Mitotic Regulation of SIRT2 by Cyclin-dependent Kinase 1-dependent Phosphorylation *

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Sirtuins are evolutionarily conserved NAD+-dependent deacetylases and ADP-ribosyltransferases involved in the regulation of cell division, apoptosis, DNA repair, genomic silencing, and longevity. Recent studies have focused on identifying target substrates for human sirtuin enzymatic activity, but little is known about processes that directly regulate their function. Here, we demonstrate that SIRT2 is phosphorylated both in vitro and in vivo on serine 368 by the cell-cycle regulator, cyclin-dependent kinase 1, and dephosphorylated by the phosphatases CDC14A and CDC14B. Overexpression of SIRT2 mediates a delay in cellular proliferation that is dependent on serine 368 phosphorylation. Furthermore, mutation of serine 368 reduces hyperploidy in cells under mitotic stress due to microtubule poisons.

The human silent information regulator 2 (Sir2)2 proteins are evolutionarily conserved from bacteria to mammals. Sir2 proteins, or sirtuins, function as protein deacetylases as well as ADP-ribosyltransferases (1–3). Mammals have seven sirtuins, which are localized to various subcellular compartments (reviewed in Ref. 4). SIRT2, which is more closely related to the yeast sirtuin Hst2p, is a cytoplasmic deacetylase that is associated with the microtubule network and targets lysine 40 of α-tubulin for deacetylation (4–6). However, the functional significance of lysine 40 acetylation and its regulation by SIRT2 have remained elusive.

Recent studies have implicated SIRT2 in the control of cellular proliferation (7, 8). In Soas2 osteosarcoma cells, SIRT2 induces a delay in mitotic exit that is dependent on its enzymatic activity (8). SIRT2 expression is reduced in a high proportion of glioma cell lines. Furthermore, replacement of SIRT2 expression in these cell lines reduced cellular proliferation as measured by colony formation assay (7). These findings suggest that SIRT2 potentially functions as a tumor suppressor gene, because it negatively regulates cellular proliferation. A role for SIRT2 during a novel mitotic stress checkpoint was identified that prevents cells from progressing into mitosis in the presence of microtubule poisons, potentially through regulation of chromatin condensation (9, 10). Consistent with these observations, SIRT2 may directly influence chromatin condensation during the G2 to M transition by regulation of Histone H4 K16 deacetylation (11).

Yeast Sir2p represses transcription at three distinct loci, including the rDNA array, where it associates with Cdc14p and Net1p in the RENT complex (12, 13). During the anaphase of mitosis, the RENT complex is released from the nucleoli and functions in the regulation of mitotic exit (12). Humans have two homologues of Cdc14p, CDC14A and CDC14B (14). CDC14B, like its yeast counterpart, is localized to the nucleoli, whereas CDC14A is targeted to the cytoplasm (15, 16). SIRT2 is phosphorylated in a cell cycle-dependent manner, and its stability is regulated by the phosphatase CDC14B (8). However, the kinase involved in SIRT2 phosphorylation, and its possible role in regulation of SIRT2 stability are unknown. In yeast, Cdc14p antagonizes Cdk1 activity by dephosphorylating several Cdk1 targets during the transition to mitotic exit (17). Here, we demonstrate that Cdk1 phosphorylates SIRT2 at serine 368, and furthermore, phosphorylation at this site is required for SIRT2-mediated delay in cell cycle progression.

EXPERIMENTAL PROCEDURES

Tissue Culture—HEK 293T, U-87 MG (HTB-14), U2OS, and HeLa cells were obtained from American Type Culture Collection, grown in Dulbecco’s modified Eagle’s medium (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (Gemini Bio-products, Woodland, CA) in the presence of penicillin, streptomycin, and 2 mM-glutamine (Invitrogen).

Plasmids and Mutagenesis—Human SIRT2 full-length and variant 2, Cdk1 and cyclin B1 cDNAs were subcloned to generate C-terminal FLAG- or hemagglutinin (HA)-tagged fusions in a derivative of the pcDNA3.1(+) backbone. Wild-type and fragments of human SIRT2 were cloned into pEGFP-C1 vector (Clontech, Mountain View, CA) by standard PCR-based strategies to generate GFP-SIRT2 full-length and deletion mutants. Site-directed mutagenesis for SIRT2 constructs was performed with QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as recommended by the manufacturer. GFP-CDC14A and CDC14B-GFP were kind gifts from Peter K. Jackson. Cyclin B1 cDNA was cloned into DsRed-C1 (Clontech) to generate RFP-cyclin B1. Full-length human SIRT2 cDNAs were...
cloned into pTrcHis vector (Invitrogen) for production of recombinant His<sub>10</sub>-SIRT2.

