Sir2 (silent information regulator 2) is a NAD\(^+\)-dependent histone deacetylase that contributes to longevity in yeast. SIRT1, a mammalian Sir2 ortholog, deacetylates histones and various transcription factors, including p53, FOXO proteins, and peroxisome proliferator-activated receptor-\(\gamma\). We found that its subcellular localization varied in different tissues of the adult mouse. Some subsets of neurons predominantly expressed SIRT1 in the cytoplasm, but ependymal cells expressed it in both the nucleus and cytoplasm. On the other hand, spermatocytes expressed SIRT1 only in the nucleus. Cardiomyocytes in the day 12.5 mouse embryo expressed SIRT1 exclusively in the nucleus, but in the adult heart, they expressed it in both the cytoplasm and nucleus. C2C12 myoblast cells expressed SIRT1 in the nucleus, but it localized to the cytoplasm after differentiation. LY294002, an inhibitor of phosphoinositide 3-kinase, strongly inhibited the nuclear localization of SIRT1 in undifferentiated C2C12 cells. In a heterokaryon assay, SIRT1 shuttled between the nucleus and cytoplasm, and leptomycin B, an inhibitor of CRM1-mediated nuclear exportation, inhibited this shuttling. Two nuclear localization signals and two nuclear export signals were identified by deletion and site-directed mutation analyses. Overexpressed nuclear (but not cytoplasmic or dominant-negative) SIRT1 enhanced the deacetylation of histone H3 in C2C12 cells. Moreover, only the nuclear form suppressed the apoptosis of C2C12 cells induced by antimitic A, an oxidative stressor. These findings indicate that nucleocytoplasmic shuttling is a novel regulatory mechanism of SIRT1, which may participate in differentiation and in inhibition of cell death.

The Sir2 (silent information regulator 2) proteins are a family of class III histone deacetylases found in organisms from bacteria to humans (1). Unlike class I and II histone deacetylases, the Sir2 family requires the cofactor NAD\(^+\) for catalytic activity (2). In yeast, Sir2 participates in heterochromatic silencing at mating-type loci (3). Sir2 extends the life span of yeast by suppressing recombination in the rDNA region and consequently reducing the formation of extrachromosomal rDNA circles (4), a cause of senescence (5). Caloric restriction extends the life span in organisms ranging from yeast to mammals, and the Sir2 family plays an essential role in this effect (6, 7).

In mammals, there are seven members of the Sir2 family, termed sirtuins, of which SIRT1 is the closest homolog of yeast Sir2. SIRT1 regulates metabolic responses in adipocytes and liver. It promotes fat mobilization in white adipocytes by repressing peroxisome proliferator-activated receptor-\(\gamma\) (8). SIRT1 deacetylates the transcription coactivator PGC1\(\alpha\), thereby inducing the expression of gluconeogenic genes and the repression of glycolytic genes (9). SIRT1 contributes to cell survival by deacetylating and thereby repressing the activity of the tumor suppressor p53 (10–12). The transcriptional activation of stress-resistance genes by FOXO proteins is also regulated by SIRT1, i.e. SIRT1 enhances the induction of manganese superoxide dismutase by FOXO4 (13) and of GADD45 by FOXO3 (14). SIRT1 also affects differentiation: it deacetylates and inhibits MyoD, a transcription factor that induces muscle differentiation, thereby suppressing the differentiation of skeletal myoblast cells (15). Fetal mice express a high level of SIRT1 (16), and SIRT1-null mice have abnormalities in various tissues, including sperm, heart, and eye (17, 18), suggesting an important role for SIRT1 in differentiation.

SIRT1 has been believed to be a nuclear protein. For example, it is localized exclusively to the nucleus in male germ cells and in COS-7 cells transfected with SIRT1 cDNA (16, 17). However, the cytoplasmic localization of SIRT1 has been reported recently in murine pancreatic \(\beta\)-cells and neonatal rat cardiomyocytes (19, 20). Dynamic changes in the subcellular localization of Drosophila Sir2 during embryonic development have also been reported (21). Thus, the subcellular localization of SIRT1 may change during development and in response to physiological and pathological stimuli. Because SIRT1 deacetylates histones and various transcription factors, its subcellular localization must affect its function.

