Conserved Enzymatic Production and Biological Effect of O-Acetyl-ADP-ribose by Silent Information Regulator 2-like NAD⁺-dependent Deacetylases*

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Silent information regulator 2 (Sir2) family of enzymes has been implicated in many cellular processes that include histone deacetylation, gene silencing, chromosomal stability, and aging. Yeast Sir2 and several homologues have been shown to be NAD⁺-dependent histone/protein deacetylases. Previously, it was demonstrated that the yeast enzymes catalyze a unique reaction mechanism in which the cleavage of NAD⁺ and the deacetylation of substrate are coupled with the formation of O-acetyl-ADP-ribose, a novel metabolite. We demonstrate that the production of O-acetyl-ADP-ribose is evolutionarily conserved among Sir2-like enzymes from yeast, Drosophila, and human. Also, endogenous yeast Sir2 complex from telomeres was shown to generate O-acetyl-ADP-ribose. By using a quantitative microinjection assay to examine the possible biological function(s) of this newly discovered metabolite, we demonstrate that O-acetyl-ADP-ribose causes a delay/block in oocyte maturation and results in a delay/block in embryo cell division in blastomeres. This effect was mimicked by injection of low nanomolar levels of active enzyme but not with a catalytically impaired mutant, indicating that the enzymatic activity is essential for the observed effects. In cell-free oocyte extracts, we demonstrate the existence of cellular enzymes that can efficiently utilize O-acetyl-ADP-ribose.

Reversible protein acetylation is emerging as a major regulatory mechanism that has been implicated in a wide range of biological signaling. One of the best known examples of reversible acetylation occurs within the amino-terminal end of core histone proteins (1). Histones are DNA-binding proteins that form the basic building blocks (nucleosomes) of chromatin. These histone amino-terminal “tails” are also the sites of other post-translational modifications that include phosphorylation, methylation, and ubiquitination (2–4). Acetylation of lysine residues on histone tails, catalyzed by histone acetyltransferases, generally correlates with transcriptional activation, whereas deacetylation of histone tails, by histone/protein deacetylases, correlates with transcriptional silencing (reviewed in Ref. 1). There are three known classes of histone/protein deacetylases, which are classified by their similarity to yeast proteins. These classes include the Rpd3-like (class I), the Hda1-like (class II), and the Sir2-like (class III) deacetylases (reviewed in Ref. 5). Class I and class II deacetylases are commonly referred to as histone deacetylases. Unique among the deacetylases, Sir2-like (class III) deacetylases, or sirtuins, are NAD⁺-dependent and are insensitive to trichostatin-A, a potent inhibitor of the histone deacetylases (reviewed in Ref. 5).

In yeast, Sir2 is required for silencing at telomeres (6–8), the mating-type loci (6, 9), and the ribosomal DNA (10–14). At the telomeres and mating-type loci, Sir2 is found in a multiprotein Sir2 complex with Sir3 and Sir4 (6, 7, 15–17). The SIR complex contributes to the stability and maintenance of the telomeric repeats (18). At the rDNA, Sir2 is associated with Net1 and Cdc14, termed the RENT (regulator of nucleolar silencing and telophase exit) complex (14, 19). The Sir2-mediated silencing at the rDNA appears to prevent or delay the formation of extrachromosomal rDNA circles, which have been shown to segregate to yeast mother cells and promote senescence (Ref. 20 and reviewed in Refs. 21 and 22). Sir2 at the silenced rDNA is linked to its function in promoting longevity. Increased dosage of the Sir2 gene resulted in increased lifespan in yeast (23) and Caenorhabditis elegans (24).

Besides silencing and lifespan extension, Sir2 has been implicated in other cellular processes including the repair of chromosomal double-strand breaks through nonhomologous end-joining (25), cell cycle progression, and chromosome stability (26). However, despite the many biological processes implicating the involvement of Sir2, its molecular function has only recently been explored (reviewed in Refs. 21 and 27–29). The first indication of Sir2 function came from the studies of cobalamin biosynthesis in Salmonella typhimurium (30). In this organism, CobB, a distant Sir2 homologue, was shown to compensate for the loss of the phosphoribosyltransferase CobT, suggesting a ribosyltransferase activity for Sir2. Initial examinations of Sir2 molecular function reported a weak ADP-riboseyltransferase activity (31, 32). Further examination, however, provided strong support for an NAD⁺-dependent deacetylase activity (33–38). The observation that histones at yeast silenced loci are hypoacetylated (39, 40) has provided a satisfying explanation for the role of Sir2 in transcriptional regulation.

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¶ The abbreviations used are: Sir2, silent information regulator 2; IPTG, isopropyl-β-D-thiogalactopyranoside; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; GVBD, germinal vesicle breakdown; BSA, bovine serum albumin; Aeh3, ARTKQTARKSTGGK(Ac)APRKQL; 1-MA, 1-methyladenine.

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12632 This paper is available on line at http://www.jbc.org
link from Sir2 function to heterochromatin structure. However, that Sir2 homologues are found in bacteria and that the cellular localization differs among the various homologues would suggest that Sir2 enzymes have additional functions.

Recent advances toward understanding the biological functions of the Sir2 family of enzymes included exploration of other potential substrates. These studies have focused on examination of known acetylated proteins as potential targets. For instance, it was recently demonstrated that Sir2 could negatively regulate the tumor suppressor p53 function in vitro and in vivo (41, 42) by deacetylating a known acetylation regulatory site (43, 44). Similarly, others have shown (45) that mouse Sir2a is capable of deacetylating the TAF6,8 component of the TATA box binding protein-containing factor, repressing RNA polymerase I transcription in vitro.