**Purification of Recombinant SIRT2**—DH5αF’IQ bacteria (Invitrogen) were transformed with pTrcHis-SIRT2 and induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside at 37 °C for 2 h. The resulting His<sub>10</sub>-tagged protein was purified as described previously (18). Recombinant protein was aliquoted and stored at −20 °C.

**Transient Transfections and Immunoprecipitations**—HEK 293T cells were transfected either by the calcium phosphate DNA precipitation method or with FuGENE 6 transfection reagent (Roche Applied Science) and lysed 48 h after transfection in low stringency lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5% Nonidet P-40, 150 mM NaCl) in the presence of protease inhibitor mixture (Complete, Roche Molecular Biochemicals, Indianapolis, IN) and phosphatase inhibitor mixtures 1 and 2 (Sigma). FLAG-tagged proteins were immunoprecipitated with anti-FLAG M2-agarose affinity gel (Sigma), for 2 h at 4 °C from 1 mg of total cell lysate measured by the Dc (dithiothreitol). Both immunoprecipitated material and recombinant SIRT2 were resuspended in 1× Laemmli buffer.

**Histone Deacetylase Assays**—Immunoprecipitated material was washed for an additional 15 min in SIRT2 deacetylase buffer (50 mM Tris-HCl, pH 9.0, 4 mM MgCl<sub>2</sub>, and 0.2 mM dithiothreitol). Both immunoprecipitated material and recombinant SIRT2 were resuspended in 100 μl of SIRT2 deacetylase buffer containing NAD<sup>+</sup> (Sigma) and <sup>3</sup>H-acetylated histone H4 peptide (amino acids 1–23) (19). The enzymatic reactions were started by addition of NAD<sup>+</sup>. Reactions were incubated for 2 h at room temperature and stopped by adding 25 μl stop solution (0.1 M HCl, 0.16 M acetic acid). Released acetate was extracted in 500 μl of ethyl acetate and vortexed for 15 min. After centrifugation at 14,000 rpm for 5 min, 400 μl of the ethyl acetate fraction was mixed with 5 ml of scintillation fluid and counted.

**Western Blotting**—Samples were separated on 10% SDS-PAGE gels and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences). Membranes were blocked with 5% blocking reagent (Bio-Rad) in Tris-buffered saline-Tween and indicated quantity of calf intestinal phosphates and incubated at 37 °C for 10 min. Membranes were washed three times 10 min in PBS plus 0.1% Tween 20. Membranes were blocked with 5% blocking reagent (Bio-Rad) and phosphatase inhibitor mixture (Complete, Roche Molecular Biochemicals, Indianapolis, IN) and phosphatase inhibitor mixtures 1 and 2 (Sigma). FLAG-tagged proteins were immunoprecipitated with anti-FLAG M2-agarose affinity gel (Sigma), for 2 h at 4 °C from 1 mg of total cell lysate measured by the Dc Protein Assay Kit (Bio-Rad). Immunoprecipitated material was washed three times for 15 min each in low stringency lysis buffer, and agarose-immune complexes were resuspended in 1× Laemmli buffer.

**In Vitro Phosphatase Assay**—Reactions were prepared in kinase assay buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 30 μM ATP in the presence or absence of 10 μCi of [γ-<sup>32</sup>P]ATP containing 3 μg of rSIRT2 and indicated quantity of Cdk1-cyclin B1 (New England Biolabs). Reactions were carried out at 30 °C for 30 min and stopped by addition of 6× Laemmli buffer to a final concentration of 1×.

**In Vitro Kinase Assay**—Reactions were prepared in kinase assay buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 30 μM ATP) in the presence or absence of 10 μCi of [γ-<sup>32</sup>P]ATP containing 3 μg of rSIRT2 and indicated quantity of Cdk1-cyclin B1 (New England Biolabs). Reactions were carried out at 30 °C for 30 min and stopped by addition of 6× Laemmli buffer to a final concentration of 1×.

**In Vitro Phosphatase Assay**—SIRT2-FLAG was immunoprecipitated from transfected HEK 293T as described above. Immunoprecipitated material was washed three times for 15 min with lysis buffer, followed by two times for 15 min with 1× NEB buffer 3 (10 mM Tris-HCl, pH 7.9, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 1 mM dithiothreitol). Immunoprecipitated material was resuspended in NEB buffer 3 with or without 10 μM sodium pyrophosphate and incubated at 37 °C for 10 min, followed by the addition of indicated units of cAMP-dependent protein phosphatase (CIP, New England Biolabs) and incubated at 37 °C for an additional 30 min. Reactions were stopped by addition of 6× Laemmli buffer to a final concentration of 1× and subjected to SDS-PAGE and Western blotting as described above. For λ-phosphatase treatments, HEK 293T cells were lysed in 50 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl in the

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presence of protease inhibitor mixture (Complete, Roche Applied Science). Lysate was treated with or without 200 units of A-phosphatase (New England Biolabs) at 30 °C for 30 min and stopped by addition of 6× Laemmli buffer to a final concentration of 1× and subjected to SDS-PAGE and Western blotting as described above.