Proteins of up to 40 kDa passively diffuse into and out of the nucleus, whereas larger proteins that exceed the diffusion limit of the nuclear membrane are actively transported via specific pathways. Nuclear importation of a protein is usually directed by a nuclear localization signal (NLS)\(^2\) in the protein’s amino acid sequence (22). The classical NLS is a cluster of basic amino acids sequence (22). The classical NLS is a cluster of basic amino acids sequence (22).
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acid residues, and a second type is the bipartite NLS, consisting of two stretches of basic amino acids separated by a spacer of 10–12 amino acid residues (23). The exportation of proteins from the nucleus is mediated by nuclear export signals (NESs) (24). The most common type of NES motif is a short stretch of amino acids containing closely spaced large hydrophobic residues such as leucine and isoleucine (25). Post-translational modifications can control the subcellular localization of a protein by modifying the function of the NLSs and/or NESs. In particular, phosphorylation is known to play an important role in this kind of modification (26).

In this study, we demonstrate that the subcellular localization of SIRT1 differs in various tissues and cells. Differentiation affects the cellular distribution of SIRT1 in cardiomyocytes. Consistent with this, SIRT1 is transported to the cytoplasm upon the differentiation of C2C12 myoblast cells. We found that the phosphoinositide 3-hydroxykinase (PI3K)-Akt pathway affects the subcellular localization of SIRT1. LY294002, an inhibitor of PI3K, induced the cytoplasmic localization of SIRT1 in C2C12 cells, whereas insulin-like growth factor-1 (IGF1) attenuated the nuclear exclusion of SIRT1 by LY294002.

We also used a heterokaryon shuttling assay to show that SIRT1 is a nucleocytoplasmic shuttling protein. We identified two NLS and two NES motifs in SIRT1 by deletion and site-directed mutation analyses. The overexpression of nuclear (but not cytoplasmic) SIRT1 significantly inhibited apoptosis induced by antisense A, a generator of reactive oxygen species. Thus, the nucleocytoplasmic shuttling of SIRT1 seems to play a crucial role in regulating the function of SIRT1.

EXPERIMENTAL PROCEDURES

Immunohistochemistry—ddY male mice (3 months old) were deeply anesthetized with pentobarbital (100 mg/ml) and perfused with 4% paraformaldehyde in 0.1 M sodium phosphate. Tissues were removed and fixed overnight in 4% paraformaldehyde, transferred to 30% sucrose overnight, mounted in OCT compound (Tissue-Tek, catalog no. 4583, Sakura Finetek), and frozen in liquid nitrogen. The samples were sliced into 10-μm thick sections on a cryostat, mounted onto clean MAS-coated slides (Matsunami adhesive silane), air-dried, and stored at −80 °C until processing. For staining with a VECTASTAIN ABC kit (Vector Laboratories), sections were pretreated with 3% H2O2 for 30 min to block endogenous peroxidase activity. The sections were washed with phosphate-buffered saline, blocked with 3% bovine serum albumin in phosphate-buffered saline for 30 min, and then incubated overnight with anti-SIRT1 antibody (16). The bound antibodies were labeled using the VECTASTAIN ABC kit or Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes) and examined by light microscopy (Olympus) or confocal microscopy (Radiance 2100, Bio-Rad).

Subcellular Fractionation and Immunoblot Analysis—Adult (3 months old) and fetal (embryonic day 12.5) ddY mouse hearts were excised under anesthesia. The hearts were washed and homogenized in ice-cold phosphate-buffered saline. The fractionation of the heart cells into cytosolic and nuclear fractions was performed using a nuclear/cytosol fractionation kit (BioVision). Whole cell lysates were obtained using CellLytic M mammalian cell lysis/extraction reagent (Sigma). For immunoblotting, anti-SIRT1 (16), anti-p300 (Upstate), anti-glyceroldehyde-3-phosphate dehydrogenase (Chemicon), anti-Akt or anti-phospho-Akt (Cell Signaling Technology), anti-total histone H3 (Upstate), or anti-acetylated or anti-non-acetylated histone H3 (Calbiochem) antibody was used.