In the current study, we have taken a distinct approach to probe the function of Sir2 enzymes. As has been shown for class I and class II deacetylases, acetylase is the direct product of this simple hydrolytic reaction (5, 46). Surprisingly, however, the yeast Sir2 and HST2 enzymes (class III) couple protein deacetylation to the formation of a novel product O-acetyl-ADP-ribose, using NAD⁺ and liberating nicotinamide in the process (37, 38). Thus acetate is not the product of this unique NAD⁺-dependent reaction. This finding raises important biological questions concerning the function of sirtuins and the role of O-acetyl-ADP-ribose. It is not clear whether this tightly coupled reaction is conserved among higher eukaryotes as these human nor is it known whether endogenous yeast complexes can produce O-acetyl-ADP-ribose. If they can, does generating O-acetyl-ADP-ribose in the cell serve a biological purpose? Here we examine these important questions using detailed enzymatic analyses and a quantitative cell microinjection assay designed to evaluate effects on the cell cycle. We demonstrate that indeed the endogenous yeast telomeric Sir2 complex, the human homologue, SIRT2, and the Drosophila homologue, dSir2 generate O-acetyl-ADP-ribose via a conserved enzymatic reaction. By using the microinjection assay, we show that both O-acetyl-ADP-ribose and Sir2 enzymes, in a dose-dependent fashion, cause a delay/block in oocyte maturation and caused a cell cycle delay/block in embryo development.

**EXPERIMENTAL PROCEDURES**

**Materials**—The plasmid containing the full-length, carboxyl-terminally histidine-tagged 
*Drosophila* Sir2 (dSir2) gene was obtained from J. Lundblad, B. Newman, and S. Smolik, Oregon Health and Sciences University. Unacylated and monoacylated H3 peptide, ART-KQTARKSTGGK(Ac)APRKQL (AcH3), corresponding to the 20 amino-acid residues of the N-terminal region of histones H3 was purchased from the Protein Chemistry Core Lab at Baylor College of Medicine. [3H]Acetyl-Coenzyme A (1.88 Ci/mmol) was purchased from PerkinElmer Life Sciences.

**Expression and Purification**—The transformation of BL21DE3-competent cells with the plasmid containing HST2 as well as the expression and purification of HST2 were as described previously (37). The plasmid containing the dSir2 gene was transformed into BL21DE3 and were grown on a 2× YT media containing 100 μg/ml ampicillin and 20 mg/liter of chloramphenicol until an A₆₀₀nm of 0.6 was obtained prior to induction with isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h. Protein purification was performed using the same protocol as reported for the purification of HST2 (37).

In short, the plasmid containing hSIRT2 or the H187A mutant was transformed into BL21DE3-competent cells. Cells were grown on 2× YT media with 100 μg/ml ampicillin until an A₆₀₀nm of 0.6 was obtained prior to induction with IPTG for 3 h. Cell lysis was accomplished using a French pressure cell with buffer consisting of 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 10 mM imidazole, and 1 mM 2-mercaptoethanol. Clarified extract was incubated with nickel-nitrilotriacetic acid-agarose for 1 h at 4 °C. The mixture was loaded into a small column, washed with 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 20 mM imidazole, and 1 mM 2-mercaptoethanol. SIRT2 was eluted using a linear gradient of 20–250 mM imidazole in 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, and 1 mM 2-mercaptoethanol. SDS-PAGE analysis was performed to confirm the presence and purity of SIRT2 in the fractions. Pooled samples were dialyzed in 50 mM Tris, pH 7.5, 10% glycerol, and 1 mM DTT and stored at −80°C until use.

**Deacetylase Assay**—The deacetylase assay employed takes advantage of the different elution profiles between substrates and products through reverse-phase high performance liquid chromatography (HPLC). Reactions were carried out with [3H]AcH3 or core histones and NAD⁺ in 50 mM Tris (pH 7.5 at 37 °C) containing 1 mM DTT. HPLC-based assays were performed as described by Tanner et al. (25). Reactions were individually injected into a Gold HPLC using a Vyداد Preparative C18 (1 × 25 cm) or a Beckman (0.46 × 15 cm) analytical C18 reverse-phase HPLC column. Upon injection, the run was isocratically using solvent A (0.05% trifluoroacetic acid/H₂O) for 1 min. The isocratic step was followed by a linear gradient of 0–20% solvent B (0.02% trifluoroacetic acid/acetonitrile) over 20 min to resolve the substrate and product peaks. Deacetylated and acetylated H3 peptides were eluted at ~5% acetonitrile. Elution of substrates and products was monitored by measuring the absorbance at 214 nm. Radioactivity of collected fractions was determined by scintillation counting.

**3H Acetylation of H3 Peptide Using p300/CBP-associated Factor**—To determine the stoichiometry of the SIRT2 reaction, unacylated H3 peptides were specifically O-acetylated at Lys-14 using CBP-associated factor. A total of 30 μM [2-3H]Acetyl-CoA (specific activity ~4500–5000 cpm/pmol) and 200 μM H3 peptide were reacted in the presence of 14.5 μM of PCAF in 5 mM DTT, 50 mM Tris, pH 7.5, at 25 °C, for 1 h. The reaction was quenched with 1% trifluoroacetic acid and was purified by reversed phase chromatography using a Beckman Biosys 510 system at 501°C with a Vyداد C18 Small Pore preparative (1 × 25 cm) column (Vyداد, Hesperia, CA) at a flow rate of 1 ml/min. The HPLC was run with 100% solvent A for 1 min followed by a 0–40% gradient of solvent B over 40 min. Lyophilized [3H]AcH3 was resuspended in 50 mM Tris, pH 7.5, and the concentration was determined using a standard curve generated by obtaining the absorbance of known concentrations of AcH3 at 220 nm. The specific activity of the [3H]AcH3 peptide stock was determined as cpm/μl and cpm/pmol using a liquid scintillation counter.

