SOD2 Functions Downstream of Sch9 to Extend Longevity in Yeast

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ABSTRACT

Signal transduction pathways inactivated during periods of starvation are implicated in the regulation of longevity in organisms ranging from yeast to mammals, but the mechanisms responsible for life-span extension are poorly understood. Chronological life-span extension in *S. cerevisiae cyr1* and *sch9* mutants is mediated by the stress-resistance proteins Msn2/Msn4 and Rim15. Here we show that mitochondrial superoxide dismutase (Sod2) is required for survival extension in yeast. Deletion of *SOD2* abolishes life-span extension in *sch9*Δ mutants and decreases survival in *cyr1:mTn* mutants. The overexpression of Sods—mitochondrial Sod2 and cytosolic CuZnSod (Sod1)—delays the age-dependent reversible inactivation of mitochondrial aconitase, a superoxide-sensitive enzyme, and extends survival by 30%. Deletion of the *RAS2* gene, which functions upstream of *CYR1*, also doubles the mean life span by a mechanism that requires Msn2/4 and Sod2. These findings link mutations that extend chronological life span in *S. cerevisiae* to superoxide dismutases and suggest that the induction of other stress-resistance genes regulated by Msn2/4 and Rim15 is required for maximum longevity extension.

WHEN microorganisms encounter an ample source of nutrients, they typically divide rapidly, reach a state of overcrowding, and then spend the vast majority of their life span in stationary phase (Werner-Washburne *et al.* 1993; Zambrano and Kolter 1996). Yeast incubated in rich glucose medium (YPD) grow rapidly by fermentation (log phase) and then switch (diauxic shift) to the postdiauxic phase as the population reaches a high density and external nutrients become depleted (Werner-Washburne *et al.* 1996). Cells grown in YPD medium continue to grow during the postdiauxic phase and then decrease metabolic rate and macromolecular synthesis by >100-fold upon entry into stationary phase. In this hypometabolic phase, yeast cells can survive for months by slowly utilizing reserve nutrients (Lillie and Pringle 1980; Werner-Washburne *et al.* 1996). By contrast, yeast wild-type strains DBY746 and SP1 grown in synthetic dextrose complete medium (SDC) normally reach maximum viability within 48 hr and maximum population density within 72 hr and survive for ~6 days (Longo *et al.* 1996, 1997; Longo 1999). Under these conditions the respiratory rate remains high for most of the life span (Figure 6). Cells switched from either YPD or SDC medium to water on day 3 instead decrease respiratory rate early and survive for as long as 3 weeks in stationary phase (Granot and Snyder 1991; Longo *et al.* 1997; Longo 1999). Thus, we make a distinction between the "postdiauxic phase" and the stationary phase: Yeast grown and maintained in SDC medium have a short, high-metabolic life span in the postdiauxic phase whereas yeast maintained in YPD or water have a long, hypometabolic life span in stationary phase. Notably, survival in SDC medium and in water appears to be related since the long-lived mutants isolated survive longer in both regimens (P. Fabrizio, L.-L. Liou and V. D. Longo, unpublished results).

We call survival in the postdiauxic and stationary phases "chronological life span" to distinguish it from the "budding life span," measured by counting the number of buds generated by a single mother cell (Jazwinski 1996; Sinclair *et al.* 1998). Although the relationship between the budding and chronological life span is not clear (Sinclair *et al.* 1998), a recent study suggests that these phenomena may be related: The replicative life span of yeast removed from stationary-phase cultures decreases progressively with chronological age (Ashrafi *et al.* 1999). Aging in the budding life span can be caused by the accumulation of rDNA circles in the nucleolus (Sinclair *et al.* 1997). By contrast, chronological life span in yeast is extended by overexpression of the human oncoprotein Bcl-2 (Longo *et al.* 1997), known to protect mammalian cells against oxidative stress (Kane *et al.* 1993), and is shortened by null mutations in either or both superoxide dismutases (Longo *et al.* 1996). The chronological life span is also extended by mutations that reduce the activities of adenylate cyclase (Cyr1) and of the serine threonine kinase Sch9

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(Fabrizio *et al.* 2001). Cyr 1 and Sch9 function in pathways that mediate glucose-dependent signaling, stimulate growth and glycolysis, and decrease stress resistance (Toda *et al.* 1988; Morano and Thiele 1999; Thevelein and de Winde 1999). Longevity extension in these mutants requires stress-resistance transcription factors Msn2 and Msn4 and the protein kinase Rim15, suggesting that increased investment in protection and repair slows down aging (Fabrizio *et al.* 2001). Furthermore, the age-dependent inactivation of the superoxide-sensitive enzyme aconitase, which is high in wild-type cells, is decreased by mutations that extend longevity (Fabrizio *et al.* 2001). A reduction in the activity of the Cyr1/PKA pathway is also implicated in the extension of the yeast replicative life span (Lin *et al.* 2000). However, stress-resistance proteins do not appear to mediate the extension of the replicative longevity since the deletion of stress-resistance transcription factors Msn2 and Msn4 does not affect replicative life span (Lin *et al.* 2000).

The yeast G-proteins Ras1 and Ras2 function upstream of Cyr1 and play overlapping roles in functions including growth, pseudohyphal development, and stress resistance (Toda *et al.* 1985; Werner-Washburne *et al.* 1993; Roberts *et al.* 1997). Yeast *ras2* null mutants at all growth stages resemble wild-type cells in stationary phase in that they accumulate glycogen and have increased thermotolerance and antioxidant defenses. The increased stress resistance in *ras2* mutants is due in part to the induction of transcription factors Msn2 and Msn4, which are inactivated by protein kinase A (PKA; Smith *et al.* 1998) downstream of Ras. Msn2 and Msn4, which are required for longevity extension in *cyr1* mutants (Fabrizio *et al.* 2001), regulate a number of genes that contain the *st*ress *r*esponse *e*lement (STRE) in their promoters (Martinez-Pastor *et al.* 1996). Among the genes reported to be regulated in this manner are those encoding for several heat-shock proteins, catalase (*CTT1*), the DNA-damage-inducible gene *DDR2* (Martinez-Pastor *et al.* 1996), and genes involved in the storage of reserve nutrients (Ruis and Schuller 1995). SODs may also be regulated by Msn2/Msn4 since the *SOD* promoters contain a STRE sequence and the expression of *SOD2* in strain JC482 lacking *RAS2* is doubled (Flattery-O'Brien *et al.* 1997). The Ras/cAMP/PKA pathway downregulates the protein kinase Rim15, which, in turn, activates the stress-resistance transcription factor Gis1 (Pedruzzi *et al.* 2000). Similarly to Msn2/Msn4, Gis1 regulates stress resistance through a postdiauxic shift (PDS) element contained in the promoter of genes including *HSP26*, *HSP12*, and *SOD2* (Flattery-O'Brien *et al.* 1997; Pedruzzi *et al.* 2000). Thus, the expression of *SOD2* may be regulated by both Msn2/Msn4 and Rim15/Gis1.

To elucidate the molecular mechanisms of aging and death in yeast we examined the role of superoxide dismutases in the life-span extension caused by mutations in the Sch9 and cAMP/PKA pathway (Fabrizio *et al.* 2001). We also investigated the role of proteins that function upstream of PKA in the regulation of longevity.

