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RGS2 Suppresses Breast Cancer Cell Growth via a MCPIP1-Dependent Pathway

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ABSTRACT

Regulator of G protein signaling 2 (RGS2) is a member of a family of proteins that functions as a GTPase-activating protein (GAP) for Gα subunits. RGS2 mRNA expression is lower in breast cancerous tissues than in normal tissues. In addition, expression of RGS2 is also lower in MCF7 (cancerous breast cells) than in MCF10A (normal breast cells). Here we investigated whether RGS2 inhibits growth of breast cancer cells. RGS2 overexpression in MCF7 cells inhibited epidermal growth factor- or serum-induced proliferation. In HEK293T cells expressing RGS2, cell growth was also significantly suppressed (In addition, exogenous expression of RGS2 in HEK293T cells resulted in the significant suppression of cell growth). These results suggest that RGS2 may have a tumor suppressor function. MG-132 treatment of MCF7 cells increased endogenous or exogenous RGS2 levels, suggesting a post-transcriptional regulatory mechanism that controls RGS2 protein levels. RGS2 protein was degraded polyubiquitinated the K71 residue, but stabilized by deubiquitinase monocyte chemotactic protein-induced protein 1 (MCPIP1), and not affected by dominant negative mutant (C157A) of MCPIP1. Gene expression profiling study showed that overexpression of RGS2 decreased levels of testis specific Y encoded like protein 5 (TSPYL5), which plays a causal role in breast oncogenesis. TSPYL5 protein expression was low in MCF10A and high in MCF7 cells, showing the opposite aspect to RGS2 expression. Additionally, RGS2 or MCPIP1 overexpression in MCF7 cells decreased TSPYL5 protein level, indicating that RGS2 stabilized by MCPIP1 have diminished TSPYL5 protein levels, thereby exerting an inhibitory effect of breast cancer cell growth. J. Cell. Biochem. 116: 260–267, 2015.

KEY WORDS: RGS2; MCPIP1; TSPYL5; GPCR; DUB

Regulator of G-protein signaling (RGS) is a negative regulator in G-protein coupled receptor (GPCR) signaling that functions as a GTPase activating protein (GAP), which acts to increase the natural GTPase activity of the Gα subunit. By increasing the GTPase activity of the Gα subunit, RGS promotes GTP hydrolysis to GDP, thus converting the Gα subunit to its inactive state and reducing its signaling ability [Hurst and Hooks, 2009]. More than 30 types of RGS or RGS-like proteins have been identified to date. These proteins have been divided into eight distinct subfamilies according to their amino acid sequence. Among them, RGS2 is included in the R4 family, which contains the most simple amino acid sequence structure [Siderovski and Willard, 2005]. Although RGS2 protein was first identified as an inhibitory proteins of G-protein signal transduction, recent studies suggest that it can have many other cellular functions.

RGS2 has protective effects against myocardial hypertrophy [Nunn et al., 2010; Tsang et al., 2010; Sjogren et al., 2012], as well as

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the ability to regulate vascular smooth muscle relaxation and blood pressure [Heximer et al., 2003; Tang et al., 2003]. We have also reported a new mechanism of RGS2 regulation of essential atherosclerosis factors such as NADPH oxidase-1 or tissue factor expressions [Lee et al., 2012; Park et al., 2013]. RGS2 was also recently reported to play a role in asthma. Moreover, decreased RGS2 expression had been found to be correlated with elevation of airway hyperresponsiveness in human monocytes, and RGS2 knockout mice showed spontaneous induction of airways hyperresponsiveness [Xie et al., 2012]. RGS2 is expressed highly in normal human cells, but its expression appears to be downregulated in cancer cells, therefore it has been suggested to play a role in cancer [Smalley et al., 2007]. RGS2 mediates pro-angiogenic function of myeloid derived suppressor cells in the tumor microenvironment [Boelte et al., 2011]. However, epigenetic repression of RGS2 promotes prostate cancer cell growth [Wolff et al., 2012]. Therefore, RGS2 function is expected to differ according to cancer types, but the details of this are still not clear. RGS2 functions are continuously expanding, and it is believed to be involved in different mechanisms related to traditional GAP activity.

Protein stability control has recently been considered as an important mechanism that regulates the functions of RGS proteins, and one of the best studied example in this aspect is RGS4. RGS4 protein is degraded by the N-end rule pathway in vitro [Davydov and Varshavsky, 2000], and is a substrate of the N-end rule pathway in vivo [Lee et al., 2005]. For example, opioid stimulation decreases RGS4 protein levels in a proteasome dependent manner [Wang and Traynor, 2011], and roles of polyubiquitination and ubiquitin conjugating enzyme in RGS4 degradation have been suggested [Lee et al., 2011]. Degradation of RGS5 and RGS13 proteins has also been reported [Bodenstein et al., 2007; Xie et al., 2010], and the RGS2 protein is stabilized by cardiotonic steroids such as ouabain or digoxin [Sjogren et al., 2012]. However, whether regulation of RGS2 protein stability may contribute to cancer cell growth has not been reported. Therefore this study was to investigate the effects of RGS2 on breast cancer cell growth, and as well as to demonstrate the underlying mechanism of growth inhibition which is the stabilization of RGS2 by deubiquitination.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

