Enhanced Gluconeogenesis and Increased Energy Storage as Hallmarks of Aging in Saccharomyces cerevisiae

Received for publication, April 19, 2001, and in revised form, June 21, 2001
Published, JBC Papers in Press, July 18, 2001, DOI 10.1074/jbc.M103509200

Stephen S. Lin, Jill K. Manchester, and Jeffrey I. Gordon

From the Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

A relationship between life span and cellular glucose metabolism has been inferred from genetic manipulations and caloric restriction of model organisms. In this report, we have used the Snf1p glucose-sensing pathway of Saccharomyces cerevisiae to explore the genetic and biochemical linkages between glucose metabolism and aging. Snf1p is a serine/threonine kinase that regulates cellular responses to glucose deprivation. Loss of Snf4p, an activator of Snf1p, extends generational life span whereas loss of Sip2p, a presumed repressor of the kinase, causes an accelerated aging phenotype. An annotated data base of global age-associated changes in gene expression in isogenic wild-type, sip2Δ, and snf4Δ strains was generated from DNA microarray studies. The transcriptional responses suggested that gluconeogenesis and glucose storage increase as wild-type cells age, that this metabolic evolution is exaggerated in rapidly aging sip2Δ cells, and that it is attenuated in longer-lived snf4Δ cells. To test this hypothesis directly, we applied microanalytical biochemical methods to generation-matched cells from each strain and measured the activities of enzymes and concentrations of metabolites in the gluconeogenic, glycolytic, and glyoxylate pathways, as well as glycogen, ATP, and NAD+. The sensitivity of the assays allowed comprehensive biochemical profiling to be performed using aliquots of the same cell populations employed for the transcriptional profiling. The results provided additional evidence that aging in S. cerevisiae is associated with a shift away from glycolysis and toward gluconeogenesis and energy storage. They also disclosed that this shift is forestalled by two manipulations that extend life span, caloric restriction and genetic attenuation of the normal age-associated increase in Snf1p activity. Together, these findings indicate that Snf1p activation is not only a marker of aging but also a candidate mediator, because a shift toward energy storage over expenditure could impact myriad aspects of cellular maintenance and repair.

Genetic studies in model organisms imply that changes in glucose and energy metabolism can alter life span (1–4), although there has been little direct biochemical analysis of this hypothesis in aging cells (5). Saccharomyces cerevisiae is an attractive model for studying how glucose and energy metabolism are linked to aging. Age-associated alterations in energy metabolism can be analyzed more readily in a unicellular eukaryote than in a multicellular organism with diverse specialized cell types (6). Mother yeast cells undergo replicative senescence, with different strains having characteristic mean and maximum life spans (7, 8). Aging is associated with readily scored phenotypic changes including sterility (9). In addition, genetic studies suggest that changes in glucose metabolism affect replicative life span. For example, increased longevity occurs with mutations affecting the glucose-responsive AMP-dependent protein kinase A pathway, hexokinase (catalyzing the first step in the glycolytic pathway), or the Snf1 pathway (10, 11).

Snf1p is a serine/threonine kinase required for the normal cellular response to glucose starvation and represents the yeast homolog of AMP kinase, a mammalian cellular “fuel gauge” (12). Snf1p is incorporated into a complex that contains Snf4p, Sip1p, Sip2p, and Gal83p. When there is limited glucose, Snf4p activates Snf1p kinase, which phosphorylates a number of target proteins including transcriptional regulators of genes involved in alternative carbon source utilization, gluconeogenesis, and respiration (12). The best characterized of these transcription factors is Mig1p, which represses many glucose-repressed genes (13). Snf1p phosphorylation of Mig1p results in Mig1p accumulation in the cytoplasm and transcriptional derepression (14). Mig1p undergoes progressive redistribution from the nucleus to the cytoplasm as wild-type (WT) cells age, consistent with a progressive generational increase in Snf1p activity (11). Loss of the Snf1p activator Snf4p produces a 20% increase in life span, whereas forced expression of SNF1 produces rapid aging (11). Loss of Sip2p also causes a rapid aging phenotype that appears to be related to increased Snf1p activity; cytoplasmic accumulation of Mig1p is augmented and the rapid aging phenotype is completely rescued by snf4Δ (11). Together, these findings suggest that Snf1p kinase inhibition (loss of Snf4p) extends life span, whereas kinase activation (loss of Sip2p) shortens life span. The effects of Snf1p kinase activation on life span must extend beyond its phosphorylation of Mig1p, because mig1Δ cells do not undergo rapid aging (11).