Pharmaco logical Treatments for Cell-cycle Arrest and Cdk1 Inhibition—For S and M phase cell-cycle arrest experiments, HEK 293T cells were treated for 15 h with 2 mM thymidine. Following treatment, cells were washed twice in PBS and replaced in fresh growth medium. Cells were then transfected with SIRT2-FLAG or the empty FLAG vector using FuGENE 6 and incubated for 8 h under standard cell culture conditions. Cells were then retreated in either 2 mM thymidine (for S phase block) or 10 μg/ml Colchicine (Sigma, M phase block) and incubated for 17 h until cells were harvested and subjected to SDS-PAGE and Western blotting as described above, or for flow cytometric analysis. For treatment of cells with Cdk1 inhibitors, HEK 293T cells were transfected with SIRT2-FLAG as described above. 24 h post transfection, cells were treated with 10 μg/ml purvalanol A (Calbiochem), 10 μM alsterpaullone (Calbiochem), or 10 μM U0126 (Cell Signaling Technologies) for 16 h until cells were harvested and subjected to SDS-PAGE and Western blotting as described above.

Time-lapse Microscopy—HeLa Cells were plated on 40-mm coverslips (Biotech) and treated for 15 h with 2 mM thymidine. Following thymidine treatment, cells were washed in PBS and grown in fresh media. Following replacement of fresh media, cells were transfected with vectors for GFP-SIRT2 and RFP-cyclin B1 and grown for in standard culture conditions for 8 h, at which time cells were retreated for 17 h in 2 mM thymidine. Cells were washed twice in PBS and grown for 6 h in fresh media, at which time cells were placed in a closed culture chamber system (Biotech) with CO2-independent media and subjected to time-lapse microscopy with images acquired every 60 s on a Nikon eclipse TE300 microscope controlled by MetaMorph software.

Hyperploidy Measurements—Polyclonal U-87 MG cells stably expressing SIRT2-FLAG proteins were subjected to 200 nM nocodazole treatment for the indicated times. At each time point, cells were harvested and fixed in ice-cold 70% ethanol and stored at −20 °C until flow cytometric analysis was performed as described previously (21).

Tandem Mass Spectrometry—Nanocapillary high-performance liquid chromatography electrospray ionization-MS and MS/MS analysis were performed utilizing an Ultimate 2000 high-performance liquid chromatography system equipped with Switchos™ and Famos™ autosampler (Dionex/LC Packings) on-line with a QSTAR XL, QqTOF hybrid mass spectrometer (Applied Biosystems/MD Sciex). The QSTAR was operated with a nanoelectrospray ion source (Protana) and PicoTip™ emitters (New Objective, Inc.). Protein digest volumes of 2–3 μl were desalted online using a Nano-Precolumn™ (Dionex/LC Packings, C18, 300-Å pore size, 5-μm particle size, 5-mm length, and 300-μm inner diameter), and peptides were separated on a self-packed column (packing material: Phenomenex Jupiter Proteo™; C12 end-capped, 4-μm particle size, 90-Å pore size, 15-cm length, and 75-μm inner diameter). The mobile phase flow rate was 250 nl/min. The column was equilibrated at 2% solvent B for 20 min before sample injection (solvent B: 80% acetonitrile/0.08% formic acid; solvent A: 2% acetonitrile/0.1% formic acid). Peptides were separated using a linear gradient of 2–50% solvent B over 20 min, followed by a column cleanup step at 95% solvent B for 5 min. Data were acquired automatically by operating the mass spectrometer in information-dependent acquisition mode, which allowed for a 1-s MS survey scan followed by two 2-s MS/MS scans. Peptides were fragmented by low energy collision-induced dissociation. Nitrogen was used as the collision gas, and the collision energy was automatically adjusted depending on the precursor ion charge state. The mass spectrometer was externally calibrated using product ions of Glu1-Fibrinopeptide B (Sigma). Data analysis was facilitated by employing BioAnalyst software (Applied Biosystems/MD Sciex) and Mascot (Matrix Science). Mass accuracy tolerance windows for Mascot searches were set at ±100 ppm and ±0.1 Da for precursor and product ions masses, respectively. The MS/MS spectra of phosphorylated peptides and their non-phosphorylated counterparts were manually verified. Selected ion chromatograms of phosphorylated and non-phosphorylated forms of peptides spanning amino acid regions 348–380 and 347–380 of SIRT2 were generated to interrogate the high performance liquid chromatography MS/MS data for the presence of precursor ions that were not automatically selected for collision-induced dissociation.