**Purification of O-Acetyl-ADP-ribose from Beckman Biosys 510 HPLC system**—A Vyداد C18 (1.0 × 25 mm) small pore preparative column (Vydad, Hesperia, CA) were used for the purification of O-acetyl-ADP-ribose. Compounds from the enzymatic reaction were separated using a gradient system comprising of solvent A and solvent B using a constant flow rate of 4 ml/min. All mobile phases were filtered through a Millipore 0.20-μm nylon filter (Millipore Corp., Bedford, MA) prior to use. Upon injection of the sample (up to 1 ml), the HPLC was run isocratically for 5.0 min followed by a 0–40% gradient of solvent B over a 20-minute period with the detector set at 260 nm. The gradient was then increased to 40% solvent B over a 20-min period. At 21 min into the run, the detector was switched to 214 nm to detect acetylated and deacetylated H3 peptide. The product derived from NAD⁺ had a retention time (Rf) of 15.5 min, while the acetylated and deacetylated H3 peptide had retention times of 28.1 and 28.8 min, respectively. O-Acetyl-ADP-ribose was collected directly from the HPLC after passage through the detector, frozen at ~80 °C, and lyophilized. Samples were stored in a desiccator under argon prior to use.

**Purification of [3H]-Labeled Chicken Core Histones and the Yeast Telomeric Complex**—Chicken core histones and the yeast telomeric complex were purified according to the protocol described previously (47). A yeast strain carrying a His₆-HA epoetin tag at the amino terminus of the SIR2 gene under its own promoter, at its endogenous locus, was generated previously (yeast strain ROY 1515; see Ref. 47). The epitope-tagged Sir2p was functionally active in silencing assays (47). Briefly, whole cell extract was loaded into an SP-Sepharose cation exchange column, and proteins were eluted with varying KCl concentrations. The Sir2-containing complex, which eluted with 350 mM KCl, was then loaded into a covalent-TALON affinity column and eluted with increasing concentrations of imidazole. The Sir2-containing fraction was then subjected to an anion-exchange chromatography using Q-Sepharose column and eluted with increasing KCl concentrations. Fractions containing the telomeric complex were identified by the presence of Sir4, but not Net1, in a Western blot analysis. The fractions containing the telomeric complex were then subjected to gel filtration chromatography using Superose 6B column. The size of the telomeric complex was determined to be ~800 kDa. For the final step in the purification,
Generation and Biological Effects of O-Acetyl-ADP-ribose

RESULTS AND DISCUSSION

Conservation of Catalytic Mechanism and of O-Acetyl-ADP-ribose Formation—Yeast Sir2 (SIR2) has been found associated with Sir3 and Sir4 in multiprotein complexes at the telomeres and the mating type loci (6, 7, 15, 16) while existing with Net1 and Cdc14 (RENT complex) at the ribosomal DNA (14, 19). The functional significance of these different complexes is not known. As briefly discussed in the Introduction, recent evidence (33–38) indicates that Sir2 and other sirtuins are potent NAD+ -dependent protein/histone deacetylases. By using recombinant bacterially expressed enzymes, analyses with ySir2 and an additional homologue in yeast, HST2, have shown that the enzymes utilize NAD+ and an acetylated substrate to carry out deacetylation and the coupled production of the novel compound, O-acetyl-ADP-ribose (37, 38). The unique and unanticipated production of O-acetyl-ADP-ribose raises important questions about this conserved and relatively large family of proteins. Is the catalytic mechanism requiring NAD+ consumption and the production of O-acetyl-ADP-ribose evolutionarily conserved? Also it is not known whether Sir2 endogenous complexes catalyze this reaction (i.e. generation of O-acetyl-ADP-ribose), as only bacterially expressed recombinant proteins have been characterized to date.

To address whether endogenous Sir2-containing complexes produce O-acetyl-ADP-ribose, yeast Sir2 telomeric complex was purified from yeast (47) and utilized in an in vitro deacetylation assay. Purification of Sir2-containing telomeric complex involved five chromatographic steps (see "Experimental Procedures" and Ref. 47), resulting in ~800-kDa complex containing ~4 major polypeptides as described previously (47). Fractions containing the telomeric complex were identified by the presence of Sir4, but not Net1, using Western blot analysis. By performing HPLC-based (reversed-phase) deacetylation assays, we have shown that recombinant yeast HST2 generates O-acetyl-ADP-ribose, which can be resolved from the various reactant/products and quantified (37). Here, by employing 3H-acetylated chicken core histones as substrate, we demonstrated that the native yeast telomeric complex was able to produce O-acetyl-ADP-ribose in the presence of NAD+ (Fig. 1A). No deacetylation or the production of O-acetyl-ADP-ribose was observed in the absence of NAD+. Consistent with the previously reported NAD+ dependence of the reaction (33–35, 38), By using this assay, we have demonstrated previously that O-acetyl-ADP-ribose elutes at approximately ~5% acetonitrile and free acetate elutes at ~1% acetonitrile. With the telomeric Sir2 complex, the vast majority of radioactivity eluted where authentic O-acetyl-ADP-ribose elutes (Fig. 1A); however, there appeared to be small but significant radioactivity where free acetate would normally elute. Two different events could explain the formation of acetate. The presence of free acetate may be due to a slight uncoupling of the NAD+ cleavage and deacetylation reaction or, more likely, may be due to the non-enzymatic hydrolysis of O-acetyl-ADP-ribose once produced by the enzyme. Because of the small quantities of telomeric complex available, longer incubation times were required to generate sufficient quantities of O-acetyl-ADP-ribose to detect, allowing sufficient time for the O-acetyl-ADP-ribose produced to hydrolyze. To test the hypothesis of solvent-derived cleavage of O-acetyl-ADP-ribose, large quantities of [3H]O-acetyl-ADP-ribose were produced using HST2 and the synthetic peptide [3H]AcH3. The [3H]acytetyl-ADP-ribose was purified by HPLC, lyophilized, and its stability tested under the identical conditions utilized for the deacetylation assays. By using HPLC to resolve the products, we observed ~18% hydrolysis over 3 h at 37 °C. The results from these control experiments are consistent with the observation that significant background hydrolysis of O-acetyl-ADP-ribose occurred during the long deacetylation assays necessary for the Sir2 complex.

