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Substrate-specific Activation of Sirtuins by Resveratrol*

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Resveratrol, a small molecule found in red wine, is reported to slow aging in simple eukaryotes and has been suggested as a potential calorie restriction mimetic. Resveratrol has also been reported to act as a sirtuin activator, and this property has been proposed to account for its anti-aging effects. We show here that resveratrol is a substrate-specific activator of yeast Sir2 and human SirT1. In particular, we observed that, *in vitro*, resveratrol enhances binding and deacetylation of peptide substrates that contain Fluor de Lys, a non-physiological fluorescent moiety, but has no effect on binding and deacetylation of acetylated peptides lacking the fluorophore. Consistent with these biochemical data we found that in three different yeast strain backgrounds, resveratrol has no detectable effect on Sir2 activity *in vivo*, as measured by rDNA recombination, transcriptional silencing near telomeres, and life span. In light of these findings, the mechanism accounting for putative longevity effects of resveratrol should be reexamined.

Sir2-family proteins (sirtuins) are Class III protein deacetylases conserved from prokaryotes to mammals (1, 2). Sirtuins have been implicated in several important cellular processes, including genomic stability (3, 4), DNA repair (5–7), transcriptional silencing (8–11), p53-mediated apoptosis (12, 13), and adipogenesis (14). In addition, Sir2-orthologs have been shown to promote longevity in yeast (4), worms (15), and flies (16), supporting the hypothesis that sirtuins may act as evolutionarily conserved regulators of aging (17).

One cause of aging in yeast is the accumulation of extrachromosomal rDNA circles in the mother cell nucleus (18). Extrachromosomal rDNA circles are self-replicating and asymmetrically segregated to the mother cell during mitosis, leading to greatly elevated levels in aged cells. The formation of extrachromosomal rDNA circles requires homologous recombination between rDNA repeat units (19, 20) in a manner antagonized by yeast Sir2 (3). Deletion of Sir2 increases rDNA recombination and shortens life span, whereas overexpression has the opposite effect (4). In addition to promoting rDNA stability, Sir2 acts with Sir3 and Sir4 to repress transcription at the silent mating loci (10, 21) and near telomeres (11). Sir2, independently of Sir3 and Sir4, also represses the transcription of genes transcribed by DNA polymerase II that are integrated into the rDNA (8, 9).

The ability of Sir2 to repress transcription, promote rDNA stability, and increase life span requires functional histone deacetylase activity (22). Histone deacetylation by Sir2 proceeds by a novel reaction in which NAD^+ is consumed, resulting in the production of *O*-acetyl-ADP-ribose and nicotinamide (23–25). Nicotinamide cleavage occurs prior to transfer of the acetyl group (26), and nicotinamide has been shown to inhibit Sir2-dependent deacetylation both *in vitro* and *in vivo* at a concentration of ~5 mM (27).

Recently, there has been much interest in characterizing small molecules that modify the ability of sirtuins to deacetylate substrate proteins. In addition to nicotinamide, several specific inhibitors of Sir2 have been described, including splitomicin (28), splitomicin analogues (29, 30), sirtinol (31), and several highly potent and selective inhibitors of SirT1 uncovered by high throughput screening (32). Sir2 inhibitors are effective at blocking Sir2-dependent transcriptional repression *in vivo*, although the effect on life span has not been examined. Activators of sirtuins have also been sought, with limited success. One report (33) describes several polyphenolic compounds that increase the catalytic activity of human SirT1. Of these, resveratrol, an agent found in red wine, increased deacetylation of a modified p53 peptide substrate ~13-fold for SirT1 and 2-fold for yeast Sir2 (33).

The ability of resveratrol to activate Sir2 *in vivo* has been examined by growing yeast cells in the presence of 10–500 μM concentration of this compound (33). This treatment is reported to increase life span by up to 100% in the PSY316 strain background and reduce rDNA recombination by 5-fold in the W303 strain background. These phenotypes were attributed to presumed activation of Sir2 by resveratrol. Paradoxically, however, no effect on Sir2-dependent transcriptional silencing at telomeres or rDNA was observed in response to resveratrol (33). More recently, resveratrol has also been reported to modestly increase life span in both flies and worms in the presence, but not in the absence, of the Sir2 orthologs dSir2 and Sir-2.1 (34).

Calorie restriction is the only intervention known to increase

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life span in yeast, worms, flies, and mammals (35), and resveratrol has been proposed to be a potential CR[1] mimetic (36). The mechanism by which CR increases replicative life span in yeast had been thought to require activation of Sir2 (37, 38); however, it was recently discovered that life span extension by CR is independent of Sir2, as long as extrachromosomal rDNA circle levels are kept low (39). Because the molecular mechanism by which CR increases life span in yeast remains unknown, we wished to determine whether resveratrol was involved in this process. Here we describe the results of experiments carried out in three different strain backgrounds to test the *in vivo* efficacy of resveratrol as an activator of yeast Sir2. We also report biochemical data demonstrating that resveratrol is a substrate-specific activator of Sir2 orthologs.

MATERIALS AND METHODS

Strains and Media—Three yeast strain backgrounds were employed in this analysis: W303R, BY4742, and PSY316. W303R is as described (4). BY4742 was obtained from Research Genetics and is as described (39). Three variants of PSY316 were used for this analysis: PSY316AR, PSY316AT, and PSY316AUT. PSY316AR is as described (38) and contains the *ADE2* gene integrated into an rDNA repeat. PSY316AT has *ADE2* integrated near a telomere and was generously provided by D. Sinclair (33). PSY316AUT has both *URA3* and *ADE2* integrated near the telomeres. Strains overexpressing *SIR2* were constructed by genomic integration of an extra copy of *SIR2* at the *LEU2* locus, as described (4). Strains lacking *SIR2* were created by replacing the *SIR2* open reading frame with *HIS3* using a PCR-generated disruption construct, as described (39).