RESULTS

Generation of Multiple Isoforms of SIRT2 by Alternative Translational Start Sites—After transfection of HEK 293T cells with a SIRT2 construct, the SIRT2 protein migrated as at least four different species by SDS-PAGE (Fig. 1A). Lysates of mock-transfected cells were analyzed alongside lysates from SIRT2-FLAG to confirm that the proteins recognized by the FLAG antiserum resulted exclusively from the expression of the SIRT2-FLAG construct. Based on expressed sequence tag sequences obtained from human genome databases, SIRT2 is expressed as two isoforms resulting from alternative splicing. In variant 1, all exons are spliced contributing to a translated protein of 389 amino acids. Variant 2 arises when the second exon is spliced out, placing a stop codon within exon 3 in-frame with the first ATG. Translation is then initiated at the second ATG (amino acid 38 of the full-length SIRT2). The SIRT2 used in our experiments corresponds to variant 1, and we suspected that both ATGs were being utilized for translation upon expression of this cDNA in HEK 293T cells (Fig. 1B). To test this possibility, we mutated the second ATG into alanine. The two lower molecular weight forms of SIRT2 were no longer present, indicating that both the first and second ATGs are utilized for translation from the exogenously expressed mRNA (Fig. 1C). These results indicate that exogenously expressed SIRT2 can be represented in vivo as two isoforms. Bands of the same size are observed for endogenous SIRT2 (Fig. 1E) suggesting that both ATGs (at position 1 and 38) are utilized for translation of the endogenous protein as well.
SIRT2 Is a Phosphoprotein—Each of the two major isoforms expressed from the exogenous SIRT2-FLAG plasmid migrated as two species. To determine if phosphorylation was responsible for the observed multiple migratory species, immunoprecipitated SIRT2-FLAG was treated in vitro with CIP. Incubation of SIRT2-FLAG with increasing concentrations of CIP resulted in a faster migratory species, indicating that phosphorylation is responsible for the various species observed by SDS-PAGE (Fig. 1D). As a control, we performed the in vitro phosphatase assay with the phosphatase inhibitor sodium pyrophosphate (Na₄P₂O₇) to confirm that the loss of the slower migratory species was due to the activity of CIP (Fig. 1D). To determine if endogenous SIRT2 is phosphorylated, we treated cell lysates with H9261-phosphatase. Following phosphatase treatment, we observed a slower migrating form of SIRT2 consistent with SIRT2 being phosphorylated in vivo (Fig. 1E).

To determine which amino acids are phosphorylated in SIRT2, we probed immunoprecipitated SIRT2-FLAG with antibodies specific for phosphorylated serine, threonine, and tyrosine. SIRT2 reacted strongly with the anti-phosphoserine, to a lesser degree with the anti-phosphothreonine, and did not react with the anti-phosphotyrosine antiserum (data not shown). To confirm that the anti-phosphoserine antibody was recognizing specifically a phosphorylated protein, we treated immunoprecipitated SIRT2-FLAG with or without CIP followed by Western blotting with anti-phosphoserine antisera. The anti-phosphoserine signal observed on immunoprecipitated SIRT2-FLAG was absent following CIP treatment (Fig. 1G). These results confirm phosphorylation of SIRT2 and suggest that SIRT2 is phosphorylated on serine residue(s).

SIRT2 Is Phosphorylated on Serine 368—To determine the site of SIRT2 phosphorylation, we generated deletion mutants of SIRT2 fused to the C terminus of GFP. GFP-SIRT2 deletion mutants were transfected with GFP-SIRT2 deletion mutants. Cell lysates were separated by SDS-PAGE and probed by Western blotting with an antiserum specific for GFP. A, schematic diagram of SIRT2 and amino acids 360–389, indicating potential sites of phosphorylation, and comparing the region surrounding serine 368 with Cdk1 consensus sequence. Cdk1 360–389 cells were mock transfected or transfected with SIRT2-FLAG wild-type or potential phosphorylation site mutants. Cell lysates were separated by SDS-PAGE and probed by Western blotting with antisera specific for anti-FLAG and anti-phosphoserine.
nine phosphorylation sites (Fig. 2B). Mutation of each serine and threonine residue in this segment showed that serine 368 modification caused a change in mobility of SIRT2, consistent with phosphorylation of this site (Fig. 2C). However, because a low intensity slowly migrating band remained for the mutant S368A, we suspect that serine 368 is not the only site leading to migratory differences in SIRT2 (Fig. 2C).

