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Overexpression of SIRT5 confirms its involvement in deacetylation and activation of carbamoyl phosphate synthetase 1

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ABSTRACT

SIR2 protein, an NAD-dependent deacetylase, is localized to nucleus and is involved in life span extension by calorie restriction in yeast. In mammals, among the seven SIR2 homologues (SIRT1-7), SIRT3, 4, and 5 are localized to mitochondria. As SIRT5 mRNA levels in liver are increased by fasting, the physiological role of SIRT5 was investigated in liver of SIRT5-overexpressing transgenic (SIRT5 Tg) mice. We identified carbamoyl phosphate synthetase 1 (CPS1), a key enzyme of the urea cycle that catalyzes condensation of ammonia with bicarbonate to form carbamoyl phosphate, as a target of SIRT5 by two-dimensional electrophoresis comparing mitochondrial proteins in livers of SIRT5 Tg and wild-type mice. CPS1 protein was more deacetylated and activated in liver of SIRT5 Tg mice than in wild-type. In addition, urea production was upregulated in hepatocytes of SIRT5 Tg mice. These results agree with those of a previous study using SIRT5 knockout (KO) mice. Because ammonia generated during fasting is toxic, SIRT5 protein might play a protective role by converting ammonia to non-toxic urea through deacetylation and activation of CPS1.

Introduction

SIR2 protein is an NAD-dependent deacetylase [1]. In yeast, increasing the dosage of the SIR2 gene extends life span, whereas disruption of the SIR2 gene shortens it [2]. SIR2 determines life span not only in yeast but also in *Caenorhabditis elegans* [3] and *Drosophila melanogaster* [4]. Mammals have seven SIR2 homologues, SIRT1-7 [5]. SIRT1 deacetylates and regulates the activities of many proteins in the nucleus. SIRT1 upregulates expression of gluconeogenic genes and downregulates glycolytic genes through deacetylation of PPARγ coactivator-1α and FOXO1 [6,7].

SIRT3, SIRT4, and SIRT5 proteins are known to be localized to mitochondria [8]. *In vitro*, SIRT3 protein deacetylates and activates mitochondrial enzymes such as glutamate dehydrogenase, isocitrate dehydrogenase 2, and acetyl CoA synthetase 2 (AceCS2) [9–11]. While SIRT4 does not have NAD-dependent deacetylase activity, it does have ADP-ribosyl transferase activity. SIRT4 inhibits insulin secretion by repression of glutamate dehydrogenase activity through ADP-ribosylation [12,13]. SIRT5 protein also exhibits NAD-dependent deacetylase activity on histone H4 peptide *in vitro* [13]. Recently, Nakagawa et al. reported that SIRT5 interacts with carbamoyl phosphate synthetase 1 (CPS1), and that deacetylated CPS1 is decreased and CPS1 activity is downregulated in livers of SIRT5 knockout (KO) mice [14].

During fasting or starvation, circulating amino acids are derived mainly from catabolism of skeletal muscle, and are used in gluconeogenesis in liver to maintain blood glucose levels. The ammonia co-generated in liver from these amino acids is toxic; the urea cycle detoxifies this ammonia by converting it to non-toxic, water-soluble urea, which is readily excreted from kidney [15,16].

CPS1 is the mitochondrial protein that catalyzes the first step of the urea cycle, the condensation of ammonia with bicarbonate to form carbamoyl phosphate [15,16]. Patients with CPS1 deficiency exhibit lethally severe hyperammonemia in the neonatal period [16], which suggests a critical role for CPS1 in the urea cycle.

In the present study, to investigate the physiological role of SIRT5, we generated SIRT5-overexpressing transgenic (SIRT5 Tg) mice and attempted to identify the target protein of SIRT5 regulation in liver. We show here that SIRT5 protein might regulate urea production by deacetylation and activation of mitochondrial CPS1, complementing the previous study of SIRT5 KO mice [14].

Materials and methods

Animal Experiments: The mice were housed in an air-controlled (temperature 25 °C) room with dark–light cycle (10 h; 14 h).

