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Characterization of Murine SIRT3 Transcript Variants and Corresponding Protein Products

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Abstract

SIRT3 is one of the seven mammalian sirtuin homologs of the yeast SIR2 gene. SIRT3 possesses NAD⁺-dependent protein deacetylase activity. Recent studies indicate that the murine SIRT3 gene expresses different transcript variants, resulting in three possible SIRT3 protein isoforms with various lengths at the N-terminus: M1 (aa 1–334), M2 (aa 15–334), and M3 (aa 78–334). The transcript variants 1 and 3 can only produce M3 protein, while M1 and M2 proteins are translationally initiated from different in-frame ATG sites in transcript 2. Here we report that three transcript variants of the mouse SIRT3 gene are broadly expressed in various mouse tissues. By expressing these SIRT3 isoforms in HEK293 cells through transient transfection, we confirmed recent reports that two longer murine SIRT3 proteins (M1 and M2) are targeted to mitochondria with higher efficiency than the shorter M3 isoform. Additionally, the M1 and M2 proteins are processed into a mature form. Using Edman degradation we identify Ile38 (majority) or Val42 as the N-terminal amino acid of the mature M1 isoform, and Met78 or Val79 as the N-terminal amino acid of the M3 isoform. Interestingly, we found that even upon mutation of the M2 ATG site in the M1 cDNA, a processed mature protein could still be produced. In terms of deacetylase activity, we found that although only the mature protein derived from M1 or M2 proteins were active against acetylated peptide substrates, all three forms had equal deacetylase activity towards a full-length native protein substrate, acetyl CoA synthetase 2.

Keywords

SIRT3; TRANSCRIPT VARIANTS; DEACETYLASE

SIRT3 is one of the seven mammalian sirtuins (SIRT1 to SIRT7) [Guarente, 2005], and together with SIRT4 and SIRT5, reside in the mitochondria [Michishita et al., 2005], although there are also reports indicating SIRT3 may enter nucleus [Scher et al., 2007; Nakamura et al., 2008]. Functionally, SIRT3 was found to deacetylate and activate acetyl-CoA synthetase 2 (ACS2), an important mitochondrial enzyme involved in generating acetyl-CoA from acetate [Hallows et al., 2006; Schwer et al., 2006]. Furthermore, glutamate dehydrogenase, isocitrate dehydrogenase 2, Ku70, Foxo3a, electron transport chain complex I subunit NDUFA9 ATP synthase and succinate dehydrogenase were reported to be SIRT3 substrates [Ahn et al., 2008; Jacobs et al., 2008; Schlicker et al., 2008; Sundaresan et al., 2008; Ivy et al., 2009; Sundaresan et al., 2009; Cimen et al., 2009]. Mice deficient in SIRT3

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exhibit a dramatic increase in acetylation levels of mitochondrial proteins [Lombard et al., 2007]. In addition, SIRT3 knockout mice have decreased levels of ATP in various tissues [Ahn et al., 2008] and reduced AMPK activation and PGC-1 α expression in the skeletal muscle [Palacios et al., 2009]. The anti-genotoxic stress effect of the NAD⁺ synthesis enzyme, Nampt, is mediated by SIRT3 and SIRT4 [Yang et al., 2007]. SIRT3 may function as a tumor suppressor to maintain mitochondrial integrity and metabolism during stress. SIRT3 deficient mice develop breast cancer [Kim et al., 2010]. SIRT3 responds to cold stimulation and activates the adaptive thermogenesis program in brown adipose tissue [Shi et al., 2005]. In the heart, SIRT3 also protects against cardiac hypertrophy through deacetylation of FOXO3a [Sundaresan et al., 2009] and LKB1 [Pillai et al., 2009]. In the liver, SIRT3 is activated by fasting to deacetylate long chain acyl CoA dehydrogenase (LCAD) to increase hepatic fatty acid oxidation [Hirschey et al., 2010].

