Mouse Sir2 Homolog SIRT6 Is a Nuclear ADP-ribosyltransferase*

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Members of the Sir2 family of NAD-dependent protein deacetylases regulate diverse cellular processes including aging, gene silencing, and cellular differentiation. Here, we report that the distant mammalian Sir2 homolog SIRT6 is a broadly expressed, predominantly nuclear protein. Northern analysis of embryonic samples and multiple adult tissues revealed mouse SIRT6 (mSIRT6) mRNA peaks at day E11, persisting into adulthood in all eight tissues examined. At the protein level, mSIRT6 was readily detectable in the same eight tissue types, with the highest levels in muscle, brain, and heart. Subcellular localization studies using both C- and N-terminal green fluorescent protein fusion proteins showed mSIRT6-green fluorescent protein to be a predominantly nuclear protein. Indirect immunofluorescence using antibodies to two different mSIRT6 epitopes confirmed that endogenous mSIRT6 is also largely nuclear. Consistent with previous findings, we did not observe any NAD⁺-dependent protein deacetylase activity in preparations of mSIRT6. However, purified recombinant mSIRT6 did catalyze the robust transfer of radiolabel from [³²P]NAD to mSIRT6. Two highly conserved residues within the catalytic core of the protein were required for this reaction. This reaction is most likely mono-ADP-ribosylation because only the modified form of the protein was recognized by an antibody specific to mono-ADP-ribosylation. Surprisingly, we observed that the catalytic mechanism of this reaction is intra-molecular, with individual molecules of mSIRT6 directing their own modification. These results provide the first characterization of a Sir2 protein from phylogenetic class IV.

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Mouse Sir2 homologs in worm and fly also promote longevity, although probably by different molecular mechanisms (8–10). Mammalian genomes contain seven Sir2 homologs, termed sirtuins (SIRTs). Of these, SIRT1, orthologous to the yeast Sir2, is the best characterized and has the broadest substrate specificity (11). A nuclear protein, SIRT1 deacetylates several substrates in vivo, including MyoD, p53, and FOXO transcription factors, thereby affecting cell differentiation and survival under stress (11–13). Recently, mouse SIRT1 was reported to inhibit the mobilization of fatty acids in white adipose tissue by repressing PPARγ, linking Sir2 proteins to the physiology of caloric restriction in mammals (14).

Of the remaining SIRTs (SIRT2–7), an in vivo substrate has only been identified for the cytoplasmic SIRT2 (15). This substrate, β-tubulin, is specifically deacetylated by SIRT2 (15), although the biological consequences of this reaction are unclear.

Yeast Sir2 and its orthologs, including mouse SIRT1 and bacterial CobB, catalyze the tightly coupled cleavage of NAD⁺ and protein deacetylation, producing nicotinamide and 2-O-acetyl-ADP-ribose reaction products (16). Whereas Sir2 proteins are generally thought to be NAD-dependent protein deacetylases, most also display a less robust mono-ADP-ribosyltransferase activity in vitro (17–20). Mutations in phylogenetically conserved residues within the catalytic core of Sir2 and SIRT1 have failed to separate the two enzymatic activities (21). The initial observation that ADP-ribosylation by Sir2 is stronger on acetylated substrates (22), combined with subsequent mechanistic studies (18, 20), has led to the conclusion that the deacetylase and phospho-ADP-ribosyltransferase activities of Sir2 proteins are coupled (16).

Using sequence similarity, eukaryotic Sir2 genes have been divided into four broad phylogenetic classes (23), known as classes I–IV (Fig. 1). SIRT1, SIRT2, and SIRT3 are class I, SIRT4 is class II, and SIRT5 falls within the predominantly prokaryotic class III. Finally, mammalian SIRT6 and SIRT7 are class IV sirtuins (23). In vitro studies indicate that human SIRT1, SIRT2, SIRT3, and SIRT5 possess NAD-dependent histone deacetylase activity (15), whereas SIRT4, SIRT6, and SIRT7 fail to deacetylate ³H-labeled acetylated histone H4 peptide (15). The lack of detectable deacetylase activity in these SIRTs may result from their specificity for targets other than those tested, or it may indicate an enzymatic activity other than deacetylation inherent in these sirtuins.