By having established that endogenous yeast Sir2 generates the unique product O-acetyl-ADP-ribose, we then attempted to demonstrate whether other distantly related Sir2 homologues, including one for human, could accomplish this reaction and generate O-acetyl-ADP-ribose. The seven human homologues of Sir2 (SIRT1–SIRT7) have not been well characterized. The few enzymatic assays that have been published on human SIRT2 (51, 52) and SIRT1 (42) have relied on observing loss of acetyl groups from substrates or from the detection of free acetate. If indeed the human sirtuins generate O-acetyl-ADP-ribose instead of acetate, then these assays were inappropriate. Alternatively, perhaps these Sir2 homologues do not couple deacetylation to the generation of O-acetyl-ADP-ribose. Thus it was not clear whether all sirtuins would generate O-acetyl-ADP-ribose as a primary product. We therefore examined the putative Sir2 homologues from Drosophila, dSir2, and from human, hSIRT2. By using recombinant bacterially expressed protein, histone deacetylase assays were performed, and the production of O-acetyl-ADP-ribose was determined (Fig. 1, B and C). Both hSIRT2 and dSir2 were shown to produce O-acetyl-ADP-ribose during the NAD+ -dependent deacetylation of the synthetic peptide AcH3. Only negligible traces of free acetate were detected in these assays. hSIRT2 was also capable of NAD+ -dependent deacetylation and O-acetyl-ADP-ribose formation using chicken core histones. Thus, the unique reaction involving the production of O-acetyl-ADP-ribose appears to be evolutionarily conserved.

Although the tight coupling of the yeast enzyme-catalyzed reactions has been examined (36–38), a detailed stoichiometric analysis of a human homologue has not been established. In fact, a complete analysis of products has not been demonstrated for any of the human homologues. Here we examined the human SIRT2 enzyme and determined the complete stoichiometry of the reaction. Limiting concentrations of NAD+ were reacted with SIRT2 at saturating levels of [3H]AcH3 peptide. Aliquots of the reaction were quenched at various time points until complete NAD+ conversion was attained. The amounts of deacetylated peptide and O-[3H]acetyl-ADP-ribose were then quantified by HPLC and liquid scintillation counting. Progress curves with three different concentrations of NAD+ showed that the amount of deacetylated H3 formed after complete reaction corresponds to the amount of the initial
NAD+/H11001 concentrations (Fig. 2A), indicating that there is a 1:1 stoichiometry between the amount of peptide deacetylated and the amount of NAD+/H11001 consumed. By quantifying the amount of O-acetyl-ADP-ribose formed, we also demonstrated that there is a 1:1:1 molar ratio between the NAD+/H11001 consumed and the O-acetyl-ADP-ribose and deacetylated peptide generated (Fig. 2B). These results suggest that the human SIRT2 tightly couples the cleavage of NAD+/H11001 to the deacetylation of substrate and the formation of O-acetyl-ADP-ribose. Previous reports (36–38) on the yeast Sir2 and HST2 indicated that the yeast enzymes catalyze a 1:1:1 stoichiometry. Taken together, these results suggest that the human SIRT2 tightly couples the cleavage of NAD+ to the deacetylation of substrate and the formation of O-acetyl-ADP-ribose. Previous reports (36–38) on the yeast Sir2 and HST2 indicated that the yeast enzymes catalyze a 1:1:1 stoichiometry. Taken together, these results

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For the dSir2 reaction, 1 mM NAD+ and 150 µM [3H]AcH3 were reacted in 50 µL of dSir2 for 30 min. Substrates and products were separated using a Vydac preparative (1 × 25 cm) C18 reversed phase column and using the following gradient: 0% solvent B for 1 min followed by 0–8% solvent B for over 20 min, and 8–28% solvent B for 35 min. Radioactivity of collected fractions was determined by scintillation counting.

**Fig. 1.** Deacetylation reaction of yeast telomeric complex (A), SIRT2 (B), and dSir2 (C). For the yeast telomeric reaction, 200 µM NAD+ and 2 µg of chicken core histones were reacted with 2 µg of yeast telomeric complex for 1.5 h. For deacetylation reaction using SIRT2, 200 µM NAD+ and 2 µg of chicken core histones were reacted in 1 µM SIRT2 for 30 min. For both the yeast telomeric complex and SIRT2, substrates and products were separated using a Beckman (0.46 × 15 cm) analytical C18 reversed phase HPLC column using the following gradients: 0% solvent B for 1 min and 0–20% solvent B for over 20 min.

**Fig. 2.** Stoichiometry between NAD+ consumption, peptide deacetylation (A), and O-acetyl-ADP-ribose formation (B). Fixed NAD+ concentrations and 100 µM [3H]AcH3 were reacted with 1 µM SIRT2, and reactions were quenched at various time points until complete NAD+ consumption was attained. Samples were injected into the Beckman C18 reversed phase column, and the concentrations of deacetylated H3 peptide and 3H-labeled O-acetyl-ADP-ribose were quantified as described under “Experimental Procedures.” The stoichiometry between the amounts of NAD+ consumed and H3 peptide deacetylated is shown in A. The amounts of deacetylated H3 peptide and O-acetyl-ADP-ribose formed at completion under various NAD+ concentrations are shown in B.
suggest a strong conservation of the reaction mechanism among sirtuins. The fact that the production of O-acetyl-ADP-ribose appears to be conserved in all sirtuins (ranging from bacteria, yeast, Drosophila, and human) suggests a potentially important biological role for O-acetyl-ADP-ribose. Next we set out to test if O-acetyl-ADP-ribose may harbor important biological activity.

Phenotypic Effects of Sir2 Enzymes and Their Product O-Acetyl-ADP-ribose—The Sir2 family of enzymes tightly couple histone/protein deacetylation to the formation of O-acetyl-ADP-ribose, a compound with no described physiological role. It is well established that class I and II histone deacetylases do not require NAD+ coenzymes and instead produce acetate, the expected product of normal deacetylase reactions (5, 46). We proposed (37) that O-acetyl-ADP-ribose may have a unique cellular function(s) that may be linked to Sir2 and sirtuins gene silencing or other physiological activity. The produced O-acetyl-ADP-ribose may have an important signaling or metabolic role by serving as a source for generating metabolic products or by serving directly as a substrate or cofactor for other enzymes/proteins that may utilize this novel product to elicit the proper cellular response. To begin to test these ideas, we initiated biological function studies employing a sensitive, quantitative microinjection assay.