Abbreviations: SIR2, silent information regulator 2; CPS1, carbamoyl phosphate synthetase 1; Tg, transgenic; KO, knockout.

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Animal care and procedures were approved by the Animal Care Committee of Kyoto University.

Isolation of total RNA and quantitative RT-PCR: Total RNA was isolated from livers, kidneys and hearts of 11 week-old C57BL/6 mice using Trizol (Invitrogen), and cDNA was prepared by reverse transcriptase (Superscript II; Invitrogen) with an oligo (dT) primer. SIRT5 mRNA levels were measured by real-time quantitative RT-PCR using ABI PRISM 7000 Sequence Detection System (Applied Biosystems). SIRT5 mRNA levels were corrected for β-actin mRNA levels. The mouse sequences of forward and reverse primers to evaluate SIRT5 expression were 5′-GTCATCACCCAGAACATCGA-3′ and 5′-ACGTGAGGTCGCAGCAAGCC-3′; respectively. The mouse sequences of forward and reverse primers to evaluate β-actin expression were 5′-TTGCAGCTCCTTCGTTGC-3′ and 5′-CACGAT GGAGGGGAATACAG-3′, respectively. SYBR Green PCR Master Mix (Applied Biosystems) was prepared for PCR run. The thermal cycling conditions were denaturation at 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min.

Plasmid construction: The expression vectors for SIRT5 protein with (pCMV5aSIRT5-FLAG) and without (pCMV5aSIRT5) FLAG tag at the C-terminus were constructed as follows. The coding region of mouse SIRT5 cDNA was cloned by PCR using mouse liver cDNA prepared as described above. The PCR fragments were subcloned into pFLAG-CMV-5a (Sigma).

Antibody: SIRT5 polyclonal antibody was produced by immunizing a rabbit with synthetic peptide CGKTLPEALAPHETE, corresponding to 15 C-terminal amino acid residues of mouse SIRT5 protein. The antibody was purified by HiTrap NHS-activated HP kit (GE Healthcare) and gel filtration column. Western blotting analysis was performed using the obtained anti-SIRT5 or anti-FLAG (Sigma) antibodies.

Generation of SIRT5-overexpressing transgenic mice: SIRT5 cDNA with FLAG tag sequences was inserted to the *Eco*RI site of transgenic plasmid pIns-1 [17], and the human insulin promoter of pIns-1 was then replaced by the CAG promoter derived from pCAGGS plasmid [18]. The transgene cassettes were excised from the resulting plasmid by digestion with *Not*I and *Xho*I, and the linearized cassettes were microinjected to fertilized eggs of C57BL/6 inbred mice (PhoenixBio CO., Ltd. Hiroshima, Japan). Since the two lines revealed similar data, all additional experiments were performed using line #38.

Preparation of mitochondria: The livers of C57BL/6 mice were homogenized in isotonic buffer (PBS containing 0.25 M sucrose) containing protease inhibitors (Complete, EDTA free: Roche) with potter homogenizer. The mitochondria were prepared as described previously [8].

Two-dimensional electrophoresis and identification of protein: Liver mitochondria were prepared as described previously [8] and lysed with rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte (BIO-RAD), 0.001% bromophenol blue), and applied to ReadyStrip IPG Strip (BIO-RAD) and separated by isoelectric focusing electrophoresis with a range pH 5 to pH 8 using PROTEAN IEF cell (BIO-RAD). The IPG Strip was then subjected to SDS–polyacrylamide gel electrophoresis. The obtained gel was stained with SYPRO Ruby protein gel stain kit (invitrogen), and the protein was visualized and analyzed using Typhoon 9210 (GE healthcare). The protein at the indicated spot was isolated and analyzed using MALDI-TOF-MS (APRO Life Science Institute, Inc.).

Immunoprecipitation: Mitochondria lysed with PBS containing 1% Triton X-100 were incubated with anti-CPS1 (Santa Cruz) or anti-acetylated lysine (Cell Signaling) antibody for 16 h at 4 °C. Protein G Sepharose (GE healthcare) was then added and incubation was continued for 3 h. The resin was washed and boiled with SDS sample buffer (0.2 M Tris, 10% sucrose, 10% SDS, 5 mM EDTA). The sample was analyzed by western blotting with anti-CPS1 antibody.