Human SIRT3 gene encodes a 399 aa protein with a predicted molecular weight of 44 kDa. It is synthesized as an enzymatically inactive protein present on the mitochondrial inner membrane, with a 25 amino acid mitochondrial localization sequence at its N-terminus [Onyango et al., 2002; Schwer et al., 2002]. It is cleaved by matrix-processing peptidase to become a 28 kDa enzymatically active mature form in the mitochondrial matrix [Schwer et al., 2002]. Initially the mouse SIRT3 protein was first reported to be a 257 amino acid protein, corresponding to residues 143–399 of human SIRT3 [Yang et al., 2000]. This form of mouse SIRT3 lacks the N-terminal 142 amino acid residues necessary for the mitochondrial localization for the human counterpart, although both mitochondrial targeting and nuclear localization signal sequences have been identified in 143–165 aa and 278–302 aa, respectively, in this form of murine SIRT3 protein [Nakamura et al., 2008].

Recently, the existence of alternative mouse SIRT3 transcripts has been proposed and confirmed [Cooper and Spelbrink, 2008; Cooper et al., 2009; Jin et al., 2009; Bao et al., 2010]. One of the murine SIRT3 transcript variants (Fig. 1A) was found to encode two longer protein isoforms (the M1 and M2 isoforms as illustrated in Fig. 1B) which were shown to be mitochondrial proteins and possess deacetylase activity. However, the mitochondrial localization and the deacetylase activity of the original shorter isoform (M3 as shown in Fig. 1B) are still controversial [Cooper et al., 2009; Jin et al., 2009; Bao et al., 2010]. Furthermore, the enzymatic activities of these murine SIRT3 protein variants against whole native protein substrates have not been measured. In this study, we examined the mRNA expression patterns of three SIRT3 transcript variants in murine tissues. In addition, we determined the precise N-terminus of the mature protein derived from M1 and M3. Finally, we measured the deacetylase activity of the long and short murine SIRT3 proteins using intact ACS2 as substrate.

METHODS

SIRT3 KNOCKOUT MICE

Mice in which the *Sirt3* gene was targeted by gene trapping were obtained from the Texas Institute for Genomic Medicine (Houston, TX, USA). Briefly, these mice were created by generating embryonic stem (ES) cells (Omnibank no. OST341297) bearing a retroviral promoter trap that functionally inactivates one allele of the *Sirt3* gene. Sequence analysis indicated that retroviral insertion occurred in the intron preceding coding exon 1 (Accession: NM_022433). Targeted 129/SvEvBrd ES cells were injected into C57BL/6 albino blastocysts. The chimeras (129/SvEvBrd) were then crossed with C57BL/6 albinos to produce the heterozygotes. Heterozygotes were then mated and the offspring were genotyped using PCR as reported [Palacios et al., 2009].

SIRT3 GENE EXPRESSION

RNA was isolated and quantified from the indicated mouse tissue and reverse transcribed into cDNA using Super-Script III reverse transcriptase kit (Invitrogen). The specific sense primer for the expression of transcript 1, 2 and 3 is: 5'-TCAGACTGTGGGGTCCGGGAGGTGTTA-3', 5'-GACTGTGGGGTCCGGGAGGTGG-3' and 5'-GGCGTTTGGCGAGGACTA-3', respectively; a common reverse primer is used: 5'-CAACATG AAAAAGGGC-3'.