In this report, we have used three isogenic strains, WT, sip2Δ (rapid aging phenotype), and snf4Δ (extended life span

This paper is available on line at http://www.jbc.org

□ The online version of this article (available at http://www.jbc.org) contains Supplemental Material that includes the annotated list of genes, identified by DNA microarray analysis, as showing age-associated changes in expression, plus detailed protocols for the microanalytical biochemical assays used in this study.

† To whom correspondence should be addressed: Dept. of Molecular Biology and Pharmacology, Washington University School of Medicine, Box 8103, 660 S. Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-7243; Fax: 314-362-7047; E-mail jjgordon@molecool.wustl.edu.

* This work was supported by in part by National Institutes of Health Grant AI38200 and by pre-doctoral support (for S. S. L.) from the American Federation for Aging Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
phenotype), as models to explore the relationship between glucose metabolism and aging. High density oligo-based microarrays were employed to compare first transcriptional responses as these cells aged. Microanalytic biochemical methods were then used to correlate observed transcriptional responses of genes involved in glucose and energy metabolism with the activities of several key enzymes and metabolites in the gluconeogenic and glycolytic pathways. Finally, because caloric restriction extends the life spans of diverse eukaryotes (10, 15–17), we compared glucose metabolism in calorically restricted and normally fed WT yeast as they aged. Our data indicate that aging in yeast is accompanied by a shift of glucose metabolism away from glycolysis and toward gluconeogenesis and energy storage. This shift is deferred by removing Snf4p or by caloric restriction.

**Experimental Procedures**

**Strains**

Isogenic strains YB332 (WT; S288CMA7aNMT1ura3-52his3 Δ200ade2-101lyz2-801leu2 3, 112), YB810 (sip2::HIS3), and YB614 (snf4::HIS3) are described in Ref. 11.

**Isolation of Young and Older Yeast Cell Populations**

The sorting procedure is based on the finding that when surface proteins of a mother yeast cell are labeled with biotin, the daughter does not inherit the biotinylated proteins, because their surface is generated de novo at the budding site (9). This allows mothers to be isolated from their descendants by streptavidin-magnetic bead sorting. To obtain de novo not inherit the biotinylated proteins, because their surface is generated by magnetic bead sorting (11). To obtain.

**Microarray-Based Gene Expression Analysis**

**DNA Microarray Analysis**

Generation 0–1 and 7–8 cells were obtained from the same culture by magnetic bead sorting (n = 3 independent cultures/strain). Sorted young or old cells (∼5 × 10^5/sort) were immediately incubated in YPD at 25°C for 20 min (temperature equilibration step), recovered by centrifugation, and washed three times in ice-cold PBS. Cell pellets were frozen in liquid N2, dried under vacuum, lysed by adding RLT buffer (RNeasy kit; Qiagen; 200 µl buffer/cell pellet), and RNA was purified (RNA binding column protocol from Qiagen). For each strain, equivalent amounts of RNA from generation-matched cells from the triple sorts were pooled. Two cRNA target sets were independently prepared from each RNA pool (20 µg of RNA/pool). Each set of cRNA targets was hybridized to duplicate high-density oligonucleotide-based Ye98 GeneChips (Affymetrix; www.affymetrix.com) according to the manufacturer’s protocols. The overall fluorescence intensity across each chip was scaled to a target intensity of 2500 using Affymetrix GeneChip software. Pairwise comparisons of expression levels in old versus young cells were performed for each strain. Differences of 2-fold or greater were considered significant if they met three criteria: the mRNA was called “present” by the GeneChip software, in both the difference was reported as “increased” or “decreased,” and the difference was observed in duplicate microarray hybridizations.