Cell Culture and Transfection—COS-7, C2C12, and L929 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% fetal bovine serum at 37 °C with 5% CO2. Differentiation of the C2C12 cells was induced by supplementing DMEM with 2% horse serum. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) or a NucleoFector kit (Amaza) following the manufacturers’ instructions. The cells received one of the following treatments: (i) 2% horse serum for 24 h, (ii) 10 nM leptomycin B (LMB) for 3 h, (iii) 10 or 30 μM LY294002 and/or 30 nM IGF1 for 24 h, or (iv) 100 μM antimycin A for 12 h. The cells were then fixed in 4% paraformaldehyde, stained with Hoechst 33342, and observed by confocal microscopy.

Heterokaryon Shuttling Assays—Semiconfluent COS-7 cell monolayers were transfected with full-length SIRT1-enhanced green fluorescent protein (EGFP), its site-directed mutants, or p53-EGFP using Lipofectamine 2000 and then incubated for 24 h in DMEM supplemented with 10% fetal bovine serum. Untransfected L929 cells were stained with Hoechst 33342 for 15 min, washed, and then incubated in DMEM with 10% fetal bovine serum. Twenty-four hours later, the L929 cells were harvested. L929 cells (5 × 105) were then added to the transfected COS-7 cells in a 12-well dish and incubated overnight at 37 °C. The co-cultures were incubated with 100 μg/ml cycloheximide for 1 h, and the cells were fused by the addition of 5% polyethylene glycol 4000 for 3 min and then washed with phosphate-buffered saline. The fused cells were further incubated with 100 μg/ml cycloheximide in the presence or absence of LMB (10 μM) for 3 h before analysis.

Plasmid Construction—To construct the SIRT1-EGFP fusion protein, the coding region of mouse SIRT1 cDNA lacking its stop codon was cloned into the vector pEGFP-N3 (Clontech) at the Xhol and Sall sites and in-frame with the EGFP coding region. The deletion mutants were constructed with PCR-derived SIRT1 fragments and pEGFP-N3 using the XhoI and SalI sites and in-frame with the EGFP coding region.

Assays for Apoptosis and Lactate Dehydrogenase Activity—Apoptotic cells were detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining using an apoptosis in situ detection kit (Wako). The lactate dehydrogenase activity of the culture medium was assayed in a spectrophotometer (Bio-Rad) using CytoTox 96 (Promega Corp.).

Statistical Analysis—Differences in the data were tested by one-way analysis of variance. The Student-Newman-Keuls method was used for the post hoc test.

RESULTS

Subcellular Localization of SIRT1—We first examined the subcellular localization of SIRT1 in adult mouse brain and testis. Positive staining with anti-SIRT1 antibody was identified in
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FIGURE 1. Differential distribution of SIRT1 in various mouse tissues. A, sections of mouse brain (striatum (panel a) and subventricular zone of the lateral ventricle (panel b)) and testis (panel c) were stained with anti-SIRT1 antibody. B, sections of fetal (embryonic day 12.5) and adult (3 month olds) mouse hearts were stained with anti-SIRT1 antibody and Hoechst 33342. The merged images are shown on the right. C, the cytoplasmic (Cyto) and nuclear (Nuc) fractions of fetal and adult mouse hearts were subjected to Western blot analysis. Staining of SIRT1, p300 (a marker for the nuclear fraction), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; a marker for the cytoplasmic fraction) is shown. D, shown is the quantitative data from three separate series of experiments.

some neuron-like cells of the striatum, where SIRT1 was expressed in the cytoplasm (Fig. 1A, panel a), and in ependymal cells, where it was expressed in both the cytoplasm and nucleus (panel b). On the other hand, spermatocytes and round spermatids expressed SIRT1 only in their nuclei (Fig. 1A, panel c).