PLASMID CONSTRUCTION

The full-length of SIRT3(1–334), SIRT3(15–334) cDNA was generated by PCR with the sense primer 5'-ATAGAATTCATGGCGCTTGACCCTC-3' and 5'-ATAGAATTCATGGCGCTAAGCGGTCG, respectively, and reverse primer: 5'-ATAGAATTCTCTGTCCTGTCCATCC-3'. The PCR product was then sub-cloned into pcDNA3.1 or pcDNA3.1-Flag vector (in which the flag tag is inserted into *EcoRI* and *XhoI*). The pcDNA3.1-SIRT3(78–334) and pcDNA3.1-SIRT3(78–334)-Flag were previously described [Yang et al., 2010]. The human SIRT3-Flag expression construct was kindly provided by Dr. Eric Verdin (University of California, San Francisco). To generate the M1 mutant with Met15 changed to Ala, the following sense primer (5'-AGAATTCATGGCGCTTGACCCTCTAGGCGCCGTCGTCCTGCAGAGCATCGCGGC GCTAAG-3') was paired with the above-mentioned reverse primer for PCR. The PCR product was then sub-cloned into pcDNA3.1-Flag vector at the *EcoRI* sites. To generate GST-SIRT3 fusion protein, the construct, GST-SIRT3 (38–334), GST-SIRT3(78–334) and its enzymatic inactive mutant: GST-SIRT3-N164A(78–334) were amplified by PCR with the sense primer: 5'-ATAGAATTCATATCCCTCTGTGTGGG-3' and 5'-ATAGAATTCATGGTGGGGGCGGC-3', respectively, and the reverse primer showed as above. The resulted PCR products were inserted into pGEX4T-1 through the *EcoRI* site. All expression constructs were verified by DNA sequencing.

CELL CULTURE

Human embryonic kidney cells (HEK293), human cervical cancer cells (HeLa), and murine fibroblasts (NIH3T3) are all commonly used for gene expression studies with high efficiency of transient transfection. These cells were cultured in DMEM supplemented with 10% bovine calf serum (Hyclone, Logan, UT), 100 IU/ml penicillin and 100 µg/ml streptomycin, at 37°C and 5% CO₂. HEK293 cells were transfected as indicated [Yang et al., 2010] and the transfected cells were lysed in cell lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 20 mM NaF, 1 mM Na₃VO₄, 5 mM EDTA, and 0.5% NP-40) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany).

IMMUNOBLOT

For immunoblotting, protein samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The blot was probed with a mouse monoclonal anti-Flag M2-Peroxidase at 1:3,000 (Sigma, St. Louis, MO), rabbit polyclonal anti-SIRT3 antibody at a 1:3,000, a monoclonal anti-acetylated-lysine antibody at 1:3,000 (Cell Signaling Technology, Danvers, MA). The secondary antibody was Affinipure Rabbit Anti-Mouse IgG, or Goat Anti-Rabbit IgG (Jackson Immuno Research, West Grove, PA) all at a 1:10,000 dilution, followed by development with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biochemicals, Rockford, IL) according to the protocol provided by the manufacturer.

IMMUNOFLUORESCENCE

For immunofluorescent detection, cells were grown on coverslips in 6-well plates. Following transfection for 1–2 days, cells were fixed using 4% paraformaldehyde in DMEM medium for 30 min. This was followed by three washes in PBS and lysis for 30 min with 0.05% Triton X-100 in PBS. Cells were then blocked with 15% BSA for 1–2 h at room temperature and were incubated with primary antibody (anti-Flag, 1:200, Sigma; anti-MnSOD, 1:500, Cayman Chemical) and secondary antibodies (goat anti-mouse-Dylight 488 or goat anti-rabbit-Dylight 649, 1:500, Jackson Immuno Research) in PBS with 3% BSA for overnight or 1 h. Slides were mounted using Fluoromount-G (SouthernBiotech, Birmingham, AL). Image acquisition was carried out using an Olympus IX-70 confocal microscope.

GST-SIRT3 FUSION PROTEIN EXPRESSION

GST-SIRT3 fusion protein constructs were transformed into BL21 strain of *E. coli* and the fusion proteins were induced with 0.1 mM IPTG at 30°C for 3 h. The bacteria were collected by centrifugation and the resulted pellets were lysed with bacterial lysis buffer (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.1% Triton X-100, and 2% glycerol). Fusion proteins were purified by affinity chromatography, using glutathione-agarose beads (Sigma) and eluted with elution buffer (20 mM glutathione, 150 mM NaCl, 50 mM Tris/HCl, pH 8.0). The GST-fused proteins were dialyzed and stored at –80°C. To remove GST, thrombin was added according to the manufacture's instruction and the digestion was evaluated by SDS-PAGE. The expression vector for ACS2-His was kindly provided by Dr. Eric Verdin (University of California, San Francisco) and the proteins were expressed and purified as reported [Schwer et al., 2006].