Analysis of serine 368 and surrounding residues for potential kinase consensus sequences indicates that serine 368 could represent a potential phosphorylation site for CKI, GSK3, PKC, and Cdk1 (Fig. 2B). To determine the phosphorylation status of exogenous SIRT2 during the cell cycle, we arrested cells in S phase or M phase. In agreement with a possible role for Cdk1 in SIRT2 phosphorylation, the phosphorylation of exogenous SIRT2 was cell-cycle-dependent, because SIRT2 phosphorylation increased during mitotic arrest (Fig. 3A). Endogenous SIRT2 was also hyperphosphorylated during mitotic arrest (Fig. 3B). These results indicate that SIRT2 phosphorylation is, in part, dependent on the stage of the cell cycle.

To determine if SIRT2 is phosphorylated by Cdk1 in vivo, we transfected cells with vectors encoding SIRT2-FLAG wild-type, S368A mutant, or the empty vector (FLAG) and tested the effect of purvalanol A, an inhibitor of Cdk1 activity. Phosphorylation of SIRT2 was reduced by purvalanol A treatment as demonstrated by an increase in the amount of faster migrating SIRT2 (Fig. 3C). As expected, no change was observed for the SIRT2 S368A mutant (Fig. 3C). Purvalanol A was recently demonstrated to target the kinase MEK1 in addition to Cdk1. To rule out the possibility that MEK1 was targeting SIRT2 at serine 368, we tested the effect of purvalanol A, alsterpaullone (a second unrelated Cdk1 inhibitor), and U0126 (an MEK1 inhibitor) on the phosphorylation status of endogenous SIRT2. Consistent with our previous results, we found that both Cdk1 inhibitors caused a decrease in SIRT2 phosphorylation, but the MEK1 inhibitor (U0126) did not have any effect on SIRT2 migration (Fig. 3D). These results suggest that SIRT2 is a target for phosphorylation in vivo by Cdk1.

Cdk1 Regulates Phosphorylation of SIRT2 on Serine 368 in Vitro—To determine if Cdk1 can phosphorylate SIRT2, we expressed His6-tagged SIRT2 wild-type and S368A in Escherichia coli, purified the proteins and incubated them with increasing concentrations of Cdk1-cyclin B1 complex in the presence of [γ-32P]ATP in vitro (Fig. 4A). Cdk1-cyclin B1 efficiently phosphorylated wild-type recombinant SIRT2 in a dose-dependent manner. The S368A mutant SIRT2 protein was not phosphorylated.

To confirm that serine 368 was the target for phosphorylation by Cdk1, we incubated recombinant SIRT2 in the presence or absence of Cdk1-cyclin B1. Incubation with Cdk1 resulted in a slower mobility of SIRT2 when analyzed by SDS-PAGE (Fig. 4B). Both SIRT2 bands, phosphorylated and unphosphorylated, were excised and subjected to in-gel digestion with trypsin. The resulting protein fragments were analyzed by tandem mass spectrometry (liquid chromatography-MS/MS). The electrospray MS/MS spectrum of the triply charged molecular ion 763.4 derived from the phosphorylated peptide amino acids 348–370 was generated. The sequence of the peptide was fully confirmed by overlapping ladders of the b and y ion series. The assignment of phosphorylation to serine 368 is based upon detection of ion y3 carrying a phosphate group (Fig. 4C). The majority of the fragment ions could be matched to the predicted product ions; many internal product ions were also observed. The completeness of the liquid chromatography-MS/MS spectrum gives us confidence that SIRT2 phosphorylation by Cdk1 in vitro occurs at serine 368 and that SIRT2 is a substrate for Cdk1 in vitro. Similar results were obtained with SIRT2-FLAG expressed in HEK 293T cells (data not shown).
Co-localization of SIRT2 and Cdk1-Cyclin B1—To determine whether SIRT2 and Cdk1-cyclin B1 co-localize in vivo, we examine the subcellular localization of both proteins using indirect immunofluorescence and antisera against endogenous SIRT2 and cyclin B1. We observed that both proteins were enriched on the centrosome during prophase (indicated by P on Fig. 5A) and on spindle fibers during metaphase (indicated by “M” on Fig. 5A). The merged images indicate that both endogenous SIRT2 and cyclin B1 are co-localized on these structures (Fig. 5A).

To further define the localization of SIRT2 and cyclin B1 during mitosis, we co-transfected GFP-tagged SIRT2 (GFP-SIRT2) and DsRed-tagged cyclin B1 (RFP-cyclin B1), and followed these cells with time-lapse microscopy (Fig. 5B). SIRT2 was diffusely localized throughout the cytoplasm, whereas cyclin B1 was localized predominantly on the centrosome as cells progressed in G2. Interestingly, just before and during centrosome separation, both SIRT2 and cyclin B1 were co-localized on the centrosome (Fig. 5B). These results indicate that a fraction of cellular SIRT2 co-localizes with cyclin B1 on the centrosome early into mitotic entry and that both proteins are concentrated on the spindle fibers during metaphase.