Determination of CPS1 activity: Livers of 8–12 week-old SIRT5 Tg and wild-type mice fasted for 16 h were homogenized [19] and CPS1 activities were assayed as described by Fahien and Cohen [20]. Briefly, the reaction was started by adding the supernatant obtained above (enzyme source) to the assay mixture containing 2.5 mM phosphoenopyruvate, 0.2 mM NADH, 10 mM NH_4Cl, 100 mM $KHCO_3$, 5 mM ATP, 10 mM $MgSO_4$, 10 mM *N*-acetylglutamate, 10 U/ml pyruvate kinase (SIGMA), 12.5 U/ml of lactate dehydrogenase (SIGMA), and 50 mM glycylglycine (pH 7.6) at room temperature, and the decrease in absorbance at 340 nm was measured. The initial velocity of the reaction was directly proportional CPS1 activity. One unit of CPS1 activity corresponded to oxidation of 1 μmol of NADH/min at room temperature.

Measurement of hepatic urea production: Hepatocytes of 8–12 week-old SIRT5 Tg and wild-type mice fasted for 16 h were isolated as described previously [21]. Obtained hepatocytes (1.5×10^5) were incubated at 37 °C in humidified atmosphere (5% CO_2) in 2 ml Krebs ringer buffer with 25 mM $NaHCO_3$, 10 mM NH_4Cl, and 5 mM ornithin–HCl [22]. Incubation was stopped by placing the cells on ice, followed by centrifugation at 4 °C for 10 min at 600*g*. The supernatant was removed and urea concentration was measured by diacetyl monoxime methods [23,24], the cells were lysed with 0.1% SDS, and the protein concentration was determined (Bio-Rad Protein Assay Kit).

Statistical analysis: Values are expressed as means ± SEM. Statistical analysis was performed unpaired Student's *t*-test. *P* values <0.05 were considered significant.

Results

Upregulation of SIRT5 mRNA levels by fasting

Expression levels of SIRT1 mRNA are known to be increased in liver and heart by fasting [25]. However, it is unclear whether the expression levels of SIRT5 are regulated by nutrient conditions. To evaluate alteration of SIRT5 mRNA expression levels in different nutrient conditions, total RNA was extracted from organs including liver, kidney, and heart in C57BL/6 mice fed *ad libitum*, fasted for 24 h, or refed for 24 h after 24-h fasting, and quantitative RT-PCR was carried out. SIRT5 mRNA levels in liver were increased 2.4-fold by fasting ($N = 4$, $P < 0.01$) and were decreased to fed condition levels by refeeding ($N = 4$, $P < 0.05$), but were unchanged in kidney or heart (Fig. 1), suggesting an important role of SIRT5 in liver.

Generation of SIRT5 Tg mice

To clarify the function of SIRT5 *in vivo*, we generated SIRT5-overexpressing transgenic (SIRT5 Tg) mice in which expression of mouse SIRT5 fused with FLAG tag at the C-terminus was driven by the CAG promoter (Fig. 2A). Southern blot analysis was performed for genotyping (Fig. 2B), and two independent SIRT5 Tg mouse lines were established on the C57BL/6 inbred background (Fig. 2C). One of these, mouse line #36, was generated with a low copy number of transgene; the other, mouse line #38, was generated with a high copy number of transgene. SIRT5 Tg mice showed no gross anatomical or reproductive defects. In addition, no histological abnormality was observed by light microscopic analysis in all of the organs examined. To investigate the expression of SIRT5 protein, an antibody specific for mouse SIRT5 was raised in a rabbit against a synthetic peptide corresponding to 15 C-terminal amino acid residues (CGKTLPEALAPHETE) of mouse SIRT5, which shows no similarity to other members of the SIRT family. To ascertain specificity of the antibody, mitochondrial proteins were prepared from COS7 cells transfected with the expression plasmid encoding mouse SIRT5 protein fused with FLAG tag,