IN VITRO DEACETYLATION ASSAY

A single acetylated peptide derived from the SIRT3 substrate AceCS2 (EILVVKRLPKTRSG- K_{Ac} -VMRLLRKKITSEAQ, K_{Ac} is acetylated lysine) and the protein: mature form of SIRT3 (38–334) and MBP-fused short form of SIRT3(78–334) were provided by Dr. Lei Jin [Jin et al., 2009]. SIRT3 enzymatic activity was assessed by measuring the amount of nicotinamide produced during the deacetylation reaction using the PNC1-OPT Assay [Hubbard et al., manuscript in preparation]. Each reaction contained 200 μ M NAD⁺, 100 μ M peptide substrate, and the indicated amount of SIRT3 enzyme. Deacetylation reactions were run for 1 h at 37°C. Reactions were incubated in the presence of developing reagent [Hubbard et al., submitted] for 1 h prior to taking fluorometric measurements (excitation at 420 nm and emission at 455 nm).

RESULTS

SIRT3 GENE AND TRANSCRIPTS

So far, three murine SIRT3 transcript variants have been reported [Yang et al., 2000]. The differences between the three transcripts occur within the first two exons of the SIRT3 gene (Fig. 1A). Transcripts 1 and 2 both contain exon 1b followed by exon 2. The difference between them is that transcript 1, which matches Genebank database sequence BC025878, contains an additional 8-bp insertion at the 5' end of exon 2 that is absent from transcript 2, which matches the EMBL database sequences BI155417 and BY253536. Transcript 3, which matches Genebank database sequence AF299338, consists of exon 1a as the first exon. Transcript 2 encodes an open-reading frame (ORF) which includes three possible translation start sites at Met1, Met15, and Met78, yielding theoretical protein products of 334aa (M1), 320aa (M2), and 257aa (M3) respectively (Fig. 1B). Transcript 3 encodes only an ORF for the 257aa M3 protein (Fig. 1B). For transcript 1, with the 8-bp insertion, the

ATG starting sites within exon 1b will result in premature termination (Fig. 1B) and only M3 can be produced. Thus, transcripts 1 and 3 can produce M3 SIRT3 protein only, whereas transcript 2 can potentially generate all three isoforms of SIRT3.

Although transcript 2 was shown to be expressed in numerous mouse tissues including the brain [Jin et al., 2009], the tissue distribution patterns of transcript 1 and transcript 3 have not been analyzed. To address this issue, three 5' primers specific to each of the transcripts were used to pair with a common 3' primer for RT-PCR detection of the three murine SIRT3 transcript variants. The specificity of the primers was confirmed using cDNA for each transcript variant (Fig. S1A). These primers were then used to analyze the expression of all three murine SIRT3 transcript variants in various mouse tissues by RT-PCR. As shown in Figure 1C, all three transcripts can be detected in all of the tissues tested from wild-type mouse. Liver from SIRT3 knockout mouse was used as a negative control for detection. All three transcripts are expressed at relatively high levels in liver, kidney, heart, brain, and brown adipose. This is in agreement with our previous Northern blot analysis [Shi et al., 2005] and Western blot detection of murine SIRT3 protein expression [Palacios et al., 2009]. The relatively similar expression level of transcripts 2 and 3 is also consistent with the cloning result in a previous report [Cooper et al., 2009].