MATERIALS AND METHODS

Yeast strains and plasmids used in this study: The yeast strains used in this study are listed in Table 1. Strains lacking *RAS2*, *SOD2*, and *MSN2/MSN4* were produced by one-step gene replacement using disruption plasmids pRAS2::LEU2 (Kataoka *et al.* 1984), pSOD2::TRP1 (Gralla and Valentine 1991), pt32-ΔXB::HIS3 (Estruch and Carlson 1993), and pAS26 (Smith *et al.* 1998). All disruptions were verified by PCR analysis or Southern blot. Overexpressor plasmids were constructed in multicopy vectors YEp351 and YEp352 as follows: YEp351-CTT1 was constructed by inserting a 3.9-kb *Bam*HI-*Hin*dIII fragment containing the *CTT1* gene into the *Sal*I site of YEp351. YEp351-SOD2, provided by D. Kosman, contains a 2-kb genomic *Bam*HI fragment inserted into YEp351. YEp352-SOD1 was constructed by ligating a 2-kb *SOD1* *Sph*I fragment into the *Sph*I site of YEp352. All the genes described above are driven by their natural promoters. These plasmids were used to construct strains overexpressing *CTT1*, *SOD1*, and *SOD2* alone and in combinations in both the SP1 and the DBY746 backgrounds.

All DNA and RNA manipulations were performed using standard techniques. Yeast transformants were obtained by the lithium acetate method (Gietz *et al.* 1992).

Northern analysis: RNA filters were prehybridized with 100 μg/ml of salmon sperm DNA at 42° for 3 hr in buffer containing 1% SDS, 50% formamide, 5× SSC, and 5× Denhardt's solution and then incubated overnight with a ^{32}P-labeled 2-kb *Bam*HI SOD2 fragment. After hybridization the filters were washed in the following manner: twice in 2× SSC, 0.1% SDS (2 min and 5 min) at 42°, and twice in 0.1× SSC, 0.1% SDS (10 min and 30 min) at 60°. The filters were exposed, developed, and scanned using the phophorImager system (Molecular Dynamics, Sunnyvale, CA).

Media, growth conditions, and postdiauxic phase survival: Unless stated otherwise, all experiments were performed in liquid media in SDC medium with 2% glucose, supplemented with amino acids, adenine, and uracil, as well as a fourfold excess of the supplements tryptophan, leucine, histidine, lysine, and methionine. Overnight cultures were grown in selective media, inoculated into flasks with a flask volume/medium volume ratio of 5:1, and grown at 30° with shaking at 220 rpm. Maximum population density is normally reached after 72 hr of growth in SDC medium. The maximum size of the viable population was ~10^7 cells/ml.

To determine the number of viable yeast, starting at day 3, 10-μl aliquots were removed from each flask and serially diluted. Each aliquot was then plated twice onto YPD (2% glucose as carbon source) plates for a total of 2 or 4 platings/population/day. Serial dilutions were performed to plate ~100 viable organisms per plate. Viability is defined as the ability of a single organism to reproduce and form a colony within 48 hr (colony forming units, or CFU). The time-dependent loss of CFUs was compared to the protein concentration in the medium, as measured by Bradford assay, which should correlate with increased cell damage and lysis. Viability was also measured by a live/dead fluorescent assay following the manufacturer's instructions for stationary-phase cells (Molecular Probes, Eugene, OR). The percentage of live cells was determined by counting red/green cells by fluorescence microscopy after staining with the FUN-1 dye.

Survival in the presence of mitochondrial superoxide-generating agents was tested by adding 1 mm paraquat or 1 μm antimycin A to the yeast cultures after 24 hr growth in SDC

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
DBY746	*MAT*α *leu 2-3, 112 his3Δ1 trp1-289 ura 3-52 GAL*$^+$	Mikus and Petes (1982)
SP1	*MAT***a** *leu2 his3 ura3 trp1 ade8 can1*	CSHL collection
KP-2	SP1 *ras2::URA3*	Toda *et al.* (1985)
PF101	DBY746 *cyr1::mTn*	Fabrizio *et al.* (2001)
EG252	DBY746 *ras2::LEU2*	This study
PF102	DBY746 *sch9::URA3*	Fabrizio *et al.* (2001)
EG110	DBY746 *sod2::TRP1*	Liu *et al.* (1992)
PF103	DBY746 *msn2::HIS3 msn4::LEU2*	Fabrizio *et al.* (2001)
PF104	DBY746 *ras2::LEU2 sod2::TRP1*	This study
PF105	DBY746 *cyr1::mTn sod2::TRP1*	This study
PF106	DBY746 *sch9::URA3 sod2::TRP1*	This study
PF107	DBY746 *ras2::LEU2 msn2::HIS3 msn4::LEU2*	This study
TK161-R2V	SP1 *RAS2*^{val19}	Toda *et al.* (1985)
CC103	DBY746 *coq3::LEU2*	Do *et al.* (1996)
DO103	DBY746 *atp2::LEU2*	This study (C. Clarke)

medium. Survival was also tested in the presence of the inhibitors of superoxide generation FCCP (4 μM) or NaCN (0.25 mM) added at time "0."

A linear regression model was adapted to estimate the days of 50% survival for each sample. Then Wilcoxon survival analysis was performed to compare the 50% survival of strains. Bonferroni adjustment was applied for pairwise comparison. All analyses were two-sided tests determined at a significance level of 0.05.

The significance of the difference in aconitase activity and reactivation was calculated by two-tailed Student's *t*-tests.

Oxygen consumption: Cellular oxygen uptake was measured at 30° in a 4-ml stirred chamber using a YSI model 53 biological oxygen monitor (Yellow Springs Instruments) following the manufacturer's directions. Cells were cultured in SDC medium and incubated for the indicated time before aliquots were removed and tested for oxygen consumption. Cells were kept in the medium in which they had been growing, and conditions that resembled the flask environment (30° and stirring) were maintained in the chamber.

Superoxide dismutase and catalase activity assays: Superoxide dismutase assays were performed by using the method of auto-oxidation of 6-hydroxydopamine (Heikkila and Felicitas 1976). For separate measurement of CuZnSod and MnSod, inhibitors were used to inhibit or inactivate the respective enzyme, and the individual activities were calculated accordingly (Geller and Winge 1984). To determine MnSOD activity, 1 mM KCN, which inhibits 95% of the CuZnSod activity, was added to the reaction mix. CuZnSod activity was measured as described above in extracts treated with 2% SDS for 1 hr at 37° to inactivate MnSod. The SDS was removed by incubation with 0.3 M KCl for 30 min at 4° followed by centrifugation at 20,000 × *g* for 10 min. Catalase activity was determined by monitoring the disappearance of hydrogen peroxide spectrophotometrically at 240 nm in 50 mM potassium phosphate buffer, pH 7.0 at 25°.