To initiate our investigation on the possible function of O-acetyl-ADP-ribose, we microinjected purified O-acetyl-ADP-ribose into starfish (A. miniata) immature oocytes. Historically, echinoderm development has been a biochemically tractable and well defined system to assess biological function of proteins, bio-active compounds, and for detailed analyses of the cell cycle (53–55). The ability to perform quantitative microinjection assays and to observe gross morphological changes and effects on the cell cycle provide for a general ease of assessing potential bio-active compounds such as O-acetyl-ADP-ribose.

Initially, we surmised that if O-acetyl-ADP-ribose produced by sirtuins was involved in some aspect of their biological function(s), then microinjection of O-acetyl-ADP-ribose into a maturating oocyte may have a phenotypic effect on the maturation process or on the developing embryo.

Immature oocytes were microinjected with HPLC-purified O-acetyl-ADP-ribose or with a buffer control. The oocytes were induced to mature by the addition of 1-methyladenine (1-MA; maturation hormone) and assessed for developmental alterations. Immature oocytes are arrested in prophase of meiosis I and, in response to 1-MA, are triggered to synchronously begin meiotic maturation (54). This is easily visualized by breakdown of the oocyte nucleus (the germinal vesicle) and polar body extrusion ((56) see Fig. 3A). If they are not fertilized, they undergo apoptosis within ~24 h (57, 58). Compared with control injections (Fig. 3A), oocytes injected with O-acetyl-ADP-ribose between 0.32 and 5 mM final concentration (Table I) exhibited a delay or a complete block in oocyte maturation as assessed by germinal vesicle breakdown (GVBD) and polar body extrusion (see Fig. 3C and D, for representative data). At cytoplasmic concentrations below 0.32 mM, no visible effect on meiotic maturation was observed (Fig. 3B and Table I). At a very high concentration (16 mM), O-acetyl-ADP-ribose resulted in immediate cell death. Thus, the O-acetyl-ADP-ribose injections were indeed causing a definable phenotype, where higher concentrations caused a complete block in oocyte maturation and lower doses caused a delay in maturation. The dose dependence of these effects suggests a specific physiological event; however, given the relatively long incubation times of these assays (23–30 min post-injection before the addition of hormone and including up to 24-h observations), there was a possibility that O-acetyl-ADP-ribose was being consumed or metabolized in the cell, resulting in diminished efficacy at lower doses.

### Table I

| Final concentration of O-Ac-ADP-ribose | No effect | 1–2-h delay | Complete block | Immediate death |
|--------------------------------------|-----------|-------------|----------------|-----------------|
| 10 mg/ml (16 mM)                     | 2/2       |             |                |                 |
| 3.0 mg/ml (5 mM)                     | 2/3       | 6/6         | 1/3            |                 |
| 1.7 mg/ml (3 mM)                     | 1/23      | 22/23       |                |                 |
| 1.0 mg/ml (1.6 mM)                   | 10/16     | 6/16        |                |                 |
| 0.5 mg/ml (0.8 mM)                   | 7/8       | 1/8         |                |                 |
| 0.2 mg/ml (0.32 mM)                  | 16/16     |             |                |                 |
| 0.1 mg/ml (0.16 mM)                  | 8/8       |             |                |                 |

| Final concentration of ADP-ribose    | No effect | 1–2-h delay | Complete block | Immediate death |
|-------------------------------------|-----------|-------------|----------------|-----------------|
| 1.0 mg/ml (1.6 mM)                  | 8/8       |             |                |                 |
| 0.5 mg/ml (0.8 mM)                  | 8/8       |             |                |                 |
| 0.1 mg/ml (0.16 mM)                 | 8/8       |             |                |                 |

**Fig. 3. Microinjection of O-acetylated ADP-ribose inhibits starfish oocyte maturation.** Immature oocytes were incubated with the maturation hormone 1-MA (A) or microinjected with varying amounts of purified O-acetyl-ADP-ribose and then incubated with 1-MA (B–D). The calculated final concentration (millimolar) of O-acetyl-ADP-ribose in the oocyte cytoplasm is indicated at the left, and the arrow points to a small oil droplet indicating an injected oocyte. Oocytes were monitored microscopically for maturation over time (the oocytes are shown at 60 and 120 min and 24 h post-hormone application). The control oocyte underwent germinal vesicle breakdown by 35 min post-hormone addition. By 24 h, the matured, unfertilized oocyte underwent apoptosis, as expected (see text). Low concentrations of O-acetyl-ADP-ribose (B) did not affect either the morphology or the timing of maturation events. However, higher concentrations of the compound either delayed (C) or completely prevented (D) the maturation process. GV, germinal vesicle. See Table I for complete tabulation of results.
To explore the possibility that the injected O-acetyl-ADP-ribose might be enzymatically metabolized over time, we generated cell-free extracts of immature oocytes and monitored the rate of conversion of added O-acetyl-ADP-ribose. Cell-free extracts were necessary, as the detection of O-acetyl-ADP-ribose would be extremely difficult in individually injected oocytes. O-[3H]Acetyl-ADP-ribose was generated enzymatically and purified using HPLC (see “Experimental Procedures”). Lyophilized O-acetyl-ADP-ribose was then dissolved in PBS buffer (containing 1% Triton X-100) and added to starfish soluble-protein extracts prepared in the same buffer. At various time intervals, aliquots were removed and subjected to HPLC analysis. Fractions were collected, and the loss of O-[3H]acetyl-ADP-ribose was quantified by liquid scintillation counting. Percent hydrolysis of O-[3H]acetyl-ADP-ribose was determined by comparing radioactivity of fractions to a t = 0 time point.