Resveratrol—Resveratrol was purchased from Biomol Research Laboratories, Inc. or Sigma Inc., as noted. For all *in vivo* experiments in yeast, resveratrol was dissolved at a concentration of 100 mg/ml in ethanol and stored at −20 °C. Me_2SO was tested as a solvent for yeast life span experiments in BY4742 and PSY316AR, with no significant difference from ethanol observed (not shown). The SIRT1 fluorescent activity assay/drug discovery kit was purchased from Biomol Research Laboratories, Inc. and used according to the manufacturer's instructions.

Telomere Silencing and rDNA Recombination—Transcriptional silencing of the *ADE2* marker in PSY316AT was monitored by streaking or patching cells on YPD (1% yeast extract, 2% peptone, 2% glucose). Low levels of *ADE2* expression result in enhanced color formation. Transcriptional silencing of the *URA3* marker in PSY316AUT was determined by survival on medium supplemented with 5-fluoroorotic acid, which is toxic to cells expressing *URA3* (40). Cells were cultured in liquid YPD or YPD supplemented with 100 μM resveratrol overnight, diluted to an appropriate density in water, and plated onto synthetic complete (SC) or 5-fluoroorotic acid media, containing or lacking resveratrol, as noted. Percent survival was calculated as the number of colonies arising on 5-fluoroorotic acid medium divided by the number of colonies arising on SC medium. Recombination in the rDNA of strain W303R was estimated by determining the rate at which the *ADE2* marker gene is lost from the rDNA array. This was accomplished by growing cells overnight in YPD or YPD supplemented with 100 μM resveratrol, plating the cells onto YPD or YPD supplemented with 100 μM resveratrol, and counting the number of half-red/half-white colonies that arose, which indicates loss of the *ADE2* marker during the first cell division after plating. The rate of rDNA marker loss is calculated as the number of half-red/half-white colonies divided by the total number of colonies formed (4).

Yeast Life Span Assays—Yeast life span analysis was carried out as described (33), with the exception that strains were coded such that the researcher performing the life span experiment did not know the identity of the strains being analyzed. Resveratrol and control medium were prepared the night prior to starting each experiment. Cells were grown in the presence of compound overnight (~12 h), prior to micromanipulation of virgin cells for life span analysis. For the experiment in Fig. 3*D*, a slightly modified protocol was used in which the resveratrol stock solution and media were prepared using only glass containers. *p* values for life span assays were generated by a Wilcoxon Rank-Sum test. Statistical significance was determined with a *p* value cutoff of 0.05.

[^{3}H]Acetate Release Assays—The histone deacetylase assay was performed using bacterially expressed and purified GST fusion proteins as previously described (28). For deacetylation assays chemically acetylated [^{3}H]acetyl-H4 peptide was incubated with or without 500 μM NAD^+, 0.1 μg of GST-enzyme in a buffer containing 150 mM NAD, 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 2.5% glycerol, and 0.5% Me_2SO. After 3 h of incubation at 37 °C the reaction was quenched by addition of 5 μl of 1 N HCl with 0.15 N acetic acid. Released [^{3}H]acetic acid was extracted with 400 μl of ethyl acetate and counted in 5 ml of scintillation fluid.

[^{14}C]Nicotinamide Release Assays—SirT1 activity was also measured by monitoring [^{14}C]nicotinamide release as described (41). Deacetylase reactions were carried out in a total volume of 16 μl containing 25 mM Tris acetate (pH 7.5), 250 mM sodium acetate, 2.7 mM potassium acetate, 1 mM magnesium chloride, 0.05% Tween 20 (v/v), and 65 ng of bacterially expressed His-tagged human SIRT1 enzyme. NAD^+, [*carbonyl*-^{14}C]NAD^+, and peptide substrate were included at concentrations described in the figure legends. The reactions were initiated by the addition of enzyme. The p53-peptide is a partial human p53 sequence comprised of residues His-368 through Lys-386 with an acetylated lysine at position 382 (BIOSOURCE International, NH_2-HLKSKKGQSTSRHK(K-Ac) LMFK-OH) resuspended in water to a concentration of 10 mM and stored at −20 °C. The H4 peptide was obtained from Upstate Cell Signaling Solutions (SGRGKGGKGLGKGGA(K_{-Ac})-RHRC), Catalog number 12-346. Fluor de Lys substrates were obtained from Biomol Research Laboratories, Inc. Resveratrol was prepared in Me_2SO and added to a final concentration ranging from 0.1 to 200 μM. The Me_2SO concentration in each reaction was <2% to avoid enzyme inactivation. Released [^{14}C]nicotinamide product was isolated using filtration-based chromatography with boronate resin (Pierce) and counted in a liquid scintillation counter. Raw data were analyzed using non-linear regression with GraphPad Prism 4.02.

RESULTS

Sir2-independent life span extension by CR has been observed in the long-lived BY4742 strain background; however, the majority of reports examining CR in yeast have used the shorter lived PSY316 strain background (39). CR by growth on low glucose, or by several genetic models, reproducibly increases life span in PSY316 by ~35% (37, 38, 42–44), whereas growth in the presence of 10–100 μM resveratrol is reported to enhance life span by up to 100% in this strain background (33).

