Molecular Cell, Vol. 17, 595–601, February 18, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.molcel.2004.12.032

Chemical Activation of Sir2-Dependent Silencing by Relief of Nicotinamide Inhibition

Short Article

Anthony A. Sauve,[1,2] Robyn D. Moir,[1,2] Vern L. Schramm,[1,*] and Ian M. Willis[1,*]
[1]Department of Biochemistry
Albert Einstein College of Medicine
1300 Morris Park Avenue
Bronx, New York 10461

Summary

Sir2 is a nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylase involved in gene silencing and longevity. Cellular stresses affect Sir2 activity, but the mechanisms of Sir2 regulation are debated. Nicotinamide has been proposed as a physiological regulator that inhibits Sir2 deacetylase activity by chemical reversal of a covalent reaction intermediate. We demonstrate a chemical strategy to activate Sir2-dependent transcriptional silencing and present evidence that the endogenous level of nicotinamide limits Sir2 activity in wild-type (wt) yeast cells. Nicotinamide inhibition of Sir2 is antagonized in vitro by isonicotinamide, which causes an increase in Sir2 deacetylation activity. Isonicotinamide also substantially increases transcriptional silencing at Sir2-regulated loci in wt strains and in strains lacking key NAD^+ salvage pathway enzymes (*PNC1* and *NPT1*). Thus, a nicotinamide antagonist is a Sir2 agonist in vitro and in vivo.

Introduction

Yeast Sir2 is a class III histone deacetylase that uses NAD^+ to deacetylate acetyllysine residues at the N-terminal tails of histones H3 and H4 in chromatin (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000). Sir2 function is necessary for the formation and spreading of heterochromatin and for transcriptional silencing at the silent mating type loci at telomeres and in the rDNA repeat (Rusche et al., 2003). Elevated *SIR2* gene dosage increases transcriptional silencing and genome stability and leads to extension of yeast replicative lifespan (Kaeberlein et al., 1999). Calorie restriction and high osmolarity also increase yeast lifespan through Sir2-dependent pathways (Lin et al., 2000; Kaeberlein et al., 2002). These stimuli upregulate Sir2 catalytic activity without increasing the level of Sir2 protein (Anderson et al., 2002). However, the mechanism of upregulation and the endogenous regulator(s) of Sir2 activity remain controversial. Nicotinamide (Bitterman et al., 2002; Anderson et al., 2003; Gallo et al., 2004), reduced nicotinamide adenine dinucleotide (NADH) (Lin et al., 2003, 2004), and NAD^+ (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000; Lin et al., 2000) have each been proposed as a principal regulator of Sir2 catalysis in vivo. Cellular stress is believed to lower the concentrations of inhibitory Sir2 regulators (Bitterman et al., 2002; Anderson et al., 2003; Gallo et al., 2004; Lin et al., 2003, 2004), although the actual concentrations of these metabolites in cells are controversial and nicotinamide concentrations in yeast cells have not been reported. Because calorie restriction increases lifespan in organisms from yeast to primates and sirtuins affect lifespan and cell survival in multicellular eukaryotes (Tissenbaum and Guarente, 2001; Vaziri, et al., 2001; Luo et al., 2001), the question of Sir2 regulation is currently at the forefront of Sir2 biology (Hekimi and Guarente, 2003). An understanding of Sir2 regulation in terms of its inhibition by nicotinamide can be used to develop strategies for chemical control of Sir2 activity that provide direct modulation of sirtuin function independent of genetic methods.

In this work, knowledge of the enzymatic mechanism of Sir2 was used to test the proposed mechanism for nicotinamide inhibition of deacetylation. This led to the identification of isonicotinamide as an antagonist of nicotinamide inhibition and as an activator of Sir2 deacetylase activity. Consistent with the biochemical effects of isonicotinamide on Sir2 activities, the compound was found to be a potent activator of Sir2-dependent transcriptional silencing in yeast. Thus, a chemical strategy to activate Sir2 catalysis in vivo is demonstrated. These results coupled with measurements of cellular nicotinamide demonstrate that the level of nicotinamide in wt yeast cells limits Sir2 activity and predict that changes in the nicotinamide concentration in response to altered cell physiology will affect Sir2 function.

Results and Discussion

Blocking the Nicotinamide Binding Site Relieves Nicotinamide Inhibition of Sir2

Sir2 deacetylation chemistry yields nicotinamide, the lysine amino group, and the unusual metabolite 2′-*O*-acetylADPR (Sauve, et al. 2001, Figure 1A). The catalytic mechanism is initiated by formation of a long-lived peptidyl-imidate intermediate. Nicotinamide achieves binding equilibrium with the imidate-enzyme complex and can react to regenerate acetyllysine and NAD^+ (Sauve and Schramm, 2003; Jackson et al., 2003) in a nicotinamide exchange reaction (base exchange, Figure 1A, k_4). Base exchange depletes the imidate intermediate during normal steady-state turnover, causing nicotinamide inhibition of deacetylation (Sauve and Schramm, 2003; Jackson et al., 2003). These findings are consistent with the proposal that changes in nicotinamide concentration in vivo may regulate Sir2 function (Anderson et al., 2003; Gallo et al., 2004). The incomplete inhibition of Sir2 by nicotinamide indicates that the 2′-hydroxyl of the ribose ring and nicotinamide react independently with the peptidyl-imidate intermediate (Figure 1A; Sauve and Schramm, 2003). For example, deacetylation of an N-terminal histone H4 peptide is inhibited by nicotinamide (K_i = 110 μM), but 14% of the activity is resistant

*Correspondence: vern@aecom.yu.edu (V.L.S.); willis@aecom.yu.edu (I.M.W.)
[2]These authors contributed equally to this work.