From the cell-free extract experiments (Fig. 4), we would predict that 0.3 mM of injected O-acetyl-ADP-ribose would be completely consumed in only 30 min. Recall that after injection of O-acetyl-ADP-ribose, the oocytes are allowed to recover for 25–30 min before the addition of maturation hormone, allowing enough time for lower doses (<0.3 mM) to be almost completely metabolized. The net result would be a lack of phenotype. Although our experiments offer strong support for the existence of enzymes that utilize O-acetyl-ADP-ribose, we do not yet know the fate of the O-acetyl-ADP-ribose molecule following its disappearance. The enzymatic activity is not likely to be a general esterase, as inclusion of multiple protease/esterase inhibitors had no effect on the ability of the protein extract to consume O-acetyl-ADP-ribose (Fig. 4).

Thus, it is not possible at this point to distinguish if the observed effects on maturation are due to a direct effect of O-acetyl-ADP-ribose or of a metabolic breakdown product. We considered the possibility that O-acetyl-ADP-ribose was being converted to a metabolite that has a known effect on oocyte maturation or cell division. In sea urchin eggs, ADP-ribose-gated calcium cyclase converts NAD+ to cyclic ADP-ribose (cADP-ribose) and nicotinamide (59). Acting as a second messenger, cADP-ribose mobilizes calcium from internal stores, as demonstrated in homogenates (60–62) and via microinjection (48, 61, 63). When injected into sea urchin eggs, 100 nM ranges of cADP-ribose trigger some degree of calcium release (63), whereas 1–10 µM ranges of cADP-ribose result in reproducible calcium release from internal egg stores (48, 61). It also has been reported that injection of cADP-ribose into the germinal vesicle of immature starfish oocytes triggers a calcium transient, which promotes maturation in the absence of 1-MA (64, 65). However, we did not detect evidence of calcium transients upon injection of O-acetyl-ADP-ribose into the starfish oocytes or into sea urchin eggs (n = 8; data not shown), suggesting that O-acetyl-ADP-ribose was not exerting its effect via conversion to cADP-ribose and mimicking its effect of triggering calcium release.

We also considered the use of ADP-ribose as a control for comparison to O-acetyl-ADP-ribose. Interestingly, recent studies have uncovered that there are ADP-ribose-gated calcium channels in ascidian oocytes (66, 67) and in mammalian cells (68, 69), implicating ADP-ribose as a second messenger. Other evidence from these studies suggests that metabolized NAD+ is the ultimate in vivo source of generated ADP-ribose (67, 68). As a consequence of these recent findings, we were initially concerned that NAD+ metabolites like ADP-ribose may not be a proper control to O-acetyl-ADP-ribose when comparing the observed in vivo effects. Importantly, ADP-ribose does not mimic the effect of cADP-ribose in sea urchin eggs, even at high doses (millimolar range) (60, 61, 63). However, calcium release was the focus of these studies, and it has not been reported if ADP-ribose has an effect on other aspects of development, such as maturation or cell division. In the current study, we observed that injection of ADP-ribose into immature starfish oocytes also delayed/inhibited maturation (Table I) compared with control (buffer alone) injections. To our knowledge, this is the first published observation on the effects of ADP-ribose.

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8 M. T. Borra, F. J. O'Neill, M. D. Jackson, B. Marshall, E. Verdin, K. R. Foltz, and J. M. Denu, unpublished observations.
injection into starfish oocytes. Thus, given that similar cellular effects were observed between O-acetyl-ADP-ribose and ADP-ribose, it is possible that O-acetyl-ADP-ribose is exerting its effects by being converted to ADP-ribose, which then affects maturation either directly or indirectly through a common metabolite of ADP-ribose. It is important to point out that the cellular concentrations of endogenous ADP-ribose are currently not known. Alternatively, the two compounds may exhibit the same phenotypic effect via different mechanisms. It will be important to determine the fate of O-acetyl-ADP-ribose in cells in order to begin to address this issue, especially because its turnover appears to be quite rapid (Fig. 4). Given that we have not been able to detect calcium rises in starfish or sea urchin eggs injected with either O-acetyl-ADP-ribose or ADP-ribose (see above), it is unlikely that the observed effects are via opening of ADP-ribose-gated calcium channels. Regardless, the dosage dependence and comparisons to sham (buffer alone) injections suggest that the effect of O-acetyl-ADP-ribose is specific, whether it is direct or via conversion to a subsequent metabolite.

To complement the O-acetyl-ADP-ribose injection studies, purified sirtuin enzymes were microinjected in place of O-acetyl-ADP-ribose. We hypothesized that if acetylated protein substrates were available in the oocyte, then microinjected enzyme would provide a more constant source (or perhaps a more localized source) of O-acetyl-ADP-ribose. Yeast HST2 and human SIRT2 were used for these studies. As a control, a catalytically impaired mutant of SIRT2 (H187A) was employed to ensure that any observed effects were due to the catalytic activity of the enzymes. The H187A mutant used for these studies displayed ~3000-fold lower catalytic efficiency compared with wild type enzyme (data not shown). When the corresponding histidine residue in ySir2 was mutated to tyrosine, the resulting H364Y mutant displayed reduced enzymatic activity and could not complement a Sir2 silencing defect in yeast (32). Similarly, in reports on the x-ray structures of two Sir2 homologues (Sir2-Af1 (70) and SIRT2 (51)), mutation of this residue to alanine caused a significant loss in activity. The Sir2-Af1 structure bound to NAD° revealed that this histidine residue was hydrogen-bonded to 3'-hydroxyl of the nicotinamide ribose (70), implicating this residue in NAD° binding and/or catalysis.

The effects of HST2 and human SIRT2 (wild type and mutant) enzymes on starfish oocyte maturation were assessed by microinjection (Table II; Fig. 5). *A. miniata* immature oocytes were microinjected with the given enzymes in PBS. The maturation hormone was applied (10 μM) 25–30 min post-injection, and oocytes were monitored for GVBD and polar body extrusion. Non-injected and control-injected oocytes showed synchronous GVBD within 35–45 min (depending on the batch of oocytes). The number of oocytes per treatment (n) is given. Data are from four separate batches of oocytes. All oocytes for a given treatment responded in the same way unless indicated otherwise (e.g., see 200 nM HST2 injection).