HEK293FT cells were cultured in DMEM supplemented with L-glutamine (200 mg/L, Hyclone, Logan, UT), 100 U/ml penicillin and streptomycin, and 10% FBS (Invitrogen, Carlsbad, CA). MCF7 cells were cultured in Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS) containing L-glutamine, antibiotics and 10% FBS. MCF10A cells were cultured in Mammary Epithelial Cell Growth Medium containing L-glutamine, antibiotics and 10% FBS. These cells were transiently transfected with vector containing RGS2 or other genes using nucleoporation reagents from Lonza (Allendale, NJ), Trans-IT®-293 (Mirus Bio, Madison, WI) or Lipofectamine® LTX-Plus reagents (Invitrogen).

REAGENTS

α-RGS2 antibody was purchased from Abnova (Walnut, CA). α-testis specific Y encoded like protein 5 (TSPYL5) antibody and Protein A/G Plus-agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). α-Flag M2 antibody, α-β-actin antibody, α-Flag M2 affinity gel and N-ethylmaleimide (NEM) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). α-HA and α-GFP antibodies were obtained from Cell Signaling Technology (Danvers, MA). MG-132 was acquired from Calbiochem (La Jolla, CA), recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN) and reverse-transcription polymerase chain reaction (RT-PCR) kits were obtained from Takara Bio (Japan).

PLASMIDS

Flag-tagged RGS2 expression plasmid was generated by inserting mouse RGS2 coding fragment into pFLAG-CMV2 vector at the *HindIII* and *BamHI* sites. GFP-tagged RGS2 expression plasmid was generated by inserting mouse RGS2 coding fragment into pEGFP-C1 vector at the *HindIII* and *BamHI* sites. Flag-tagged MCPIP1 expression plasmid was generated by inserting mouse MCPIP1 coding fragment into pFLAG-CMV2 vector at the *HindIII* and *KpnI* sites. GFP-tagged MCPIP1 expression plasmid was generated by inserting mouse MCPIP1 coding fragment into pEGFP-C1 vector at the *Hind*III and *KpnI* sites. RGS2 serial point mutants and MCPIP1 point mutant (C157A) were generated using the QuickChanges II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) with Flag-RGS2 or Flag-MCPIP1 as a template. TSPYL5 construct was a gift from Dr. TY Kim (Seoul National University, Korea). Ligated vectors were transformed into DH5α cells and plasmid sequences were confirmed by Sanger sequencing.

ISOLATION OF TOTAL RNA FROM BREAST TISSUES

Breast tissue samples were placed in a screw cap tube, after which Zirconia beads (BioSpec Products, Bartlesville, OK) were added and the samples were incubated with at least 10 volumes of TRIzol. Next, samples were immediately homogenized in a homogenizer (Precellys 24, Bertin Technologies, France) for at least 30 s at 6,800 rpm until the sample was uniformly homogeneous. The homogenates were then added chloroform and shaken vigorously for 15 s, after which they were incubated at room temperature for 10 min. Next, the supernatants were carefully transferred to a new tube and the RNA was isolated using isopropanol, washed with 75% ethanol, and dissolved in TE buffer.

GENERATION OF RGS2 INDUCIBLE CELL LINES

The human RGS2 coding fragment was subcloned into mammalian expression vector, pcDNA4/TO/myc-HisB (Invitrogen), which contains the zeocin resistance gene. The recombinant plasmid construct, pcDNA4/TO/myc-HisB/hRGS2, was confirmed by restriction enzyme digestion. Next, HEK293T cells were grown in 100 mm culture dishes until approximately 70–80% confluences, at which point they were cotransfected with pcDNA4/TO/myc-HisB/hRGS2 plasmid DNA and pcDNA6/TR (Invitrogen), which has a blasticidin resistance gene using Lipofectamine reagents in serum-free OPTI-MEM (Gibco BRL, Carlsbad, CA). To enrich the transfected cells, fresh DMEM

containing 25 μg/ml zeocin and 1.25 μg/ml blasticidin was replaced 18 h later and cells were grown under selection conditions for more than two weeks. Cells resistant to both zeocin and blasticidin were then subjected to serial dilution to isolate clones. A total of 13 clones were obtained, and RGS2 expression was confirmed by immunoblot using RGS2 antibody. RGS2 expression of the isolated clones was induced in the presence of doxycycline.

REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

Total RNA was extracted from cells using TRIzol (Invitrogen). First-strand cDNA was synthesized from 1 μg total RNA with random primers, oligo-dT, and reverse transcriptase (Takara Bio). The cycling conditions for PCR were 95°C for 5 min, followed by 26–33 cycles at 95°C for 1 min, 63°C for 1 min, and 72°C for 1 min. For semi-quantitative PCR, target gene expression was normalized to β-actin transcription.