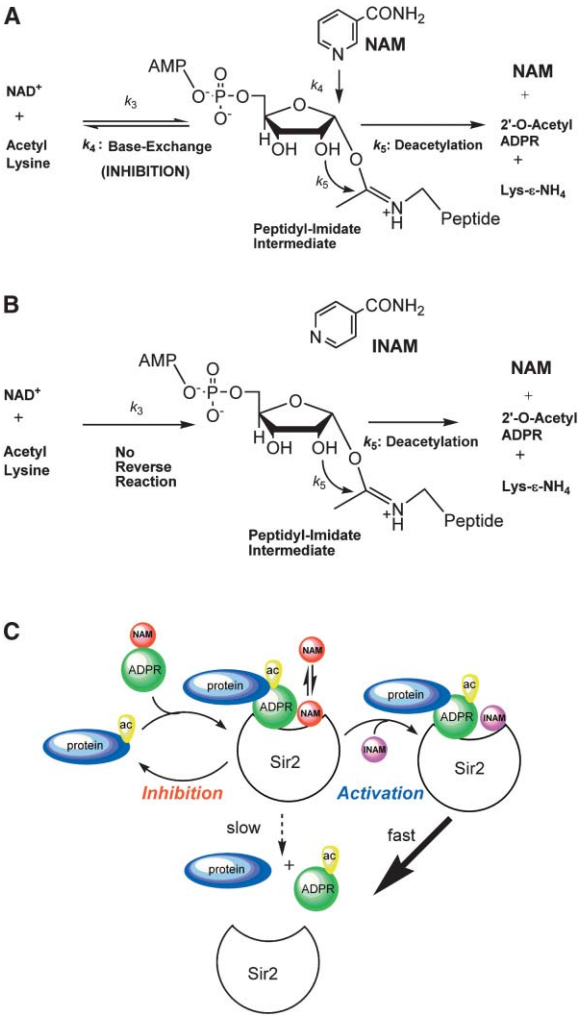

Figure 1. Schematic Representation of ADP-Ribosyl-Imidate Reactivity in Sir2-Catalyzed Base Exchange and Deacetylation Reactions

(A) Nicotinamide (NAM) and 2′-hydroxyl attacks occur on opposite faces of the ribose moiety leading to chemical competition between base exchange and deacetylation. Nicotinamide inhibition of deacetylation results from chemical reversal of the imidate intermediate.

(B) Proposed action of isonicotinamide (INAM) as a ligand at the nicotinamide binding site. Base exchange is not possible because the nitrogen atom is in an unreactive position. Efficient deacetylation occurs due to the chemical independence of the deacetylation and base-exchange reactions.

(C) Scheme for reaction of acetylated histone (active chromatin; blue and yellow) with NAD^+ (green and red) and Sir2 in yeast cells in the presence of isonicotinamide. Endogenous nicotinamide (red) competes for the ADPR-imidate (blue, yellow, and green) Sir2 complex with exogenous INAM (pink). Because the INAM complex with Sir2 cannot react to reform substrates and INAM does not inhibit deacetylation, the complex metabolizes forward to form deacetylated histones (silent chromatin) and 2′-AADPR.

to inhibition (Figure 2B). This limit establishes the partitioning of the imidate-enzyme complex between base exchange and deacetylation reactions and is described by the ratio of rate constants k_4 and k_5 (Figure 1A, Sauve and Schramm, 2003). Thus, the chemical mechanism of Sir2 predicts that a nicotinamide antagonist bound in the nicotinamide site could selectively inhibit the base-exchange reaction and thereby increase the deacetylation rate (Figure 1B, Sauve and Schramm, 2003).

To evaluate this prediction, we determined the effect of isonicotinamide, a nonreactive nicotinamide isostere, on Sir2 base exchange and deacetylation rates. Isonicotinamide increased the apparent K_m value for base exchange without significantly affecting V_{max} (Figure 2A), consistent with a specific competitive effect on nicotinamide binding and a noncompetitive effect on NAD^+ and peptide binding (Figure 1C). The K_i for isonicotinamide is 68 mM based on these curves (Figure 2A). Isonicotinamide concentrations up to 100 mM inhibit base exchange but do not substantially affect rates of deacetylation in the absence of nicotinamide (Figure 2B). Nicotinamide inhibits deacetylation (Figure 2B) with good agreement between K_i (deacetylation) and K_m (exchange) at 0 mM isonicotinamide (Sauve and Schramm, 2003). Because isonicotinamide does not inhibit deacetylation but competitively inhibits base-exchange, isonicotinamide is predicted to relieve nicotinamide inhibition of deacetylation. As predicted, the K_i (deacetylation) values for nicotinamide increased with the isonicotinamide concentration (Figure 2B). Thus, isonicotinamide directly antagonizes nicotinamide inhibition of deacetylation by competitive inhibition with nicotinamide in the base-exchange reaction.

Activation of Sir2 Deacetylase Activity at Physiological Nicotinamide Concentrations

Nicotinamide concentrations as low as 50 μM are predicted to inhibit Sir2 catalysis in cells (Anderson et al., 2003; Gallo et al., 2004). We examined the effect of isonicotinamide on base-exchange and deacetylation activity in the presence of 125 μM [carbonyl-^{14}C]nicotinamide, which is within the proposed physiological range (see below). Base exchange is inhibited with increasing isonicotinamide concentration. Conversely, deacetylation activity increased by as much as 45% over the same isonicotinamide concentration range (Figure 2C). The inhibition of base exchange and the activation of deacetylation under these conditions suggest that functional control of Sir2 by nicotinamide can be relieved by isonicotinamide binding to the imidate-enzyme intermediate (Figures 1B and 1C).