We had found that, unlike the case for the BY4742 or W303R strains, overexpression of *SIR2* fails to increase life span in PSY316AR (39). One possible explanation for this apparent discrepancy is that Sir2 activity is not increased in response to elevated *SIR2* dosage in PSY316. We therefore used PSY316AT and PSY316AUT variants, with *ADE2* or both *ADE2* and *URA3*, respectively, integrated near a telomere, to assess Sir2 activity in response to *SIR2* overexpression. PSY316AT and PSY316AUT are congenic to PSY316AR, except for the location of the *ADE2* and *URA3* marker genes (see "Materials and Methods"). As previously seen for PSY316AR (39), overexpression of *SIR2* had no effect on life span in PSY316AT (Fig. 1*A*). Sir2-dependent silencing of both the *URA3* (Fig. 1*B*) and *ADE2* (Fig. 1*C*) genes integrated near telomeres was increased, however, indicating that Sir2 activity was elevated in these cells. Therefore, increased Sir2 activity due to overexpression of the protein failed to increase life span in the PSY316 genetic background.

Because resveratrol is reported to increase the life span in PSY316AT, but activation of Sir2 does not increase life span in this strain, we speculated that resveratrol might be acting as a CR mimetic and enhancing yeast longevity by a Sir2-independent mechanism. We therefore tested the effect of resveratrol on the life span in BY4742, a strain background in which the longevity effects of CR and Sir2 are separable (39). At a final concentration of either 10 or 100 μM, resveratrol obtained from Biomol failed to significantly increase either mean or maximum life span (Fig. 2*A*). Similarly, no effect on life span was observed using a second source of resveratrol obtained from Sigma (Fig. 2*B*). We were concerned that perhaps the resveratrol used for these experiments had degraded or otherwise lost

[1] The abbreviations used are: CR, calorie restriction; FdL, Fluor de Lys.

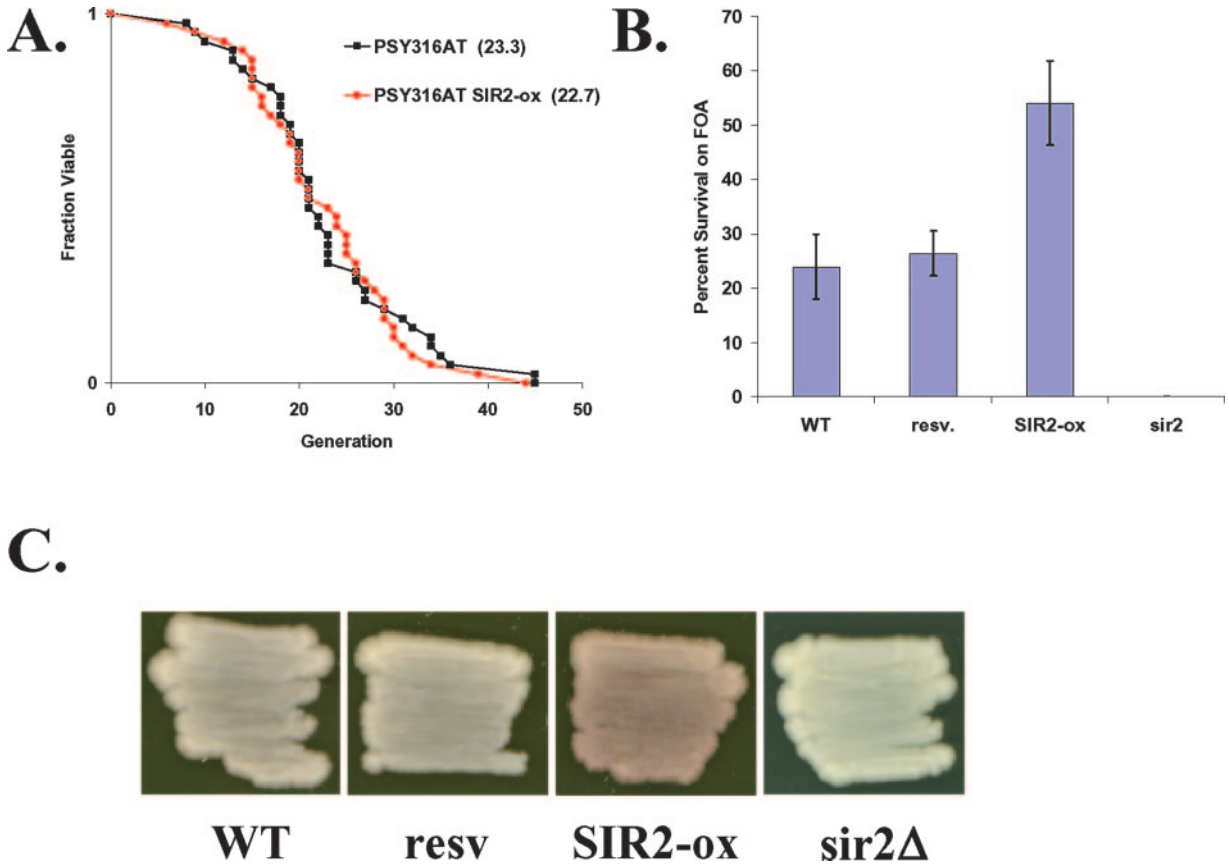

FIG. 1. **Effect of Sir2-overexpression on silencing and life span in PSY316.** *A*, overexpression of Sir2 fails to significantly increase life span in PSY316AT ($p = 0.9$). Life spans were determined for PSY316AT (■) and PSY316AT SIR2-ox (●). Mean life span for each strain is shown in parentheses. *B*, transcriptional silencing of *URA3* integrated near telomeres is enhanced by overexpression of *SIR2* but not by resveratrol in PSY316AUT. *C*, transcriptional silencing of *ADE2* integrated near telomeres is enhanced by overexpression of *SIR2*, but not by resveratrol (*resv*), in PSY316AT. *WT*, wild type. *FOA*, 5-fluoroorotic acid.

the ability to activate Sir2. We examined this possibility by using the SIRT1 fluorescent activity assay/drug discovery kit, which utilizes the Fluor de Lys method for determination of *in vitro* activity of SirT1. Based on results obtained with this assay, we found that the resveratrol from both sources activated SirT1 4–8-fold at a concentration of 100 μM (Fig. 2*C*). This magnitude of activation is consistent with that previously reported (33) and is comparable with control resveratrol included with the assay kit.