LUCIFERASE REPORTER ASSAY

HEK293FT cells were seeded at 3×10^5 cells per well and cultured overnight in 6-well plates. The cells were then transfected with the indicated plasmids, together with NF-κB-dependent firefly luciferase construct. At 24 h after transfection, 1 ng/ml of TNF-α was added to the media. The cells were then incubated for another 6 h before they were collected for dual specific luciferase reporter gene assays, which were conducted according to the manufacturer's protocol. Data shown represent three independent experiments performed in triplicate.

CELL VIABILITY

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction or trypan blue exclusion assays. For MTT assay, cells were transfected with EGFP control or RGS2-EGFP plasmid DNA. At 48 h after transfection, MTT solution was added following medium removal, after which cells were incubated for 6 h at 37°C. Formazan crystals in the viable cells were solubilized with dimethy sulfoxide, and the absorbance at 550 nm was determined. For trypan blue exclusion assay, both floating and attached cells were harvested from dishes and collected by centrifugation at 800 g for 5 min. Cell pellets were then resuspended in PBS and mixed with trypan blue (0.2%), after which viable (unstained) and dead (stained) cells were counted using a hemocytometer.

IN VIVO UBIQUITINATION ASSAY

HEK293FT cells were transfected with HA-ubiquitin as well as other plasmids as indicated. After 24 h, the cells were harvested using NP-40 lysis buffer (50 mM Tris-HCl; pH 8.0, 1.5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40) containing protease inhibitor cocktail and 2 mM NEM. To denature the proteins, the cell lysates were incubated with 1% SDS and 1 mM dithiothreitol at 65°C for 20 min. The total cell extracts were then directly subjected to immunoblot analysis with specific antibody or further immunoprecipitated using specific antibodies or α-Flag M2 affinity gel as indicated and then analyzed by immunoblot with HA antibody.

WESTERN-BLOT ANALYSIS

Cells were cultured and transfected with targeted gene. After which cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail). Proteins were then separated on 8% reducing SDS–PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) in 20% methanol, 25 mM Tris, and 192 mM glycine. Membranes were blocked with 5% non-fat dry milk and incubated overnight with primary antibody at 4°C before washing, after which they were incubated by 1 h with horseradish-peroxidase conjugated secondary antibody, subjected to further wash steps, and then developed with an enhanced chemiluminescence system (SuperSignal® West Femto, Thermo Scientific, IL).

COIMMUNOPRECIPITATION

For in vitro coimmunoprecipitation, cells were transfected with plasmid encoding specific genes such as RGS2. At 24 h after transfection, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 1.5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, and protease inhibitor cocktail). Cellular debris was removed by centrifugation at 12,000 g for 10 min at 4°C. Next, the lysates were incubated with α-Flag M2 affinity gel. Coimmunoprecipitation assays were performed using α-Flag M2 affinity gel according to the manufacturer's instruction. After extensive washing, immunoprecipitated proteins were resolved by 8–10% SDS–PAGE and analyzed by immunoblotting with Flag, GFP, or specific antibodies. Membranes were developed using the enhanced chemiluminescence system.

MICROARRAY AND DATA ANALYSIS

MCF7 cells were electroporated with wild-type RGS2 or GFP vector. At day 3 after culture, total RNA were obtained and used for microarray analysis, which was performed by D&P Biotech (South Korea). Briefly, 1 μg RNA was used to generate biotin-labeled single-stranded cDNA by in vitro transcription. Then, the biotinylated single-stranded cDNA was hybridized to an Affymetrix Human HG 1.0 ST Array GeneChip (Affymetrix, Santa Clara, CA). Each stained probe array was scanned with a GeneChip® Scanner 3000 (Affymetrix) 7G at 570 nm. The signal intensity of the gene expression level was calculated by Expression Console™ software, Version 1.1 (Affymetrix). To get rid of false positive signal, we applied cut-off value of 300 in the detection signal. Gene ontology was analyzed using NetAffyx (http://www.affymetrix.com/estore/index.jsp).

RESULTS

LOSS OF RGS2 EXPRESSION IN BREAST CANCER TISSUES AND CELL LINE

We compared the expression of RGS2 mRNA in normal and cancerous human breast specimens to determine whether RGS2 may play a role in breast cancer development. RT-PCR revealed that RGS2 was highly expressed in normal breast tissues. However, cancerous tissues expressed significantly lower levels of RGS2 mRNA than normal tissues (Fig. 1A). To further validate this finding, we used human monocytes, a model system routinely used to examine gene

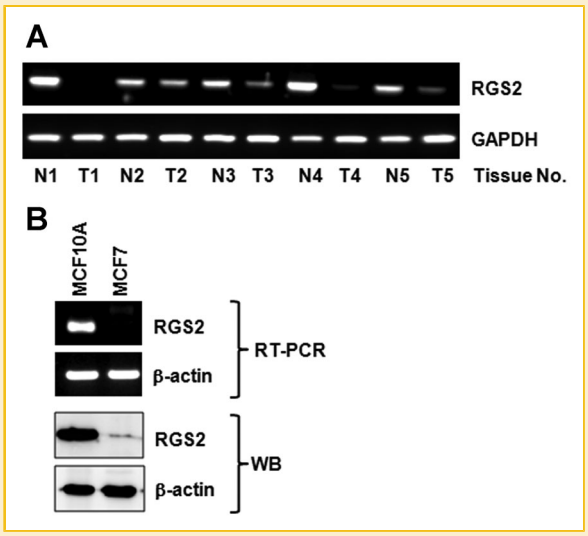

Fig. 1. Loss of RGS2 expression in human breast cancer tissues and cell lines. A: RT-PCR of the RGS2 in breast tissues of normal mammary gland and breast cancer were determined and normalized to a GAPDH control. B: MCF10A and MCF7 cell lines were cultured and RGS2 mRNA expression was determined by RT-PCR and normalized to a β-actin control. RGS2 protein expression was determined by immunoblotting using RGS2 Ab. The data are representative of five independent experiments (N, normal tissue; T, tumor tissue).

