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 extra-chromosomal rDNA circles in the mother cell nucleus (18). Extra-chromosomal 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 extra-chromosomal DNA 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 deacetylase 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 mimic (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, PSY316AAT, and PSY316AUT. PSY316AR is as described (38) and contains the ADE2 gene integrated into an rDNA repeat. PSY316AAT and PSY316AUT are as described (38) and contains the ADE2 gene integrated into an rDNA repeat. The ADE2 gene in PSY316AR is integrated into the telomeres. Strains overexpressing SIR2 in the presence of compound overnight (prepared the night prior to starting each experiment). Cells were grown in liquid YPD or YPD supplemented with 100 mM acid, which is toxic to cells expressing Sir2. We also report biochemical data demonstrating that resveratrol is a substrate-specific activator of Sir2 orthologs.

**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 SIR2 and SIR2 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. 1A). Sir2-dependent silencing of both the URA3 (Fig. 1B) and ADE2 (Fig. 1C) 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 mimic 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 Biolom failed to significantly increase either mean or maximum life span (Fig. 2A). Similarly, no effect on life span was observed using a second source of resveratrol obtained from Sigma (Fig. 2B). We were concerned that perhaps the resveratrol used for these experiments had degraded or otherwise lost

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1 The abbreviations used are: CR, calorie restriction; FdL, Fluor de Lys.
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 \( \mu M \) (Fig. 2C). 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. 3A). In contrast to the prior report (33), we also found that resveratrol had 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. 2C), 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 \(^3\)Hacetate release from the histone H4 substrate using SirT1, SirT2, or Sir2 enzymes (Fig. 4A). 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.

Discussion

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

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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, [14C]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 [14C]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, [14C]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, [14C]nicotinamide cpm released as a function of NAD⁺ concentration. NAD⁺ (10% [carboxyl-14C]NAD⁺) was varied from 1 to 1000 μM. The FdL-p53 (○) or p53 peptide (■) concentration was fixed at 30 μM. Km values (Table I) were computed by non-linear least squares fitting using Prism 4.0. Data are expressed as the mean ± S.E.

| Substrate Peptide | Peptide Km (μM) | NAD⁺ Km (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. 3B), 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. 6A). It is, however, possible that resveratrol modifies the affinity of Sir2 orthologs toward a subset of in vitro 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.
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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