A Nicotinamide Antagonist Increases Silencing by Sir2 in Yeast

Isonicotinamide is expected to increase gene silencing at Sir2-regulated loci if the normal endogenous level of nicotinamide inhibits Sir2 function in vivo (Figure 1C). To address this prediction, we examined the effect of isonicotinamide on the expression of reporter genes integrated at each of the chromosomal loci that are subject to Sir2-dependent transcriptional silencing. Silencing of a telomeric *URA3* gene (TEL-VIIL-*URA3*) confers resistance to 5-fluoroorotic acid (5-FOA). Isonicotinamide increased silencing of the telomeric *URA3* gene, as indicated by the >10-fold increase in colony growth on FOA-containing medium (Figure 3A). Notably, isonicotinamide had no effect on colony survival on nonselective medium. In agreement with the competitive binding mechanism (Figure 1), addition of isonicotin-

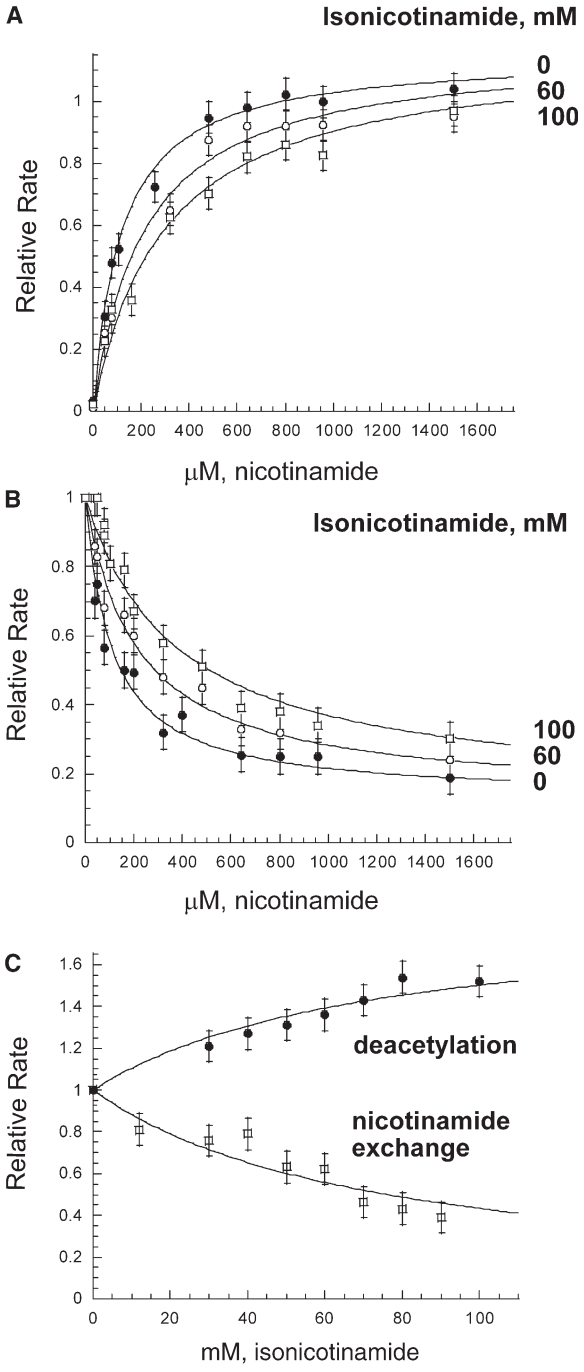

Figure 2. The Sir2 Catalyzed Base-Exchange Rate and Deacetylation Rate of a Histone H4 N-Terminal Peptide Was Measured as a Function of [carbonyl-^{14}C]nicotinamide Concentration

(A) Nicotinamide base-exchange rate was measured at different concentrations of isonicotinamide. The increase in apparent K_m for exchange is due to competitive inhibition by isonicotinamide with nicotinamide binding. Concentrations of isonicotinamide are 0, 60, and 100 mM. K_m values for nicotinamide of 115 ± 15, 210 ± 17, and 295 ± 25 μM and V_{max} values of 1.15 ± 0.04, 1.11 ± 0.06, and 1.10 ± 0.06, respectively, were determined from best fits of the points to the Michaelis-Menton equation. These values determine a K_i for isonicotinamide of 68 ± 11 mM.

(B) Deacetylation rate was measured as a function of ^{14}C nicotinamide concentration in reactions containing the same isonicotinamide concentrations used in (A). Inhibition curves are fit to the equation for partial inhibition: relative rate = 1 − f([I]/(K_i + [I])), where relative rate is defined on a scale of one based on the uninhibited rate (see Experimental Procedures). The value of f (the residual or uninihibited rate) was determined to be 0.14 ± 0.05. K_i values for nicotinamide were determined to be 110 ± 10, 212 ± 15, and 370 ± 40 μM at 0, 60, and 100 mM isonicotinamide, respectively, and reflect binding competition between nicotinamide, an inhibitor of deacetylation, and isonicotinamide, which is not an inhibitor of this reaction. From these values, a binding constant for isonicotinamide was determined to be 54 ± 12 mM.

(C) Base exchange and deacetylation rates were measured as a function of isonicotinamide concentration at a fixed, physiologically relevant concentration of nicotinamide (125 μM), which is inhibitory for Sir2-catalyzed deacetylation (K_i = 110 μM). The theoretical maximal extent of activation under these conditions is 1.87 ± 0.15. The binding constant for isonicotinamide determined from both the top and bottom curves in (C) is 70 ± 10 mM. For all three experiments (A–C) the average determined isonicotinamide binding constant is 64 ± 11 mM, placing it within experimental error for all three measurements.

Error bars are ± 7.5% of the absolute values as described in the Experimental Procedures.

amide to nicotinamide-containing medium, which inhibits its silencing (Bitterman et al., 2002), generated an intermediate growth phenotype (Figure 3A). The enhanced silencing effect of isonicotinamide on this telomeric reporter gene was especially pronounced (>10^3-fold) in a *dot1*Δ strain, which is defective in histone H3-lysine 79 methylation. In this strain, silencing is reduced by dispersion of the Sir proteins from the telomeres (van Leeuwen et al., 2002). Thus, enhanced telomeric silencing caused by isonicotinamide in the *dot1*Δ strain serves to demonstrate the Sir2 specificity of the effect. Silencing of a second telomeric marker in these strains (*ADE2* integrated at TEL-VR) was also increased by isonicotinamide (data not shown).

