304 *Biochemistry* **2010,** *49,* 304–311
DOI: 10.1021/bi901627u

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Regulation of Succinate Dehydrogenase Activity by SIRT3 in Mammalian Mitochondria[†]

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Received September 17, 2009; Revised Manuscript Received December 9, 2009

ABSTRACT: A member of the sirtuin family of NAD^+-dependent deacetylases, SIRT3, is identified as one of the major mitochondrial deacetylases located in mammalian mitochondria responsible for deacetylation of several metabolic enzymes and components of oxidative phosphorylation. Regulation of protein deacetylation by SIRT3 is important for mitochondrial metabolism, cell survival, and longevity. In this study, we identified one of the Complex II subunits, succinate dehydrogenase flavoprotein (SdhA) subunit, as a novel SIRT3 substrate in SIRT3 knockout mice. Several acetylated Lys residues were mapped by tandem mass spectrometry, and we determined the role of acetylation in Complex II activity in SIRT3 knockout mice. In agreement with SIRT3-dependent activation of Complex I, we observed that deacetylation of the SdhA subunit increased the Complex II activity in wild-type mice. In addition, we treated K562 cell lines with nicotinamide and kaempferol to inhibit deacetylase activity of SIRT3 and stimulate SIRT3 expression, respectively. Stimulation of SIRT3 expression decreased the level of acetylation of the SdhA subunit and increased Complex II activity in kaempherol-treated cells compared to control and nicotinamide-treated cells. Evaluation of acetylated residues in the SdhA crystal structure from porcine and chicken suggests that acetylation of the hydrophilic surface of SdhA may control the entry of the substrate into the active site of the protein and regulate the enzyme activity. Our findings constitute the first evidence of the regulation of Complex II activity by the reversible acetylation of the SdhA subunit as a novel substrate of the NAD^+-dependent deacetylase, SIRT3.

Reversible acetylation of mitochondrial proteins is critical for regulation of many biological processes, including oxidative phosphorylation and the Krebs cycle (*1*−*7*). Flavoprotein of the succinate dehydrogenase complex (Complex II SdhA subunit) was identified as one of the acetylated proteins of mouse liver mitochondria in two independent high-throughput mappings of acetylated proteins by tandem mass spectrometry (*1*, *7*). Complex II or succinate dehydrogenase (SDH) is found as an inner membrane-bound enzyme complex, and it is the only enzyme that participates both in the Krebs cycle and in oxidative phosphorylation in mitochondria. It has four different protein subunits: hydrophilic subunits SdhA[1] and SdhB facing the matrix side of the inner membrane and hydrophobic subunits SdhC and SdhD tethering the complex in the phospholipid membrane. SdhA is a 70 kDa large flavoprotein subunit containing covalently bound FAD and substrate binding site for the point of entry of electrons into Complex II. SDH plays such an important role in the mitochondria that severe deficiency of this enzyme is incompatible with life. However, point or milder mutations in the C-terminal domain of SdhA lead to Leigh syndrome and various neurodegenerative disorders (*8*). Mutations of the other SDH subunits containing Fe−S cofactors have been associated with generation of reactive oxygen species causing tumor formation (*9*).

Post-translational modifications of SdhA by phosphorylation at Tyr residues and acetylation at lysine residues were previously reported (*1*, *7*, *10*). Interestingly, six acetylated lysine residues in SdhA were mapped in the LC−MS/MS analysis of well-fed rat mitochondria in two independent studies (*1*, *7*). However, neither enzymes responsible for reversible acetylation and phosphorylation nor the regulatory roles of these post-translational modifications in SdhA or Complex II activity are known. Several members of the class III histone deacetylases (sirtuins), SIRT3, SIRT4, and SIRT5, have been found to reside in mitochondria (*6*, *11*, *12*). Sirtuins use NAD^+ as a cosubstrate; both SIRT3 and SIRT4 are required to maintain cell survival after genotoxic stress in a NAD^+-dependent manner, and genetic variations in the human SIRT3 gene have been linked to longevity (*13*, *14*). We have previously shown that the level of SIRT3 expression in adipose tissue is increased by caloric restriction and cold exposure (*1*, *15*). Mitochondrial acetyl-CoA synthetase 2 and glutamate dehydrogenase (GDH) are the two key metabolic enzymes regulated through deacetylation by SIRT3 (*3*, *6*, *16*). Thus, SIRT3 was determined to be the major deacetylase that modulates mitochondrial function in response to the [NADH]/[NAD^+] ratio by regulating the activity of key metabolic enzymes (*6*, *12*, *16*, *17*).

In addition to metabolic enzymes, nuclear-encoded subunits of the electron transport chain complexes and ribosomes responsible for the synthesis of 13 essential proteins of the oxidative phosphorylation were found to be regulated by reversible acetylation (*1*).

[†]This work was supported National Institutes of Health grants to Q.T. (R01DK075978) and E.C.K. (R01GM071034).

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[1]Abbreviations: 2D-gel, two-dimensional gel electrophoresis; FAD, flavin adenine dinucleotide; K562, human chronic myelogenous leukemia; LC−MS/MS, liquid chromatography and tandem mass spectrometry; NAD^+, nicotinamide adenine dinucleotide; SdhA, succinate dehydrogenase subunit A; sirtuin or SIRT, silent information regulator two (Sir2) homologue.

 Published on Web 12/10/2009

In our recent studies, we demonstrated that the mitochondrial ribosomal protein MRPL10 is acetylated and its deacetylation by the NAD^+-dependent deacetylase SIRT3 regulates mitochondrial protein synthesis (*18*). Additionally, Complex I subunit NDUFA9 is also identified as a SIRT3 substrate, and acetylation and deacetylation of this protein is proposed to regulate and maintain basal ATP levels in mammalian mitochondria (*17*). However, the contribution of Complex II acetylation was overlooked on oxidative phosphorylation and ATP production in the same study (*17*).