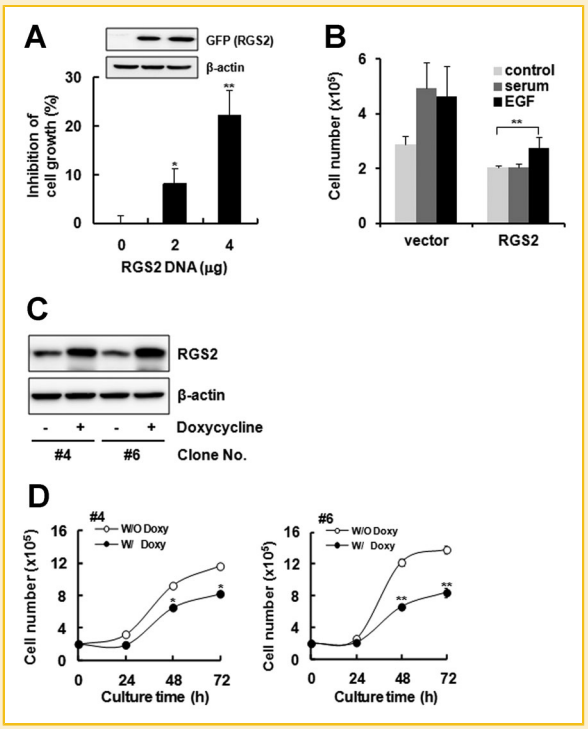

Fig. 2. Suppression of cell growth by RGS2. A: MCF7 cells were transfected with vector or increasing concentration of GFP-tagged RGS2 plasmids for 48 h. RGS2 quantity in lysates of MCF7 cells assessed by immunoblotting with GFP Ab. Cell viability was measured by MTT reduction assay. B: Serum-starved MCF7 cells were transfected with either vector or GFP-tagged RGS2 plasmids, then stimulated with serum or EGF for 48 h. Cell viability was measured by trypan blue exclusion assay. C,D: HEK293T cells were cotransfected with pcDNA4/TO/myc-HisB/hRGS2 plasmid DNA and pcDNA6/TR. RGS2 protein of the cells was induced by doxycycline treatment and determined by immunoblotting using RGS2 Ab. Cell viability was measured by trypan blue exclusion assay. Data represent mean ± SD, and experiments were repeated five times in duplicate, $^{*}P < 0.05$, $^{**}P < 0.01$by two-tailed Student's *t*-test.

expression and its relationship to human disease. We also found a significant decrease in RGS2 mRNA in monocytes isolated from samples of peripheral blood with cancer compared to normal (data not shown). We then compared the expression of RGS2 mRNA and protein in MCF10A (a non-tumorigenic breast epithelial cell line) and MCF7 (a non-invasive breast cancer cell line) cells. RGS2 expression was dramatically downregulated in MCF7 than MCF10A cells (Fig. 1B). RGS2 expression was also low several other breast cancer cell lines such as MDA-MB-231 and MDA-MB-361 (data not shown). These results suggest that the RGS2 expression is down-regulated in breast cancer cells.

RGS2 SUPPRESSES THE GROWTH OF MCF7 CELLS

In view of these findings, we considered the possibility that RGS2 might normally function to suppress breast cancer cell growth or survival. Therefore, we examined the effects of RGS2 on the survival of a human breast cancer cell line. Transient expression of enhanced GFP-tagged RGS2 significantly inhibited the growth of MCF7 cells in a dose-dependent manner (Fig. 2A). RGS2 overexpression also significantly suppressed proliferation of MCF7 cells in response to EGF or FBS (Fig. 2B). To further investigate how RGS2 affects proliferation of MCF7 cells, we attempted to stably express RGS2 protein in MCF7 cells. However, we failed to obtain these cells, because RGS2 expressing cells died over time. Therefore, we tested the growth of HEK293T cells stably expressing RGS2. Among the thirteen clones of cells transduced with a plasmid that contains a RGS2 coding sequence under the control of a doxycycline inducible promoter, two clones expressed RGS2. The expression of RGS2 was correlated with attenuated cell proliferation (Figs. 2C and D). Collectively, these results suggest that RGS2 negatively regulates the growth mammalian cells.