The effect of isonicotinamide on Sir2 activity at the silent mating-type loci was measured in an *HMR::TRP1* strain by growth on medium lacking tryptophan (Trp−) (Figure 3B). Silencing of *TRP1* decreases growth on Trp− media. Consistent with the ability of isonicotinamide to increase the activity of Sir2, growth on Trp− medium was reduced significantly (10^3- to 10^4-fold) compared to medium lacking the compound. Conversely, the decrease in silencing caused by nicotinamide resulted in increased growth on Trp− medium. Neither compound altered the growth phenotype of an isogenic *sir2*Δ strain. Thus, as demonstrated at telomeric loci (Figure 3A), the effects of nicotinamide and isonicotinamide are specific for Sir2 under these assay conditions. Isonicotinamide also increased Sir2 activity at *HML* as indicated by the enhanced growth of *HML::URA3* strains (UCC3515 and UCC4574; Singer, et al., 1998) on FOA-containing medium (data not shown).

Sir2 also localizes to the nucleolus where it functions to propagate a specialized chromatin structure on the rDNA (Rusche et al., 2003). The silencing of RNA pol II-transcribed genes inserted into the rDNA array is sensitive to *SIR2* gene dosage (Smith et al., 1998; Fritze et al. 1997) and is decreased by nicotinamide (Bitterman et al., 2002; Anderson et al., 2003; Gallo et al., 2004). The increased resistance (5- to 10-fold) of an *RDN1::URA3* strain to FOA indicates that isonicotinamide increases silencing at the rDNA locus (Figure 3C). Thus, isonico-

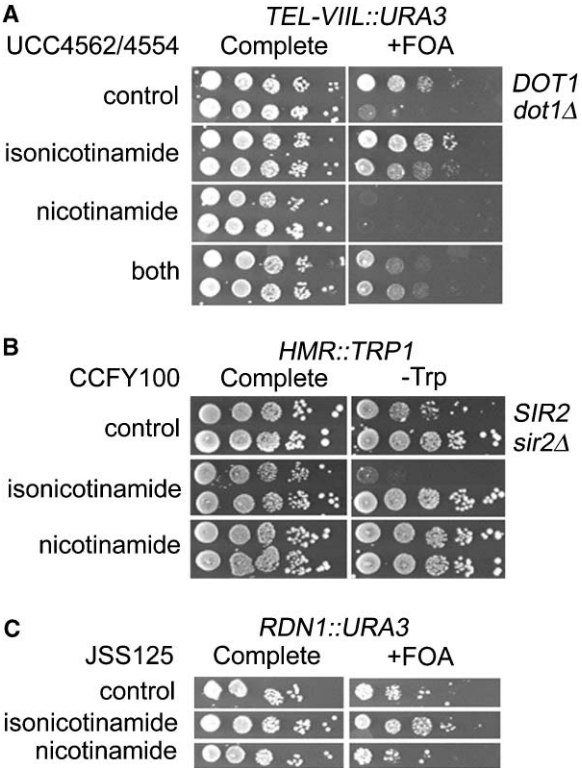

Figure 3. Isonicotinamide Increases Silencing at Sir2-Regulated Loci

10-fold serial dilutions of yeast strains were spotted onto control (YPD) or selective media containing 25 mM isonicotinamide, 5 mM nicotinamide, both compounds, or neither compound. Photographs were taken after incubation for 2 to 3 days.

(A) Silencing at telomere VIIL was monitored by *URA3* expression through increased survival on media containing FOA. Isogenic strains UCC4562 (*DOT1*) and UCC4554 (*dot1*Δ) (Singer et al., 1998) are shown in each panel (top and bottom rows, respectively).

(B) Silencing at the *HMR* locus was detected by *TRP1* expression through decreased survival on media lacking tryptophan. The phenotype of strain CCFY100 (Roy and Runge, 2000) (top row) is compared to an isogenic *sir2*Δ derivative (bottom row).

(C) Silencing of *URA3* expression at the rDNA locus of strain JSS125(S3) (Smith and Boeke, 1997) was assayed on FOA-containing media.

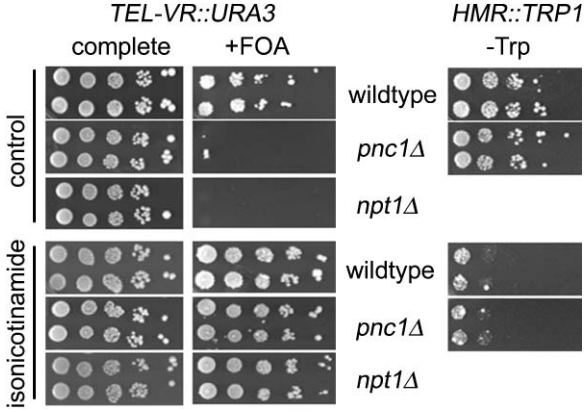

Figure 4. Activation of Silencing by Isonicotinamide Does Not Require *PNC1* or *NPT1*

10-fold serial dilutions of strain CCFY100 and isogenic *npt1*Δ and *pnc1*Δ derivatives were spotted onto YPD or selective media with or without 25 mM isonicotinamide. Silencing at TEL-VR::*URA3* is reported by survival on FOA-containing media and at *HMR*::*TRP1* is detected by reduced growth on media lacking tryptophan.

tinamide increases the activity of Sir2 in vivo at all three types of silent loci, albeit to different extents, consistent with the fact that different reporter genes were employed (van Leeuwen and Gottschling, 2002) and the differential sensitivity of the loci to perturbations in silencing (Gallo et al., 2004). For the telomeric and *HM* loci, the effect of isonicotinamide was demonstrated by using multiple reporter genes in both positive and negative selection assays.