We next examined whether differentiation alters the subcellular localization of SIRT1. SIRT1 was localized exclusively to the nucleus in the cardiomyocytes of day 12.5 embryos (Fig. 1B). In contrast, the myocytes of adult hearts showed diffuse cytoplasmic and nuclear staining for SIRT1 (Fig. 1B). To verify the difference in SIRT1 localization, subcellular fractions of hearts from fetal (embryonic day 12.5) and adult mice were analyzed by Western blotting. The 120-kDa SIRT1 band was detected only in the nuclear fraction in the embryonic hearts. In contrast, the intensity of the band was ~8 times higher in the cytoplasmic fraction than in the nuclear fraction in the adult hearts (Fig. 1, C and D).

We next examined whether SIRT1 might translocate from the nucleus to the cytoplasm in response to the differentiation stimulus (Fig. 2A, right panels). These data suggest that SIRT1 might be a nucleocytoplasmic shuttling protein.

Because other nucleocytoplasmic shuttling proteins such as p53 and the FOXO proteins are regulated by phosphorylation, we thought that the subcellular distribution of SIRT1 might also be controlled by kinases. We examined the effect of various kinase inhibitors on the localization of SIRT1-EGFP in C2C12 cells and found that LY294002, an inhibitor of PI3K, excluded SIRT1-EGFP from the nucleus dose-dependently (Fig. 2B, panel a) (data not shown). In control cells, the nuclear distribution of SIRT1-EGFP was not affected by IGF1, an upstream signal of the PI3K cascade. When IGF1 was added to cells treated with LY294002, SIRT1-EGFP was located in the nucleus of many of them (Fig. 2B). The effect of IGF1 and LY294002 on the PI3K cascade was confirmed by analyzing the phosphorylation of Akt, a downstream target of PI3K. Indeed, LY294002 inhibited phosphorylation of Akt dose-dependently, which was partially reversed by co-administration of IGF1 (Fig. 2B, panel b). These results indicate that the subcellular localization of SIRT1 may be regulated by the PI3K signal cascade.

To detect the nucleocytoplasmic shuttling of SIRT1, we next performed a heterokaryon shuttling assay using COS-7 and L929 cells, both of which express SIRT1 exclusively in the nucleus. COS-7 cells transfected with SIRT1-EGFP were fused with untransfected L929 cells, which were stained with Hoechst 33342. If SIRT1 shuttled between the nucleus and the cytoplasm in the heterokaryon, SIRT1-EGFP should be found not in the cytoplasm of the L929 cell but in the nucleus of the COS-7 cell (Fig. 2C). To confirm this, we fused COS-7 and L929 cells, both of which express SIRT1 exclusively in the nucleus. The specificity of LMB in our experiments was examined by its effect on p53, a well known
nucleocytoplasmic shuttling protein, the nuclear exportation of which depends on CRM1 (28). LMB abolished the nucleocytoplasmic shuttling of p53-EGFP (Fig. 2C, panel b), confirming that LMB actually inhibits CRM1-dependent nuclear exportation. These observations suggest that SIRT1 is exported by an NES-dependent mechanism.

Identification of the NLSs in SIRT1—NLS-containing proteins are transported into the nucleus. We screened the amino acid sequence of SIRT1 to identify potential NLSs, which consist of a cluster of basic amino acids. Two were found in amino acids 31–38 and 223–230 (hereafter referred to as NLS1 and NLS2, respectively) (Fig. 3A). To determine whether NLS1 and NLS2 are functional, we constructed a series of SIRT1-EGFP deletion mutants and expressed them in COS-7 cells (Fig. 3A). Wild-type SIRT1-EGFP and the deletion mutants containing both NLS1 and NLS2 (SIRT1-(1–230) and SIRT1-(31–737)) were expressed exclusively in the nucleus (Fig. 3A, panels a, c, and d, respectively). However, the deletion of either NLS1 (SIRT1-(223–737) and SIRT1-(223–737)) (Fig. 3A, panels e and f, respectively) or NLS2 (SIRT1-(1–38)) (Fig. 3A, panel b) resulted in the distribution of the EGFP signal to both the cytoplasm and nucleus. When both NLS1 and NLS2 were deleted (SIRT1-(231–737)), the SIRT1-EGFP mutant was localized mostly to the cytoplasm (Fig. 3A, panel g). These results demonstrate that both NLS1 and NLS2 are functional and that the complete nuclear localization of the protein requires both NLSs.