Here, we confirm that one of the subunits of Complex II, SdhA, is indeed a highly acetylated protein and is a novel SIRT3 substrate as shown in SIRT3 knockout mice using various proteomics techniques. We have also determined the SIRT3-dependent activation of Complex II in wild-type mice and in cells overexpressing SIRT3. Our results reported in this study suggest a more global role for SIRT3 in regulating oxidative phosphorylation by reversible acetylation of the Complex II subunit SdhA and, therefore, ATP production in mammalian mitochondria.

MATERIALS AND METHODS

Isolation of Mitochondria from Mouse Liver and Enrichment of Complex II. SIRT3 knockout mice were obtained from the Texas Institute for Genomic Medicine (Houston, TX). Briefly, these mice were produced by generating embryonic stem (ES) cells (Omnibank entry OST341297) bearing a retroviral promoter trap that functionally inactivates one allele of the *Sirt3* gene, as described previously (*19*). Liver tissue obtained from $Sirt3^{+/+}$, $Sirt3^{+/-}$, and $Sirt3^{-/-}$ mice was resuspended in an isotonic mitochondrial buffer (MB) [210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES-KOH (pH 7.5)], supplemented with protease inhibitors (1 mM phenylmethanesulfonyl fluoride and 50 μg/mL leupeptin), and then homogenized in a Dounce homogenizer (Wheaton) on ice. The suspension was centrifuged at 400*g* on a microcentrifuge (ThermoForma) at 4 °C. This procedure was repeated twice, and supernatants were centrifuged at 10000*g* and 4 °C for 10 min to pellet mitochondria. After the mitochondrial pellets were lysed in a buffer containing 0.26 M sucrose, 20 mM Tris-HCl (pH 7.6), 40 mM KCl, 20 mM $MgCl_2$, 0.8 mM EDTA, 0.05 mM spermine, 0.05 mM spermidine, 6 mM β-mercaptoethanol, and 1.6% Triton X-100, mitochondrial lysates were loaded onto 34% sucrose cushion and centrifuged at 100000*g* and 4 °C for 16 h. The cushion layers enriched for acetylated proteins were precipitated with acetone.

2D-gel and Immunoblotting Analysis. Acetone-precipitated protein pellets were resuspended in Destreak rehydration buffer (Amersham Biosciences Inc.) and loaded onto the IPG strips (pI 3−10) (Bio-Rad Laboratories, Inc.). IPG strips were rehydrated overnight and run on the Ettan IPGphor (Amersham Biosciences Inc.) according to the manufacturer's protocols. The first-dimension IPG strips were equilibrated in 6 M urea, 0.375 M Tris-HCl (pH 8.8), 2% SDS, 20% glycerol, and 2% (w/v) DTT for 10 min. The strips then were equilibrated in the equilibration buffer containing 2.5% (w/v) iodoacetamide and loaded onto the second-dimension SDS−PAGE gel. The gels were either stained with Coomassie Blue or transferred to a PVDF membrane to be probed with *N*-acetyllysine antibody at a 1:3000 dilution or SIRT3 antibody at a 1:1000 dilution (Cell Signaling Technology Inc.), a monoclonal SdhA (Complex II subunit 70 kDa Fp) antibody at a 1:5000 dilution (MitoSciences Inc.), or β-actin antibody at a 1:5000 dilution (Abcam Inc.). The secondary antibody was ImmunoPure Antibody goat anti-mouse IgG (Pierce Biochemicals Inc.) at a 1:5000 dilution, goat anti-rabbit IgG at a 1:5000 dilution, or Affinipure rabbit anti-mouse IgG, rabbit anti-goat IgG, or goat anti-rabbit IgG (Jackson Immuno Research), each at a 1:10000 dilution, followed by development with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biochemicals Inc.) according to the protocol provided by the manufacturer.

Mass Spectrometric Identification and Mapping of Acetylation Sites. SDS−PAGE bands and 2D-gel spots corresponding to acetylated proteins were excised and digested in-gel with trypsin prior to liquid chromatography tandem mass spectrometry (LC−MS/MS) analysis. The LC−MS/MS analyses were performed with an LTQ mass spectrometer equipped with a nano-electrospray ionization source and Surveyor MS Pump Plus HPLC system and Surveyor Micro AS autosampler (ThermoFisher Co.). The in-gel tryptic digests (3−5 μL) were injected and loaded onto a peptide trap (MiChrom peptide CapTrap, C8 like resin, 0.3 mm × 1 mm, 5 μm) over 3 min at a rate of 10 μL/min for online desalting and concentration. The peptide trap was then placed in line with the analytical column, a PicoFrit column (0.075 mm × 150 mm) packed in-house with Supelco's Wide Bore C18 (5 μm, 300 Å) resin. The column was eluted at a rate of 250 nL/min using a gradient that consisted of 0.1% formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The peptides were eluted by ramping solvent B to 40% over 30 min. Tandem MS spectra were acquired for ions above a predetermined intensity threshold using automated data-dependent acquisition. The spectra were processed and searched against the protein sequence database Swiss-Prot using locally maintained Mascot 2.2 (Matrix Science) and Proteome Discoverer 1.0 (ThermoFisher) search engines to identify proteins and modifications. Mass tolerances were 3 and 2 amu for precursor and product ions, respectively. Up to two missed cleavages were allowed for digestion by trypsin, and methionine oxidation (+16) and lysine acetylation (+42) were considered as variable modifications.