TRANSIENT EXPRESSION OF M1, M2, AND M3 MURINE SIRT3 ISOFORMS IN CELLS

To study the expression of the three murine SIRT3 protein isoforms in cells, transcript 2 cDNA was used to generate M1 protein, while a truncated transcript 2 starting from the ATG site which encodes Met15 was used to express M2. M3 SIRT3 protein was expressed from transcript 1 as reported previously [Shi et al., 2005]. For the convenience of detection, a Flag tag was added to the C-terminus of each protein. These constructs were transiently transfected into HEK293 cells and the expression of murine SIRT3 isoforms was detected by Western blot using either an antibody against the C-terminus of SIRT3 or the C-terminal Flag tag. As shown in Figure 2A, the SIRT3 M3 protein showed only one band, while multiple bands were detected for SIRT3 M1 or M2 proteins. These bands most likely correspond to SIRT3 proteins at various stages of maturation. Flag tagged M1 precursor proteins appear to be 34–35 kDa and the Flag tagged M2 precursor proteins have an apparent size of 32–33 kDa. A lower band indicated by an arrow appeared in cells expressing either M1 or M2, and this was purported to be the mature form of SIRT3 [Cooper et al., 2009; Jin et al., 2009]. Whereas M1 or M2 expressing cells produced the similar levels of SIRT3 precursor proteins, the mature protein was more abundant in cells expressing M2 (Fig. 2A), indicating the M2 proteins are likely to be processed more efficiently. A similar banding pattern was observed in NIH3T3 fibroblasts transfected with these constructs (data not shown).

Human SIRT3 was reported to be cleaved by the matrix processing peptidase (MPP) between residues 101 and 102 [Schwer et al., 2002]. Based on the sequence alignment between human SIRT3 and murine SIRT3, the murine SIRT3 38–334aa is equivalent to the human SIRT3 mature form. To determine the actual N-terminus of the mature protein derived from M1 SIRT3 protein, transiently expressed M1 and M2 were immunoprecipitated with anti-Flag antibody and resolved by SDS-PAGE and stained with Coomassie blue (Fig. 2B). The band corresponding to the mature SIRT3 was cut and sequenced by Edman degradation. N-terminal sequencing data suggest that most of the mature SIRT3 protein starts at Ile38, while a small amount of mature SIRT3 protein starts at Val42. The expressed M3-Flag protein was also purified using anti-Flag agarose beads and the N-terminus was determined by Edman degradation. The mature M3 proteins had mostly Val79 at the N-terminus. A minority of M3 proteins retained N-terminal Met78.

Since both M1 and M2 proteins can be translated from transcript 2, and in cells transfected with the M1 construct a band located at the same position as the M2 precursor can be seen (Fig. 2A), it was proposed that this band is possibly M2 protein translationally initiated from the Met15 in the M1 sequence [Cooper et al., 2009]. Consequently, the mature protein in cells transfected with the M1 expressing construct might only be produced from the M2 protein but not the M1 protein. To clarify this issue, a M1 mutant with M15A substitution was generated to prevent M2 translation initiation. As shown in Figure 2C, a band which corresponds to the M2 precursor was still produced. The mature form of SIRT3 is also produced (indicated by an arrow), although at a much lower efficiency than M2. We did find a processed band disappeared in M1-M15A expressing cells (indicated by a *), suggesting it might be derived from M2.

SUB-CELLULAR LOCALIZATION OF THREE MURINE SIRT3 PROTEIN ISOFORMS

To determine the sub-cellular localization of the three murine SIRT3 isoform proteins, we transfected NIH3T3 fibroblasts with Flag-tagged SIRT3 expressing constructs. The overexpressed SIRT3 proteins were detected by immunofluorescence using anti-Flag antibody. Mitochondrial compartments were visualized with an anti-MnSOD antibody, as MnSOD proteins are mitochondrially localized and used as a mitochondria indicator. As shown in Figure 3, both M1 and M2 SIRT3 exhibited predominant mitochondrial localization. However, the SIRT3 M3 proteins appeared aggregated with only partial mitochondrial localization. These findings are consistent with previous reports that M1 and M2 possess an N-terminal mitochondrial localization signal and are mitochondrial proteins [Cooper et al., 2009; Jin et al., 2009]. Similar patterns of sub-cellular localization were observed when HEK293 or HeLa cells were used to express these murine SIRT3 constructs (data not shown).