Resveratrol was previously reported to increase life span by up to 100% in the short lived PSY316AT strain (33). The inability of resveratrol to increase life span in BY4742 suggested that resveratrol might act in a strain-specific manner. To determine the generality of resveratrol as a putative CR mimetic, we tested the effects of resveratrol on life span in W303R, another short lived strain commonly used in yeast aging research. Unlike in PSY316, overexpression of *SIR2* is known to increase life span in W303R (4). However, similar to our results with BY4742, we were unable to detect any significant increase in either the mean or maximum life span of W303R mother cells in response to resveratrol (Fig. 3*A*). In contrast to the prior report (33), we also found that resveratrol had no significant effect on rDNA recombination in W303R (Fig. 3*B*).

Given that we found no longevity effect from resveratrol treatment in either BY4742 or W303R, we wished to reproduce the previously observed (33) life span extension by resveratrol in PSY316. We observed a marginal increase in life span in response to resveratrol at 10 μM ($p = 0.16$) in the PSY316AR strain (Fig. 3*C*); however, the magnitude of the effect was much reduced compared with that seen by Howitz *et al.* (33) (12% increase in mean RLS *versus* 60–100% reported). Similarly, we observed only a slight effect on life span in the PSY316AT strain (7% increase, $p = 0.29$) (Fig. 3*D*). Consistent with the prior report (and unlike the case for overexpression of *SIR2*), we also found that resveratrol has no effect on Sir2-dependent transcriptional silencing in this strain (Fig. 1, *B* and *C*).

Because we were unable to detect significant phenotypic changes associated with resveratrol in several yeast strain backgrounds (Figs. 1–3), yet we had verified that the resveratrol used for these experiments was active by the Fluor de Lys (FdL) assay *in vitro* (Fig. 2*C*), we wished to further examine the biochemical interaction between sirtuins and resveratrol. The Fluor de Lys assay is a relatively new biochemical method for measuring deacetylation of a chemically modified acetylated peptide substrate coupled to aminomethylcoumarin. Upon deacetylation, the aminomethylcoumarin group is proteolytically cleaved resulting in fluorescence (45).

To further evaluate the properties of resveratrol as a putative sirtuin activator, we utilized a well characterized deacetylation assay in which the acetyl group of histone H4 substrate is radiolabeled with ^{3}H (see Refs. 26 and 46). This assay has been used to measure the relative deacetylase activity of different mutant forms of yeast Sir2, and the level of Sir2 deacetylase activity is known to correlate with silencing in yeast, rDNA recombination, and life span phenotypes (22, 28). Surprisingly, in this context, we did not find any stimulatory effect of resveratrol on NAD^+-dependent [^{3}H]acetate release from the histone H4 substrate using SirT1, SirT2, or Sir2 enzymes (Fig. 4*A*). Interestingly, although resveratrol had a large stimula-

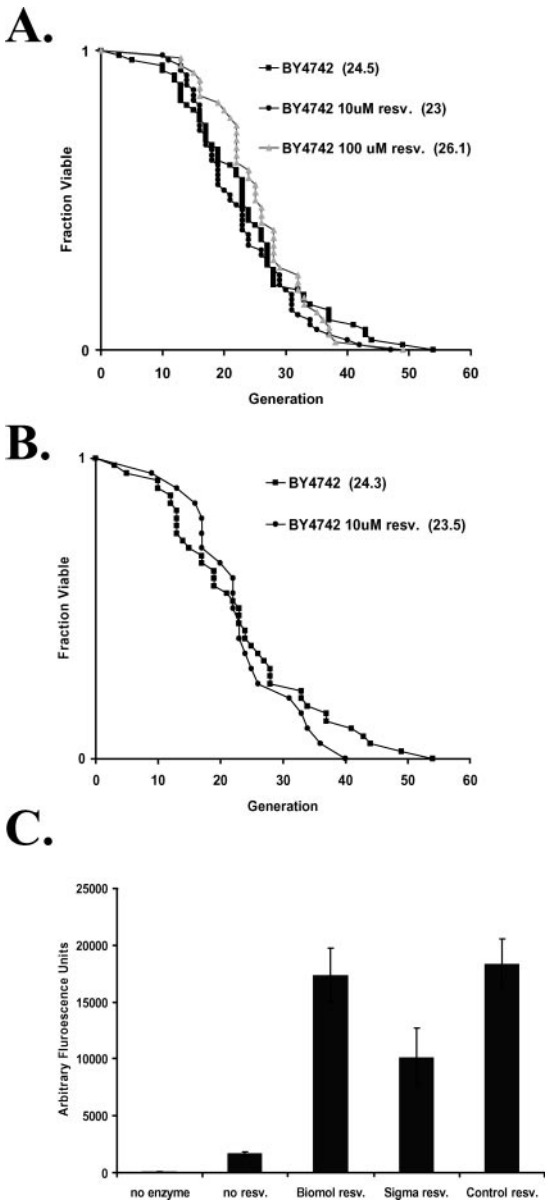

FIG. 2. **Resveratrol has no significant effect on life span in BY4742.** *A*, resveratrol obtained from Biomol Inc. fails to significantly alter life span in BY4742 at either 10 μM ($p = 0.6$) or 100 μM ($p = 0.2$). Life spans were determined for BY4742 mother cells grown in the absence of drug (■), or in the presence of either 10 μM (●) or 100 μM (▲) resveratrol (*resv*). Mean life span for each strain is shown in parentheses. *B*, resveratrol obtained from Sigma Inc. also fails to significantly alter life span in BY4742. Life spans were determined for BY4742 mother cells in the absence (■) or presence (●) of 10 μM resveratrol. *C*, resveratrol used for life span experiments was tested for activity using the SIRT1 fluorescent activity assay/drug discovery kit from Biomol Inc. Deacetylation of the FdL-p53 peptide by SirT1 is enhanced by 100 μM resveratrol purchased from either Biomol Inc. or from Sigma Inc. Control resveratrol is resveratrol included with the kit.

tory effect on SirT1 using the Fluor de Lys assay and FdL-p53 substrate, resveratrol failed to activate SirT2 in either assay (Fig. 4*B*). SirT2 is most closely related to the yeast homolog of Sir2, Hst2. These data suggest that rather than being a general activator of sirtuins, resveratrol specifically stimulates Sir2 orthologs (*e.g.* yeast Sir2 and human SirT1), and it does so in a substrate-specific manner.