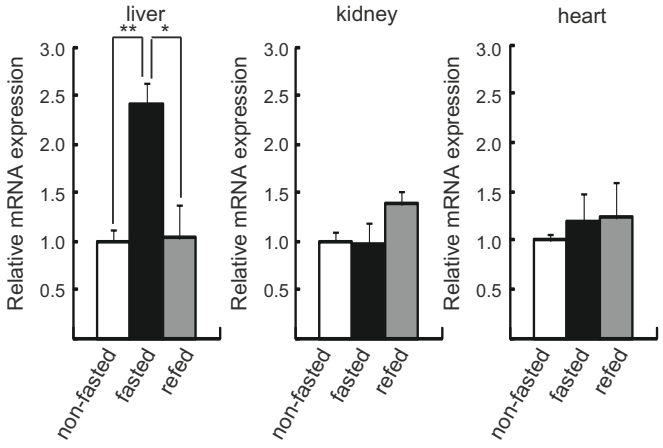

Fig. 1. Alteration of expression levels of SIRT5 mRNA by fasting and refeeding. Total RNA was isolated from livers, kidneys and hearts of 11 week-old C57BL/6 mice. Mice were divided into three groups: non-fasted ($N = 4$, open bars), fasted ($N = 4$, filled bars), and refed ($N = 4$, gray bar). The non-fasted group was fed *ad libitum*, the fasted group was fasted for 24 h, and the refed group was fasted for 24 h followed by normal chow for 24 h. The expression levels of SIRT5 mRNA were estimated by quantitative RT-PCR. SIRT5 mRNA levels were corrected for β-actin mRNA levels. Values are means ± SEM. $^{*}P < 0.05$. $^{**}P < 0.01$.

and western blotting was performed using the obtained anti-SIRT5 antibody or anti-FLAG antibody. A single band at ~32 kDa corresponding to the molecular weight of SIRT5–FLAG protein was detected with each antibody (Fig. 2D), demonstrating that this antibody specifically recognizes SIRT5 protein. Western blot analysis of mitochondrial proteins prepared from livers of the two SIRT5 Tg mouse lines using the anti-SIRT5 antibody showed that both endogenous 32-kDa SIRT5 protein and a SIRT5–FLAG protein with a molecular size somewhat larger were present, and that expression of SIRT5–FLAG protein was more abundant than that of endogenous SIRT5 protein in both Tg mouse lines (Fig. 2E).

Identification of CPS1 as a target of SIRT5 protein

Since SIRT5 mRNA levels were significantly upregulated in liver by fasting, we attempted to identify the protein that is modified by SIRT5 protein in liver. We hypothesized that deacetylation of the SIRT5 target protein might be facilitated by overexpression of SIRT5 in SIRT5 Tg mice. Because NAD-dependent deacetylase converts acetylated lysine to lysine of the target protein, its isoelectric point should shift to a higher pH value. Therefore, we performed two-dimensional electrophoresis to compare mitochondrial proteins prepared from livers of SIRT5 Tg mice and wild-type littermates; the protein samples were applied for isoelectric focusing electrophoresis, and separated by SDS–PAGE followed by staining with SYPRO Ruby. One of the proteins that newly appeared in SIRT5 Tg liver (Fig. 3A, indicated by arrow) was isolated, treated with trypsin, and analyzed by MALDI-TOF-MS (Fig. 3B). The sequences identified by mass spectrometry covered the N-terminal segment of carbamoyl phosphate synthetase 1 (CPS1) (Table. 1), suggesting that the protein is CPS1. CPS1 is a mitochondrial protein