Aconitase activity and reactivation: Cells were inoculated at an OD_{600} of 0.1 in SDC medium and harvested at the indicated times. Whole-cell extracts were obtained by glass bead lysis under argon in 50 mM Tris pH 7.2, 150 mM NaCl, 5 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride with an equal volume of 0.5 mm acid-washed glass beads and vortexing for six cycles of 30 sec followed by 2 min of cooling. After centrifugation, the supernatants were aliquoted, flash frozen, and stored at −70°. Because of the instability of 4iron-4sulfur (4Fe-4S) clusters in air, the extraction procedures were performed as rapidly as possible. Furthermore, aliquots kept at −70° were thawed only immediately before the assay. Aconitase activity was measured spectrophotometrically as described (Racker 1950). Briefly, the linear absorbance change at 240 nm (*cis*-aconitate disappearance) was followed in a reaction mixture containing 1 mM *cis*-aconitate, 0.5 M NaCl, and 0.1 M Tris pH 7.4. For 4Fe-4S cluster reactivation experiments, 1 mM ferric sulfate ($FeSO_4$) and 1 mM sodium sulfide (Na_2S) were added to the cuvette containing all the reagents required for the aconitase assay. Alternately, extracts were preincubated for 30 min with 50 mM dithiothreitol, 0.2 mM Na_2S, and 0.2 mM ferric ammonium sulfate. Activity was measured as described above.

RESULTS

The role of Sod2 in life-span extension: Transcription factors Msn2/Msn4 and Gis1, the latter regulated by Rim15, can activate a variety of stress-resistance genes through either a STRE or a PDS element. Among the promoters containing both a STRE and a PDS element is that of *SOD2* (Flattery-O'Brien *et al.* 1997; Pedruzzi *et al.* 2000). Thus, Sod2 may function downstream of stress-resistance transcription factors Msn2/Msn4 and Gis1 to extend longevity. To test this hypothesis we deleted *SOD2* in the *cyr1::mTn* (PF101) and *sch9*Δ (PF102) strains. *sod2*Δ and *sch9*Δ*sod2*Δ double mutants (EG110 and PF106) survived similarly to wild-type cells, suggesting that Sod2 is required for the threefold longer life span of *sch9*Δ mutants (Figure 1A). The deletion of *SOD2* also reduced life-span extension in *cyr1::mTn* mutants (Figure 1B). Double *sod1*Δ*sod2*Δ mutants were not studied since the deletion of both *SOD*s causes a major decrease in life span. The viability for each strain is reported as a percentage of the viability on day 3 for the same strain.

At days 3 and 5 the viability for *sod2*Δ mutants is

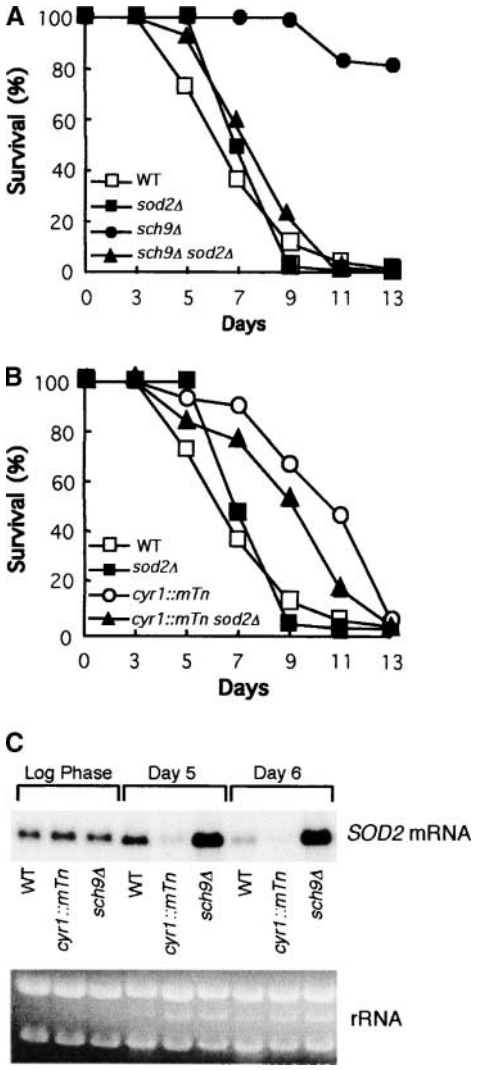


FIGURE 1.—Mitochondrial Sod (Sod2) is required for the chronological life-span extension of *sch9*Δ (PF102) and *cyr1::mTn* (PF101) mutants. (A) Survival of the wild type (DBY746), *sod2*Δ (EG110), *sch9*Δ (PF102), and *sch9*Δ lacking *SOD2* (PF106). (B) Survival of wild type and *cyr1::mTn* (PF101) and *cyr1::mTn* lacking *SOD2* (PF105). The average of two independent experiments with duplicate samples is shown for A and B. (C) Northern blot of RNA prepared from exponentially growing, day 5, or day 6 cultures of wild type, *cyr1::mTn*, and *sch9*Δ mutants probed for *SOD2*. Compared to wild-type controls, *SOD2* expression in *sch9*Δ mutants was 3.5- and 8-fold higher at days 5 and 6, respectively. Equal RNA loading was confirmed by ethidium bromide staining after electrophoresis (bottom). The experiment was performed twice with similar results.

~100%. Notably, when the survival experiments were performed in 250-ml flasks, instead of the 50-ml flasks used in this study, *sod2*Δ mutants lost 20–40% of the viability by day 3 (LONGO *et al.* 1999). Although the flask volume/medium volume ratio of 5:1 is maintained in both large and small flasks, the larger flask may affect the oxygen levels to which cells are exposed.

To determine whether the *cyr1::mTn* and *sch9*Δ mutations affect the expression of *SOD2*, we monitored the age-dependent levels of *SOD2* mRNA in these mutants. The deletion of *SCH9*, but not of the *cyr1::mTn* mutation, caused a major age-dependent induction of *SOD2*, as determined by Northern blot analysis (Figure 1C). *SOD2* expression in *sch9*Δ mutants was 3.5- and 8-fold higher than that in wild-type cells at days 5 and 6, respectively. The low levels of *SOD2* mRNA in *cyr1::mTn* mutants may be explained by the early decrease in oxygen consumption rates in these mutants (Figure 6), since the expression of the mitochondrial *SOD2* should decrease with the decrease in metabolic rates. This may also explain why the deletion of *SOD2* did not abolish the life-span extension in *cyr1::mTn* mutants (Figure 1B).

TABLE 2

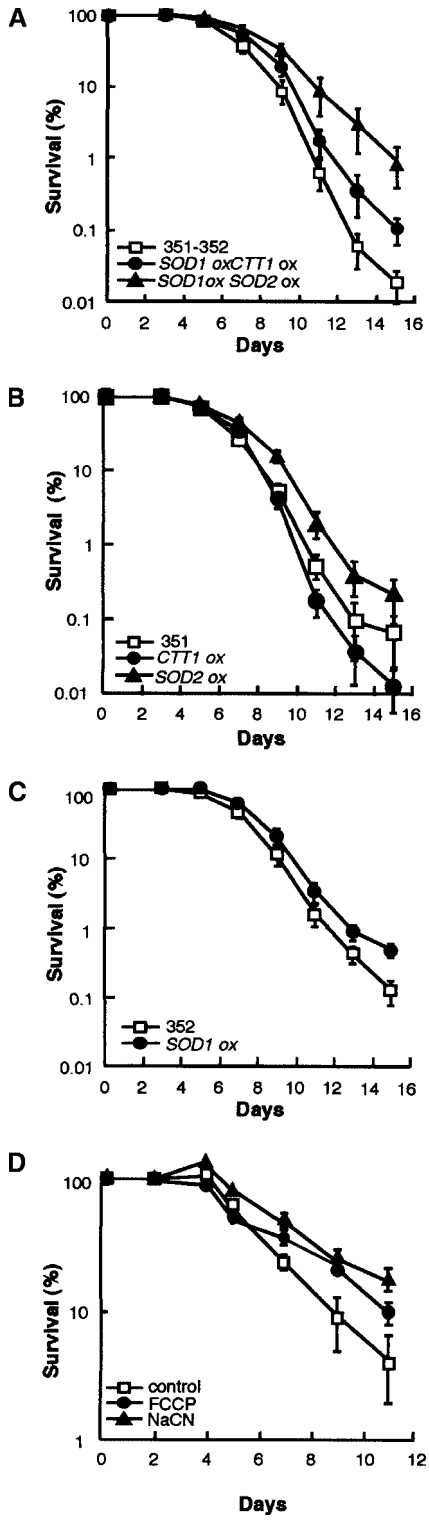
Specific activities of Sod1, Sod2, and catalase