Activation of Silencing by Isonicotinamide Is Not Dependent on NAD$^+$ Salvage Pathway Enzymes

Increased expression of the nicotinamidase encoded by *PNC1* has been observed in response to a variety of stress conditions (see Anderson et al., 2003) and is proposed to occur in calorie-restricted cells (Anderson et al., 2003). Overexpression of *PNC1* can enhance Sir2-dependent silencing, extend lifespan, and suppress the inhibitory effect of exogenous nicotinamide on these processes (Anderson et al., 2003; Gallo et al., 2004). To address whether enhanced silencing by isonicotinamide results from induction of *PNC1* as opposed to a direct effect on Sir2 (Figure 1B), we examined the effect of isonicotinamide in a *pnc1*Δ strain. Consistent with other studies (Gallo et al., 2004; Sandmeier et al., 2002), deletion of *PNC1* generates a silencing defect at a telomeric *URA3* gene (TEL-VR-*URA3*, Figure 4). This defect was readily reversed by the addition of isonicotinamide, as indicated by the pronounced ($>10^4$-fold) increase in colony growth on FOA-containing medium (Figure 4). Similarly, silencing at the *HMR* locus (*HMR::TRP1*) in the *pnc1*Δ strain was strongly enhanced by isonicotinamide and produced a dramatic (10^3-fold) reduction in growth on Trp− medium (Figure 4). These data demonstrate that isonicotinamide activation of Sir2 activity in vivo is independent of Pnc1.

In the NAD$^+$ salvage pathway, deamidation of nicotinamide by Pnc1 produces nicotinic acid, which is converted into the corresponding mononucleotide by the product of the *NPT1* gene. Deletion of *NPT1* lowers the intracellular NAD$^+$ concentration 2- to 3-fold, weakens transcriptional silencing, and abolishes lifespan extension by calorie restriction (Smith et al., 2000; Lin et al., 2000, 2004; Sandmeier et al., 2002). Nonetheless, isonicotinamide enhances the expression of a telomeric reporter gene in an *npt1*Δ strain (Figure 4). Thus, isonicotinamide activation of Sir2 is not dependent on Npt1 and occurs despite decreased NAD$^+$ levels. The ability of isonicotinamide to enhance transcriptional silencing in the presence and the absence of key NAD$^+$ salvage enzymes (Figures 3 and 4), together with the mechanistic knowledge of its action in antagonizing nicotinamide base exchange (Figures 1 and 2), provides compelling evidence that the endogenous level of nicotinamide limits Sir2 deacetylase activity under normal cellular conditions. Moreover, the data suggest that nicotimanide inhibition of Sir2 in vivo is incomplete in wt cells. This conclusion is supported by the fact that exogenous nic-

Table 1. Nicotinamide Levels in Yeast

Strain CCFY100	Cell Number ($\times 10^9$)	^{16}O Nicotinamide pmol[a]	μM[b] (cellular)	μM[b,c] (nuclear)
Wt	3.2	1150	9.6 $\pm$ 0.4	155 $\pm$ 14
pnc1Δ	3.2	14500	110	1800

[a] Total pmol of nicotinamide in cell lysates (see Experimental Procedures).
[b] Cellular and nuclear nicotinamide concentrations assume a uniform distribution.
[c] Nuclear nicotinamide concentration assumes that all the nicotinamide is in the nucleus.

otinamide or the absence of an NAD^+ salvage pathway, both of which are predicted to increase endogenous nicotinamide levels, inhibit Sir2 function, and thus enable substantially greater effects of isonicotinamide in antagonizing this inhibition (Figures 3A and 4). Accordingly, changes in endogenous nicotinamide levels resulting from altered cell physiology that may occur with the induction of *PNC1* under various stress conditions (Gallo et al., 2004 and references therein), are expected to regulate Sir2 activity. We note that these findings do not exclude the possibility that Sir2 activity may also be affected by changes in the NAD/NADH ratio (Lin et al., 2004).

Measurement of Cellular Nicotinamide Concentrations

Although genetic studies support a role for nicotinamide as an endogenous inhibitor of Sir2 activity (Anderson et al., 2003), quantitative determinations of nicotinamide levels in yeast cells have not been reported. To directly address this issue, ^{18}O nicotinamide was synthesized (Kolodziejska-Huben et al., 2002) and employed as an internal standard to measure nicotinamide in yeast cell lysates derived from wt and *pnc1*Δ strains (Table 1). Briefly, this was achieved by calculating the ratios of ^{16}O and ^{18}O nicotinamide peaks obtained by mass spectrometry of high-performance liquid chromatography (HPLC)-purified perchloric acid extracts (see Experimental Procedures). Nicotinamide formation in yeast is known to arise from enzymatic NAD^+ breakdown, but its distribution in the cell is unknown. Nonetheless, significant nuclear concentrations are expected based on the relative abundance of NAD^+-dependent deacetylases in this compartment (Ghaemmaghami et al., 2003; Huh et al., 2003). By assuming that nicotinamide is either distributed uniformly throughout the cell or restricted to the nucleus, we determined nicotinamide concentration limits of 10 μM and 150 μM, respectively, for the wt strain (Table 1). Importantly, within this concentration range, endogenous nicotinamide can impact Sir2 activity because the reported IC50 values for Sir2 inhibition range from 50 to 120 μM (Bitterman et al., 2002; Sauve and Schramm, 2003; Jackson et al., 2003).