We confirmed these findings using site-directed mutations introduced into NLS1 and/or NLS2 in full-length SIRT1-EGFP. Mutating the two adjacent arginines in NLS1 (amino acids 37 and 38) to alanine residues (mtNLS1) produced a protein that was equally distributed to the nucleus and cytoplasm (Fig. 3B, panel b). Similarly, mutating the four basic amino acids in NLS2 (amino acids 227–230) to alanines (mtNLS2) yielded an equal distribution of SIRT1-EGFP to the cytoplasm and nucleus (Fig. 3B, panel c). The combination of mtNLS1 and mtNLS2 (mtNLS) resulted in the complete cytoplasmic localization of the protein (Fig. 3B, panel d). Thus, both NLS1 and NLS2 are involved in the nuclear importation of SIRT1.
Identification of the NESs in SIRT1

Because LMB inhibited the nuclear exportation of SIRT1 (Fig. 2C), SIRT1 is likely to contain an NES, which consists of a largely hydrophobic leucine-rich sequence (29). To map the location of the NES(s) in SIRT1, another series of SIRT1-EGFP deletion mutants was constructed and introduced into COS-7 cells (Fig. 4). Because SIRT1 deletion clones that contained the two NLSs showed exclusively nuclear localization (Fig. 3A), it was difficult to analyze the exportation activity of an NES. Hence, we used deletion clones containing only NLS2, which had much less nuclear importation activity than did both NLSs together (Fig. 4A).

In this series of experiments, several mutants showed considerable cell-to-cell variability in their subcellular distributions. Therefore, we divided the expression patterns of mutant clones into three groups: cells expressing mutant SIRT1-EGFP in the nucleus alone, in both the nucleus and cytoplasm, and in the cytoplasm alone; and the percentage of each group in the total number of transfected cells was compared (Fig. 4). When the C-terminal region of SIRT1 was sequentially deleted, SIRT1-(223–737), SIRT1-(223–489), SIRT1-(223–474), and SIRT1-(223–445) all exhibited similar subcellular distributions, with ∼15% of the cells expressing the protein in the cytoplasm alone (Fig. 4B, panels b–e, respectively). However, SIRT1-(223–354) was not detected in the cytoplasm alone in any cell (Fig. 4B, panel f), indicating that the nuclear exportation activity of this mutant is very weak. Thus, amino acids 355–445 might contain an NES. Because SIRT1-(223–277) showed a similar subcellular distribution compared with SIRT1-(223–354) (Fig. 4B, panel g), amino acids 278–354 are unlikely to contain an NES.

We next examined whether there is another NES domain in the N-terminal region by sequential deletion of the N terminus (Fig. 4A, panels h–j). SIRT1-(136–262) was expressed in the cytoplasm alone in 17% of the cells (Fig. 4B, panel h). In contrast, neither SIRT1-(191–262) nor SIRT1-(204–262) was expressed in the cytoplasm alone (Fig. 4B, panels i and j, respectively), indicating that another NES might lie between amino acids 136 and 190.
Identification of the NESs—Regions 136–190 and 355–445 contain clusters of hydrophobic amino acids, i.e., LLLTDGLL and VDLLIVI (hereafter referred to as NES1 and NES2, respectively). To investigate whether NES1 and NES2 are functional, we examined the effect of LMB on the distribution of SIRT1-(136–262) and SIRT1-(223–445) (Fig. 5B, panels b and e, respectively). LMB significantly increased the number of cells that expressed SIRT1-(136–262) or SIRT1-(223–445) in the nucleus from 3 to 10% and from 4 to 22%, respectively. In addition, LMB decreased the number of cells expressing SIRT1-(136–262) or SIRT1-(223–445) in the cytoplasm from 17 to 11% and from 14 to 0%, respectively (Fig. 5B, panels b and e). These findings suggest that both amino acids 136–262 and 223–445 contain functional NESs.