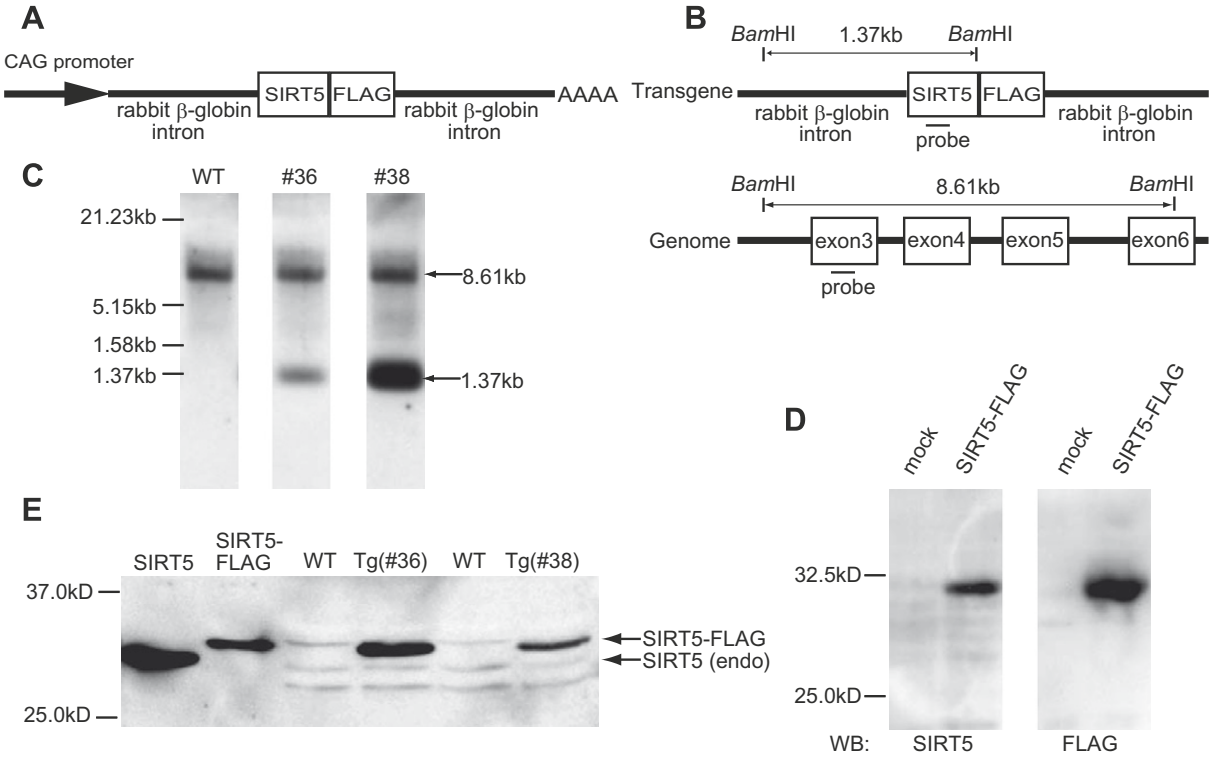

Fig. 2. Generation of SIRT5-overexpressing transgenic (SIRT5 Tg) mice and anti-SIRT5 antibody. (A) Scheme of the transgene used to generate SIRT5 Tg mice. The CAG promoter drives expression of mouse SIRT5 fused with FLAG tag at the C-terminus (SIRT5–FLAG). (B) Location of probes for Southern blot analysis. The probe corresponding to the DNA sequence in exon3 of mouse SIRT5 gene detects an 8.61-kb fragment in genomic DNA and a 1.37-kb fragment in the transgene after digestion with *Bam*HI. (C) Southern blot analysis of *Bam*HI-digested genomic DNA of wild-type (left panel) and SIRT5 Tg mice (middle panel: #36 transgenic mouse line, right panel: #38 transgenic mouse line). Southern blotting was performed with the probe indicated in (B). (D) Ascertainment of anti-SIRT5 antibody. The plasmid encoding mouse SIRT5 protein fused with FLAG tag (pCMV5aSIRT5–FLAG) was transfected to COS7 cells. Mitochondrial proteins were prepared from transfected and mock-transfected cells, and western blotting was performed with anti-SIRT5 antibody (left panel) and anti-FLAG antibody (right panel). (E) Overexpression of SIRT5–FLAG protein in SIRT5 Tg mice. Mitochondrial proteins were prepared from cells transfected with the plasmid encoding mouse SIRT5 without (pCMV5aSIRT5) or with FLAG (pCMV5aSIRT5–FLAG) and from livers of SIRT5 Tg mice and wild-type littermates (#36 and #38 transgenic mouse lines), and western blot analysis was performed using anti-SIRT5 antibody. Endogenous SIRT5 (endo) and SIRT5–FLAG proteins are indicated by arrows.