Nicotinamide levels in the *pnc1*Δ strain were found to be more than 10-fold higher than those measured for the wt strain, with total cellular and nuclear concentration limits calculated as 110 and 1800 μM, respectively (Table 1). Thus, the profound deficits in silencing reported for *pnc1*Δ strains appear to be a consequence of significantly elevated nicotinamide levels on Sir2 activity as suggested previously (Anderson et al., 2003; Gallo et al., 2004). Together, the measurements of nicotinamide in wt and *pnc1*Δ strains confirm our conclusions based on the biochemical and in vivo effects of isonicotinamide, namely (1) endogenous nicotinamide levels inhibit Sir2 activity under normal conditions, and (2) stress conditions that alter nicotinamide levels can regulate Sir2 activity.

Nicotinamide Antagonism Activates Sir2 by a Different Mechanism than Resveratrol

The unusual mechanism of Sir2-catalyzed deacetylation permits unique opportunities for chemical intervention to enhance its enzymatic activity. Polyphenolic compounds such as resveratrol have been proposed to increase Sir2 deacetylation activity by changes in the Michaelis constant for both the acetylated substrate and NAD^+ (Howitz et al., 2003). In contrast, nicotinamide inhibition and isonicotinamide activation of Sir2 deacetylase activity is achieved without affecting substrate or NAD^+ binding by altering the proportion of imidate-enzyme complexes proceeding toward the deacetylated product (Figures 1C and 2). These findings suggest that combinations of mechanistically distinct small molecule activators of Sir2 may synergistically enhance deacetylase activity in vivo. Finally, we note that isonicotinamide and mechanistically similar Sir2 activators could be especially effective agonists of mammalian sirtuins, which are more potently inhibited by nicotinamide than the yeast Sir2 enzyme (Sauve and Schramm, 2003). Mouse Sir2, for example, has a biochemical potential to be activated 20-fold compared to 2.5- to 6-fold for the yeast enzyme (Figure 2B and Sauve and Schramm, 2003).

Experimental Procedures

Effects of Nicotinamide and Isonicotinamide on Sir2 Enzyme Activity

Yeast Sir2 was expressed and purified as described previously (Imai et al., 2000). Sir2-catalyzed exchange rate and deacetylation rate was measured by using an N-terminal histone H4 peptide (AGG[AcK]GG[AcK]GMG[AcK]VGA[AcK]RHSC) as a function of [carbonyl-^{14}C] nicotinamide concentration (Sauve and Schramm, 2003). Concentrations of isonicotinamide were set at 0, 60, and 100 mM, and varying concentrations of nicotinamide were used to assess base-exchange rate in 20 min incubations. HPLC separation of the reaction mixtures resolved adenosine diphosphoribose (ADPR), nicotinamide, isonicotinamide, acetyl-ADPR, and NAD. NAD peaks were collected, and radioactivity was measured by liquid scintillation counting. To ensure initial rate conditions, the amount of radioactivity in the NAD peak was always less than 10% of the nicotinamide peak. For all chromatograms, the amount of ADPR and 2′-O-acetyl adenosine diphosphoribose (AADPR) formed was determined by peak integration. Errors for each measurement did not exceed 5% of the value of the maximum rate. For each isonicotinamide concentration, curves of base exchange were constructed by best fit of the points to the Michaelis-Menton equation as a function of nicotinamide concentration with the maximal value of exchange measured in the absence of isonicotinamide assigned a value of 1.0. For de-

acetylation, the curves were fit to the equation: relative rate = 1 − f([I]/(K_i + [I])) where relative rate is defined on a scale of one based on the uninhibited rate. The constant f is the fractional inhibition attained by nicotinamide saturation, [I] is the concentration of nicotinamide, and K_i is the apparent nicotinamide inhibition constant (Sauve and Schramm, 2003).

To demonstrate activation of Sir2 activity by isonicotinamide, base-exchange and deacetylation rates were measured as above except that isonicotinamide concentrations were varied at a fixed, physiologically relevant concentration of [carbonyl-^{14}C]nicotinamide (125 μM) that is inhibitory for Sir2-catalyzed deacetylation (K_i = 110 μM). Errors for the individual measurements for both deacetylation and base exchange did not exceed 7.5% of the value of the maximum rate in each case. Values for the base-exchange reaction were fit to the equation: relative rate = 1 − ([I]/(K_i + [I])) where rates are measured on a scale of one based on the uninhibited base-exchange rate. [I] is the concentration of isonicotinamide and K_i is the apparent isonicotinamide binding constant. Deacetylation rates were fit to the equation: relative rate = 1 + f([I]/(K_i + [I])) where relative rate is defined on a scale of one based on the rate of deacetylation at 0 mM isonicotinamide. The constant f is the fractional activation attained by isonicotinamide saturation, [I] is the concentration of isonicotinamide, and K_i is the apparent isonicotinamide binding constant.

Quantitation of Nicotinamide

^{18}O nicotinamide was synthesized from ^{18}O-H_2O and 3-cyano-pyridine (Kolodziejska-Huben et al., 2002). The pure material was obtained by HPLC on a C-18 semipreparative column (Waters Delta-Pak) by elution with 50 mM ammonium acetate (pH 7.0) and peak detection at 260 nm. ^{18}O nicotinamide eluted with a retention time identical to an authentic standard. Masses for all experiments were determined by flow injection analysis with an LTQ quadruple linear ion trap mass spectrometer equipped with an ESI source (Thermo-Finnigan, San Jose, CA). Samples in 50% acetonitrile 0.1% TFA were delivered to the mass spectrometer operating in normal scan mode to detect ions in the m/z range of 100 to 150. Continuously acquired spectra detected nicotinamide as two ions (125 and 123 mass units) corresponding to the ^{18}O- and ^{16}O-labeled cations, respectively, with a relative abundance of 96.5:3.5 (96.5% ^{18}O enrichment). A 240 μM standard solution was used to prepare a 2 μM ^{18}O nicotinamide, 1 M perchloric acid solution for subsequent experiments.