These findings suggested a plausible explanation for the lack of phenotypes observed in yeast cells treated with resveratrol (Figs. 2, 3) and indicated that perhaps the Fluor de Lys group is necessary for resveratrol-mediated activation of Sir2 orthologs. To better characterize this apparent substrate specificity, we used a second radioisotopic assay to determine the effect of resveratrol on human SirT1 (41). In this assay, the nicotinamide group of NAD^+ is labeled with ^{14}C. Because deacetylation of the acetyl-lysine substrate is coupled to the breakage of the glycosidic bond in NAD^+, release of [^{14}C]nicotinamide can be used as a measure of substrate deacetylation (Fig. 5*A*).

Using the [^{14}C]nicotinamide release assay, we examined the effect of resveratrol on SirT1 deacetylation of a p53 peptide substrate or an H4 peptide substrate, either containing or lacking the Fluor de Lys group. Similar to the results seen with the ^3H-release assay, resveratrol induced concentration-dependent enhancement of deacetylation for only the substrates containing Fluor de Lys and not the native peptides (Fig. 5, *B* and *C*). Thus, activation of SirT1 by resveratrol appears to be specific for substrates containing Fluor de Lys, regardless of the assay format.

To examine whether the Fluor de Lys group alters affinity of the SirT1 enzyme for peptide substrates, we compared the K_ms of the p53 substrate with or without the Fluor de Lys group. We measured deacetylation of the p53 substrate at a range of peptide concentrations and a fixed concentration of NAD^+ (Fig. 6*A*). The K_m for Fluor de Lys containing substrate was 87.6 ± 19 μM, which is about 9-fold higher than the K_m of the native p53 peptide (10.3 ± 2.6 μM) (Table I). The maximal velocity, V_{max}, of the deacetylation reaction was not decreased by the presence of the Fluor de Lys group. We also determined the effect of the Fluor de Lys group on the K_m of NAD^+. We measured deacetylation of a fixed concentration of p53 substrate over a range of NAD^+ concentrations (Fig. 6*B*). We noted that the K_m for NAD^+ is comparable for native and Fluor de Lys substrates, 132.5 ± 33.9 *versus* 191.9 ± 21.7 μM, respectively. These data suggest that the presence of the Fluor de Lys group on the acetyl-peptide substrate specifically decreases the affinity of SirT1 for peptide but not NAD^+.

Our observation that the K_m of the FdL-p53 substrate is significantly higher than the K_m of the native p53 peptide substrate in the absence of resveratrol (Table I) suggested the possibility that resveratrol may stimulate deacetylation of substrates containing the Fluor de Lys group by increasing their affinity for SirT1 to a level comparable with native peptides. To evaluate this possibility, we carried out competition studies to determine the effect of the FdL-p53 peptide on deacetylation of the native histone H4 peptide in the presence and absence of resveratrol using the [^3H]acetate release assay. The IC_{50} of FdL-p53 on deacetylation of histone H4 peptide was 352 ± 52 μM; however, in the presence of 100 μM resveratrol, IC_{50} of FdL-p53 peptide was reduced to less than 10 μM (Fig. 7*A*). This result suggests that resveratrol increases binding affinity of Fluor de Lys peptide by more than 10-fold, which is consistent with the observed ~10-fold increase in deacetylation of p53-FdL by SirT1 in the presence of resveratrol. Competition studies of histone H4 peptide deacetylation with native p53 peptide revealed that native p53 peptide was a much better competitive substrate than FdL-p53 in the absence of resveratrol, with an IC_{50} of 9.2 ± 1.5 μM (Fig. 7*B*). Importantly, resveratrol had no effect on the ability of native p53 peptide substrate to compete with native histone H4 substrate for deacetylation by SirT1, further indicating that binding of substrates lacking Fluor de Lys is not enhanced by resveratrol. Thus, we find no evidence that resveratrol stimulates SirT1 deacetylation of substrates lacking Fluor de Lys.

DISCUSSION

The biological activities of resveratrol have been noted for at least 20 years (47). Resveratrol has been proposed to have wide