Mono-ADP-ribosylation is emerging as a common mechanism of reversible protein modification within mammalian cells. Originally described as the acting mechanism for specific bacterial toxins, ADP-ribosylation is typically performed by separate families of intra- and extracellular enzymes in vertebrates (24). Known targets of the intracellular class of enzymes include molecular chaperone GRP78/BiP, translational elongation factor 2, and β-subunit of heterotrimeric G-proteins (24). Extracellular mammalian ADP-ribosyltransferases, generally found in cells of the immune system, modify substrates important for the im-
mune response, as well as integrin α7 and the antimicrobial peptide defensin (24). In most known cases, ADP-ribosylation of arginine residues results in reversible inactivation of the substrate protein (25), although this modification may enhance certain enzymatic functions within the substrate (26).

In this report, we characterize the tissue-specific expression, subcellular localization, and in vitro enzymatic activity of mouse SIRT6. This investigation, the first detailed study of a class IV sirtuin, reveals mouse SIRT6 to be a widely expressed, predominantly nuclear protein with a robust auto-ADP-ribosyltransferase activity.

MATERIALS AND METHODS

Multiple Sequence Alignments—Sequences of mouse proteins SIRT1, SIRT4, SIRT5, and SIRT6 were aligned with the yeast Sir2p core using ClustalW. In the sirtuin phylogenetic tree, SIRT1 is class I, SIRT4 is class II, SIRT5 is class III, and SIRT6 is class IV. At each position of the alignment, identical residues are boxed and shaded in gray. Amino acids boxed in black correspond to mSIRT6 residues targeted for mutagenesis in this study.

![Sequence alignment of mouse sirtuin core domains.](image)

**FIG. 1.** Sequence alignment of mouse sirtuin core domains. Conserved core domains of mouse SIRT1, SIRT4, SIRT5, and SIRT6 were aligned with the yeast Sir2p core using ClustalW. In the sirtuin phylogenetic tree, SIRT1 is class I, SIRT4 is class II, SIRT5 is class III, and SIRT6 is class IV. At each position of the alignment, identical residues are boxed and shaded in gray. Amino acids boxed in black correspond to mSIRT6 residues targeted for mutagenesis in this study.

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The abbreviations used are: mSIRT6, mouse SIRT6; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; DAPI, 4',6-diamidino-2-phenylindole; PBS, phosphate-buffered saline; GST, glutathione S-transferase.
lysate was centrifuged at 19,000 rpm for 30 min. The supernatant was placed on a column. The column was washed extensively with PBSTG, and cells were grown at 37 °C to an optical density of 0.7. Protein expression of mSIRT6-(274–355) were transformed into BL21(DE3)-Codon Plus-RP and incubated with coverslips for 45 min. Coverslips were mounted with VectaShield mounting media containing 4',6-diamidino-2-phenylindole, dilactate (Vector, Burlingame, CA). Microscopy was performed on a Nikon Eclipse E500 fluorescence microscope. Digital images were obtained using a SPOT RT charge-coupled device camera and software using identical exposure times for comparable samples.

**Multiple Tissue Northern Blots—**Probe containing the SITR6 open reading frame was created using the Primelink Random Primer Labeling kit (Stratagene) according to the manufacturer’s instructions. The probe was labeled with 32P by ligating it into pET-GST cut with NheI and BamHI.

**Antibody Production—**pET28a-His-mSIRT6 and pET-GST-mSIRT6-(274–355) were transformed into BL21(DE3)-Codon Plus-RP cells (Stratagene) and inoculated into 4- to 2-liter cultures of LB (50 mg/ml kanamycin and 35 mg/ml chloramphenicol), respectively. The cells were grown at 37 °C to an optical density of 0.7. Protein expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM for 2 h, and the cells were harvested by centrifugation.

The cells containing the pET28a-His-mSIRT6 plasmid were resuspended in lysis buffer (0.1 M NaH2PO4, 10 mM Tris, 8 mM urea, pH 8.0) and lysed by freezing in liquid N2. The lysate was centrifuged at 19,000 rpm for 30 min, and the supernatant was incubated with 4 ml of nickel-nitrilotriacetic acid-agarose (Stratagene) for 3 h at 4 °C, loaded onto a column, and washed extensively with wash buffer (0.1 M NaH2PO4, 10 mM Tris, 8 mM urea, pH 6.4). The purified protein was eluted with elution buffer (0.1 M NaH2PO4, 10 mM Tris, 8 mM urea, pH 3.0) in 1.5-M fractions. The pH was neutralized by the immediate addition of 100 μl of 1 M Tris base. The fractions containing protein were pooled against PBS. The protein was sent to Covance Research Bioproductions for antibody production.