### Table II

*Effects of yeast HST2 and human SIRT2 enzymes on starfish oocyte maturation*

| Enzyme   | Final concentration | n  | Effect on GVBD                                  |
|----------|---------------------|----|-----------------------------------------------|
| HST2     | 25                  | 10 | No observed effect                            |
|          | 60                  | 8  | No effect 3/8; 2 h-delay in 5/8                |
|          | 72                  | 22 | Complete block                                |
|          | 200                 | 13 | Complete block in 12/13; 3-h delay in 1/13     |
| H187A    | 200                 | 10 | No observed effect                            |
|          | 770                 | 3  | No observed effect                            |
| hSIRT2   | 25                  | 5  | No observed effect                            |
|          | 60                  | 5  | 2-h delay in GVBD and polar body extrusion    |
|          | 200                 | 5  | Complete block                                |

![Fig. 5. Microinjection of yeast HST2 and hSIRT2 inhibit starfish oocyte maturation.](image)

Immature oocytes were microinjected with the indicated purified, recombinant protein (to 25 or 200 nM final concentration in the cytoplasm) and then incubated with the maturation hormone 1-MA. Control oocytes (see Fig. 3A) and oocytes injected with the catalytically inactive form of the enzyme (H187A, C) underwent germinal vesicle breakdown even at high concentrations of injected protein. Injection of 25 nM HST2 (A) had little to no effect on the morphology or timing of maturation events but at 200 nM (B) inhibited maturation (see Table II for complete tabulation of results).

transfection, because it is known precisely how much enzyme is present on a per cell basis, allowing for a dosage response evaluation. Although numerous studies have been published on the overexpression of Sir2 and homologues in yeast and mammalian cells (reviewed in Refs. 28 and 27, respectively), the
concentration of any of these overexpressed proteins in the cell has not been quantified. Although we do not know the physiological levels of sirtuins in cells, the effective dosages of the injected enzymes fall within the range of that reported for injection of other active enzymes into starfish oocytes, all of which exhibited specific effects. For example, injection of $1 \mu M$ final concentration of glutathione S-transferase-Mos protein into immature starfish oocytes activated mitogen-activated protein kinase and maintained oocyte arrest, with a clear dose dependence (71). Furthermore, injection of constitutively active human Src protein stimulated calcium release and triggered DNA synthesis in starfish eggs at 7.2 $\mu M$ final concentration but not at levels of $\leq 1.5 \mu M$ (72). Again there was a clear dose dependence, and inactive Src protein at the same concentration did not elicit an effect. Investigators also have injected proteins designed to act as dominant-interfering agents that target endogenous starfish egg enzymes. Carroll et al. (73) injected recombinant tandem SH2 domains of phospholipase C$_\gamma$, which inhibited calcium release at levels of $\leq 2 \mu M$, with complete block observed at levels of 75 $\mu M$ final concentration. Similarly, injection of Src family kinase SH2 domains at levels as low as 2.5 $\mu M$ concentration inhibited calcium release in starfish eggs. Importantly, in both of these studies, point-mutated, inactive proteins, or related proteins had no effect on calcium release. In comparison then, the reproducible effects we have observed upon injection of 60–200 nM of the sirtuin enzymes again suggests a specific effect on maturation.

To extend these observations and to examine whether these Sir2 enzymes caused any phenotypic effects on early embryonic cell division, HST2 or the H187A SIRT2 mutant were microinjected into cell division, one daughter blastomere was microinjected with the indicated enzyme. Embryos were monitored for further cell divisions over time. The non-injected daughter blastomere served as the internal control in each experiment. The number of injected embryos per treatment is given ($n$). Data for enzyme injections are from three separate batches of oocytes and three preparations of enzymes. Data from the catalytic mutant of SIRT2 H187A are also given. Data for O-acetyl-ADP ribose injections are from two batches of oocytes and two preparations of the compound (data listed in main text).

| Injected agent | Final concentration in blastomere | $n$ | Effects, next cell division | Effects, 3rd cell division | Effects, other |
|---------------|----------------------------------|-----|---------------------------|---------------------------|--------------|
| HST2          | 25                               | 5   | 4/5 normal                | Normal                    | Normaldevelopment |
|               |                                  |     | 1/5 slight delay$^a$      | Normal                    | Normaldevelopment |
|               |                                  |     | 4/10 slight delay$^a$     | Complete block            |              |
|               |                                  |     | 6/10 no division (complete block) |              |              |
| HST2          | 72                               | 10  | 2/7 slight delay$^a$      | Complete block            |              |
|               |                                  |     | 5/7 no division (complete block) |              |              |
| H187A         | 200                              | 5   | Normal                    | Normal                    | Normaldevelopment |
|               | 500                              | 5   | Normal                    | Normal                    | Normaldevelopment |

$^a$ Synchronous cell divisions occur approximately every 30 min. The slight delay observed ranged from 5 to 15 min compared with the daughter blastomere.

The reproducible delay before a complete block in the next round of cell division. Most cells injected with the lower 25 nM HST2 developed normally (Table III and Fig. 6D). The H187A mutant exhibited no phenotype at any concentration (Table III and Fig. 6C). Again, there was a clear dosage effect that paralleled the effect observed on oocyte maturation. The low concentrations necessary for the blocks, the dosage effects, and the requirement for active enzyme collectively suggest that these Sir2 enzymes are causing a specific and robust biological effect that results in cell cycle inhibition.