Cell Culture. Brown preadipocyte HIB1B cells with retroviral stable expression of murine SIRT3 (amino acids 78−334) were previously described (*15*). In addition, an alternative transcript of murine SIRT3 expressing a longer form of murine SIRT3 (amino acids 1−334) was a gift from D. Sinclair of Harvard Medical School (Boston, MA) (*20*). The full-length SIRT3(1−334) cDNA was amplified by PCR with the following primers: 5′-ATAGAATTCATGGCGCTTGACCCTC-3′ and 5′-ATAGAATTCTCTGTCCTGTCCATCC-3′. The PCR product was then inserted into the EcoRI site of the pBabe-puro-Flag vector (Flag tag inserted between the EcoRI and SalI sites of pBabe-puro). HIB1B cells with stable retroviral expression of full-length SIRT3(1−334) were established as described previously (*15*). Mitochondria were isolated from HIB1B stable cell lines expressing truncated and full-length SIRT3 grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) bovine calf serum (Hyclone, Logan, UT), 1% (v/v) penicillin/streptomycin, and puromycin (4 μg/mL) at 37 °C with 5% CO_2 in a humidified atmosphere, and those cells were routinely subcultured in the semiconfluent state.

Approximately 7×10^7 K562 (human chronic myelogenous leukemia) cells were grown in RPMI 1640 medium (Mediatech Inc.) supplemented with 10% (v/v) bovine calf serum (Hyclone), 100 IU/mL penicillin, and 100 μg/mL streptomycin, at 37 °C and 5% CO_2 in a humidified atmosphere. Cells were treated with nicotinamide (Calbiochem, San Diego, CA) or kaempferol

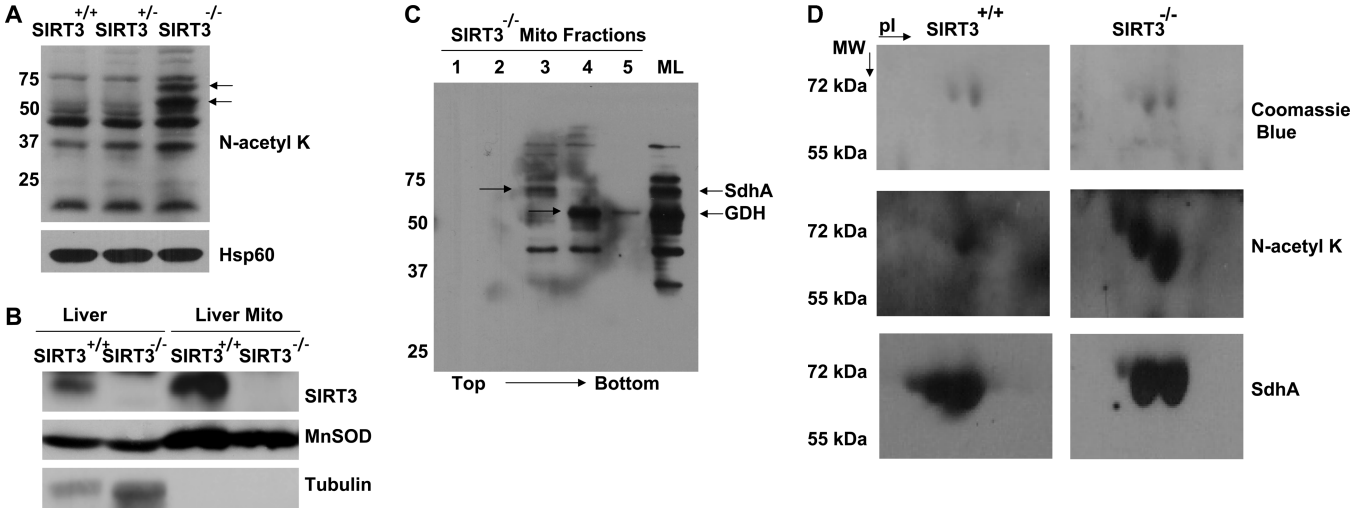

FIGURE 1: Detection of SdhA as a novel SIRT3 substrate in SIRT3 knockout mouse liver mitochondria. Acetylated proteins and SIRT3 expression in *Sirt3*$^{+/+}$, *Sirt3*$^{+/-}$, and *Sirt3*$^{-/-}$ mouse liver mitochondria were evaluated by immunoblotting analysis using various antibodies. (A) Mitochondrial lysates prepared as described in Materials and Methods were separated via SDS−PAGE, and an increased level of acetylation of mitochondrial proteins was detected by immunoblotting probed with *N*-acetyllysine (N-acetyl K) antibody. As a control for equal loading, a protein blot was developed with Hsp60 antibody. (B) SIRT3 protein levels in the liver or isolated liver mitochondria from wild-type or SIRT3 deficient mice were detected by immunoblotting analysis using SIRT3 antibody. Mitochondrial protein MnSOD and cytoplasmic protein tubulin were also detected as controls. (C) Approximately 2 mg of *Sirt3*$^{-/-}$ mouse liver mitochondrial lysate was layered on a 34% sucrose cushion and fractioned into five separate layers (the top and bottom fractions of the sucrose cushion are shown by the arrow). Equal volumes of each fraction were separated via SDS−PAGE, and acetylated proteins in each fraction were detected by immunoblot analysis. The total *Sirt3*$^{-/-}$ mouse liver mitochondrial lysate (ML) layered on the cushion was also analyzed to locate the acetylated proteins in the fractions. Arrows show the location of SIRT3 substrates glutamate dehydrogenase (GDH) and the flavoprotein subunit of succinate dehydrogenase (SdhA). (D) Approximately 50 μL of fraction 3 from *Sirt3*$^{+/+}$ or *Sirt3*$^{-/-}$ mouse liver mitochonria was separated via 2D-gel, and acetylated proteins were detected with *N*-acetyllysine antibody. The acetylated 2D-gel spots corresponding to the Coomassie Blue-stained gel spots were digested in gel and identified by mass spectrometry. The protein identification determined by mass spectrometry was confirmed by immunoblotting using SdhA antibody.

(Sigma, St. Louis, MO) for 16 or 48 h at a final concentration of 10 mM or 50 μM, respectively. For immunoblotting, cell pellets were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, and 0.1% SDS, supplemented with protease inhibitor cocktail (Sigma). After incubation on ice for 10 min, the soluble protein fraction was collected by centrifugation at 14000*g* and 4 °C for 15 min.