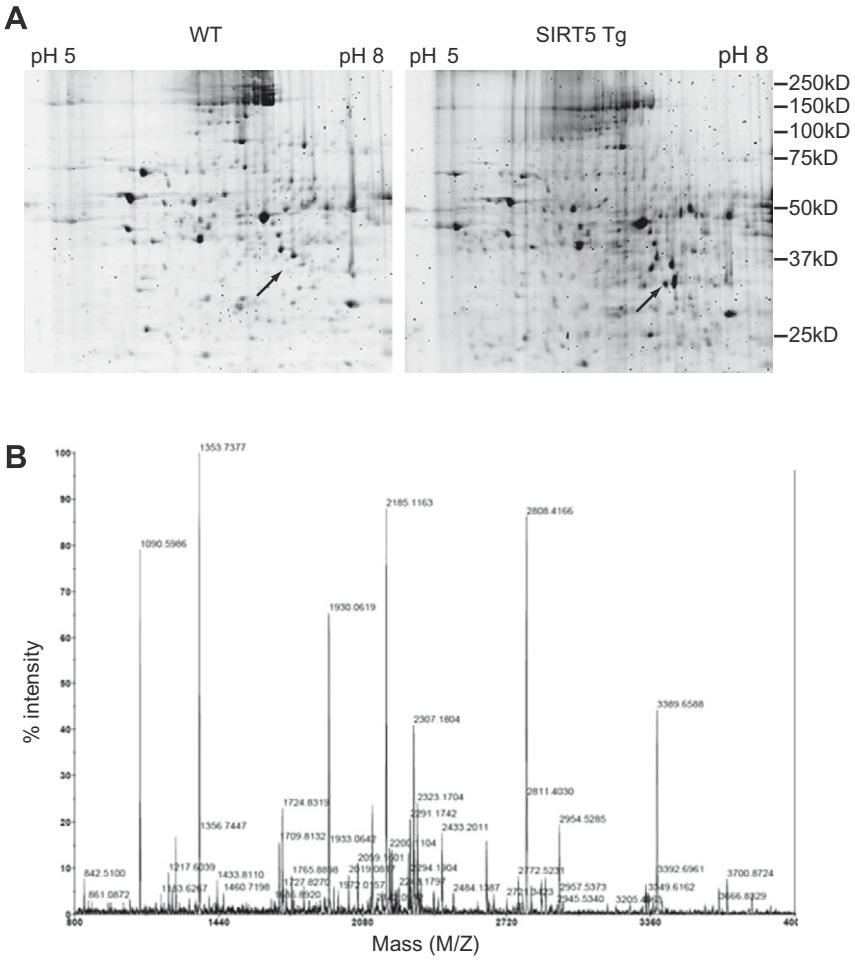

Fig. 3. Identification of the target protein of SIRT5. (A) Two-dimensional electrophoresis of mitochondrial proteins prepared from livers of SIRT5 Tg and wild-type mice. The position of the mitochondrial protein prepared from SIRT5 Tg liver identified by MALDI-TOF-MS is shown by arrow (right panel). The protein is not detected at the corresponding position (indicated by arrow, left panel) in wild-type liver. (B) MALDI-TOF-MS analysis. The mitochondrial protein prepared from SIRT5 Tg liver indicated in (A) was analyzed using MALDI-TOF-MS.

Table 1
Characterization by MALDI-TOF-MS of the target protein of SIRT5.