The cells containing the pET-GST-SIRT6-(274–355) plasmid were resuspended in PBSTG (PBS, 0.1% Tween 20, 10% glycerol, 1 mM dithiothreitol) and 0.1 g of lysozyme, incubated at 37 °C for 20 min, and frozen in liquid N2. The lysate was centrifuged at 19,000 rpm for 30 min. The supernatant was incubated with 1 ml of GST-agarose (Stratagene) for 3 h at 4 °C (without shaking) and loaded onto a column. The column was washed extensively with PBSTG, and the protein was eluted with elution buffer (50 mM Tris, 10 mM glutathione, pH 7.5). Fractions containing protein were dialyzed against PBS and sent to Covance Research Bioproductions for antibody production.

The antibodies were affinity-purified as follows. Aliquots of the proteins used to generate the antibodies were attached to 1 ml of NHS-activated Sepharose columns (Amersham Biosciences) according to the manufacturer’s instructions. 10 ml of the final bleed of each antibody was combined with 10 ml of antibody wash buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and applied to the column. Each column was washed extensively with antibody wash buffer, and the antibody was eluted with 100 mM glycine, pH 2.5. The pH was neutralized by the addition of 1 ml of 1 M Hepes, pH 7.5. The antibody-containing column was then subjected to a second round of affinity purification, but this time the antibody was dialyzed against PBS, and sent to Covance Research Bioproductions for antibody production.

**Quantitation of ADP-ribosylation—**ADP-ribosylation reactions were performed as described above, but with the following modifications. The total volume was 20 μl, including 5 μl of protein, 0.8 μl of 6.25 μM [32P]NAD, and 4 μl of 10 μM NAD. Purified reaction products were separated by SDS-PAGE, and Coomassie blue-stained bands were transferred to polyvinylidene difluoride membrane before autoradiography and subsequent immunoblot analysis. Blots were stained with Coomassie Blue, and the Coomassie Blue-stained bands and Western signals were aligned to verify that the radioative bands were SIRT6.

**mSIRT6 Expression in Mice and Embryos—**To determine the expression pattern of mSIRT6, we analyzed RNA extracts from eight adult mouse tissues by Northern blotting (Fig. 2A). Using a CDNA probe corresponding to the 5′ region of mSIRT6, expression of mSIRT6 mRNA was observed in every tissue type tested (Fig. 2A).Confirming the predictions of sequence analysis (23), mSIRT6 was detected as a single 1.0-kb transcript, suggesting that this gene is not found in alternate splice forms. When normalized to actin, the highest levels of mSIRT6 mRNA were seen in the brain, heart, and liver, with the lowest expression level observed in skeletal muscle.

To evaluate mSIRT6 levels during development, we probed Northern blots of RNA from four mouse embryonic stages from

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**RESULTS**

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different mice were included. 

panel analyzed by Western blotting using antibodies specific to mSIRT6 (the indicated wild-type mouse tissues were resolved by SDS-PAGE and analyzed by Northern blotting as described in Materials and Methods. Muscle actin found in heart and skeletal muscle samples migrated as a distinct band. B, RNA from 7-, 11-, 15-, and 17-day mouse embryos was analyzed by Northern blotting as described in A. C, protein extracts from the indicated wild-type mouse tissues were resolved by SDS-PAGE and analyzed by Western blotting using antibodies specific to mSIRT6 (top panel) or actin (bottom panel). For each tissue type, samples from four different mice were included.

Fig. 2. Analysis of mouse SIRT6 and actin expression in adult tissues and developing embryos. A, RNA was isolated from the indicated tissues in wild-type adult mice, resolved by electrophoresis, and subjected to Northern blotting. Blots were probed with 32P-labeled cDNA specific to mSIRT6 (top panel) or actin (bottom panel). Muscle actin found in heart and skeletal muscle samples migrated as a distinct band. B, RNA from 7-, 11-, 15-, and 17-day mouse embryos was analyzed by Northern blotting as described in A. C, protein extracts from the indicated wild-type mouse tissues were resolved by SDS-PAGE and analyzed by Western blotting using antibodies specific to mSIRT6 (top panel) or actin (bottom panel). For each tissue type, samples from four different mice were included.

Having established the prevalence of mSIRT6 message in embryonic and adult tissues, we wished to determine the distribution of mSIRT6 protein. Affinity-purified antiserum specific to mSIRT6 was used to probe Western blots of proteins isolated from eight adult murine tissue types (Fig. 2C). For each tissue type, samples from four individual animals were obtained and analyzed by immunoblot. As illustrated in Fig. 2C, mSIRT6 protein was detectable in all tissue types as a single 40-kDa band. As expected from Northern analysis, levels were high in brain, liver, and heart when normalized to actin protein levels. Surprisingly, mSIRT6 protein was most strongly expressed in muscle (Fig. 2C), despite the relative paucity of its transcript in this tissue. This observation might indicate increased mSIRT6 transcript stability in muscle or an enhanced rate of translation in this tissue type relative to others tested.