Dephosphorylation of SIRT2 by Human CDC14 Proteins—To investigate potential roles of CDC14A and CDC14B in the dephosphorylation of SIRT2, we co-transfected cells with expression vectors for SIRT2-FLAG and either GFP, GFP-CDC14A, or CDC14B-GFP. Extracts were probed with an antisera specific for GFP to assess expression levels of CDC14 and with anti-FLAG antiserum to detect SIRT2. Both CDC14A and CDC14B dephosphorylated SIRT2 in vivo, but the dephosphorylation by CDC14A was more complete (Fig. 6). In contrast to published observations (8), we did not observe a degradation of SIRT2 in response to co-expression of CDC14A or CDC14B.

SIRT2-mediated Reduction in Cellular Proliferation Is Dependent on Serine 368—To explore the role of phosphorylation of SIRT2 by CDK1, we first tested the possibility that it might change the protein deacetylase activity of SIRT2. We transfected HEK 293T cells with expression vectors for SIRT2-FLAG wild-type, S368A, or the catalytically inactive H187Y mutant (Fig. 7A). The immunoprecipitated SIRT2-FLAG proteins were tested for deacetylase activity in vitro using acetylated histone H4 peptide as a substrate. We found that both wild-type SIRT2 and the S368A mutant had comparable histone deacetylation activity (Fig. 7B). As predicted, SIRT2

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**FIGURE 4.** In vitro phosphorylation of SIRT2 by Cdk1 occurs on serine 368. A, increasing amounts of recombinant Cdk1-cyclin B1 (0–20 μg) were incubated in the presence or absence of recombinant wild-type or S368A SIRT2 and [γ-32P]ATP. Proteins were separated by SDS-PAGE stained with Coomassie Blue and exposed to film. B, wild-type SIRT2 was incubated in the presence or absence of Cdk1-cyclin B1 (20 μg) to phosphorylate SIRT2. Proteins were separated by SDS-PAGE and stained with Coomassie Blue. C, recombinant SIRT2 incubated with Cdk1-cyclin B1 in 8 μl was excised from the gel and subjected to in-gel digestion with trypsin, followed by analysis with tandem mass spectrometry.

**FIGURE 5.** Interaction and co-localization of SIRT2 with the Cdk1-cyclin B1 complex. A, confocal microscopy analysis of SIRT2 localization with cyclin B1 in human fibroblasts. U20S Cells were stained for SIRT2 (green) and cyclin B1 (red). Field shows a prophase cell (P) and cell in metaphase (M). B, HeLa cells grown on a coverslip were transfected with GFP-SIRT2 and RFP-cyclin B1 and synchronized by double thymidine block. Images of both GFP and RFP were acquired each minute of a cell at the G2/M transition. Four time points at 3-min intervals are shown. Images in A and B are representative staining of all cells observed undergoing mitosis.
H187A was enzymatically inactive. An additional mutant of SIRT2, S368E, was also tested for enzymatic activity using the same assay and shown to have the same enzymatic activity as wt SIRT2 (data not shown).

Next, we tested whether serine 368 plays a role in the SIRT2-mediated delay in cellular proliferation. To follow cell division we used CFSE, a cell-permeable dye that nonspecifically binds to cellular proteins but has little or no effect on cell proliferation or viability. As cells proliferate, the dye is divided evenly between daughter cells. Glioma cells (U-87 MG) were transfected with either the empty FLAG vector, SIRT2-FLAG wild type, S368A, and S368E. All cell lines where confirmed to express SIRT2-FLAG proteins (Fig. 7D). Interestingly, under these experimental conditions, expression of SIRT2-FLAG wt had little effect on proliferation, whereas the phosphomimetic SIRT2 mutant S368E induced a significant delay in cell growth (Fig. 7E). This result suggests that the S368E mutant functions in a dominant in comparison to wt SIRT2.

**Mutation of SIRT2 Serine 368 Affects Glioma Sensitivity to Mitotic Poisons**—Recently, SIRT2 was shown to function in preventing polyploidy due to bypassing a mitotic stress checkpoint in cells treated with the mitotic poison nocodazole (10). To explore a possible role of SIRT2 phosphorylation in this process, we treated U-87 MG cells overexpressing SIRT2 wt and the S368E mutant with nocodazole for 0, 24, 48, 72, and 96 h. Vector control and SIRT2-FLAG wild-type both had comparable levels of hyperploidy with prolonged nocodazole treatments. However, both the cell line expressing the SIRT2 mutant S368E showed a reduction in hyperploid cells after prolonged treatment with nocodazole (Fig. 8). These results suggest that modification of SIRT2 phosphorylation status is involved in the regulation of this mitotic checkpoint.

**DISCUSSION**

Sirtuin proteins have attracted considerable interest in recent years as regulators of critical cellular processes, including cell growth, apoptosis, and metabolism (20, 22). Here, we demonstrate a role for a post-translational modification in regulating the biological function of a human sirtuin.