Measured peptide mass (Da)	Predicted peptide sequence	Start–end
1090.5986	KVPAIYGVDTRM	157–166
1217.6039	KSLGQWLQEEKV	147–156
1353.7377	RGQNQPVLNITNRQ	316–327
1723.8272	KIEFEGQSVDFVDPNKQ	182–196
1930.0619	KEPLFGISTGNIITGLAAGAKS	287–306
2058.1598	RKEPLFGISTGNIITGLAAGAKS	286–306
2184.1154	KGQILTMANPIIGNGGAPDTTARD	90–111
2807.4071	KIEFEGQSVDFVDPNKQNLIAEVSTKD	182–206
2953.5338	KGQILTMANPIIGNGGAPDTTARDELGLNKY	90–118
3662.8010	KMKGYSFGHPSSVAGEVVFNTGLGGYPEALTDPAYKG	55–89

Mass of peptides corresponding to a tryptic digest of CPS1. The corresponding sequence and position (number of amino acid residues) in the sequence are indicated.

expressed predominantly in liver, and catalyzes condensation of ammonia and bicarbonate to carbamoyl phosphate, which is the first step in the urea cycle in liver [15,16].

CPS1 is deacetylated in SIRT5 Tg liver

Since CPS1 is known to be an acetylated protein [26], we investigated deacetylation of CPS1 protein by SIRT5. The mitochondrial protein prepared from livers of SIRT5 Tg and wild-type mice fed *ad libitum* were immunoprecipitated with an anti-acetylated lysine antibody followed by immunoblotting using anti-CPS1 antibody. Acetylated CPS1 protein levels in SIRT5 Tg ($N = 3$) mice were 40% lower than those in wild-type mice ($N = 3$), although total CPS1 expression levels were similar (Fig. 4A). To determine whether SIRT5 protein regulates CPS1 activities, livers of SIRT5 Tg and wild-type mice were homogenized and CPS1 activities were measured. CPS1 activities were significantly increased approximately 2-fold in SIRT5 Tg mice ($N = 5$, $P < 0.01$) compared to those in wild-type mice (Fig. 4B). These results demonstrate that SIRT5 protein deacetylates CPS1 and upregulates its activity in liver.

Urea production is upregulated in SIRT5 Tg hepatocytes

To verify that SIRT5 is involved in the urea cycle by regulating CPS1 activity, production of urea in primary cultured hepatocytes was evaluated. Primary hepatocytes were isolated from SIRT5 Tg and wild-type mice and incubated with ammonia, bicarbonate, and ornithine for 1 h, and the amount of urea synthesized was determined by measuring the urea concentration in the media. Hepatocytes from SIRT5 Tg mice produced more urea than those of wild-type mice (44%, $N = 4$, $P < 0.01$) (Fig. 4C), indicating that urea synthesis is upregulated by overexpression of SIRT5 in liver.