Strain	CuZnSod (Sod1) (units/mg)	MnSod (Sod2) (units/mg)	Catalase (Ctt1) (units/mg)
351-352	0.48	0	3.9
SOD1-SOD2	1.97	0.42	1.66
SOD1-CTT1	ND	ND	11.6

ND, not determined.

Superoxide dismutases and survival: To test further the role of superoxide dismutases in the survival extension of *cyr1::mTn* and *sch9*Δ mutants (Figure 1), we measured the chronological life span of yeast overexpressing antioxidant enzymes. We overexpressed various combinations of cytosolic CuZnSod (Sod1), mitochondrial MnSod (Sod2), and cytosolic catalase T (Ctt1) in wild-type strains DBY746 and SP1. The activity of both Sod1 and Sod2 increased by more than threefold in *SOD1-SOD2* overexpressors compared to that of yeast transformed with plasmid controls (Table 2). The activity of catalase was also increased by threefold in catalase overexpressors (Table 2). The overexpression of *SOD1* and *SOD2* together had the greatest effect on survival (Figure 2A). The mean chronological life span for *SOD1 SOD2* double overexpressors in the DBY746 background was increased by 33%, from 6 to 8 days ($P < 0.05$). Double overexpression of *SOD1* and *CTT1* resulted in a 10% increase in life span (Figure 2A; $P < 0.05$). The overexpression of either *SOD1* or *SOD2* alone resulted in only minor increases in mean survival, whereas the overexpression of cytosolic catalase alone slightly decreased survival (Figure 2, B and C). CuZnSod, MnSod, and catalase T were also overexpressed in the SP1 background. The overexpression of both *SOD1* and *SOD2* resulted in a modest, but significant, life-span extension in this background, with an increase of 10% in mean survival compared to control strains ($P < 0.05$; data not shown). Single overexpression of either *SOD1* or *SOD2* in SP1 did not cause a significant improvement in survival (data not shown). The role of mitochondrial superoxide in promoting loss of viability in the postdiauxic

phase was confirmed by treating wild-type cells with FCCP and NaCN, an uncoupler and an inhibitor of respiration, respectively, which are known to reduce mitochondrial superoxide generation in mammalian cells (Boveris and Chance 1973; Turrens *et al.* 1985) and yeast (Longo *et al.* 1999). These inhibitors increased viability at days 9 and 11 by two- to threefold (Figure 2D; $P < 0.05$). Since respiration is essential for long-term survival and FCCP and NaCN inhibit energy production by the mitochondria, the experiments could be carried out only to day 11.



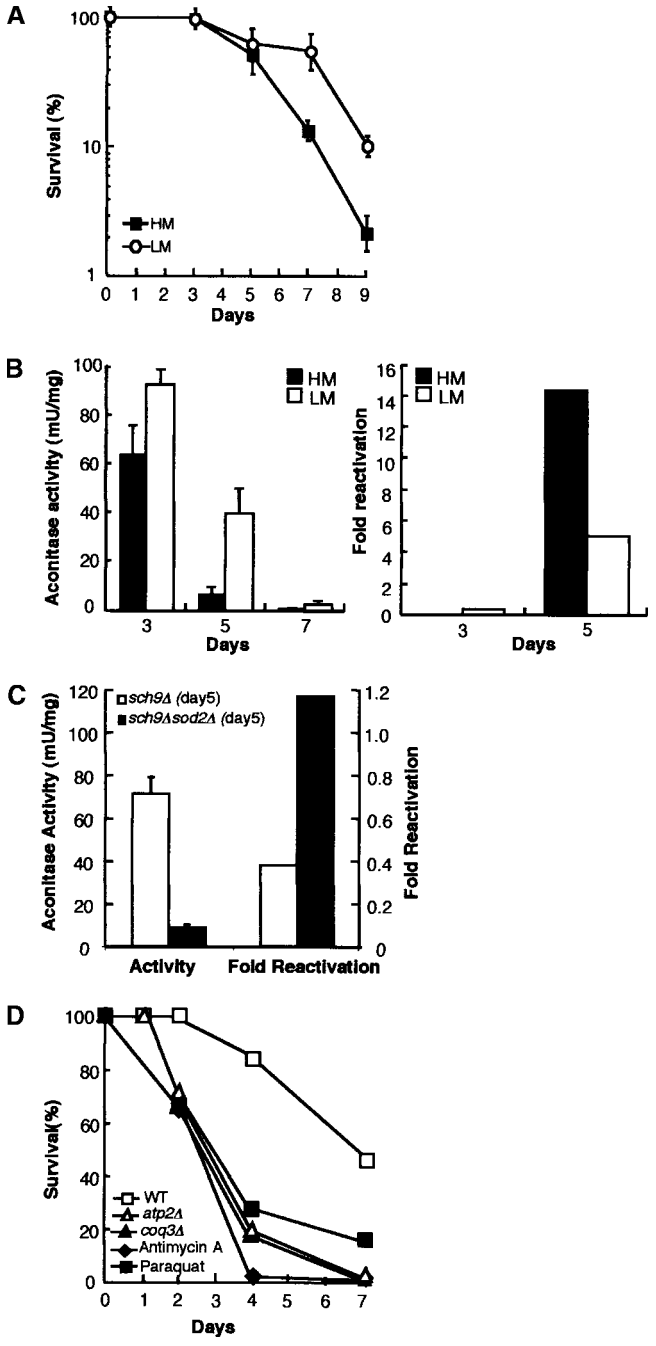
Aconitase activity and reactivation: To study further the role of superoxide in the aging and death of *Saccharomyces cerevisiae*, we measured the age-dependent activity of aconitase, a mitochondrial 4Fe-4S cluster-containing enzyme sensitive to inactivation by superoxide (Li *et al.* 1995; Longo *et al.* 1999). Using cell extracts from two experiments, we measured aconitase activity in five independent wild-type populations with a particularly high mortality rate (80% average; high mortality, or HM) and five *SOD1SOD2* overexpressors with a low mortality rate (20% average; low mortality, or LM) at day 5 (Figure 3A). Mortality rates at day 5 represent the percentage of the population that died between days 5 and 7. In both the HM and the LM groups, aconitase activity was high at day 3 (Figure 3B). At day 5 aconitase activity was sixfold higher in the LM group than in the HM group (Figure 3B), suggesting that loss of aconitase activity precedes, and may contribute to, aging and death in yeast. The partial inactivation of aconitase in the LM group at day 5 is not surprising, considering that mortality rates in this group increased in the following 4 days.