THE MATURE M1 AND M2 SIRT3 ISOFORMS AS WELL AS THE SHORTER M3 SIRT3 ARE ALL ENZYMATICALLY ACTIVE

It was reported that M3 SIRT3 is enzymatically inactive against an ACS2 peptide substrate [Jin et al., 2009]. We also compared the deacetylase activity of the mature SIRT3 derived from M1 or M2 (aa38–334) and the shorter M3 SIRT3 protein (aa78–334) using an in vitro activity assay. In agreement with Jin et al. [2009], we found the recombinant GST-SIRT3(38–334) or SIRT3(38–334) with the GST removed exhibited enzymatic activity. However, neither the shorter GST-SIRT3(78–334) nor SIRT3(78–334) without GST were active against an ACS2 acetylated peptide substrate (Fig. 4A) or an H3 acetyl K9 substrate (Supplemental Fig. 2). Considering the possibility that the shorter M3 SIRT3 isoform may require an intact, native protein substrate, we tested the deacetylase activity of the SIRT3 proteins on a known SIRT3 protein substrate, ACS2. SIRT3(38–334), SIRT3 M3(78–334) and its enzymatically inactive mutant SIRT3 M3-NA (the originally named N87A [Shi et al., 2005]) were tested for enzymatic activity. As shown in Figure 4B, at the concentration of 200 nM, SIRT3(38–334) and SIRT3(78–334) showed similar NAD⁺-dependent deacetylase activity towards the full-length ACS2 substrate. As expected, NA mutation abolished the deacetylase activity of M3 SIRT3(78–334). This result was confirmed by the Western blot detection of the diminution of acetylation level of the ACS2 protein by both the long and the short forms of murine SIRT3 proteins (Fig. 4C).

DISCUSSION

The murine SIRT3 protein was first reported to be a 257 amino acid protein which aligns with human SIRT3 residues 143–399 [Yang et al., 2000]. This initial murine SIRT3 sequence lacks the N-terminal 142 amino acid residues necessary for the mitochondria localization of its human counterpart. Thus, the presence of a longer form of murine SIRT3

was postulated [Cooper and Spelbrink, 2008]. Several recent reports confirmed the existence of longer SIRT3 isoforms that are targeted to the mitochondria with high efficiency [Cooper et al., 2009; Jin et al., 2009; Bao et al., 2010]. In the present work, we demonstrated that all three transcript variants of the murine SIRT3 gene are expressed in numerous mouse tissues. We also confirmed previous reports that the two longer murine SIRT3 proteins (M1 and M2) are targeted to mitochondria with a higher efficiency than the shorter M3 isoform. In addition, we showed that while the M3 protein is not processed, the M1 and M2 proteins are processed to a mature form. Using Edman degradation, we determined the N-terminal sequence of the mature form produced by M1 and found it is started at Ile38 or Val42, while the protein produced by M3 starts with Val79 or Met78. Moreover, we also demonstrated that mature protein can be produced from M1 cDNA independent of M2, albeit at a lower rate of maturation than SIRT3 proteins produced from M2. Furthermore, we found that both the mature murine SIRT3 derived from M1 or M2 or the shorter SIRT3 M3 protein have similar deacetylase activity when whole native substrate protein (ACS2) was used as substrate.

Most mitochondrial proteins are synthesized with N-terminal pre-sequences and typically cleaved by the mitochondrial processing peptidases [Vogtle et al., 2009]. Our N-terminal sequencing data suggested that the mature protein derived from M1 or M2, is cleaved at the Ile38, which is consistent with the cleavage site specificity of MPP [Taylor et al., 2001]. A minority of the mature form of M1 proteins start from Val42. This might be due to additional processing by other protease, such as the mitochondrial intermediate protease Oct1 [Gakh et al., 2002], functioning after MPP cleavage.

It is important to note that the multiple transcript variants of SIRT3 are not unique to mouse. They also exist in humans. Upon examination of the available human SIRT3 cDNA sequences in various databases, we found that there are two transcript variants of human SIRT3: one is the reported full-length human SIRT3 matching Genbank database sequence AF083108 and the other is similar to the mouse transcript 3 containing an 8-bp insertion (matching the Genbank database sequence AL535769). This transcript would theoretically encode a shorter version of the human SIRT3 protein, one quite similar to the M3 isoform of mouse SIRT3. This suggests that the M3 SIRT3 isoform might be functionally conserved. Although the M3 isoform of murine SIRT3 does not possess any N-terminal mitochondrial targeting signal for mitochondrial localization, an internal mitochondrial targeting sequence and nuclear localizing signal have been reported [Nakamura et al., 2008].