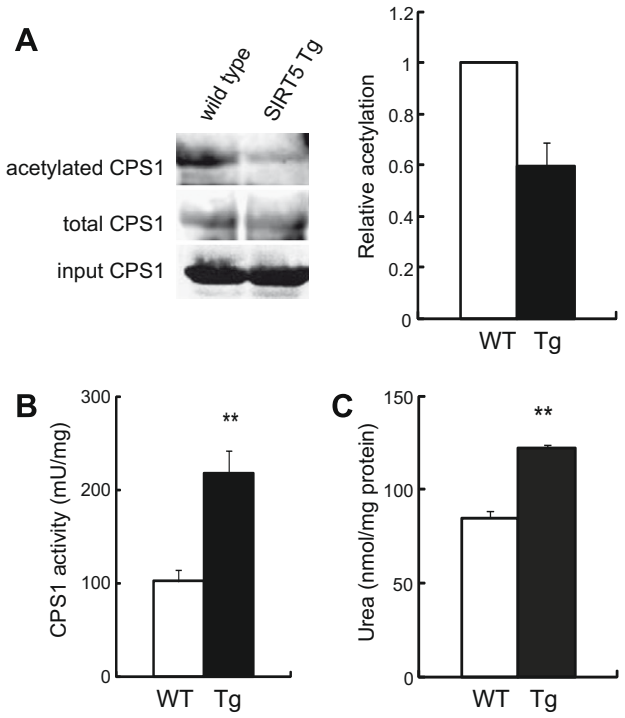

Fig. 4. Deacetylation and activation of CPS1 in livers of SIRT5 Tg mice. (A) CPS1 deacetylation in livers of SIRT5 Tg mice. The mitochondrial protein from livers of SIRT5 Tg and wild-type mice fed *ad libitum* were immunoprecipitated with anti-acetylated lysine antibody (left upper panel) and anti-CPS1 antibody (left middle panel), and immunoblotted with anti-CPS1 antibody. The right panel shows acetylation of CPS1 protein. The ratio of acetylated CPS1 protein of SIRT5 Tg liver to that of wild-type liver was calculated from densitometry of western blots ($N = 3$). (B) Activation of CPS1 activity in SIRT5 Tg liver. Livers of SIRT5 Tg ($N = 5$) and wild-type ($N = 5$) mice fasted for 16 h were homogenized and CPS1 activities were determined as described in Materials and methods. (C) Production of urea in primary cultured hepatocytes. Primary hepatocytes were isolated from SIRT5 Tg ($N = 4$) and wild-type ($N = 4$) mice fasted for 16 h and incubated with $NaHCO_3$ (25 mM), NH_4Cl (10 mM) and ornithine–HCl (5 mM) for 1 h. The amount of urea synthesized was determined by measuring the urea concentration in the media as described in Materials and methods. Values are means ± SEM. $^*P < 0.05$. $^{**}P < 0.01$.

Discussion

In the present study, SIRT5 mRNA levels in liver were found to be increased by fasting. To investigate the function of SIRT5 in liver, we established SIRT5 Tg mice and identified CPS1 as the target protein of SIRT5 by analyses of liver mitochondrial proteins using two-dimensional electrophoresis and MALDI-TOF-MS. We found that CPS1 is deacetylated and that CPS1 activity is significantly increased in the liver of SIRT5 Tg mice. CPS1 is the first and key enzyme of the urea cycle, condensing ammonia with bicarbonate to generate carbamoyl phosphate [15,16]. In hepatocytes from SIRT5 Tg mice, urea synthesis was upregulated compared to that in wild-type mice.

Which residue on CPS1 is deacetylated by SIRT5 is still unclear. The protein that we identified as a target of SIRT5 using two-dimensional electrophoresis and MALDI-TOF-MS was the N-terminal domain of CPS1 (Table. 1), suggesting that the lysine residue of CPS1 deacetylated by SIRT5 is contained in this domain. As Kim et al. reported that CPS1 has nine lysine acetylation sites [26], at least Lys55, Lys119, and/or Lys287 among these lysine residues located in the N-terminal domain might be deacetylated by SIRT5.

During fasting, circulating amino acids, especially alanine, are derived by lysis of muscle proteins, and the amino acids are catalyzed in liver by α-ketoglutarate aminotransferase to generate pyruvate and glutamate. Pyruvate is used in gluconeogenesis to maintain plasma glucose levels, and glutamate is catalyzed by glutamate dehydrogenase to form α-ketoglutarate and ammonia. Ammonia is extremely toxic; the urea cycle converts ammonia to non-toxic urea, which is readily excreted from kidney. It also has been reported that CPS1 activity is increased by fasting or calorie restriction in rodent liver [27,28]. Thus, SIRT5 protein may be involved in detoxification of ammonia during fasting through deacetylation and activation of CPS1.

Recently, Nakagawa et al. reported that CPS1 activity is downregulated in liver of SIRT5 KO mice, which exhibit hyperammonemia by fasting [14]. We show here that CPS1 activity in liver is upregulated in SIRT5 Tg mice during fasting and that urea synthesis is upregulated in SIRT5 Tg hepatocytes, complementing their data.

Suggesting the mechanism of CPS1 activation during fasting, SIRT5 mRNA expression levels in liver were increased by fasting, but protein expression levels were not altered under the same condition in wild-type mice (Supplemental Fig. 1). Nakagawa et al. reported that SIRT5 protein expression levels were not altered, but that NAD levels were elevated by fasting in wild-type mice [14], suggesting that CPS1 is not activated by an increase in SIRT5 protein levels but by activation of SIRT5 through elevation of NAD.

SIRT5 protein is highly expressed in organs other than liver, including kidney, skeletal muscle, and heart (Supplemental Fig. 1), where their functions are yet unknown. Further investigation of the pathophysiological role of SIRT5 is required.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.081.

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