RGS2 IS UBIQUITINATED AND DEGRADED

Because of RGS2 protein expression was high in MCF10A cells and low in MCF7 cells. Therefore, we tested the possibility whether its stability might be regulated by the ubiquitin proteasome system. We treated MCF7 cells with MG-132, a proteasome inhibitor, and analyze the RGS2 protein levels using immunoblotting. The result showed that MG-132 treatment increased the expression of endogenous RGS2 protein level (Fig. 3A). Treatment of MCF7 cells transiently expressing GFP-tagged RGS2 also increased the level of ectopic RGS2 (Fig. 3B). These results, together with previous published data [Bodenstein et al., 2007; Sjogren et al., 2012], suggested that RGS2 is a short-lived protein that is turned over by the proteasome. We therefore investigated whether RGS2 was ubiquitinated in cells. To test this idea, HEK293FT cells were transfected with Flag-RGS2 and HA-ubiquitin and then treated with MG-132. RGS2 was immunoprecipitated with anti-Flag beads under denaturing conditions. Immunoblotting with anti-HA revealed a fraction of RGS2 was polyubiquitinated (Fig. 3C). The RGS2 protein consists of 211 amino acids including 22 lysines. Among these, there are four

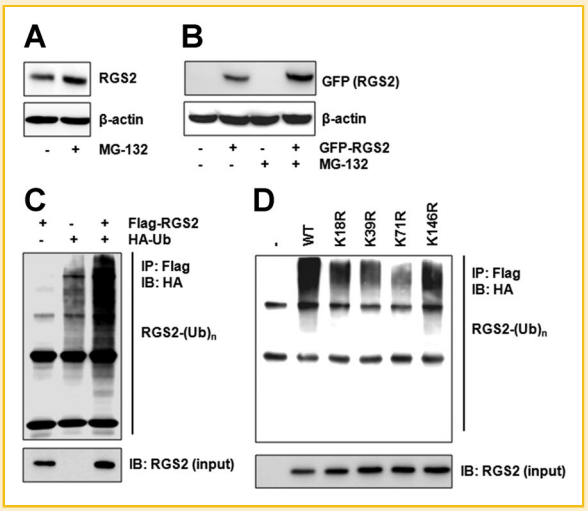

Fig. 3. Ubiquitination of RGS2 protein. A: MCF7 cells were cultured and treated with the proteasome inhibitor MG-132 (20 μM) for 6 h. RGS2 protein expression was determined by immunoblotting using RGS2 Ab. B: HEK293FT cells were transfected either vector or GFP-tagged RGS2 plasmids and treated with MG-132 for 6 h before collection. RGS2 protein expression was determined by immunoblotting using GFP Ab. C: Lysates from HEK293FT cells transiently cotransfected with Flag-tagged RGS2 and HA-tagged Ub plasmids were subjected to immunoprecipitation with anti-Flag Ab followed by immunoblot analysis with anti-HA Ab. D: HEK293FT cells were cotransfected with the indicated Flag-tagged RGS2 WT, or mutants and HA-tagged Ub plasmids, and immunoprecipitation and immunoblot analysis were performed with the indicated Abs. The data are representative of five independent experiments.

putative ubiquitination sites based on prediction using the Bayesian discrimination method [Xue et al., 2006]. To confirm that the predicted RGS2 ubiquitination sites were functional, we generated four types of RGS2 mutant (K18R, K39R, K71R, and K146R) and tested the ubiquitination of these mutants. While each of the mutants had no difference in their expression compared to wild type RGS2, K71R mutant had significant defect in ubiquitination. In addition, K18R and K39R mutants were slightly reduced to be ubiquitinated. However, K146R mutant had no change in ubiquitination (Fig. 3D). Therefore, K71 residue is appears to be the best candidate site for RGS2 ubiquitination.

MCPIP1 IS DEUBIQUITINASE TO STABILIZE RGS2 PROTEIN

To explore whether a member of deubiquitinases (DUBs) is involved in regulating the stabilization of RGS2, we expressed 40 different human DUBs in HEK293FT cells and analyzed the steady state expression of RGS2. The results revealed that MCPIP1 significantly enhanced RGS2 expression, whereas other DUBs had little effects (data not shown). To confirm the effects of MCPIP1, we transfected cells with an RGS2-expressing plasmid without or with MCPIP1-expressing plasmid. Immunoblotting showed that overexpression of MCPIP1 significantly increased the RGS2 level in HEK293FT cells (Fig. 4A). Consistent with this result, MCPIP1 expression also significantly enhanced the RGS2 protein level in MCF7 cells (Fig. 4B). We next tested whether MCPIP1 could bind RGS2 to influence its stability using coimmunoprecipitation. We found that MCPIP1 associated with RGS2 in a dose-dependent manner (Fig. 4C). Given that MCPIP1 is a deubiquitinase, we tested whether MCPIP1 regulates RGS2 protein by controlling its ubiquitination status. HEK293FT cells were transfected with HA-tagged ubiquitin and Flag-MCPIP1. Immunoblotting of whole cell lysates with HA antibody revealed that cells expressing MCPIP1 contained fewer ubiquitinated proteins than control cells (Fig. 5A). This result suggest that MCPIP1 expression has a global effect on protein ubiquitination in cells. To test whether RGS2 ubiquitination was affected by MCPIP1 expression, expression vectors encoding GFP-RGS2 and HA-ubiquitin were cotransfected into the HEK293FT cells with vector control or expression vector encoding Flag-MCPIP1. Cell lysates from the transfected cells were subject to immunoprecipitation with anti-RGS2 antibody. The precipitated materials were analyzed by immunoblotting with anti-HA to detect ubiquitinated RGS2. As shown in Figure 5B, overexpression of MCPIP1 attenuated RGS2 polyubiquitination. To confirm the MCPIP1 specificity in the decreased RGS2 ubiquitination, we constructed a catalytically inactive MCPIP1 mutant using the method described by Liang et al. [Liang et al., 2010]. This mutant converted the 157 cysteine residue in the putative catalytic domain of MCPIP1 into alanine. The mutant activity was then tested by measuring NF-κB promoter activity as described by Liang [Liang et al., 2010]. While WT MCPIP1 inhibited TNF-α-induced NF-κB activity, the C157A MCPIP1 mutant had almost no effect (Fig. 5C). We also compared the WT and mutant MCPIP1 effect on RGS2 ubiquitination. While WT MCPIP1 inhibited