Visualization of mSIRT6-GFP in Nuclei—In order to observe the subcellular localization of mSIRT6, we engineered two GFP fusion expression vectors under the control of the cytomegalovirus promoter. Both a C-terminal (mSIRT6-GFP) and an N-terminal (GFP-mSIRT6) fusion construct were transfected into NIH 3T3 cells grown on coverslips. Transfections were performed on glass coverslips in 6-well plates. 48 h after transfection, cells were washed, fixed with paraformaldehyde, stained with DAPI, and analyzed by fluorescence microscopy. The fluorescein isothiocyanate channel (left column) shows GFP fluorescence, and the DAPI channel (middle column) reveals nucleic acid localization. Images from both channels are merged in the right column. For each transfection, a representative field of cells is pictured. B, NIH 3T3 cells grown on coverslips were stained with affinity-purified rabbit polyclonal antibodies to two different mSIRT6 epitopes (full-length mSIRT6, middle row; mSIRT6 amino acids 274–355, bottom row). Samples were then stained with DAPI and Cy3-conjugated anti-rabbit IgG and visualized by fluorescence microscopy. Cy3 fluorescence is shown in the left column, DAPI-stained nucleic acids are shown in the middle column, and the merged image is shown in the right column.

Important, immunofluorescence and GFP localization studies revealed the same predominantly nuclear localization pattern for mSIRT6. We therefore conclude that the majority of mSIRT6 is found in the nucleus, whereas a small fraction may be localized to the cytoplasm.

mSIRT6 Catalyzes the Transfer of 32P from a 32P-labeled NAD Donor—Of the seven mammalian Sir2 homologs, only SIRT1, SIRT2, SIRT3, and SIRT5 have been shown to have NAD+-dependent protein deacetylase activity in vitro (15). Consistent with published reports, we did not observe any protein deacetylase activity of mouse or human SIRT6 using a variety of experimental conditions and potential substrates (data not shown).

Several sirtuins also possess a mono-ADP-ribosyltransferase activity (19). We therefore sought to test whether mSIRT6 could catalyze the transfer of radiolabel from [32P]NAD+ to histones, an assay of ADP-ribosyltransferase activity. Histone
The presence of $^{32}$P NAD was transferred to nitrocellulose membrane, and exposed to film. Proteins were separated by SDS-PAGE, purified, and incubated in the presence of $^{32}$P NAD. Reactions were purified as described before and subjected to autoradiography. Labeled GST-mSIRT6 following incubation with $^{32}$P NAD (Fig. 6B). No band was present at the corresponding position in preparations from mutant mSIRT6 (Fig. 6A), demonstrating the specificity of the antibody for the modified form of mSIRT6. This evidence strongly suggests that this modified form of mSIRT6 is mono-ADP-ribosylated. Whereas our results do not rule out the possibility that the modification of wild-type mSIRT6 occurs in E. coli before purification, the absence of signal in point mutant SIRT6 indicates that mono-ADP-ribosylation depends on the catalytic activity of mSIRT6.

Having confirmed mono-ADP-ribosylation as the nature of the modification of mSIRT6, we wished to ascertain whether this modification was catalyzed in an inter- or intra-molecular fashion. mSIRT6 and two different mutants (H133Y and S56A) were used in a molecular cis/trans test for enzymatic activity. GST-mSIRT6 was purified from E. coli and incubated in combination with His$_6$-tagged mutant and wild-type mSIRT6 in the presence of $^{32}$P NAD. Reactions were purified as described before and subjected to autoradiography. Labeled GST-mSIRT6 migrated as an ~80-kDa band in all reactions (Fig. 6B). His$_6$-tagged mSIRT6 migrated as a strongly labeled 39-kDa band (Fig. 6B). However, this band was absent from the corresponding reactions of both His$_6$-tagged mutants, demonstrating that GST-mSIRT6 was unable to ADP-ribosylate those proteins. Coomassie Blue staining of the gel prior to film exposure revealed similar amounts of protein in all three lanes (data not shown). We therefore conclude that the auto-ADP-ribosylation reaction catalyzed by mSIRT6 is intra-molecular.

**DISCUSSION**

Here we provide the first characterization of a class IV sirtuin, mouse SIRT6. This sirtuin shows a relatively weak serine deacetylase activity in preparations of mSIRT6 expressed in bacterial and mammalian cells. Surprisingly,
mSIRT6 did show a robust ADP-ribosyltransferase activity in vitro, indicating that recombinant bacterial preparations indeed contained active protein.