Complex II Enzymatic Activity Assay. Mitochondria and K562 cell pellets prepared as indicated above were lysed in a buffer containing 300 mM mannitol, 20 mM sodium phosphate (pH 7.2), 10 mM KCl, 5 mM $MgCl_2$, and 2 mg/mL dodecyl β-D-maltoside. Preincubation of varying amounts of mitochondrial or K562 cell lysates was performed in a buffer containing 300 mM mannitol, 20 mM sodium phosphate (pH 7.2), 10 mM KCl, 5 mM $MgCl_2$, 50 mM sodium succinate, and 40 mM sodium azide, prior to the addition of 50 μM 2,6-dichloroindophenolate to fully activate the succinate dehydrogenase. The Complex II enzymatic activity was recorded by monitoring the reduction of 2,6-dichloroindophenolate at 600 nm. The rate is calculated by dividing the absorbance difference between two linear points by the time point difference [rate = (absorbance 1 − absorbance 2)/(time 2 − time 1)] (*21*).

RESULTS

Succinate Dehydrogenase Is Acetylated, and SIRT3 Is Responsible for Its Deacetylation. We have recently identified acetylated and phosphorylated protein(s) of mitochondrial ribosomes using a combination of immunoblotting and capillary LC−MS/MS analysis and identified NAD^+-dependent SIRT3 as the deacetylase responsible for deacetylation of MRPL10 (*18*, *22*, *23*). Using a similar strategy, we identified acetylated proteins specifically deacetylated by SIRT3 in wild-type and SIRT3 knockout mouse liver mitochondria to determine SIRT3 substrates. For this purpose, mitochondria were isolated from SIRT3 knockout (*Sirt3*$^{-/-}$), wild-type (*Sirt3*$^{+/+}$), and heterozygote (*Sirt3*$^{+/-}$) mouse liver mitochondria. Acetylated proteins in mitochondrial lysates were detected by immunoblotting performed with *N*-acetyllysine antibody, which revealed two major protein bands at ~70 and ~55 kDa with an increased level of acetylation in SIRT3 knockout mouse mitochondrial lysate as shown by arrows (Figure 1A). Our findings suggested that these two proteins are potential substrates of NAD^+-dependent SIRT3 since they were highly acetylated in the absence of SIRT3 expression in knockout mice (Figure 1A). The lack of expression of SIRT3 in the whole liver or liver mitochondria from the SIRT3 knockout mice was confirmed by immunoblot analysis (Figure 1B).

To identify the proteins in these bands and simplify the protein content for 2D-gel separation, mitochondrial lysate obtained from SIRT3 knockout mice was fractionated on a 34% sucrose cushion containing nonionic detergent Triton X-100. Immunoblot analysis of the fractions showed that the two major acetylated proteins at 70 and 55 kDa were in fractions 3 and 4, respectively, implying the presence of these proteins in large protein complexes (Figure 1C). For the identification of 70 and 55 kDa proteins, 2D-gel was performed using fractions 3 and 4, and protein blots were probed with anti-*N*-acetyllysine antibody (Figure 1D and data not shown for fraction 4). Protein bands corresponding to acetylated proteins detected via 2D-gel were excised, digested in gel with trypsin, and analyzed by capillary LC−MS/MS for identification. The mass spectrometric analyses of the 2D-gel spots revealed the presence of the flavoprotein subunit of succinate dehydrogenase (SdhA) and glutamate dehydrogenase (GDH and data not shown) in 70 and 55 kDa protein bands, respectively (Table 1). Acetylation of glutamate

Table 1: Peptides Detected from Tryptic Digests of the Acetylated Band Detected in SIRT3$^{-/-}$ Mouse Mitochondria by LC−MS/MS Analysis

peptide sequence	m/z	Mascot score
ISQLYGDLK	1036.6	38
HTLSYVDIK	1075.6	41
WHFYDTVK	1095.6	48
NTVIATGGYGR	555.4	83
SMQNHAAVFR	589.2	66
AFGGQSLacKFGK	592.2	74
KHTLSYVDIK	602.8	57
AKNTVIATGGYGR	655.2	82
GEGGILINSQGER	666.2	84
VTLEYRPVIDK	667.3	63
TGHSLLHTLYGR	678.2	88
ANAGEESVMNLDK	698.2	98
VDEYDYSKPIQGQQK	899.8	82
VRVDEYDYSKPIQGQQK	1027.4	102
NTVIATGGYGRTYFSCTSAHTSTGDG-TAMVTR	1102.1	41

dehydrogenase and the role of SIRT3 in its deacetylation were reported previously (*16*). Therefore, we focused our efforts on determining the acetylation and deacetylation of SdhA in mitochondria obtained from SIRT3 knockout and wild-type mice. To confirm deacetylation of SdhA by SIRT3, immunoblotting and Coomassie Blue-stained gels of protein lysates were compared (Figure 1D). Even though the SdhA signals obtained by its specific antibody in both SIRT3 knockout and wild-type fractions were comparable, the magnitude of the acetylation signal significantly increased in the mitochondrial fraction from SIRT3 knockout mice (Figure 1D). This observation supports the possibility that the deacetylation of SdhA is due to the expression of endogenous SIRT3 in wild-type mouse mitochondria while the absence of SIRT3 expression in knockout mice causes hyperacetylation of the SdhA subunit (Figure 1D).

In addition to confirming the acetylation of the SdhA subunit by immunoblotting, one of the acetylated tryptic peptides was also identified with a Mascot score of 74 in the LC−MS/MS analysis of the 2D-gel spots that were previously detected. The CID spectrum of the acetylated AFGGQSLacKFGK peptide is given in Figure 2A. In high-throughput analysis of acetylated proteins from well-fed rat liver mitochondria, several other acetylated lysines were previously identified (*1*, *7*). Alignment of these acetylated peptides with the conserved regions in several other mammalian and chicken mitochondrial forms and *Escherichia coli* SdhA shows that the acetylated lysines are highly conserved in these proteins (Figure 2B). To demonstrate the location of acetylated lysines in the SdhA subunit, we modeled Complex II structure using the coordinates of the chicken mitochondrial Complex II (Figure 2C) (*24*). In this structure, conserved acetylated lysine residues (K179, K485, K498, and K538) in the mouse sequence are labeled as red surfaces in the SdhA subunit (Figure 2). All these residues are located on the hydrophilic surface of the subunit supporting the reversible acetylation of these residues by changes in the [NADH]/[NAD$^+$] ratio.