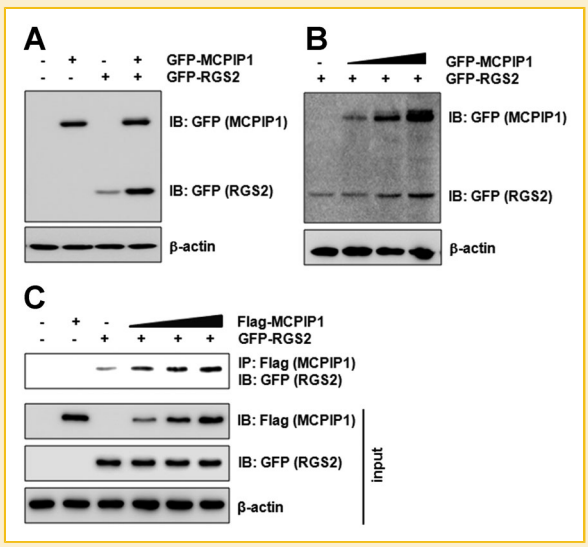

Fig. 4. Stabilization of RGS2 protein by MCPIP1. A: HEK293FT cells were cotransfected with the indicated GFP-based expression plasmids. RGS2 and MCPIP1 protein expression were determined by immunoblotting using GFP Ab. B: MCF7 cells were cotransfected with GFP-tagged RGS2 together with increasing concentration of GFP-tagged MCPIP1 plasmids. RGS2 and MCPIP1 protein expression were determined by immunoblotting using GFP Ab. C: HEK293FT cells were cotransfected with GFP-tagged RGS2 and increasing concentration of Flag-tagged MCPIP1 plasmids. Shown are immunoblots with anti-GFP Ab after immunoprecipitation with anti-Flag (upper panel), or of total cell lysates (input). The data are representative of five independent experiments.

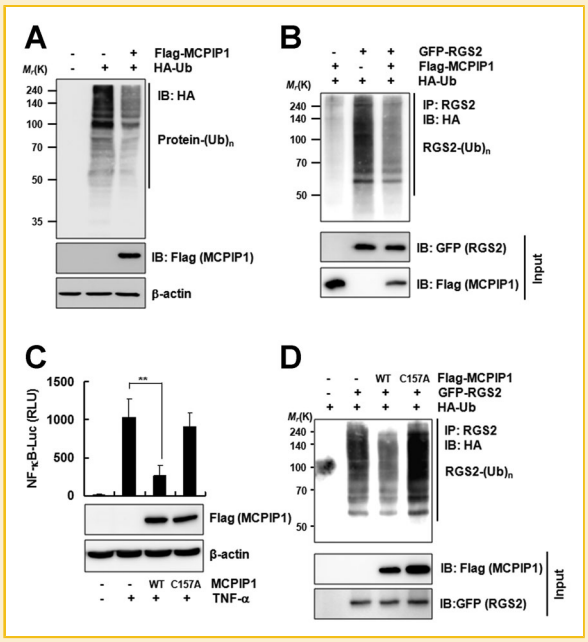

Fig. 5. Deubiquitination of RGS2 by MCPIP1. A: HEK293FT cells were transfected with vector or Flag-tagged MCPIP1 together with HA-tagged Ub plasmids. Ubiquitinated-proteins were determined by immunoblotting using anti-HA Ab. B: Lysates from HEK293FT cells transiently cotransfected with GFP-tagged RGS2, Flag-tagged MCPIP1, and HA-tagged Ub plasmids were subjected to immunoprecipitation with anti-RGS2 Ab followed by immunoblot analysis with anti-HA Ab (upper panel), or of total cell lysates (input). C: HEK293FT cells were transfected with the indicated Flag-tagged MCPIP1 WT or mutant (C157A) and luciferase reporter for NF-κB. The cells were treated for 6 h with vehicle or TNF-α (1 ng/ml). Bars show mean luciferase activity +/− s.d. (n = 5). D: Lysates from HEK293FT cells transiently cotransfected with GFP-tagged RGS2, Flag-tagged MCPIP1 WT, or MCPIP1 mutant (C157A) and HA-tagged Ub plasmids were subjected to immunoprecipitation with anti-RGS2 Ab followed by immunoblot analysis with anti-HA Ab (upper panel), or of total cell lysates (input). Data represent mean ± S.D., and experiments were repeated five times in duplicate, $^{**}P < 0.01$ by two-tailed Student's *t*-test.