Although the murine M3 SIRT3 proteins is 40 amino acid shorter at the N-terminus than the mature form derived from M1 or M2 SIRT3, the conserved enzymatic core domain is intact in the shorter M3 protein, suggesting it may maintain enzymatic activity. Interestingly, a recent report [Jin et al., 2009] showed that the M3 SIRT3 protein was enzymatically inactive when assayed using ACS2 peptide as substrate. The present work confirms this result. However, we found that when intact native ACS2 protein was used as substrate in an in vitro assay, the M3 SIRT3 (78–334) displayed similar enzymatic activity to the mature form of the longer SIRT3 (38–334) isoform (Fig. 4B). A recent report also demonstrated that re-expressing the SIRT3 M3 protein in MEF cells deficient of SIRT3 was able to suppress hyper-acetylation of mitochondrial proteins to the same extent as the re-expression of M2 proteins [Bao et al., 2010]. These results are in agreement with the notion that the SIRT3 M3 protein is enzymatically active.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Murine SIRT3 transcript variants. A: Schematic representation of the first two exons of mouse SIRT3 gene. Two ATG sites located in exon 1b used for the translation of M1 or M2 and the ATG site located in exon 2 for the translation of M3 are marked. The eight base pair insertion at the 5' of exon 2 is in black. B: The cDNA sequence of the first 2 exons of murine SIRT3 transcript variants. Exons 1a and 1b are in black and exon 2 is in grey. The 8 bp insertion is surrounded by a box. The three potential translation initiation codons are indicated in bold. The stop codon that results in the premature termination is in bold. The sense primers (primer 1–3) that were used for the specific amplification of the three transcript variants are underlined. C: The RT-PCR detection of three transcript variants of murine SIRT3 in RNA extracted from indicated tissues of wild-type (Wt) or SIRT3^{-/-} mice. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 2.

Transient expression of M1, M2, and M3 in HEK293 cells. **A:** HEK293 cells were transfected with M3-SIRT3(78–334)-Flag, M1-SIRT3(1–334)-Flag and M2-SIRT3(15–334)-Flag. After 2 days, the cells were lysed and SIRT3 protein detected by Western blot analysis using antibodies against SIRT3 (lower panel) or Flag (top panel). The mitochondria from Sirt3^{-/-} or Sirt3^{+/-} mice were used as controls. The band corresponding to the mature form of SIRT3 is marked with an arrow. **B:** Transiently expressed M1-Flag SIRT3 proteins were immunoprecipitated with anti-Flag antibody and stained with Coomassie G250. The band corresponding to the mature form of SIRT3 as indicated by an arrow was cut for Edman sequencing. **C:** HEK293 cells were transfected with M1-Flag, M2-Flag and M1-M15A-Flag. After 2 days, the cells were lysed and SIRT3 proteins were detected by Western blot analysis with anti-Flag antibody. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 3.
The sub-cellular localization of M1, M2, and M3. Flag-tagged SIRT3 isoform were expressed in NIH-3T3 cells by transient transfection. Two days later, the SIRT3 proteins were detected by immunofluorescence with anti-Flag antibody (green) and the mitochondria were labeled with anti-MnSOD antibody (red).

Fig. 4. Deacetylase activities of murine SIRT3 protein isoforms. A: The deacetylase activity of recombinant SIRT3(38–334) or SIRT3(78–334) with or without GST fusion, in the presence or absence of NAD⁺, were analyzed using acetylated ACS2 peptide as substrate. B: The deacetylase activity of recombinant SIRT3(38–334) or SIRT3(78–334), in the presence or absence of NAD⁺, were analyzed using acetylated ACS2 whole protein as substrate. C: Western blot analysis was carried out to detect acetylation level of ACS2 proteins that were deacetylated by SIRT3(38–334) or SIRT3(78–334), in the presence or absence of NAD⁺ (top panel). The protein levels of ACS2 and SIRT3 were detected with Ponceau S staining and rabbit anti-SIRT3 antibody, respectively.