To investigate further the nuclear exportation activities of NES1 and NES2, site-directed mutations were introduced into SIRT1-(136–262) and SIRT1-(223–445) (mtNES1 and mtNES2, respectively) (Fig. 5A). mtNES1 significantly reduced the percentage of cells expressing the protein in the cytoplasm alone from 17 to 4% (Fig. 5B, panels a and c). Similarly, mtNES2 significantly reduced the cytoplasmic expression of SIRT1-(223–445) from 14 to 2% (Fig. 5B, panels d and f). These findings suggest that both NES1 and NES2 have nuclear exportation activity.

We next introduced mtNES1 and/or mtNES2 into full-length SIRT1-EGFP (Fig. 6A) and analyzed the nuclear exportation activity using the heterokaryon shuttling assay (Fig. 6B). When L929 cells were fused with COS-7 cells expressing wild-type SIRT1-EGFP, 89% of the heterokaryons expressed the protein in the L929 nuclei (n/H11005 36) (Fig. 6B, panel a, arrows). The mutation of NES1 (mtNES1) slightly reduced the nuclear exportation activity, so 80% of the heterokaryons contained L929 nuclei expressing the mutant protein (n/H11005 41) (Fig. 6B, panel b, arrows). In contrast, only 7% of the heterokaryons had L929 nuclei that were positive for mtNES2 (n = 30) (Fig. 6B, panel c, arrowheads), indicating that NES2 has strong nuclear exportation activity. Combining the mutations in NES1 and NES2 (mtNES) almost abolished the nuclear exportation activity, so only 3% of the
heterokaryons had L929 nuclei that were positive for mtNES (n = 32) (Fig. 6B, panel d, arrowheads).

Functional Significance of the Nucleocytoplasmic Shuttling of SIRT1—To investigate the functional role of the nucleocytoplasmic shuttling of SIRT1, SIRT1-EGFP and mtNLS-EGFP, which resided in the nucleus and cytoplasm, respectively, were expressed in C2C12 cells (see Fig. 3B). To assess the significance of the deacetylase activity of SIRT1, we also used a dominant-negative SIRT1 mutant (H355Y-EGFP). As expected, like SIRT1-EGFP, H355Y-EGFP resided in the nucleus (see Fig. 8A). The deacetylation activities of SIRT1 and its mutants were examined by Western blot analysis using antibodies that recognize total, diacetylated (Lys9 and Lys14), or non-acetylated histone H3. The overexpression of nuclear SIRT1 (SIRT1-EGFP) markedly increased the band of non-acetylated histone H3 compared with the other mutants, and the amount of acetylated and non-acetylated histone H3 decreased reciprocally (Fig. 7). The overexpression of cytoplasmic (mtNLS-EGFP) or dominant-negative (H355Y-EGFP) SIRT1 did not affect the amount of acetylated and non-acetylated histone H3 in the C2C12 cells (Fig. 7). These results indicate that only nuclear SIRT1 can deacetylate histone H3.