Role of Hyperacetylation of SdhA in Complex II Activity. To determine the effect of acetylation on the oxidation of succinate to fumarate by Complex II activity, we measured the oxidation of 2,6-dichloroindophenolate (DCIP) in mitochondrial suspensions obtained from SIRT3 knockout and wild-type mice. First, mitochondrial suspensions obtained from these mice were separated via 12% SDS−PAGE and evaluated for the SdhA, Hsp60, and acetylation levels by immunoblotting of the same gel probed with specific antibodies. Although the same amounts of SdhA and Hsp60 were loaded in the gels, the degree of acetylation was much higher in the mitochondrial suspension from SIRT3 knockout mice than in that from wild-type mice (Figure 3A). After confirming the presence of equal amounts of SdhA in these samples, we performed the Complex II activity assays at several different amounts of mitochondrial suspensions obtained from SIRT3 knockout and wild-type mice (Figure 3B). In these assays, the activity of complex II was followed by the transfer of electrons from succinate to DCIP at 600 nm (Figure 3B). As plotted in Figure 3B, rates of reaction were measured as changes in absorbance at 600 nm over time as a function of the amount of mitochondrial suspension used in the assays. At 15 μg of mitochondria suspension, the difference between the rate of Complex II activity from SIRT3 knockout mice and wild-type mice was ~30% (Figure 3B). To demonstrate the linearity of the percent inhibition detected by the assay, different amounts of mitochondrial lysate were used; however, the percent inhibition did not change significantly above 15 μg of mitochondrial suspension. Here, the reduction of DCIP was directly related to SdhA activity since electrons from succinate are first transferred to enzyme-bound cofactor, FAD, in the SdhA subunit. For this reason, the decrease in Complex II activity can be attributed to an increased level of acetylation of SdhA in mitochondria from the SIRT3 knockout mice (Figure 3B).

Role of the Increased Level of SIRT3 Expression in Deaceylation of SdhA and Complex II Activity. The significant increase in the level of acetylation of several proteins in SIRT3 knockout mouse mitochondria (Figures 1A and 3A) prompted us to determine the effect of SIRT3 overexpression. For this purpose, we used brown preadipocyte HIB1B cells with retroviral stable expression of murine SIRT3 (amino acids 78−334) as described previously (*15*). In addition, alternative transcripts of murine SIRT3 were found recently to express proteins with extension at the N-terminus (*20*). Accordingly, we have generated HIB1B cells with retroviral expression of the long form of SIRT3 (amino acids 1−334). To determine the role of SIRT3-dependent deacetylation of mitochondrial proteins, mitochondria were isolated from HIB1B control and stable cells expressing two different forms of the SIRT3 gene. In the immunoblotting analysis performed with *N*-acetyllysine antibody, we observed a general decrease in the level of acetylation of some of the acetylated protein bands and a protein at ~70 kDa in mitochondrial lysates obtained from SIRT3 overexpression cells. This 70 kDa band overlapped with the SdhA signal in the reprobing of the blot with the SdhA antibody (Figure 4A).

Stimulation of sirtuins, class III histone deacetylases, by several polyphenolic compounds such as resveratrol and kaempferol has been suggested recently (*25*−*27*). Specifically, kaempferol treatment of the chronic myelogenous leukemia, K562, cell line has been shown to increase the level of SIRT3 expression in these cell lines (*27*). Moreover, nicotinamide is a general sirtuin inhibitor and has been shown to inhibit SIRT3-dependent deacetylation of GDH and NDUFA9 (*17*, *28*). To demonstrate the effect of SIRT3 expression on Complex II activity, we treated K562 cells with 50 μM kaempferol or 10 mM nicotinamide for either 16 or 48 h and monitored the changes in acetylation and expression of SIRT3 by immunoblotting analysis using whole cell lysates (Figure 4B). Reprobing of the membranes was performed with SdhA and Hsp60 antibodies to ensure equal amounts of