The exposure of aconitase and of other 4Fe-4S cluster-containing enzymes to superoxide causes inactivation (Fridovich 1995) due to the oxidation-dependent loss of one iron from the 4Fe-4S cluster (Flint *et al.* 1993). Superoxide-inactivated aconitase can be reactivated by incubation of cell extracts with excess Fe^{3+} and sulfide (S^{2-}; Longo *et al.* 1999). Little reactivation occurred for the HM and LM groups at day 3 (Figure 3B). By

Figure 2.—Life span of Sod overexpressors. Yeast strain DBY746 transformed with the indicated multicopy plasmids (YEp351 and YEp352), either vector only or carrying cytosolic CuZn*SOD* (*SOD1*), mitochondrial Mn*SOD* (*SOD2*), or cytosolic catalase (*CTT1*) were tested for survival as described. (A) Survival of DBY746 *SOD1CTT1* and *SOD1SOD2* double overexpressors. (B) Survival of DBY746 *CTT1* and *SOD2* single overexpressors. (C) Survival of DBY746 *SOD1* overexpressors. Each overexpressor is shown in the same figure as its specific plasmid control (YEp352 for *SOD1*; YEp351 for *SOD2* and *CTT1*). (D) Strain DBY746 was incubated in the presence of the respiratory inhibitors that reduce mitochondrial superoxide generation: FCCP (4 μM) or NaCN (0.25 mM). Viability was measured at the indicated times. To avoid the selection of strains with mutations that increase or decrease survival independently of Sods during the transformation, the experiments with each DBY746 overexpressor strain were performed between 6 and 10 times using transformants obtained from three separate transformations, all of which behaved similarly. For each graph, all experiments were averaged; bars show the standard error for each time point. Experiments with *SOD1-SOD2* and *SOD1CTT1* overexpressors in the SP1 parent strain were performed twice with double samples grown independently.

contrast, at day 5, incubation of extracts with Fe^{3+} and S^{2-} caused a 15-fold reactivation of aconitase in HM extracts and a 5-fold reactivation in LM extracts (Figure 3B), suggesting that the enzyme is present in an inactive form due to the loss of iron from its 4Fe-4S cluster. Reactivation of aconitase by >10-fold was also observed in HM and LM extracts on day 7 (data not shown).

The deletion of *SCH9* delays the age-dependent inactivation of aconitase (Fabrizio *et al.* 2001). We tested further the role of *SOD2* as a mediator of the effects of the *sch9* deletion on longevity by measuring aconitase activity and reactivation in *sch9*Δ*sod2*Δ double mutants.



At day 5, the activity of aconitase in *sch9*Δ mutants was higher than that of either the HM or the LM group (Figure 3, B and C). By contrast, aconitase activity was very low in *sch9*Δ*sod2*Δ mutants (Figure 3C). Aconitase reactivation in the presence of Fe^{3+} and S^{2-} was threefold higher in *sch9*Δ*sod2*Δ mutants than in *sch9*Δ mutants (Figure 3C). The relatively low reactivation of aconitase in *sch9*Δ*sod2*Δ compared to that in the HM and LM groups may be due to an irreversible inactivation of the 4Fe-4S cluster of aconitase caused by long-term exposure to high levels of superoxide and hydrogen peroxide (Flint and Allen 1996).

To test the effect of aconitase inactivation and loss of mitochondrial function on survival, we treated cells with agents known to inactivate aconitase in a superoxide-dependent manner (antimycin A, paraquat; Longo *et al.* 1999) and monitored the survival of mutants that are respiration deficient (*coq3*Δ, *atp2*Δ). Treatment of wild-type cells with 1 μM antimycin A or 1 mM paraquat, which increases the generation of mitochondrial superoxide and reversibly inactivates aconitase, resulted in an early viability loss (Figure 3D). These results are consistent with a role for mitochondrial superoxide in the inactivation of aconitase and the early loss of viability. The requirement for functional mitochondria during survival was confirmed by deleting *COQ3*, an enzyme involved in the biosynthesis of coenzyme Q, or *ATP2*, encoding for the β-subunit of the F_1 ATPase. Both genes are required for respiratory function (Mueller 1988; Poon *et al.* 1999). *coq3*Δ and *atp2*Δ mutants died early (Figure 3D).

Survival of *ras* mutants: In yeast, Ras1 and Ras2 activate Cyr1, which promotes aging and death (Figure 1B).

Figure 3.—Aconitase activity in wild-type (HM) and *SOD1-SOD2* overexpressors (LM). Extracts from five independent wild-type cultures with HM and five *SOD1oxSOD2ox* cultures with LM at day 5 (two studies) were assayed for aconitase activity. (A) The percentage of survival from day 3 to day 9 for the LM and HM groups. For the LM group, mortality at day 5 ranged from 0 to 0.37 (average of 0.17 ± 0.076). For the HM group, mortality at day 5 ranged from 0.44 to 0.9 (average of 0.77 ± 0.085). Values are mean ± SE. (B) Aconitase activity in the LM and HM groups expressed as milliunits/milligrams (left) and aconitase fold increase in activity in the presence of the reactivation agents Fe^{3+} and Na_2S (right). (Values are mean ± SE; $P < 0.05$ between HM and LM at day 5). (C) Aconitase activity and percentage of reactivation in the presence of reactivation agents for *sch9*Δ and *sch9*Δ*sod2*Δ strains (day 5). (Values are mean ± SE; $P < 0.05$ between *sch9*Δ and *sch9*Δ*sod2*Δ at day 5). (D) Percentage of survival of wild-type cells, of respiratory-deficient mutants *coq3*Δ and *atp2*Δ, and of wild-type cells treated with agents that increase the generation of mitochondrial superoxide (1 μM antimycin A and 1 mM paraquat). Treatment with antimycin A or paraquat causes the inactivation of aconitase (data not shown). ($P < 0.05$ for *coq3*Δ, *atp2*Δ, antimycin A, or paraquat *vs.* wild type).