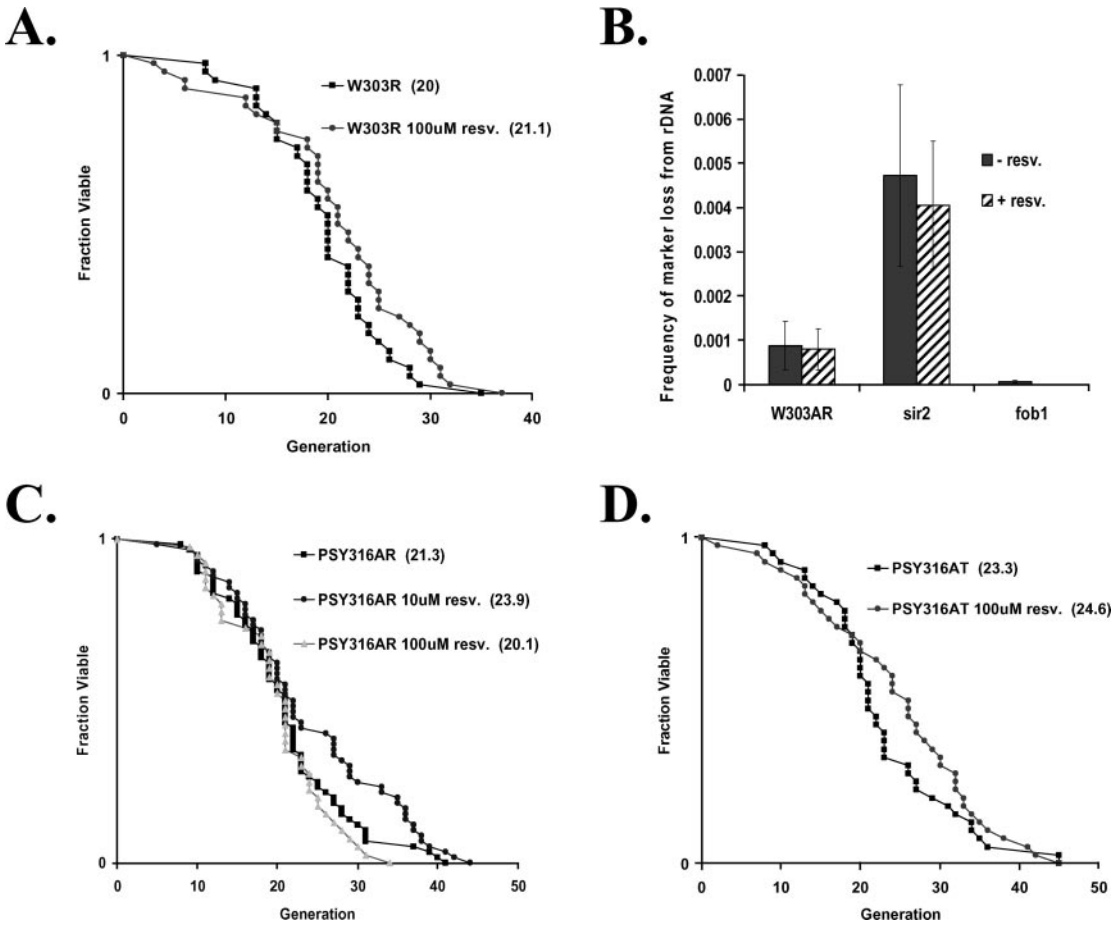

FIG. 3. **Resveratrol has no significant effect on life span or rDNA recombination in W303R and PSY316.** *A*, resveratrol (*resv*) fails to significantly alter life span in W303R ($p = 0.2$). Life spans were determined for W303R mother cells in the absence (■) or presence (●) of 100 μM resveratrol. Mean life span for each strain is shown in parentheses. *B*, growth in the presence of 100 μM resveratrol has no significant effect on rDNA recombination in the presence or in the absence of Sir2, as measured by loss of an *ADE2* marker from the rDNA array (4). As expected, deletion of *SIR2* increases rDNA recombination and deletion of *FOB1* decreases rDNA recombination. *C*, resveratrol fails to significantly alter life span in PSY316AR at either 10 μM ($p = 0.2$) or 100 μM ($p = 0.7$). Life spans were determined for PSY316AR mother cells grown in the absence of drug (■), or in the presence of either 10 μM (●) or 100 μM (▲) resveratrol. *D*, resveratrol fails to significantly alter life span in PSY316AT ($p = 0.3$). Life spans were determined for PSY316AT mother cells in the absence (■) or presence (●) of 100 μM resveratrol.

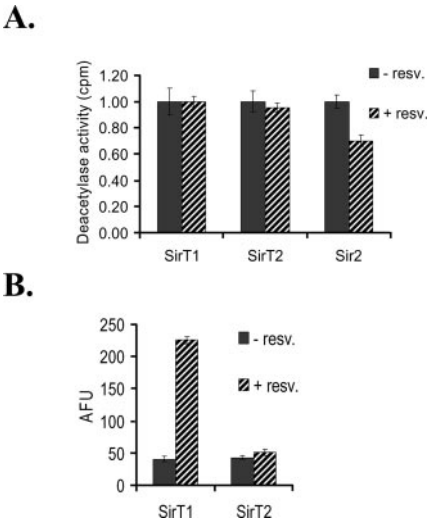

FIG. 4. **Sirtuin-specific and substrate-specific activation by resveratrol.** *A*, resveratrol (*resv*) does not stimulate deacetylation of a native H4 peptide by SirT1, SirT2, or Sir2 enzymes. Relative NAD^+-dependent deacetylase activity with and without 100 μM resveratrol of SirT1, SirT2, and Sir2 measured by the [^{3}H]acetate release assay, as described under "Materials and Methods." *B*, resveratrol stimulates deacetylation of FdL-p53 substrate (100 μM resveratrol, 25 μM NAD^+, 12.5 μM acetylated FdL-p53) by SirT1 but not SirT2 measured by the FdL assay. *AFU*, arbitrary fluorescence units.

ranging effects, including proapoptotic, fungicidal (48), chemopreventive (49), and antioxidant (50, 51) properties. Most recently, resveratrol has been suggested to activate sirtuins both *in vitro* and *in vivo* and to enhance longevity in yeast, worms, and flies (33, 34). In contrast to the prior report (33), we find that treatment of yeast cells with resveratrol failed to cause any of the phenotypes expected upon activation of Sir2, and activation of Sir2 orthologs by resveratrol *in vitro* appeared to be an artifact, as it was specific for substrates containing the non-physiological, fluorescent Fluor de Lys moiety.