To examine whether microinjection of O-acetyl-ADP-ribose into blastomeres could mimic the phenotypes observed in the enzyme studies, similar microinjection studies were performed. A. miniata oocytes were matured by addition of the hormone 1-methyladenine and then fertilized. After the first cell division, one daughter blastomere was microinjected with the O-acetyl-ADP-ribose, and embryos were monitored for further cell divisions over time. Microinjection of O-acetyl-ADP-ribose resulted in a delay or arrest in subsequent cell divisions (Fig. 6B). At a final concentration of 0.32 mM O-acetyl-ADP-ribose in the cytoplasm, 2 of 3 injected blastomeres exhibited a complete block in the next cell division, whereas the third blastomere appeared to develop normally. At a higher concentration (0.8 mM) of O-acetyl-ADP-ribose, 3 of 3 injected blastomeres exhibited a complete block in the next cell division. As in the case of oocyte maturation, injection of ADP-ribose into oocytes gave similar results (data not shown). Although we did not carry out an extensive dose dependence study on large numbers of oocytes, the results suggest that, similar to the effects observed upon active enzyme injections, O-acetyl-ADP-ribose has specific effects (direct or indirect) on early embryonic cell divisions.

**SUMMARY AND PERSPECTIVE**

Quite strikingly, injection of pure O-acetyl-ADP-ribose mimicked the oocyte maturation and cell division blocks that were observed when low nanomolar levels of active Sir2 enzymes were injected. Although we cannot distinguish between direct and indirect effects, the results provide important findings that suggest the product O-acetyl-ADP-ribose plays an integral role, if not sufficient, role in Sir2-dependent phenotypic assays. Injection of a catalytically impaired mutant of human SIRT2 displayed no phenotypic effect, indicating that the enzymatic activity is necessary for the manifestation of the maturation and developing developmental arrest.

**TABLE III**

*Effects of HST2 on early embryonic cell division*

A. miniata oocytes were matured by addition of the hormone 1-methyladenine and then fertilized. One-cell zygotes were then placed in injection chambers. After the first cell division, one daughter blastomere was microinjected with the indicated enzyme. Embryos were monitored for further cell divisions over time. The non-injected daughter blastomere served as the internal control in each experiment. The number of injected embryos per treatment is given ($n$). Data for enzyme injections are from three separate batches of oocytes and three preparations of enzymes. Data from the catalytic mutant of SIRT2 H187A are also given. Data for O-acetyl-ADP ribose injections are from two batches of oocytes and two preparations of the compound (data listed in main text).
cell division blocks. We have also provided evidence that the unique enzymatic reaction utilizing the coupled generation of O-acetyl-ADP-ribose is conserved among higher eukaryotes, namely fly and human. Additionally, we showed that endogenous telomeric yeast complex produces O-acetyl-ADP-ribose via the same reaction as that observed from isolated recombinant enzymes.

To explore Sir2 function, recent studies have focused on examination of known acetylated proteins as obvious potential substrate targets for the sirtuins and histone deacetylases. Histones were the first logical substrates to test, and indeed, Sir2 homologues are capable of in vitro deacetylation of histone proteins. However, several facts and recent observations would indicate that histones are not viable substrates for all sirtuins. Because bacteria lack histones, their Sir2 homologues must use an alternative substrate(s). The homologue AFI from *Arachaeoglobus fulgidus* could not efficiently deacetylate histones (70); acetylated BSA was used as an alternative substrate to demonstrate enzymatic activity. Non-nuclear localizations of several Sir2-like enzymes (74) further imply that nuclear acetylated histones may not be the enzymatic targets. Given these observations, investigations have attempted to link sirtuin function with other known acetylated proteins. For instance, it was recently demonstrated (41, 42) that mouse Sir2a and human SIRT1 could negatively regulate tumor suppressor p53 function in *vitro* and *in vivo*. This regulation is accomplished through the deacetylation of a lysine residue that was known to be an acetylation-dependent regulatory site (43, 44). Others (45) have shown that the mouse Sir2a is capable of deacetylating the TAF68 component of TIF-IB/SL1, repressing RNA polymerase I transcription in *vitro*. Whereas these studies have focused on the involvement between protein deacetylation and transcriptional regulation, it is important to point out that echinoderm oocyte maturation and the early embryonic cell cycle do not require new transcription (54). Instead, these events rely on maternally stored RNA and a host of signaling events that include post-translational protein modification, such as phosphorylation. Thus, the observed effect of Sir2 enzymes, and their product, on these biological processes are likely targeting post-transcriptional signaling events. A signaling cascade involving reversible protein acetylation may be required to trigger oocyte maturation and cell division, and injection of Sir2 enzymes may block this pathway by deacetylating key signaling proteins. Alternatively, specific protein deacetylation may not be critical for these effects, but instead, the production of O-acetyl-ADP-ribose alone may induce the delays/blocks. As demonstrated, O-acetyl-ADP-ribose injections could mimic the effects seen with active Sir2 enzymes, suggesting that O-acetyl-ADP-ribose may be sufficient to induce the phenotypic blocks. However, there may be a synergistic effect of both specific protein deacetylation and the *in vivo* production and utilization of O-acetyl-ADP-ribose, as only low nanomolar levels of enzyme were required. The O-acetyl-ADP-ribose may act as a direct second messenger, although we cannot rule out an indirect effect, because O-acetyl-ADP-ribose can be turned over in oocyte extracts and ADP-ribose injections resembled the effect of the O-acetyl-ADP-ribose. In this case, the products of a subsequent O-acetyl-ADP-ribose reaction(s) might elicit the biological response. For example, the O-acetyl-ADP-ribose could be utilized by enzymes that catalyze its hydrolysis to acetate and ADP-ribose or by enzymes that catalyze the transfer of either the acetyl- or ADP-ribose moiety to an acceptor protein or small molecule. Thus, O-acetyl-ADP-ribose could initiate a signaling cascade whereby additional enzymes/protein are required to convert this molecule to its ultimate bio-active form. These exciting questions will be addressed in future studies directed at identifying the acetylated protein targets of these enzymes, determining the fate of O-acetyl-ADP-ribose in cells, and identifying the enzymes/proteins that utilize this novel molecule as a substrate or ligand.

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