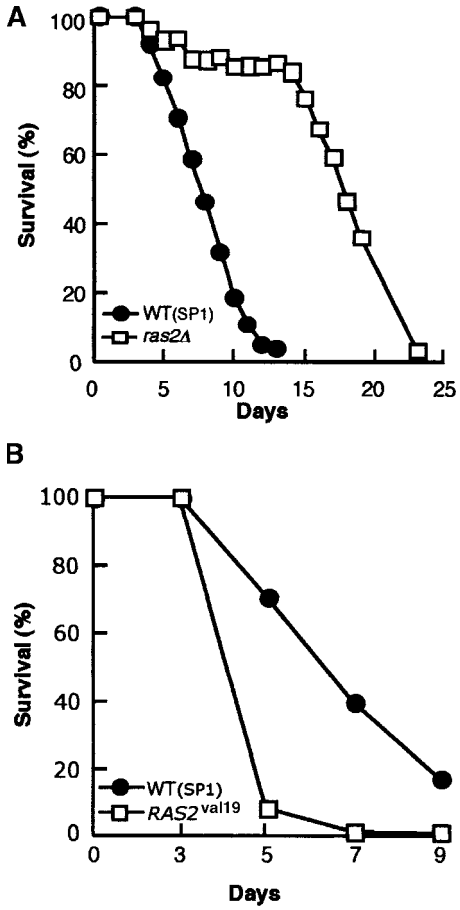


FIGURE 4.—Survival of wild-type and *ras2*Δ mutants in the postdiauxic phase. (A) The percentage of survival is shown for wild-type (SP1, solid symbols) and *ras2*Δ (KP2, open symbols) yeast populations. Experiments were performed three times with similar results. A representative experiment with the average of duplicate wild-type and *ras2*Δ populations is shown. The survival time for the *ras2* strain was significantly longer than that for wild type [$P < 0.05$; the deletion of *RAS2* causes a similar increase in life span in the DBY746 background (EG 252); data not shown]. (B) Wild-type (SP1) and $RAS2^{val19}$ mutants with constitutive active Ras2 (TK1611R2V). A representative experiment is shown. The experiment was repeated twice with similar results. The survival time for the $RAS2^{val19}$ strain was significantly shorter than that for wild type ($P < 0.05$).

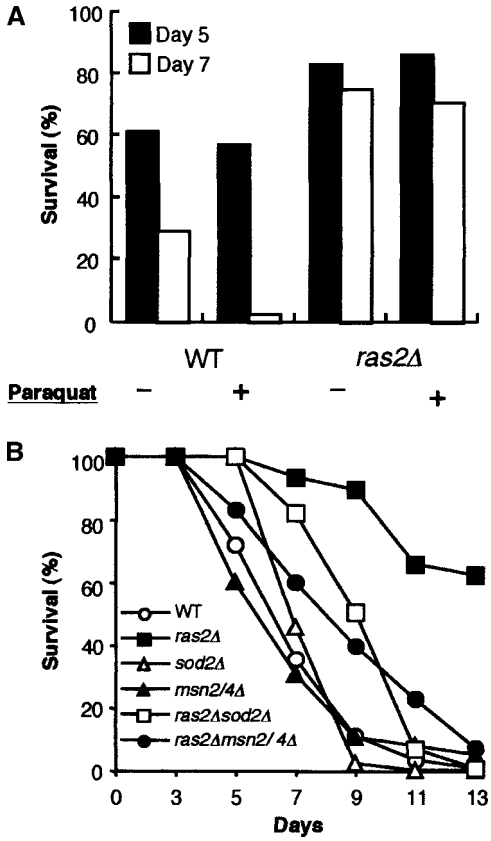


FIGURE 5.—Superoxide toxicity and survival of *ras2*Δ mutants. (A) Wild-type (DBY746) and *ras2*Δ (EG252) cells were grown in SDC. A total of 1 mM paraquat (superoxide-generating agent) was added after 24 hr. Viability was measured at days 5 and 7. A representative experiment with triplicate samples is shown. The experiment was repeated three times with similar results. (B) Chronological life span for wild-type cells (DBY746) and for mutants lacking *RAS2* alone or in combination with deletions in *MSN2/4* or *SOD2* (PF107, PF104). The experiment was performed at least twice in duplicate. The average of six to eight independent samples is shown ($P < 0.05$ for *ras2*Δ*msn2/4*Δ compared to that of *ras2*Δ and for *ras2*Δ *sod2*Δ compared to that of *ras2*Δ *or* WT).

To elucidate the longevity regulatory pathway upstream of Cyr1, we measured the life span of *ras1* and *ras2* deletion mutants. Deletion of *RAS1* in strain SP1 slightly decreased survival (data not shown), but the deletion of *RAS2* doubled survival in both the SP1 (Figure 4A) and the DBY746 background (data not shown; $P < 0.05$). To confirm the role of Ras2 in longevity, we tested strains carrying temperature-sensitive mutations in the Ras pathway. *ras1-ras2*^{ts} (lacking *RAS1* and with a temperature-sensitive mutation in *RAS2*) maintained at the restrictive but not at the permissive temperature doubled survival compared to wild-type controls (data not shown). To test the role of increased Ras2 activity on survival, we monitored the survival of mutants with constitutively active Ras2 ($RAS2^{val19}$). The activation of Ras2 caused early death (Figure 4B). The mean life span of mutants with constitutively active PKA (*bcy1*) was also decreased from 6 to <2 days (data not shown). These results suggest that a pathway that includes Ras2, Cyr1, and PKA regulates the chronological life span.

Ras2, Msn2/Msn4, and SOD2: To test whether *ras2* mutants are resistant to oxidative stress during aging we treated mutant strains with the superoxide-generating agent paraquat. *ras2* mutants (EG252) retained >70% of the initial viability after a 7-day treatment with paraquat (1 mM) compared to the 5% survival rate for paraquat-treated wild-type controls (DBY746; Figure 5A). To test the role of stress-resistance genes in the extended longevity of *ras2*Δ mutants, we deleted transcription factors Msn2 and Msn4 in *ras2*Δ (PF107). The deletion of *msn2*Δ*msn4*Δ abolished the effect of *ras2*Δ on longevity

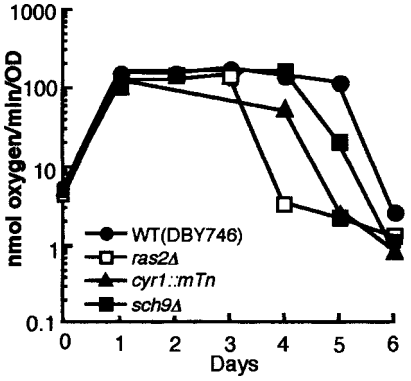


FIGURE 6.—Metabolic rates for the long-lived mutants. Oxygen consumption for wild-type strain DBY746 and *ras2*Δ, *cyr1::mTn*, and *sch9*Δ mutants generated in the DBY746 background (EG252, PF101, PF102) is displayed. The average of three independent samples for each strain is shown. Oxygen consumption was also determined for strain SP1 and *ras2*Δ mutants generated in the SP1 background (KP-2). The rate of oxygen consumption remained high until day 6 for both SP1 and KP-2 (data not shown). Day 0 represents the point at which the cells reach an OD_{600} of 1.

(Figure 5B, $P < 0.05$). The role of Msn2/Msn4 in mediating longevity extension in both *ras2*Δ and *cyr1::mTn* mutants (FABRIZIO *et al.* 2001) suggests that Ras2 and Cyr1 function in the same pathway to downregulate stress resistance and promote senescence.

To test whether superoxide dismutases function downstream of the Ras2/PKA/Msn2/Msn4 pathway to regulate survival extension in *ras2*Δ mutants, we deleted *SOD2* in *ras2*Δ mutants (*ras2*Δ*sod2*Δ, PF104). The survival of *ras2*Δ mutants was shortened by the deletion of *SOD2* (Figure 5B; $P < 0.05$). However, *ras2*Δ*sod2*Δ survived 30% longer than wild-type cells ($P < 0.05$), confirming that the induction of other systems is important for survival extension. To test whether increasing superoxide protection could extend further the survival of *ras2*Δ mutants, we overexpressed both *SOD1* and *SOD2* in *ras2*Δ mutants. *ras2*Δ *SOD1oxSOD2ox* mutants survived for slightly shorter periods than *ras2*Δ mutants, indicating that *ras2*Δ cells have optimized their protection against superoxide toxicity (data not shown).