We conclude that mSIRT6 is a mono-ADP-ribosyltransferase for the following reasons. First, the purified protein can catalyze the transfer of radiolabel from \[^{32}\text{P}\]NAD, a reaction also catalyzed by other Sir2 family members (17, 19, 22) and previously shown to be mono-ADP-ribosylation (19). Second, this activity requires the catalytic function of mSIRT6 because two different point mutations in phylogenetically invariant residues predicted to abolish enzymatic activity eliminated the transfer of label from NAD to mSIRT6. Third, only the enzymatically modified form of mSIRT6 is recognized by an anti-ADP-ribose antibody specific to mono-ADP-ribosylated proteins. Finally, the transfer of label from \[^{32}\text{P}\]NAD to mSIRT6 is accomplished by an intra-molecular mechanism, indicating that SIRT6 may use ADP-ribosylation as a way to auto-regulate its activity.

Recombinant mSIRT6 expressed in E. coli and incubated with NAD was able to incorporate a quantity of ADP-ribose...
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Fig. 6. Auto-ADP-ribosylation of mSIRT6. A, equal amounts of recombinant mSIRT6 and mutant mSIRT6-H133Y protein were incubated with [32P]NAD− and blotted to nitrocellulose as described in the Fig. 4 legend. The blot was probed with antibody specific to mono-ADP-ribose, stripped, and reprobed with antibody specific to mSIRT6 as a control. B, GST-SIRT6 was incubated with [32P]NAD− and either mSIRT6 (lane 1), mSIRT6-S56A (lane 2), or mSIRT6-H133Y (lane 3). Reactions were analyzed by autoradiography as described in the legend to Fig. 4C. Coomassie Blue staining of the gel prior to film exposure revealed similar amounts of recombinant protein in all three lanes (data not shown).

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equivalent to 15% of the moles of mSIRT6 present in the reaction. This level of ADP-ribosylation is impressive, considering the intra-molecular nature of the reaction, by which a molecule of SIRT6 is only active on a single substrate molecule. We failed to observe a band shift resulting from ADP-ribosylation, suggesting that the number of amino acid residues ADP-ribosylated on mSIRT6 is very small. Assuming a single ADP-ribosylation per molecule of SIRT6, our results indicate a strong 15% activity in our recombinant protein preparations.

Mouse SIRT6 is broadly expressed at the RNA and protein levels throughout development and during adulthood in at least eight different tissue types, indicating broad expression throughout the organism. The SIRT6 protein is particularly abundant in brain and liver. Interestingly, mSIRT6 protein expression is highest in muscle, even though mRNA levels in this tissue are low relative to those of actin. This might indicate increased SIRT6 protein stability in muscle compared with other tissue types. Alternatively, the disproportionate amount of SIRT6 protein observed in muscle may result from an increased rate of translation of SIRT6 mRNA in this tissue type.

Localization studies of mSIRT6 using N- and C-terminal GFP fusions as well as antibodies to two different epitopes showed that expression of mSIRT6 was largely nuclear, with only a diffuse presence in cytoplasm. Interestingly, endogenous levels of SIRT6 do not permeate certain intra-nuclear regions, probably corresponding to the nucleolus (Fig. 3B). The only other mouse class IV sirtuin, mSIRT7, is localized to the nucleolus.2 We therefore speculate that mSIRT6 and mSIRT7, despite extensive sequence homology (39%), have non-overlapping functions within the cell. Like SIRT6, SIRT7 has not been shown to possess any in vitro protein deacetylase activity (15).

Several ADP-ribosyltransferases are capable of reversible auto-modification resulting in altered enzymatic activity. Pseudomonas ExoS, a bifunctional enzyme, is a Rho GTPase-activating protein as well as an ADP-ribosyltransferase (25). Auto-ADP-ribosylation at arginine 146, observed in vitro and in vivo, reduces GTPase-activating protein activity (25), suggesting a mechanism for intra-molecular regulation. In mammals, auto-modification of ADP-ribosyltransferase 5 converts the protein from NADase to transferase (26), again suggesting a mechanism for regulation of enzyme activity. We speculate that SIRT6 might regulate its own activity in vivo by ADP-ribosylation of specific residues required for activity. At this time, we have not yet identified the physiological targets of SIRT6. Unlike class I sirtuins including yeast Sir2 and SIRT1, SIRT6 appears to be highly specific to SIRT6. Unlike class I sirtuins including yeast Sir2 and SIRT1, SIRT6 might regulate its own activity in vivo.

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