RGS2 ubiquitination, C157A MCPIP1 did not (Fig. 5D). These results suggest that DUB activity of MCPIP1 might be able to stabilize RGS2.

RGS2 DOWN-REGULATES TSPYL5 EXPRESSION

After confirming that MCPIP1 regulates RGS2 protein stability, we investigated how it inhibits MCF7 cell growth. To accomplish this, we performed microarray analysis using MCF7 cells that overexpressed RGS2 transiently. The data revealed that many genes expression were changed. Among them, the expression of TSPYL5 was significantly decreased (data not shown). We focused on TSPYL5 because it is known to accelerate MCF7 cell growth by regulation of p53 and p21 expression [Epping et al., 2011]. TSPYL5 protein expression was compared in the two cell lines used in the RGS2 expression experiments. Unlike RGS2, TSPYL5 expression was absent in MCF10A cells, while it was high in MCF7 cells (Fig. 6A), suggesting that RGS2 may negatively regulate the expression of TSPYL5. Indeed, RGS2 overexpression reduced TSPYL5 protein expression in a dose-dependent manner (Fig. 6B). MCPIP1 overexpression, which stabilizes the RGS2 level, also reduced TSPYL5 protein expression (Fig. 6C). Taken together, these results suggest that RGS2 stabilized by MCPIP1 reduces TSPYL5 expression, and that reduced TSPYL5 might explain the observed effect on breast cancer cell growth by RGS2 overexpression.

DISCUSSION

Hormone receptors are major indicators of breast cancer, yet ~30% of hormone receptor positive breast cancers still show resistance to endocrine therapy [Clark et al., 1984; Garcia-Becerra et al., 2012]. Therefore, the existence of new indicators other than hormone receptors in breast cancer progression is highly possible. The results of our study suggest that RGS2, stabilized by a DUB MCPIP1 in mammary epithelial cells, reduces TSPYL5 expression and acts as a tumor suppressor.

It is already known that the GPCR-stimulated pathway acts as a critical mediator in oncogenic signaling. GPCR activates heterotrimeric G-proteins, which mediate extracellular signals to the cell interior. RGS is a protein family that accelerates deactivation of G-proteins, and RGS2 has the simplest structure among the RGS. Although the best known function of RGS2 is regulation of blood pressure through GPCR signaling [Tang et al., 2003], some reports

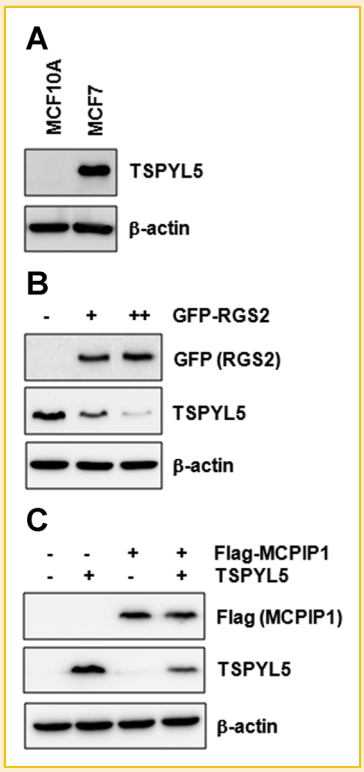

Fig. 6. Down-regulation of TSPYL5 by MCPIP1-RGS2 pathway. A: MCF10A or MCF7 cells were cultured and TSPYL5 protein expression was determined by immunoblotting using TSPYL5 Ab and normalized to a β-actin control. B: MCF7 cells were transfected with increasing concentration of GFP-tagged RGS2 plasmids followed by immunoblot analysis with anti-GFP or anti-TSPYL5 Abs. C: MCF7 cells were transfected with Flag vector or Flag-tagged MCPIP1 plasmids followed by immunoblot analysis with anti-Flag or anti-TSPYL5 Abs. The data are representative of five independent experiments.

have shown that changes of in the RGS2 expression in transcript level are correlated with carcinogenesis; thereby RGS2 is expected to play a role in cancer cells as well. RGS2 transcript was reduced in ovarian cancer, prostate cancer, and acute myeloid leukemia [Sethakorn and Dulin, 2013]. Our results also showed low levels of RGS2 transcript in breast cancer tissue and cell lines compared to normal tissue and non-cancerous cell line, indicating the protective function of RGS2 in cancer development. In addition, although we do not know the specific mechanism, RGS2 transcript from the blood monocytes of breast cancer patients was lower than that of healthy individuals (data not shown). These results suggest that RGS2 transcript level is important for breast cancer regulation. Conversely, Smalley et al. [2007] reported that RGS2 transcript is expressed in high levels in breast cancer [Smalley et al., 2007]. However, the previous study also showed that RGS2 expression was lower in breast cancer cell line than in normal cells, and that although RGS2 expression was high in solid breast cancer tissue, but they did not compare with normal tissue.