Yeast cells, grown in YC synthetic media (van Leeuwen and Gottschling, 2002) to early log phase, were lysed by glass bead breakage in a minibeadbeater in 2 volumes 1 M perchloric acid containing 2 μM ^{18}O nicotinamide. Breakage was monitored by light microscopy and was greater than 90%. Yeast cell number was determined by optical density and confirmed by haemocytometer counting. The lysate was cleared by centrifugation, neutralized, and injected on a C-18 semipreparative column. Nicotinamide fractions were collected at 23–25 min, lyophilized, and redissolved in 10 μl 50% acetonitrile, 0.1% TFA for direct infusion ESI-MS. Peak areas were determined by integration of total ion at mass 122.8–123.2 amu and at 124.8–125.2 amu, and the peak ratios were determined. ^{16}O pmol = (123 ion area/125 ion area − 0.035) × ^{18}O pmol. Appropriate blanks and standards were analyzed together with the spent media, which was found to contain 0.08 μM and 1.0 μM nicotinamide for the wt and *pnc1*Δ strains, respectively. An average nuclear volume for an asynchronous cell population of 2.46 μm^3 (Winey et al., 1997) and an average cellular volume of 42 μm^3 (Jorgensen et al., 2002) were used to calculate nicotinamide concentrations assuming even distribution throughout either the nucleus or the entire yeast cell.

Yeast Strains and Media

Yeast strains were grown at 30°C on YC or HC synthetic media (van Leeuwen and Gottschling, 2002) supplemented (or not) with nicotinamide (5 mM) or isonicotinamide (25 mM), where indicated. *NPT1*, *SIR2*, and *PNC1* were deleted by one-step, PCR-mediated gene replacement of the entire coding region with NatMX4 (Goldstein and McCusker, 1999) amplified from p4339 (Tong et al. 2004). All gene deletions were confirmed by diagnostic PCR. Strains were grown to log phase (OD < 3.0) in synthetic complete liquid media, washed in H_2O, and resuspended at OD 0.5 to prepare 10-fold serial dilutions in H_2O. Each dilution (7 μl) was spotted with a multichannel pipettor onto solid media, and plates were incubated for 2–3 days at 30°C before photography. Silencing phenotypes were evaluated on synthetic complete and test media (± nicotinamide and/or isonicotinamide) prepared from the same media batch at the same time under identical growth conditions as recommended (van Leeuwen and Gottschling, 2002).

Silencing Assays

Telomeric silencing was reported at TEL-VIIL::*URA3* and at TEL-VR::*ADE2* in isogenic strains UCC4562*(DOT1)* and UCC4554*(dot1*Δ*)* (Singer et al., 1998) and at TEL-VR::*URA3* in CCFY100 (Roy and Runge, 2000). A decrease in *URA3* expression was detected by increased survival on HC media containing 0.1% 5-FOA (Toronto Research Chemicals). Silencing of *ADE2* expression was monitored by colony color on YC media. The accumulation of red pigment, indicating a block in adenine biosynthesis, was developed in grown colonies by shifting the plates to 4°C for a further 4 days. Silencing at the *HM* loci was detected by a decrease in *TRP1* or *URA3* expression. *HMR* silencing was measured at *HMR*::*TRP1* in CCFY100 and an isogenic *sir2*Δ derivative by the failure to grow on HC media lacking tryptophan. *HML* silencing was monitored by survival on FOA-containing media at *HML*::*URA3* in strain UCC3515 (Singer et al., 1998). Silencing at the rDNA locus was also assayed by FOA survival in strain JSS125(S3) (Smith and Boeke, 1997).

Acknowledgments

We thank Dan Gottschling and Kurt Runge for strains and Fang Wang for mass analysis. The LTQ mass spectrometer was obtained under a shared instrument grant to Dr. Ruth Hogue Angeletti. This work was supported by National Institutes of Health grants to V.L.S. and I.M.W.

Received: July 7, 2004
Revised: November 16, 2004
Accepted: December 14, 2004
Published: February 17, 2005

References

Anderson, R.M., Bitterman, K.J., Wood, J.G., Medvedik, O., Cohen, H., Lin, S.S., Manchester, J.K., Gordon, J.I., and Sinclair, D.A. (2002). Manipulation of a nuclear NAD^+ salvage pathway delays aging without altering steady-state NAD^+ levels. J. Biol. Chem. *277*, 18888–18890.

Anderson, R.M., Bitterman, K.J., Wood, J.G., Medvedik, O., and Sinclair, D.A. (2003). Nicotinamide and PNC1 govern lifespan extension by calorie restriction in *Saccharomyces cerevisiae*. Nature *423*, 181–185.

Bitterman, K.J., Anderson, R.M., Cohen, H.Y., Latorre-Esteves, M., and Sinclair, D.A. (2002). Inhibition of silencing and accelerated aging by nicotinamide, a putative regulator of yeast Sir2 and human SIRT1. J. Biol. Chem. *277*, 45099–45107.

Fritze, C.E., Verschueren, K., Strich, R., and Easton Esposito, R. (1997). Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA. EMBO J. *16*, 6495–6509.

Gallo, C.M., Smith, D.L., Jr., and Smith, J.S. (2004). Nicotinamide clearance by Pnc1 directly regulates Sir2-mediated silencing and longevity. Mol. Cell. Biol. *24*, 1301–1312.

Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., and Weissman, J.S. (2003). Global analysis of protein expression in yeast. Nature *425*, 737–741.

Goldstein, A. L. and McCusker, J. H. (1999). Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. Yeast *15*, 1541–1553.

Hekimi, S., and Guarente, L. (2003). Genetics and the specificity of the aging process. Science *299*, 1351–1354.

Howitz, K.T., Bitterman, K.J., Cohen, H.Y., Lamming, D.W., Lavu, S.,

Wood, J.G., Zipkin, R.E., Chung, P., Kisielewski, A., Zhang, L.L., et al. (2003). Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nature *425*, 191–196.