We found that exogenously expressed SIRT2 can be translated from two different ATG codons, one at the initial translational start, and the second at the ATG codon at amino acid 38. Cloning of the mouse gene suggested that translation could occur from the second ATG, because it is preceded by a perfect Kozak consensus sequence (23). Our data suggest that the differential translation of exogenously expressed SIRT2 cDNA, in addition to the observed multiple variants that exist for the endogenous SIRT2, may be the result of either alternative splicing or alternative translational start sites. In future experiments, it will be of interest to assess the role of the 5′- or 3′-untranslated regions, which are absent in our transient expression vectors, in determining which ATG site is utilized. None of our current assays for SIRT2 showed any differences between the two variants.

We also demonstrated that each isoform of SIRT2 migrated as two different species on SDS-PAGE. When larger quantities of protein were analyzed by Western blotting, we observed a
Regulation of cell-cycle progression by SIRT2 is dependent on serine 368 phosphorylation. A, HEK 293T cells were mock transfected or transfected with wild-type, H187Y, or S368A SIRT2-FLAG. Cellular lysates were separated by SDS-PAGE and probed by Western blotting with antisera specific for FLAG. B, SIRT2-FLAG proteins from lysates in A were immunoprecipitated with anti-FLAG antibody and utilized in an in vitro deacetylation assay using an acetylated histone peptide substrate. Data are representative of three independent experiments. C, glioma cells were mock transfected or transfected with a control vector (FLAG), SIRT2 wild-type, SIRT2 S368A, or SIRT2 S368E (all FLAG-tagged), with DsRed as a marker for transfected cells. Transfected cells were stained with CFSE and replated. Cells were harvested at 48 h after staining and analyzed by flow cytometry. D, U-87 MG cell lines were transfected and selected to generate polyclonal cell lines expressing vector control (FLAG), SIRT2-FLAG wild type, S368A, and S368E. Cell lysates were separated by SDS-PAGE and probed by Western blotting with antisera specific for FLAG. E, polyclonal U-87 MG cells from D were plated in 12-well plates at 5000 cells per well. Wells were harvested at 48-h intervals, and the total cells per well were counted. Error bars represent ± S.D. of cell counts from three wells, and data are representative of three independent experiments. *p = 0.01.

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third, slower migratory species (data not shown). Treatment of SIRT2 in vitro with CIP showed that SIRT2 is targeted for phosphorylation. We mapped the primary phosphorylation site of SIRT2 resulting in the observed migratory shift to serine 368. We believe that phosphorylation of serine 368 is not the only post-translational modification of SIRT2. Indeed, the S368A mutant did not migrate as a single band by SDS-PAGE, indicating that a second site of modification, possibly another phosphorylation site, exists. Furthermore, this additional phosphorylation might also be induced during mitosis, because an even slower migratory species is observed in cells arrested in M phase of the cell cycle.

Bioinformatic analysis suggested that serine 368 could serve as a phosphorylation site for a number of kinases. We found that serine 368 represented a consensus phosphorylation site for the mitotic kinase Cdk1. SIRT2 phosphorylation in a mitosis-dependent manner was previously reported by Dryden and colleagues (8), but the phosphorylation site or potential kinases were not identified. We found that Cdk1 can regulate the phosphorylation of serine 368 both in vitro and in vivo, indicating that the mitotic phosphorylation of SIRT2 is caused, at least in part, by Cdk1 activity. Furthermore, we observed that SIRT2 and Cdk1-cyclin B1 are found on similar mitotic structures, including the centrosome and the mitotic spindle during the metaphase.

Additionally, we found that SIRT2, like other Cdk1 targets, can be dephosphorylated by the phosphatases CDC14A and CDC14B. In contrast to a published report (8), we did not observe any degradation of SIRT2 by the 26 S proteasome in response to CDC14B overexpression. Due to the complete loss of protein in their experiments, Dryden et al. could not determine if SIRT2 is in fact dephosphorylated by CDC14B. However, different cell lines were examined in the two studies: an osteosarcoma cell line Soas2 was used in the Dryden study (8), whereas we used HEK 293T cells (this study). These differences may reflect cell-type-specific effects of CDC14A and CDC14B on SIRT2 stability.