The PSY316 strain used by Howitz *et al.* (33) is unique in that overexpression of *SIR2* fails to increase replicative life span in this background (39). The mechanism underlying this important difference is currently unknown; however, it would be surprising if a small molecule, such as resveratrol, increased life span by activating Sir2 in a strain that is insensitive to elevated Sir2 dosage. One potential explanation for this paradox is that increased dosage of Sir2 fails to result in increased Sir2 activity in PSY316. This is clearly not the case, though, as overexpression of *SIR2* resulted in enhanced transcriptional silencing of marker genes inserted near telomeres (Fig. 1, *B* and *C*). Resveratrol, on the other hand, failed to increase Sir2-dependent transcriptional silencing (Fig. 1, *B* and *C* (33)), further suggesting that yeast Sir2 is not activated *in vivo* by resveratrol. A less straightforward possibility is that overexpression of *SIR2* in PSY316 enhances Sir2 activity at telomeres

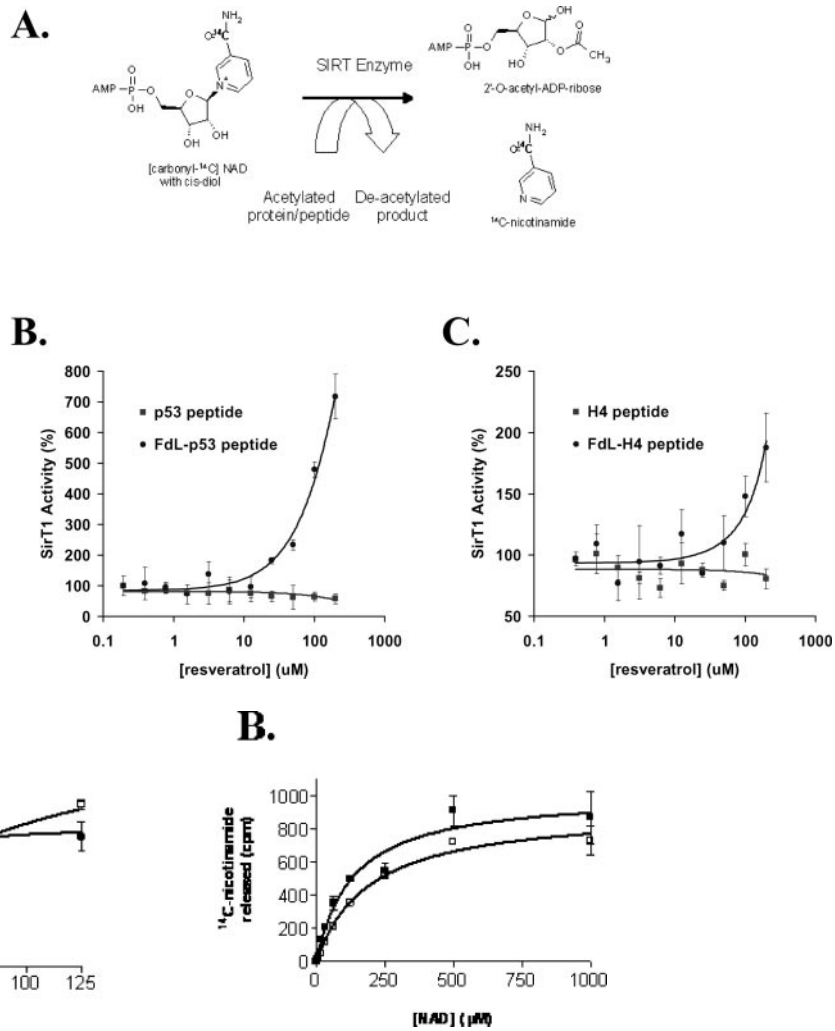

FIG. 5. **Resveratrol activates SirT1 deacetylation of Fluor de Lys-containing peptides but not native peptide substrates.** SirT1 deacetylase assays were performed as described under "Materials and Methods." Dose-response assays were performed with 0.4–200 μM resveratrol added to a reaction mixture containing 60 μM NAD^+. *A*, [^{14}C]nicotinamide release assay. *B*, comparison of SirT1 activation by resveratrol in reactions containing either FdL-p53 peptide (●) or p53 peptide substrate (■) and NAD^+. *C*, comparison of SirT1 activation by resveratrol in reactions containing either FdL-H4 (●) or H4 (■) peptide substrate and NAD^+. Percent activity of the enzymatic reaction in the presence of activator is calculated as follows. Percent activity = 100 × ((+) resveratrol)/((−) resveratrol control). Where (+) resveratrol and (−) resveratrol control are the [^{14}C]nicotinamide released (cpm) in the presence and absence of resveratrol, respectively. Data are expressed as the mean ± S.E.

FIG. 6. **SirT1 enzyme kinetic parameter determination.** *A*, [^{14}C]nicotinamide counts/min (cpm) released as a function of peptide substrate concentration. FdL-p53 (□) or p53 peptide (■) concentration was varied from 0.5 to 125 μM. The NAD^+ concentration was fixed at 90 μM. *B*, [^{14}C]nicotinamide cpm released as a function of NAD^+ concentration. NAD^+ (10% [*carbonyl*-^{14}C]NAD^+) was varied from 1 to 1000 μM. The FdL-p53 (□) or p53 peptide (■) concentration was fixed at 30 μM. K_m values (Table I) were computed by non-linear least squares fitting using Prism 4.0. Data are expressed as the mean ± S.E.

TABLE I
FdL decreases SirT1 substrate affinity

SirT1 K_m values were determined for acetylated p53 substrate containing (FdL-p53) or lacking (p53) the FdL group at 90 μM NAD^+. SirT1 K_m values for NAD^+ were determined at 30 μM p53 or FdL-p53 peptide.