SIRT1 represses apoptosis in response to oxidative stress (10). We therefore examined the effect of the subcellular localization and activity of SIRT1 on the cell death induced by oxidative stress in C2C12 cells using antimycin A, which inhibits complex III of the mitochondrial electron transport chain and increases the formation of toxic superoxide anion (O2·-). C2C12 cells were transfected with EGFP, SIRT1-EGFP, mtNLS-EGFP, or H355Y-EGFP and treated with 100 μM antimycin A for 12 h, and the apoptotic cells were then detected by TUNEL staining (Fig. 8A). Of the EGFP-expressing cells, 40.4 ± 5.5% were positive for TUNEL staining. SIRT1-EGFP significantly reduced

FIGURE 5. Identification of NES1 and NES2 in deletion mutants of SIRT1. A, diagrammatic representation of the site-directed mutations. Amino acid sequences of mutant NESs (mtNES1 and mtNES2) are shown. The red boxes represent NES1 and NES2, and the blue boxes represent NLS2. B, schematic diagrams of the EGFP fusion constructs on the left. Red and pink boxes represent wild-type (wt) and mutant NESs, respectively. COS-7 cells were transfected with the corresponding plasmids and incubated in DMEM for 48 h with (-) or without (+) treatment with 10 nM LMB for 3 h before analysis. The percentages of cells expressing SIRT1-EGFP in the nucleus alone (n), in both the nucleus and cytoplasm (n+c), and in the cytoplasm alone (c) are indicated. At least 200 cells were counted for each group. Representative pictures of EGFP and Hoechst 33342 staining and the merged images are shown on the right. C, quantitative data from three separate series of experiments. *, p < 0.05 versus cytoplasmic in wild-type NES1; **, p < 0.05 versus nuclear in wild-type NES1; †, p < 0.05 versus cytoplasmic in wild-type NES2; ‡, p < 0.05 versus nuclear in wild-type NES2.
the number of TUNEL-positive cells, given that only 4.4 ± 0.7% of the SIRT1-EGFP-expressing cells were positive for TUNEL staining ($p < 0.05$) (Fig. 8B). Neither the cytoplasmic (mtNLS-EGFP) nor the dominant-negative (H355Y-EGFP) mutant showed this anti-apoptotic effect (Fig. 8B). The protective effect of nuclear SIRT1 was further examined by measuring the activity of lactate dehydrogenase released into the culture medium. Treatment with antimycin A increased the lactate dehydrogenase activity to 544 ± 54% of that before treatment, and this increase was significantly suppressed by the expression of nuclear SIRT1 (294 ± 26%, $p < 0.05$) (Fig. 8B). Nuclear SIRT1 was apparently less effective in inhibiting lactate dehydrogenase release than in protecting the cells from apoptosis as evaluated by TUNEL staining. However, at least 50% of the lactate dehydrogenase released into the culture medium was probably from untransfected cells because the transfection efficiency was ~50%. As before, neither cytoplasmic nor dominant-negative SIRT1 showed a protective function against lactate dehydrogenase release. Thus, only nuclear SIRT1 exhibited a cell-protective function against oxidative stress in C2C12 cells.

**DISCUSSION**

The subcellular localization of SIRT1 differed from cell to cell in vivo; some cells showed nuclear expression of SIRT1, but others expressed it in the cytoplasm alone or in both the nucleus and cytoplasm. The subcellular localization of SIRT1 was regulated by at least two NLSs and two NESs in its amino acid sequence and was affected by the PI3K signaling cascade. We found that only nuclear SIRT1 was functional in deacetylating histone H3 and suppressing the cell death induced by antimycin A.

*Drosophila* Sir2 has been shown to change its subcellular localization dynamically during development: it is first expressed in both the cytoplasm and nucleus; it then relocates to inhabit the cytoplasm alone; and finally, it is redistributed again to reside in both locations (21). We found that *Drosophila* Sir2 has two potential NLSs in amino acids 197–203 and 791–797 and at least one possible NES sequence in amino acids 420–426. *Caenorhabditis elegans* Sir2.1 also contains a possible NLS and NES at amino acids 436–442 and 320–326, respectively. Because the NES sequences of *Drosophila* Sir2 and
Sir2.1 are mostly identical to that of NES2 in mouse SIRT1, these NESs are probably functional. Interestingly, yeast Sir2 contains not only two possible NLSs, but also NES-like sequences that are very similar to NES1 and NES2 of mouse SIRT1. Thus, the nucleocytoplasmic shuttling of Sir2 proteins may be a mechanism that is conserved from vertebrates to yeast.