Age-dependent metabolic rates: We characterized further the chronological life span and tested whether survival extension is associated with an early decrease in metabolic rates by measuring oxygen consumption in long-lived mutants. In two wild-type strains (DBY746 and SP1), respiration was low when the cells were actively growing in log phase, increased during the diauxic shift, and remained high until day 5 or 6 (Figure 6 and data not shown). In *sch9*Δ mutants, the age-dependent oxygen consumption was similar to that of wild-type cells (Figure 6). Metabolic rates in the DBY746 background decreased 48 hr earlier in *ras2*Δ and *cyr1::mTn* mutants than in wild-type cells. However, in the SP1 background the age-dependent oxygen consumption for *ras2*Δ was similar to that of wild-type cells (data not shown). Neither *SOD1SOD2* nor *SOD1CTT1* overexpression had significant effects on the age-specific metabolic rates compared to DBY746 plasmid controls (data not shown). These results suggest that an early decrease in metabolic rates is associated with certain mutations that extend survival, but is not required for longevity extension.

Survival in the reproductive and postreproductive phase: The chronological life span in the postdiauxic phase is measured by monitoring the ability of a cell to form a colony within 3 days of incubation on complete medium (CFUs). We tested whether the loss of CFUs correlates with the death of the organism. We measured the concentration of proteins released into the medium by dead and damaged wild-type DBY746-plasmid control cells and by the longer-lived *SOD1SOD2* double overexpressors. The increase in protein concentration in the medium of both strains began within 2 days of the major loss of CFUs at day 10 (Figure 7). The protein concentration in the medium of *SOD1SOD2* overexpressors, which survive 2 days longer, increased 2 days later and remained lower throughout the study compared to wild-type controls (Figure 7A). We also measured viability by staining cells with a fluorescent dye at days 3–7. Approximately 20% of the cells were dead at days 3 and 5 whereas 70% were dead at day 7 (Figure 7B). Taken together, these results suggest that the loss of the ability to form a colony is followed by death and lysis and is a valid method to estimate the total chronological life span of yeast (including the postreproductive phases).

DISCUSSION

Signal transduction proteins that regulate longevity have been identified in several organisms including yeast, worms, flies, and mice (KENYON 2001; LONGO and FABRIZIO 2002). However, the mechanisms responsible for longevity extension in these model systems are poorly understood. This study shows that expression of mitochondrial *SOD2* is required for the longevity extension caused by mutations that decrease the activity of the Ras/Cyr1/PKA and Sch9 pathways and confirms that superoxide toxicity plays an important role in yeast aging and death. However, *SOD2* overexpression is not sufficient for maximum survival, suggesting that other genes regulated by stress-resistance transcription factors Msn2/Msn4 and Gis1 contribute to longevity extension.

Our previous studies showed that the genes regulated by stress-resistance transcription factors and kinases, including Msn2, Msn4, and Rim15, mediate chronological life-span extension in yeast (FABRIZIO *et al.* 2001). The *SOD2* promoter contains an STRE element regulated by Msn2/Msn4 and a PDS element regulated by transcription factor Gis1, which functions downstream of Rim15 (FLATTERY-O'BRIEN *et al.* 1997; PEDRUZZI *et al.* 2000). The reduced life span of *ras2*Δ *sod2*Δ, *cyr1::mTn*

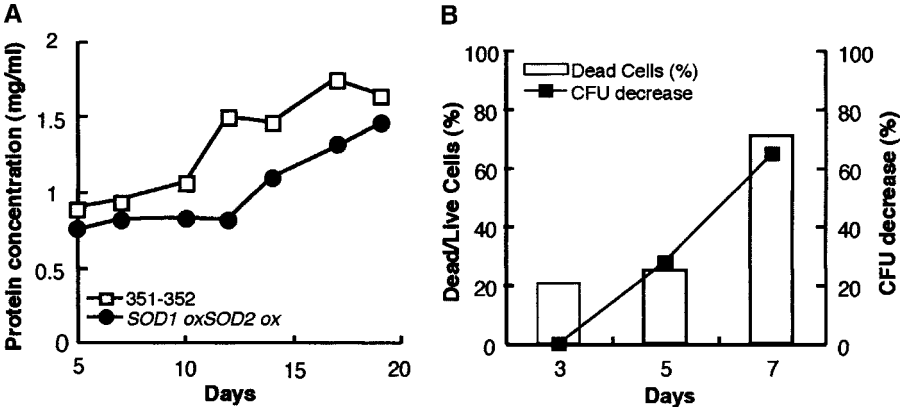


FIGURE 7.—Age-dependent viability loss. (A) Concentration of proteins released into the medium by wild-type controls (DBY746 351-352) or *SOD1SOD2* double overexpressors. (B) Comparison of viability using the CFU method or the live/dead fluorescent probe for yeast (FUN-1; Molecular Probes).

*sod2*Δ, and *sch9*Δ *sod2*Δ double mutants observed in this report indicates that longevity is extended in part by inducing *SOD2* expression. The increase in *SOD2* expression in *sch9*Δ mutants supports this conclusion. By contrast, the low expression of *SOD2* at days 5 and 6 in *cyr1::mTn* mutants is surprising considering that an increase in *SOD2* expression has been demonstrated in both *ras2*Δ mutants and *cyr1* temperature-sensitive mutants maintained at the restrictive temperature (FLATTERY-O'BRIEN *et al.* 1997). However, the downregulation of mitochondrial respiration in *cyr1* mutants at day 5 may cause an early decrease in Sod2 and in other enzymes that protect mitochondria against oxidative damage (Figure 6). The early entry of *cyr1::mTn* mutants in a hypometabolic state, which decreases oxygen consumption by >100-fold, is expected to reduce superoxide generation and may explain the limited effect of the *SOD2* deletion on the extended survival of *cyr1::mTn* mutants.