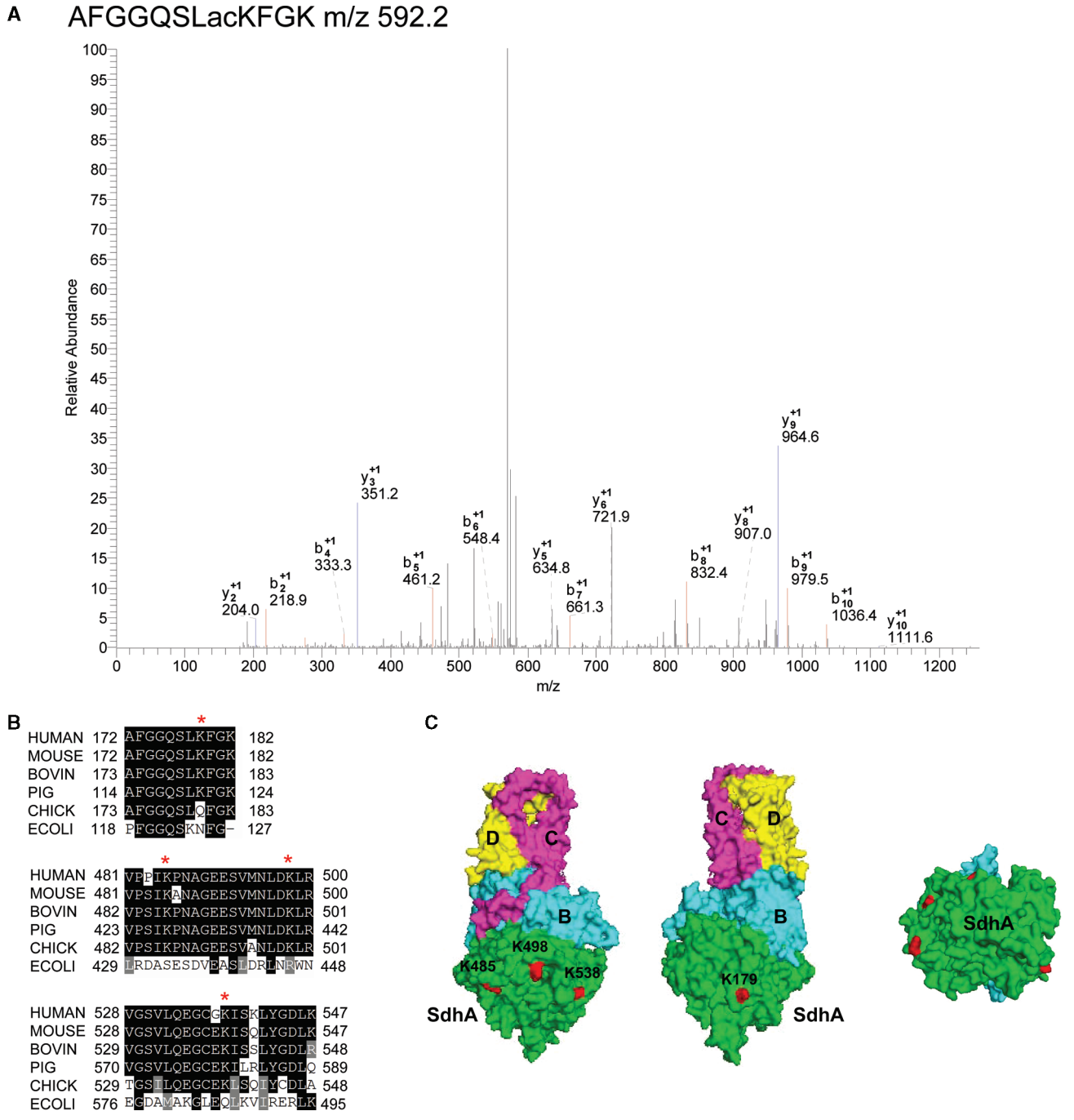

FIGURE 2: Acetylation of SdhA at conserved K179, K485, K498, and K538 residues. (A) CID spectrum of the acetylated peptide detected via LC−MS/MS analysis of 2D-gel spots of SdhA from SIRT3 knockout mouse mitochondria. (B) Primary sequence alignment of acetylated peptides from mice SdhA and its homologues from different species. The human, bovine, pig, chicken, and *E. coli* SdhA were aligned with acetylated peptides of mouse SdhA. An asterisk denotes the acetylated Lys residues detected in the LC−MS/MS analysis. The alignment was created with CLUSTALW in Biology Workbench and displayed in BOXSHADE. (C) Crystal structure model of chicken SdhA (Protein Data Bank entry 1YQ3) representing all four subunits: SdhA (green), SdhB (cyan), SdhC (yellow), and SdhD (pink). The conserved Lys residues found to be acetylated in mouse SdhA (denoted with asterisks in panel B) are colored red.

protein loading in the SDS−PAGE gels. Consistent with the increased level of expression of SIRT3 in kaempherol-treated cells, the overall acetylation level of proteins decreased compared to that in the control and nicotinamide-treated cells (Figure 4B). In addition to the detection of overall changes in acetylation of proteins in K562 cells, we fractionated the cell lysates treated with kaempferol and nicotinamide along with untreated cells on a 34% sucrose cushion containing 1.6% Triton X-100 to enrich for SdhA protein. Similar to the pattern obtained in fractionation of mouse liver mitochondria (Figure 1C), SdhA remained associated and sedimented with the rest of the Complex II subunits in fractionation of kaempferol- and nicotinamide-treated cells as confirmed by immunoblot analyses (Figure 4C). Especially in the nicotinamide-treated and control cells, the acetylated protein signal (indicated by arrows) overlapped with the SdhA signal in the reprobing of the membranes with the specific SdhA antibody. On the other hand, the level of acetylation of SdhA was significantly reduced in kaempferol-treated cells, despite the strong SdhA signal obtained with the Sdh antibody in the reprobing. Interestingly, the acetylation signal

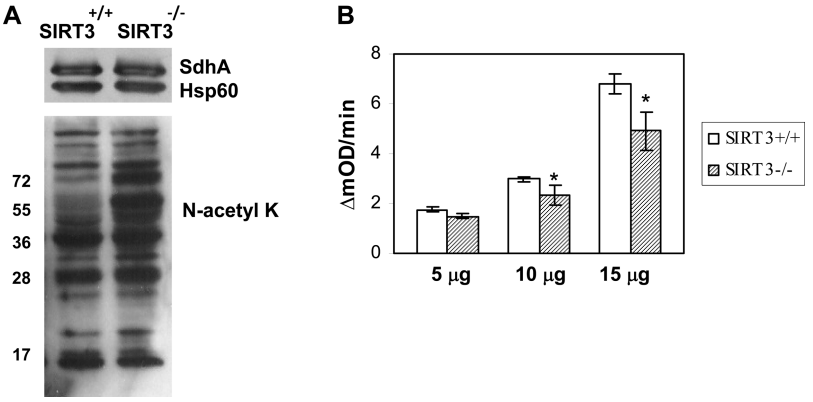

FIGURE 3: Regulation of succinate dehydrogenase (Complex II) activity by acetylation of SdhA. Hyperacetylation of SdhA decreases Complex II activity in SIRT3 knockout mice. (A) Equal amounts of lysates obtained from Sirt3$^{+/+}$, *Sirt*3$^{+/-}$, and *Sirt*3$^{-/-}$ mouse liver mitochondria were separated by 12% SDS−PAGE and probed with *N*-acetyllysine (N-acetyl K), SdhA, and Hsp60 antibodies. (B) Complex II activity was measured as the rate of DCIP reduction, monitored at 600 nm using different amounts of mitochondrial lysates from *Sirt*3$^{+/+}$ and *Sirt*3$^{-/-}$ mouse liver mitochondria. Asterisks denote $p < 0.05$.