Our experiments did not reveal a role for phosphorylation in regulating SIRT2 deacetylase activity. The mutation of serine 368 to alanine, which cannot be phosphorylated, or to a glutamic acid (S368E), which potentially could mimic phospho-
leads to slower progression of transfected cells through mitotic exit. This function of SIRT2 is dependent on its enzymatic activity. Furthermore, overexpression of SIRT2 in glioma cells reduces colony formation when cells are grown in selection medium (7, 8). We found that overexpression of SIRT2 reduces cell proliferation, which was not observed with expression of the S368A mutant. Our results confirm that SIRT2 expression is anti-proliferative, and furthermore, we find this function of SIRT2 is dependent on phosphorylation at serine 368. Interestingly, both SIRT2 wild-type and the SIRT2 mutant S368A exhibited similar deacetylase activity when assayed on a histone peptide substrate in vitro, as well as α-tubulin in vivo (data not shown). Therefore, the loss of SIRT2-dependent regulation of cell proliferation observed with the S368A mutant is not likely to occur as a result of a change of SIRT2 enzymatic activity. However, we cannot exclude the possibility that phosphorylation of serine 368 might affect the activity of SIRT2 on other unidentified acetylated substrates.

Consistent with phosphorylation of serine 368 being necessary for a delay in cellular proliferation in glioma cells, we found that expression of a phosphomimetic at serine 368 negatively regulates cellular proliferation of the glioma cell line U-87 MG. We did not observe a delay in proliferation of U-87 MG cells stably expressing wild-type SIRT2-FLAG in our extended time course as we had observed in the CFSE proliferation assay. If phosphorylation of SIRT2 at serine 368 plays a dominant role in slowing proliferation, as suggested by the phenotype of the SIRT2 S368E mutant, it is possible that clones containing a low level of SIRT2 phosphorylation were favored during the selection of polyclonal stable cells expressing wild type SIRT2.

Finally, we found that cells overexpressing a phosphomimetic mutant of SIRT2-FLAG (S368E) showed a decrease in hyperploidy in response to prolonged exposure to mitotic poisons. We did not observe a reduction in hyperploid cells in response to overexpression of wild-type SIRT2 as was previously described (10), an observation that may be due to the use of a different glioma cell line. The U251 MG cells used by Inoue et al. (10) contain a mutated p53, resulting in a majority of the cells bypassing the mitotic stress checkpoint, whereas U-87 cells contain a normal p53 protein. In our assay the phosphomimetic SIRT2 mutant S368E caused a reduction in hyperploidy when cells are exposed to nocodazole. Understanding how SIRT2 and its phosphorylation by Cdk proteins influences specific aspects of the cell-cycle and mitotic checkpoints will be of interest to pursue in the future considering a potential role for SIRT2 in glioblastoma formation.

A master regulator switch surrounding the kinase Cdk1 controls cell cycle progression at the G2 to M transition. Cdk1 activity is involved in the regulation of cellular events associated with entrance into mitosis, including centrosome separation, Golgi fragmentation, chromosome condensation, nuclear envelope breakdown, and spindle assembly (25). Our results suggest that regulation of SIRT2 by Cdk1-mediated phosphorylation may function at several distinct steps during mitosis. First, Cdk1 is active on the centrosome, where SIRT2 is localized during centrosome separation. Second, both proteins are found on the mitotic spindle, and Cdk1 may regulate SIRT2 function on the spindle fiber and microtubule acetylation on that structure. Interestingly, centrosome separation and spindle fiber assembly/function were not significantly altered, perhaps because of redundancy with HDAC6, or lack of checkpoint control in tumor cells.

Recent data suggest that CDC14B functions in the regulation of microtubule dynamics. Expression of a mutated CDC14B with cytoplasmic localization drives the formation of bundled microtubules (26). This may occur during mitosis when CDC14B is released from the nucleoli. During telophase and cytokinesis, microtubules form into a highly stable bundled structure known as the mid-body. By regulating SIRT2, CDC14B may influence the formation of these bundles. However, cells lacking SIRT2 or expressing SIRT2 mutants have not shown changes in microtubule structure during mitosis. Thus, SIRT2 may be dispensable for this function, which might be fulfilled by HDAC6 in the absence of functional SIRT2. In addition, tubulin may not be the primary target for SIRT2 during mitosis, and SIRT2 may regulate the acetylation status, and
thus the function of other proteins during mitosis. Because SIRT2 enzymatic activity is required for a delay in cell proliferation, SIRT2 may have targets that are not necessary for mitotic progression but are sufficient to regulate the rate of mitosis. The recent demonstration that SIRT2 shows preference for histone H4-acetylated residue lysine 16 and the observation that this lysine residue is deacetylated during mitosis (G2/M transition) could account for our observations and will be tested in future experiments (11).

We find that SIRT2 is a target for the mitotic kinase Cdk1, and its phosphorylation by Cdk1 is required for SIRT2 to mediate a delay in cell-cycle progression. Along with previous studies (8), our findings illustrate a new mechanism for regulating mammalian sirtuins in the control of cell cycle progression. Furthermore, with the recent interest in identification of sirtuin activators, these results suggest that utilization of sirtuin activators that specifically target SIRT2 could serve as potential anticancer therapy.

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