Inhibition of breast cancer cell growth does not only occur in response to RGS2. Indeed, other RGS proteins have shown inhibitory effects of cancer cell growth. For example, RGS6 is known as a mediator for p53 activation that inhibits cell growth via its powerful apoptotic action [Huang et al., 2011]. Surprisingly, RGS6 did not require GAP activity of G-protein to induce apoptosis [Maity et al., 2011]. RGS16 is also known to inhibit breast cancer cell growth through phosphoinositide 3-kinase (PI3K) signaling [Liang et al., 2009]. A new mechanism of RGS proteins was suggested based on the observation that the N-terminus of RGS16 binds with the p85α subunit of PI3K, demonstrating that regulating cell growth is not related to the GAP activity of RGS16. The results of present study showed that RGS2 directly interacted with MCPIP1, suggesting that its regulation of cell growth may be unrelated to the GAP activity of RGS2. In other words, the signaling method that occurs via the interactions of RGS2 and its mediating proteins will be important in cell growth regulation. Moreover, the expected ubiquitination lysine site is not a GAP domain of RGS2; therefore it might independently act GAP activity. RGS protein regulating cancer is consistently increasing, but each type acts through a very different mechanism; accordingly more research is required.

Our transient overexpression and inducible overexpression analyses indicated that RGS2 suppresses cell growth. It is high likely that suppression of MCF7 cell growth by RGS2 can be used to target TSPYL5 protein because RGS2 overexpression decreased TSPYL5 protein level, which is used as a prognostic marker of breast cancer. Gene expression profiling is useful to predicting the severity of breast cancer patients, and TSPYL5 is one of the genes discovered by this method. TSPYL5 is also among the genes in the MammaPrint 70 gene prognostic expression signature that predicts clinical outcome of breast cancer [van ′t et al., 2002]. The genomic location of the TSPYL5 gene is chromosome 8q22, a region that is frequently amplified in breast cancer [Hu et al., 2009; Li et al., 2010]. Epping et al. confirmed that TSPYL5 overexpression accelerates breast cancer cell growth, and the role of ubiquitin-specific protease 7 (USP7), a DUB, in this process. TSPYL5 interacts with USP7, increases p53 ubiquitination, and eventually suppresses p53 level in MCF7 cells [Epping et al., 2011]. Our results also indicate the importance of TSPYL5 in the inhibition of breast cancer cell growth by RGS2. The authors of the aforementioned studies suggested that USP7 targets TSPYL5 and regulates breast cancer cell growth, but we suggest a different type of DUB. Specifically, we believe that MCPIP1, a new DUB family, targets the RGS2 protein that seems to act as a upstream regulator of TSPYL5.

The human genome encodes at least 98 DUBs. Based on their domain structures, DUBs can be divided into five families: ubiquitin carboxy-terminal hydrolases, USPs, ovarian tumor proteases, Machado–Joseph disease proteases, and JAMM/MPN domain-containing metallopeptidases (JAMMs). Each of these families contains specific, but conserved DUB domains. However, MCPIP has recently been considered a sixth DUB family based on its necessity for specificity of function [Fraile et al., 2012]. Although MCPIP1 contains an ubiquitin-associated (UBA) domain that can interact with ubiquitinated protein in the N-terminus, only a few target proteins are currently known. Therefore, the discovery of RGS2 as a new target of MCPIP1 is very important. Our results show that MCPIP1 stabilizes RGS2 protein by direct interaction. While reports of RGS2 ubiquitination are new, the possibility is not surprising since, in addition the RGS4/5, the RGS2 N-terminal residue contains a sequence relating to the N-end rule pathway, which is responsible for proteasomal degradation [Bodenstein et al., 2007]. However, ubiquitin E3 ligase mediating RGS2 degradation has yet to be identified. We also could not identify an E3 ubiquitin ligase accelerating RGS2 ubiquitination. Instead, we confirmed the function of MCPIP1, which is responsible for RGS2 deubiquitination.

MCPIP1 is a gene identified in human peripheral blood monocytes treated with MCP-1 [Zhou et al., 2006]. This gene is powerfully induced by proinflammatory molecules such as TNF, IL-1β, LPS, and pam3CSK4 [Liang et al., 2008a,b; Matsushita et al., 2009; Skalniak et al., 2009; Kasza et al., 2010]. MCPIP1 has been suggested as a negative regulator of macrophage activation [Liang et al., 2008b], and a mouse study showed that MCPIP1 deficiency can provide various phenotypes such as severe anemia, autoimmune response, and severe inflammatory response [Miao et al., 2013]. Nevertheless the underlying mechanism of action of MCPIP1 is poorly understood, although it has been suggested that it may control mRNA stability of IL-6 and IL-12p40 by RNase activity [Uehata and Akira, 2013] and protein stability of NF-κB signaling mediators by DUB activity. Our results suggest that MCPIP1 stabilizes RGS2 protein level, and that C157 residue plays an important role regulating DUB activity. We also assume that RGS2 protein will regulate breast cancer cell growth through TSPYL5 protein.

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