In this study, the introduction of either mtNES1 or mtNES2 into deletion mutants decreased the cytoplasmic expression of the protein (Fig. 5), indicating that both NESs are functional. However, the heterokaryon assay showed that only mtNES2 (but not mtNES1) abolished the nuclear export activity (Fig. 6B). These findings suggest that NES1 might be masked in full-length SIRT1, but it might have been unmasked by the deletion of certain amino acids, which may cause a conformational change. Inhibition of PI3K by LY294002 induced cytoplasmic localization of SIRT1 (Fig. 2B, panel a), indicating that the PI3K cascade is active in unstimulated C2C12 cells. In fact, we found that Akt was phosphorylated in control C2C12 cells (Fig. 2B, panel b). Therefore, phosphorylation by some kinase downstream of PI3K may mask NES in full-length SIR1. Alternatively, the affinity of NES1 for CRM1 may be weaker than that of NES2 and its activity insufficient to induce nuclear export in the presence of two functional NLSs.

We demonstrated that nuclear SIRT1 suppressed the cell death induced by oxidative stress in C2C12 myoblast cells in vitro. Therefore, the nuclear transport of SIRT1 may act as an endogenous protective mechanism in some pathological conditions such as heart failure and neurodegenerative disease. Because PI3K is activated in heart failure (30), SIRT1 may translocate into the nucleus and suppress apoptosis in failing hearts. The mechanism by which SIRT1 protects these cells was not explored in this study. However, one possible downstream mediator is the tumor suppressor p53 because SIRT1 is reported to deacetylate and inhibit p53, resulting in the suppression of cell death induced by H$_2$O$_2$, in some cells (10). Manganese superoxide dismutase participates in the detoxification of reactive oxygen species. Because SIRT1 stimulates the induction of manganese superoxide dismutase by FOXO4 (13), the cellular resistance against oxidative stress may also be mediated by the induction of manganese superoxide dismutase. The precise mechanisms of how nuclear SIRT1 protects cells against oxidative stress warrant further investigation.

FOXO1, FOXO3, and FOXO4 are phosphorylated by Akt and exported from the nucleus, resulting in their transcriptional repression (31–33). SIRT1 has been shown to regulate the transcriptional activation of stress-resistance genes by FOXO proteins (13, 14). However, it is unlikely that SIRT1 and FOXO proteins physically interact under an active Akt signal.
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because we found that the transport direction of SIRT1 by Akt was completely opposite that of FOXO proteins. This contradiction might be reconciled by a recent study (34). DAF16, a forhead family transcription factor in C. elegans, translocates into the nucleus upon heat shock stress and interacts with Sir2.1 even when it is already phosphorylated by Akt (34). Interestingly, DAF16 mutated at all known Akt phosphorylation sites is transcriptionally inactive despite its constitutively nuclear localization (35). Thus, both Akt signal and other stress stimuli may be required for SIRT1-FOXO interaction and transactivation.

The significance of the cytoplasmic localization of SIRT1 is unknown. Nuclear exportation of class II histone deacetylases activates transcription factors that are repressed by these histone deacetylases (36). The nuclear exclusion of SIRT1 and its cytoplasmic localization may also act physiologically to modulate the negative regulation of transcription factors by SIRT1. For example, because SIRT1 binds to and inhibits MyoD, a key transcription factor for muscle development (15), the exportation of SIRT1 from the nucleus may be important for the activation of MyoD in muscle differentiation. Our finding that SIRT1 was excluded from the nucleus upon the differentiation of C2C12 cells (Fig. 1C) is in agreement with this idea.

In conclusion, the nucleocytoplasmic shuttling of SIRT1 represents a novel mechanism for its functional regulation. As the nuclear translocation of SIRT1 increases the cellular resistance to apoptosis, modification of the nucleocytoplasmic shuttling of SIRT1 to favor its nuclear translocation might provide a new therapeutic approach for oxidative stress-related diseases.

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