Huh, W.K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S., and O'Shea, E.K. (2003). Global analysis of protein localization in budding yeast. Nature *425*, 686–691.

Imai, S., Armstrong, C.M., Kaeberlain, M., and Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature *403*, 795–800.

Jackson, M.D., Schmidt, M.T., Oppenheimer, N.J., and Denu, J.M. (2003). Mechanism of nicotinamide inhibition and transglycosidation by Sir2 histone/protein deacetylases. J. Biol. Chem. *278*, 50985–50998.

Jorgensen, P., Nishikawa, J.L., Breitkreutz, B.J., and Tyers, M. (2002). Systematic identification of pathways that couple cell growth and division in yeast. Science *297*, 395–400.

Kaeberlein, M., McVey, M., and Guarente, L. (1999). The Sir2/3/4 complex and Sir2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. Genes Dev. *13*, 2570–2580.

Kaeberlein, M., Andalis, A.A., Fink, G.R., and Guarente, L. (2002). High osmolarity extends life span in *Saccharomyces cerevisiae* by a mechanism related to calorie restriction. Mol. Cell. Biol. *22*, 8056–8066.

Kolodziejska-Huben, M., Kaminski, Z., and Paneth, P. (2002). Synthesis of ^{18}O-labelled nicotinamide. J. Labelled Comp. Radiopharm. *45*, 1–6.

Landry, J., Sutton, A., Tafrov, S.T., Heller, R.C., Stebbins, J., Pillus, L., and Sternglanz, R. (2000). The silencing protein Sir2 and its homologs are NAD-dependent deacetylases. Proc. Natl. Acad. Sci. USA *97*, 5807–5811.

Lin, S.J., Deffossez, P.A., and Guarente, L. (2000). Requirement of NAD and *SIR2* for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. Science *289*, 2126–2128.

Lin, S.J., Kaeberlein, M., Andalis, A.A., Sturtz, L.A., Defossez, P.A., Culotta, V.C., Fink, G.R., and Guarente, L. (2003). Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. Nature *418*, 344–348.

Lin, S.J., Ford, E., Haigis, M., Liszt, G., and Guarente, L. (2004). Calorie restriction extends yeast life span by lowering the level of NADH. Genes Dev. *18*, 12–16.

Luo, J., Nikolaev, A.Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001). Negative control of p53 by Sir2a promotes cell survival under stress. Cell *107*, 137–148.

Roy, N., and Runge, K.W. (2000). Two paralogs involved in transcriptional silencing that antagonistically control yeast life span. Curr. Biol. *10*, 111–114.

Rusche, L.N., Kirchmaier, A.L., and Rine, J. (2003). The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu. Rev. Biochem. *72*, 481–516.

Sandmeier, J.J., Celic, I., Boeke, J.D., and Smith, J.S. (2002). Telomeric and rDNA silencing in Saccharomyces cerevisiae are dependent on a nuclear NAD^+ salvage pathway. Genetics *160*, 877–889.

Sauve, A.A., and Schramm, V.L. (2003). Sir2 regulation by nicotinamide results from switching between base exchange and deacetylation chemistry. Biochemistry *42*, 9249–9256.

Sauve, A.A., Celic, I., Avalos, J., Deng, H., Boeke, J.D., and Schramm, V.L. (2001). Chemistry of gene silencing: the mechanism of NAD^+-dependent deacetylation reactions. Biochemistry *40*, 15456–15463.

Singer, M.S., Kahana, A., Wolf, A.J., Meisinger, L.L., Peterson, S.E., Goggin, C., Mahowald, M., and Gottschling, D.E. (1998). Identification of high-copy disruptors of telomeric silencing in Saccharomyces cerevisiae. Genetics *150*, 613–632.

Smith, J.S., and Boeke, J.D. (1997). An unusual form of transcriptional silencing in yeast ribosomal DNA. Genes Dev. *11*, 241–254.

Smith, J.S., Brachmann, C.B., Pillus, L., and Boeke, J.D. (1998). Distribution of a limited Sir2 protein pool regulates the strength of yeast rDNA silencing and is modulated by Sir4p. Genetics *149*, 1205–1219.

Smith, J.S., Brachmann, C.B., Celic, I., Kenna, M.A., Muhammad, S., Starai, V.J., Avalos, J.L., Escalante-Semerena, J.C., Grubmeyer, C., Wolberger, C., and Boeke, J.D. (2000). A phylogenetically conserved NAD^+-dependent protein deacetylase activity in the Sir2 protein family. Proc. Natl. Acad. Sci. USA *97*, 6658–6663.

Tissenbaum, H.A., and Guarente, L. (2001). Increased dosage of a *sir-2* gene extends lifespan in *Caenorhabditis elegans*. Nature *410*, 227–230.

Tong, A.H., Lesage G., Bader G.D., Ding H., Xu H., Xin X., Young J., Berriz G.F., Brost R.L., Chang M., et al. (2004). Global mapping of the yeast genetic interaction network. Science *303*, 808–813.

van Leeuwen, F., and Gottschling, D.E. (2002). Assays for gene silencing in yeast. Methods Enzymol. *350*, 165–186.

van Leeuwen, F., Gafken, P.R., and Gottschling, D.E. (2002). Dot1p modulates silencing in yeast by methylation of the nucleosome core. Cell *109*, 745–756.

Vaziri, H., Dessain, S.K., Ng Eaton, E., Imai, S.I., Frye, R.A., Pandita, T.K., Guarente, L., and Weinberg, R.A. (2001). hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell *107*, 149–159.

Winey, M., Yarar, D., Giddings, T.H., Jr., and Mastronarde, D.N. (1997). Nuclear pore complex number and distribution throughout the Saccharomyces cerevisiae cell cycle by three-dimensional reconstruction from electron micrographs of nuclear envelopes. Mol. Biol. Cell. *8*, 2117–2132.