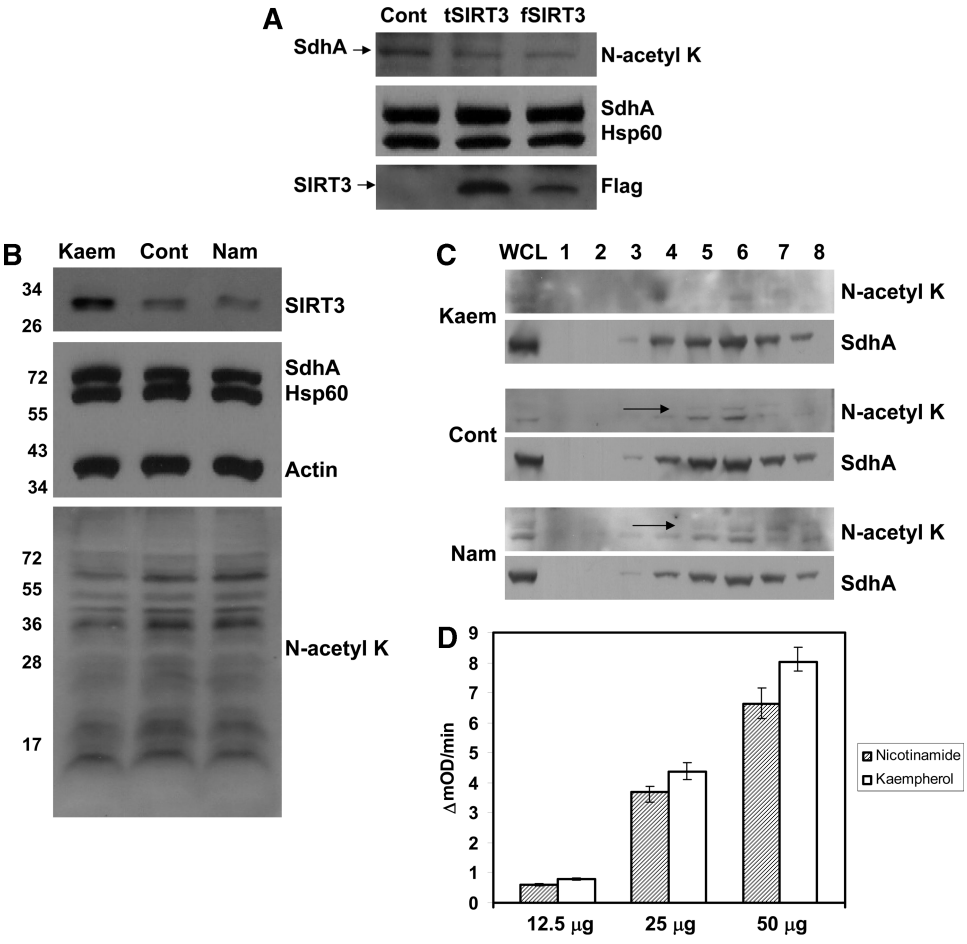

FIGURE 4: Role of SIRT3 overexpression in SdhA deacetylation and Complex II activity. Overexpression of SIRT3 in HIB1B cells increases Complex II activity by deacetylation of SdhA. (A) Mitochondria from control and HIB1B cells stably expressing Flag-tagged truncated (tSIRT3) and full-length (fSIRT3) forms were isolated, and ~20 μg of the mitochondrial lysate from each cell line was separated via 12% SDS−PAGE. Immunoblotting analyses were performed with antibodies described in the legend of Figure 3A and Flag-tagged antibody. (B) Immunoblotting analysis of K562 cell lysates obtained from control cells (Cont) and cells treated with nicotinamide (Nam) and kaempferol (Kaem). Approximately 20 μg of control and treated K562 cell lysates from each sample was loaded onto a 12% SDS−PAGE gel, and immunoblotting was performed as described above. Actin and Hsp60 blots were shown to ensure equal loading in the protein lanes. (C) Equal amounts (~2 mg) of control and treated K562 cells lysates (WCL) were layered on a 34% sucrose cushion and fractionated into eight 1 mL aliquots after high-speed centrifugation. Equal volumes of each fraction (1−8) were precipitated with acetone and loaded on 12% SDS−PAGE gels for immunoblot analysis. Arrows show the location of acetylated protein overlapping with the SdhA signal. (D) Complex II activity was monitored using different amounts of kaempferol- and nicotinamide-treated K562 cell lysates. The analysis was conducted in triplicate, and values are means ± the standard deviation.

coming from the lower band was also affected by kaempferol and nicotinamide treatments (Figure 4C).

Again, to determine the role of SdhA acetylation on Complex II activity, we performed Complex II enzyme activity assays using

whole cell lysates obtained from nicotinamide- and kaempferol-treated K562 cells, which revealed that Complex II was ~20% more active in kaempferol-treated cells compared to the Complex II activity from nicotinamide-treated cells (Figure 4D). The Complex II activity in control cells was similar to the activity of nicotinamide-treated cells (data not shown).