The double overexpression of *SOD1SOD2* but not of *SOD1* and *CTT1* or of each gene alone extends survival by 30%. Although Sod1 is found mainly in the cytosol, it also reaches the mitochondrial intermembrane space (OKADO-MATSUMOTO and FRIDOVICH 2001) and may protect against the superoxide generated in mitochondria and released into both the matrix and the intermembrane space (HAN *et al.* 2001). Therefore, both *SOD1* and *SOD2* may extend life span by protecting yeast against mitochondrial superoxide, although increased protection against cytosolic superoxide is also likely to be important for long-term survival (LONGO *et al.* 1996). The association between mortality increase and aconitase reactivation is consistent with a role for superoxide-dependent mitochondrial damage in yeast aging and death. In fact, aconitase inactivation and reactivation are lower in long-lived mutants than in wild-type yeast (Figure 3; FABRIZIO *et al.* 2001). The ability of iron and sulfur to restore aconitase activity indicates that the enzyme is present in the cells in a form that can be reactivated, most likely in a 3Fe-4S cluster form, suggesting that iron has been released from aconitase (GARDNER *et al.* 1995). This release of iron from the 4Fe-4S cluster may further increase oxidative damage by promoting the generation of the strong oxidant hydroxyl radical by Fenton chemistry (FRIDOVICH 1995). Therefore, superoxide may promote aging by decreasing the activity of an essential TCA cycle enzyme and by promoting the generation of highly reactive free radicals. Evidence for the role of superoxide and mitochondrial damage in decreasing survival was provided by the effect of paraquat and antimycin A on life span. These agents, which promote the generation of superoxide and are particularly toxic to *sod2*Δ mutants (LONGO *et al.* 1999), decreased the survival of wild-type cells (Figure 3D). Mutations that cause respiratory deficiency also caused early death. These results are consistent with a role for high levels of mitrochondrial superoxide and the loss of mitochondrial function in aging and death. Although Sod2 is required for survival extension, the induction of antioxidant protection can account only for part of the longevity extension caused by mutations in the Ras/cAMP and Sch9 pathways. In fact, the effect of the overexpression of both *SOD1* and *SOD2* on life span is much smaller compared to that caused by *ras2*Δ or *sch9*Δ mutations. Furthermore, *ras2*Δ and *cyr1::mTn* mutants lacking *SOD2* survive for shorter periods than the single mutants, but survive for longer periods than wild type, suggesting that other systems contribute to life-span extension.

The similarities between the genes and pathways involved in the regulation of chronological longevity in yeast and higher eukaryotes are remarkable. In yeast, the downregulation of glucose signaling by *ras2*, *cyr1*, and *sch9* mutations increases longevity and resistance to oxidative stress and heat shock (LONGO 1999; FABRIZIO *et al.* 2001). In the *cyr1* mutants chronological life-span extension is mediated by the stress-resistance transcription factors Msn2 and Msn4, which induce the expression of genes encoding for several heat-shock proteins, catalase (*CTT1*), the DNA-damage-inducible gene *DDR2*, and *SOD2* (THEVELEIN and DE WINDE 1999; FABRIZIO *et al.* 2001). Analogously, in worms, mutations

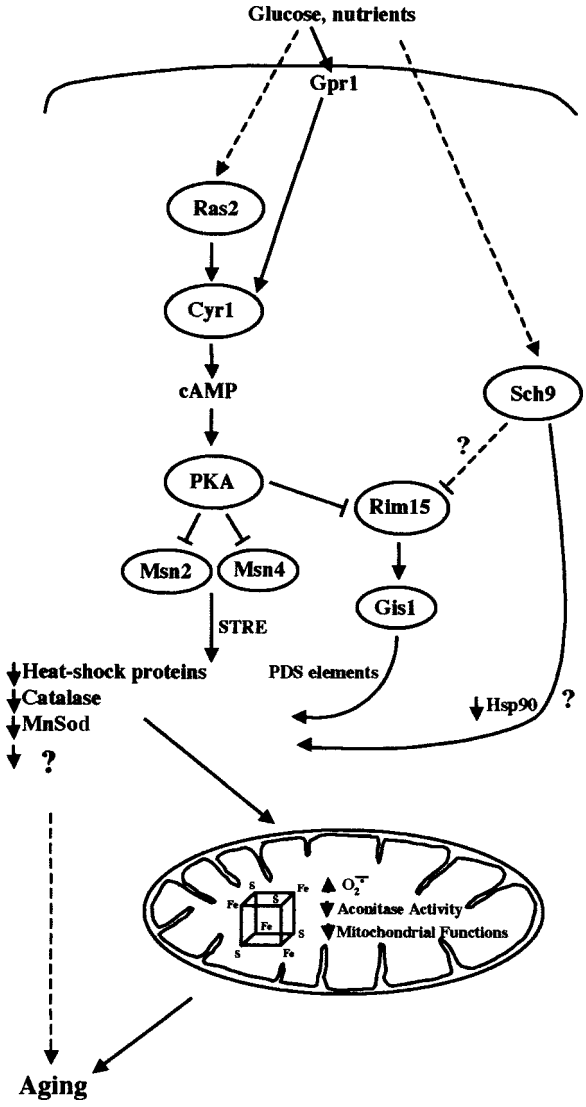


FIGURE 8.—Model for the regulation of stress resistance and aging in yeast. Glucose activates the Cyr1/cAMP/PKA pathway, in part, via the G-protein-coupled receptor Gpr1 and activates Sch9 by an unknown mechanism. Cyr1/cAMP/PKA inactivates stress-resistance transcription factors Msn2 and Msn4, which regulate the expression of many stress-resistance genes including those encoding for heat-shock proteins, catalase, and Sod2. Activation of Sch9 results in a major decrease in stress resistance either via Rim15/Gis1 and/or Hsp90 or via an unidentified effector. Mutations that decrease the activity of Ras2 (*ras2*Δ), Sch9 (*sch9::mTn* and *sch9*Δ), or Cyr1 (*cyr1::mTn*) extend the chronological life span by activating stress-resistance proteins Msn2, Msn4, and Rim15, by decreasing the levels of mitochondrial superoxide, by delaying aconitase inactivation, and by other unknown mechanisms.

in the signal transduction genes *age-1* and *daf-2* extend survival by 65–100% (JOHNSON 1990; KENYON *et al.* 1993) and increase thermotolerance and antioxidant defenses (LARSEN 1993; LITHGOW *et al.* 1995; KIMURA *et al.* 1997), apparently through stress-resistance transcription factor DAF-16 (LIN *et al.* 1997). In yeast, chronological life-span extension is associated with decreased mitochondrial superoxide generation and aconitase inactivation and requires mitochondrial Sod2. Analogously, among the genes regulated by the worm *daf-2* pathway are several heat-shock proteins and mitochondrial SOD (HONDA and HONDA 1999; CHERKASOVA *et al.* 2000). The yeast Ras/Cyr1/PKA pathway downregulates glycogen storage and genes involved in the switch to the hypometabolic stationary phase and to the dormant spore state (WERNER-WASHBURNE *et al.* 1996; BOY-MARCOTTE *et al.* 1998). The worm *daf-2* pathway also regulates the storage of reserve nutrients (fat and glycogen) and the switch to the hypometabolic dauer larvae state (KENYON *et al.* 1993; MORRIS *et al.* 1996; KIMURA *et al.* 1997). Thus, yeast and worms appear to regulate stress resistance and longevity by modulating the activity of similar proteins and pathways (LONGO 1999; KENYON 2001; LONGO and FABRIZIO 2002). Recent results suggest that analogous pathways may also regulate stress resistance and aging in Drosophila and mammals (KENYON 2001; LONGO and FABRIZIO 2002).

In conclusion, this report provides evidence for the existence of yeast prosenescence pathways activated by glucose and other nutrients and downregulated by starvation (Figure 8). These pathways, which include Ras2/Cyr1/PKA and Sch9, downregulate stress-resistance transcription factors Msn2/Msn4 and Gis1 and consequently downregulate the expression of many stress-resistance genes (Figure 8), including mitochondrial *SOD2*. The combination of high respiratory rates and low protection against superoxide in old yeast results in aconitase inactivation and mitochondrial damage, which is likely to play a major role in aging and death. However, the induction of additional stress-resistance systems appears to be required for maximum longevity extension. It will be important to identify additional mediators of longevity extension in yeast and to determine whether an analogous starvation-dependent pathway regulates longevity in mammals.

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