Substrate	Peptide K_m	NAD^+ K_m
	μM	μM
p53 peptide	10.3 ± 2.6	132.5 ± 33.9
FdL-p53 peptide	87.6 ± 19.7	191.9 ± 21.7

but not at rDNA loci and that resveratrol has the opposite effect. This also seems unlikely, however, because Howitz *et al.* (33) report that resveratrol fails to enhance rDNA silencing, and we showed that resveratrol failed to decrease rDNA recombination (Fig. 3*B*), two measures of Sir2 activity at the rDNA.

Our discovery that activation of Sir2 orthologs by resveratrol *in vitro* is substrate-specific raises the important question whether any biologically relevant sirtuin substrates are responsive to resveratrol *in vivo*. To date, there is limited evidence on this matter. In our *in vitro* studies, activation of sirtuins by resveratrol was specific for peptide substrates containing the Fluor de Lys group. Resveratrol was isolated as a SirT1 activator in a screen using the FdL-p53 peptide substrate, and the presence of the Fluor de Lys group is sufficient to decrease the affinity of SirT1 for an acetylated peptide. Taken together, these observations are consistent with the model that resveratrol is a specific suppressor of the Fluor de Lys-associated decrease in substrate affinity (Fig. 6*A*). It is, however, possible that resveratrol modifies the affinity of Sir2 orthologs toward a subset of *in vivo* targets or that resveratrol causes sirtuins to deacetylate substrates *in vivo* that are normally low affinity targets. In this regard, it is interesting to note that a few studies have reported data consistent with, although by no means conclusively demonstrating, sirtuin-dependent *in vivo* effects of resveratrol (14, 52, 53). A precise characterization of the *in vitro* and *in vivo* parameters of sirtuin activation by resveratrol will be an important focus for future studies.

Our results also raise important questions regarding the utility of resveratrol as an anti-aging drug or calorie-restriction mimetic. Resveratrol has been reported to increase life span in both worms and flies in the presence, but not in the absence, of Sir2 orthologs (34). The inability to demonstrate enhanced longevity in a mutant background is consistent with a mechanism involving the mutated protein (sirtuins, in this case); however, causal interpretation of this type of negative result is confounded by the likelihood that the mutation itself causes unknown physiological changes. In fact, there is no direct evidence suggesting that resveratrol can activate sirtuins *in vivo* in either *Caenorhabditis elegans* or *Drosophila melanogaster*.

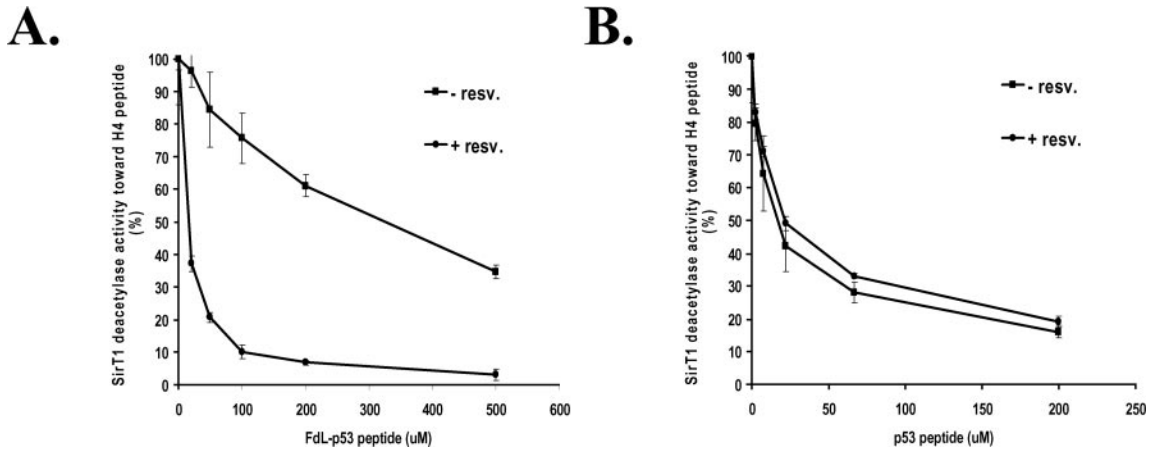

FIG. 7. **Competition of H4 peptide deacetylation by SirT1 with FdL-p53 or native p53 peptide.** Histone H4 deacetylation, measured by [^{3}H]acetate release, in the presence of a range of concentrations of the FdL-p53 peptide substrate (*A*) or native p53 peptide substrate (*B*), with and without 100 μM resveratrol (*resv*). Histone H4 deacetylation in the presence of the competitor substrate is normalized to H4 deacetylation without the competitor.

There are, however, several examples of other antioxidant compounds reported to increase longevity in invertebrates (*e.g.* Refs. 54–61), suggesting that life span extension by resveratrol could result from its antioxidant properties rather than its putative sirtuin-activating properties. In addition to its antioxidant properties, resveratrol has also been reported to specifically inhibit the mammalian mitochondrial ATPase (62–64) as well as to inhibit mitochondrial respiratory capacity at complex III through competition with coenzyme Q (65). These activities of resveratrol may be particularly relevant to its longevity-promoting effects in *C. elegans*, as several mutants with decreased mitochondrial function have been reported to increase life span in this organism (66, 67), and decreased coenzyme Q levels accomplished either through dietary (68) or genetic (69) manipulations have a similar effect.

To date, the myriad biological effects of resveratrol (*e.g.* see Ref. 70) have been neglected in aging-related research, in favor of models interpreting phenotypes in terms of sirtuin activation. We present here evidence that resveratrol is a substrate-specific activator of Sir2-orthologs and that the ability of resveratrol to enhance deacetylation of sirtuin substrates *in vivo* needs to be reexamined. We suggest that further studies of the longevity promoting properties of resveratrol should consider the full spectrum of biological processes likely to be altered by this important compound. This will allow for a mechanistic understanding of the effects of resveratrol on the life span of invertebrates, and perhaps, mammals.

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