DISCUSSION

Mitochondria are required for the production of more than 90% of the ATP required for survival of eukaryotic cells in oxidative phosphorylation. Regulation of oxidative phosphorylation and Krebs cycle components by post-translational modifications has already been established (*1*, *7*, *29*, *30*). ADP/ATP and [NADH]/[NAD^+] ratios are important for regulation of these pathways either by post-translational modifications such as phosphorylation and acetylation or by allosteric regulation. Regulation of mitochondrial function by phosphorylation has been known for a long time; however, the recent progress in identification of mitochondrion specific NAD-dependent sirtuins such as SIRT3, SIRT4, and SIRT5 revealed the importance of the [NADH]/[NAD^+] ratio in regulation of protein and enzyme function in post-translational modifications by reversible acetylation (*14*, *28*). One of the best-characterized mitochondrial NAD^+-dependent deacetylases, SIRT3, has been known to regulate activities of several metabolic enzymes and Complex I subunit NDUFA9 by deacetylation (*17*). Moreover, we have recently discovered its pivotal role in the regulation of mitochondrially encoded proteins of oxidative phosphorylation by mitochondrial protein synthesis by specific deacetylation of a ribosomal protein MRPL10 (*18*).

In this study, comparison of acetylated proteins in wild-type and SIRT3 knockout mouse mitochondria has led us to a novel substrate for SIRT3, the flavoprotein of succinate dehydrogenase complex (SdhA), along with a known substrate, glutamate dehydrogenase. SdhA is one of the hydrophilic subunits of the succinate dehydrogenase involved in the Krebs cycle and oxidative phosphorylation in mammalian mitochondria. Previously, in two independent high-throughput surveys of the acetylated proteins of rat liver, several acetylated peptides were mapped from SdhA (*1*, *7*), while it was reported as an unacetylated protein in a comprehensive study of SIRT3-dependent deacetylation of Complex I subunit NDUFA9 (*17*). However, the role of acetylation in the enzyme activity and the deacetylase responsible for this modification were not determined previously.

We believe that the data presented here convincingly clarify the discrepancy reported in the literature and demonstrate that SIRT3 is indeed the major mitochondrial deacetylase controlling the oxidative phosphorylation by reversible lysine acetylation (*16*, *17*). In the comparison of 2D-gel immunoblotting of $SIRT3^{-/-}$ and $SIRT3^{+/+}$ mouse liver mitochondria, SdhA was found to be hyperacetylated in the absence of SIRT3; however, it is possible that the degree of acetylation in wild-type mice is regulated by availability of acetyl-CoA and/or NADH levels in the mitochondria. For this reason, we have not observed complete deacetylation of SdhA in the wild-type mouse liver mitochondrial lysates (Figures 1D and 3A). More importantly, we have shown the effect of hyperacetylation on Complex II activity in $SIRT3^{-/-}$ liver mitochondria (Figure 3B). Interestingly, the Complex II activity in SIRT3 knockout mice was ~30% lower than that of the wild type, possibly due to incomplete deacetylation of SdhA in the wild-type mice (Figure 3). Previously, none of the Complex II subunit proteins were reported as acetylated proteins for the immunocaptured Complex II components in SIRT3 knockout mice (*17*). This discrepancy could be due to the sample preparation used by Ahn et al. as they determined the acetylation of Complex II components after immunocapturing of the complex (*17*). In addition to changes in SdhA acetylation and Complex II activity in $SIRT3^{-/-}$ and $SIRT3^{+/+}$ mouse mitochondria, we have shown a decrease in SdhA activity while an increased level of acetylation was observed in cells treated with a general deacetylase inhibitor, nicotinamide. In contrast, kaempferol treatment of the same cell line caused an increase in the level of expression of SIRT3 and deacetylation of SdhA accompanied by a 20% increase in Complex II activity possibly due to SIRT3-dependent deacetylation of SdhA. Surprisingly, the changes in acetylation of SdhA did not completely inhibit the Complex II activity. As proposed previously, it is likely that only a minor proportion of the protein is acetylated or acetylation only partially regulates the enzyme activity even though mitochondrial protein hyperacetylation is dramatic in SIRT3 knockout mice (*16*). Additionally, conserved acetylated lysine residues in mammalian SdhA are located on the surface of the protein, away from the active site of the enzyme. Therefore, it is feasible to expect that acetylation of the positively charged residues on the surface of the enzyme might either slightly change the affinity of the enzyme for its negatively charged substrate, succinate, or induce conformational changes to reduce the activity of the enzyme (Figure 2B).

Regulation of Complex II activity by reversible acetylation of the SdhA subunit relates how oxidative phosphorylation and Krebs cycle components are regulated by metabolite levels in mammalian mitochondria. In the case of high levels of reduced cofactors such as NADH and $FADH_2$ present in the mitochondria, there is no need for further oxidation of acetyl-CoA in the Krebs cycle for generation of these cofactors to support oxidative phosphorylation. Thus, it would be reasonable to suggest that acetylation of SdhA just slows the Krebs cycle, as this process will also cause accumulation of acetyl-CoA in the mitochondria. On the other hand, when the NAD^+ level increases in the mitochondria, SIRT3 and other NAD^+-dependent deacetylases will be activated and deacetylate SdhA and other acetylated components of the Krebs cycle. In agreement with stimulation of catalytic activities of metabolic enzymes such as glutamate dehydrogenase and acetyl-CoA synthetase 2 by deacetylation, deacetylation of SdhA also stimulates Complex II or succinate dehydrogenase activity to promote the Krebs cycle for the generation of reduced NADH and $FADH_2$, as they are the electron donors for ATP synthesis in oxidative phosphorylation. Another potential regulation of Complex II activity is by phosphorylation of the SdhA subunit as it was found to be phosphorylated by Fgr tyrosine kinase in vitro (*10*). Given its importance in oxidative phosphorylation, it could be suggested that this enzyme can be regulated through cooperation or interplay between these two different post-translational modifications at varying metabolite levels. Moreover, in the case of complete inhibition of the complex, succinate accumulation resulting from the decreased SdhA activity may cause deleterious effects in the cell due to the absence of additional mitochondrial metabolic enzymes that can metabolize succinate (*8*, *9*).

ACKNOWLEDGMENT

We thank Dr. David Sinclair for providing the full-length murine SIRT3 cDNA.

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