactor that mediates the interaction between transcriptional coactivator CBP and Pol II complex (12). And RHA also activates CREB- and NF-κB-dependent transcription. Therefore, we think that RHA is involved in cell proliferation. In addition, GCIP did not interact with RHA, and GCIP did not inhibit the interaction between RHA and Pol II. Therefore, we hypothesize that the inhibitory effect of GCIP on cell growth could be due to competition for binding to CBP with RHA.

Recent studies have suggested that HAT inhibitors represent important anti-inflammatory therapies (39,40). FLS reportedly induce several inflammatory cytokines. A recent study demonstrated that NF-κB regulates IL8 expression via CBP (41). Besides, CBP inhibition induces a TNF-α-dependent increase in NF-κB function and target gene expression in LPS-stimulated FLS (42). NF-κB and JUN may also affect the regulation of FLS proliferation by recruiting CBP (7,8,43). In addition, CBP regulates cell growth via several transcription factors, including CREB (9,10), JUN, Fos proto-oncogene, AP1 transcription factor subunit (44), and a variety of nuclear hormone receptors (11). In this study, GCIP was identified applying a yeast two-hybrid screening to a library of FLS-derived cDNAs. GCIP partially colocalized with CBP and repressed CREB- and NF-κB-dependent transcription by interacting with CBP. Therefore, GCIP may be a key factor in synovial cell outgrowth and could be a promising diagnostic and therapeutic target. Further analysis is needed to resolve the role of GCIP in FLS growth, and to determine whether GCIP modulation is a viable strategy to repress RA-associated synovial cell overgrowth. Nevertheless, our findings also indicate that the coactivator CBP is a functional target for GCIP in the regulation of cell proliferation. This suggests that GCIP targets not only bHLH proteins but also the coactivator CBP, which unveils a novel inhibitory mechanism for an HLH protein.

Acknowledgements

The authors would like to thank Dr Minako Nakazawa (Institute of Medical Science, St. Marianna University School of Medicine) for insightful comments, and Ms. Sanae Shinkawa, Ms. Yukari Nakagawa, Ms. Makiko Yui and Ms. Etsuko Nakamura (Institute of Medical Science, St. Marianna University School of Medicine) for technical assistance. The authors would like to thank Mr. Yu Nakatani (Misato Marine Hospital) for collecting patients' samples and aiding in performing the experiments.

Funding

The present study was supported in part by the Japan Society for the Promotion of Science KAKENHI (grant nos. 23659176, 26670479, 16H05157 and 18K06972). This study was also supported in part by funds provided through a program of the Strategic Research Foundation at Private Universities (grant nos. S1411011 and 2014-2018) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT). Partial funding was also provided via grants from the Naito Foundation, Natural Science Scholarship Daiichi-Sankyo Foundation of Life Science, Mitsubishi, Tanabe Pharma Corporation, Bureau of Social Welfare and Public Health, Academic contribution of Pfizer, Eisai, Santen Pharmaceutical, Abbvie, AYUMI Pharmaceutical Corporation, Takeda Science Foundation, AstraZeneca (R&D Grant 2013), and the ONO Medical Research Foundation and Industry-university cooperation (BioMimetics Sympathies Inc.).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HF, SA and TN conceived the project and designed the experiments. HF, SA and TN performed the experiments and analyzed the data. HF and TN wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The human experimental protocols in the present study (approval nos. 2728 and 2729) were approved by the Ethics Review Committee of Tokyo Medical University. Written informed consent was obtained from all patients prior to the collection of joint tissue samples. In addition, we confirm that all the experiments were performed in accordance with the relevant guidelines and regulations.

Patient consent for publication

Consent for publication was obtained from patients.

Competing interests

The authors declare that they have no competing interests.

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