**Microanalytic Biochemical Assays**

**General Comments about Enzyme Cycling**—This sensitive, versatile, and well established analytic method (18) allows the levels of enzymes, metabolites, and nucleotides to be measured through reactions that generate reduced or oxidized forms of NAD or NADP. The usefulness of employing NAD and NADP for analytic purposes has been described by Lowry and Passoneau (18). This method was adapted for S. cerevisiae as outlined below.

**Preparation of Cellular Extracts**—Generation 0–1 and 7–8 cells were recovered by sorting at 4°C. Cell number was defined by hemocytometer. Cells from each sort were divided into three aliquots of 10^7 cells each, one for RNA extraction (and subsequent DNA microarray profiling of gene expression), another for enzyme assay, and the other aliquot for measurement of cellular metabolites. Cells were incubated at 25°C in YPD for 20 min, followed by three washes in PBS (4°C). After the final wash, cell pellets were quickly frozen in liquid nitrogen and freeze-dried under vacuum for 4 days at −35°C.

To prepare lysates for assaying cellular enzyme activities, freeze-dried cells were incubated for 1 min at 25°C in 1 ml of extraction buffer (20 mM phosphate buffer, pH 7.4, 0.02% bovine serum albumin, 0.5 mM EDTA, 5 mM β-mercaptoethanol, 25% glycerol, and 0.5% Triton X-100). The lysates were stored at −80°C until further use. With the exception of fructose-6-phosphate kinase, which was measured in fresh lysates, all other enzymes studied were stable in lysates even after several cycles of freezing and thawing.

To measure cellular metabolites, 500 µl of ice-cold 0.05 M NaOH/1 mM EDTA was added to 10^7 freeze-dried cells, and the extract was quickly divided into two 200-µl aliquots. 200 µl of 0.1 M HCl was added to one of the aliquots to generate an acid extract. The remaining alkaline extract plus the acid extract were incubated at 60°C for 30 min to destroy endogenous enzyme activities and pyridine nucleotides. The alkaline extract was neutralized by adding 100 µl of a solution containing 100 mM Tris-HCl, pH 5.1, and 0.05 M HCl. The acid extract was neutralized with 100 µl of 0.4 M Tris base. All extracts were stored at −80°C prior to use.

**Enzyme Cycling**—The low levels of some enzyme activities, metabolites, and nucleotides in the extracts required that NAD+ or NADPH, generated in the primary analytic reaction, be amplified through a series of cycling steps. For NAD+ or NADPH, each cycling step involved coupled reactions (Scheme I). The scheme used for NADPH is shown in Scheme II. Cycling was performed in 10 × 75-mm borosilicate glass tubes. After an appropriate number of cycles were performed, the reaction was terminated by heating at 100°C for 5 min. Once the samples were cooled to room temperature, 1 ml of indicator reagent was added to convert the cyclized product (malate in the case of NAD+ cycling, Scheme III), and 6-phosphogluconate in the case of the NADPH cycling) to NADH or NADPH, respectively.

The NAD+ cycling indicator step involved addition of 1 ml of malate reagent (50 mM amino-methylpropanol (pH 9.9), 5 mM l-glutamate (pH 9.9), 0.2 mM NAD+, 5 µg/ml malic dehydrogenase (3000 units/mg protein; Sigma), and 2 µg/ml glutamate oxaloacetate transaminase (200 units/mg; Roche Molecular Biochemicals)). The NADPH cycling indicator step was initiated by adding 1 ml of 6-phosphogluconate reagent (50 mM imidazol-HCl (pH 7.0), 25 mM acetic acid, 1 mM EDTA, 30 µM ameso-
nium acetate, 5 mM MgCl₂, 0.1 mM NAD⁺, and 2.5 µg/ml 6-phosphogluconate dehydrogenase (20 units/mg; Sigma)).

Indicator reactions were incubated for 10 min at 25 °C. The NADH generated from malate or the NADPH produced from 6-phosphogluconate was measured fluorometrically (excitation at 365 nm, emission monitored at 460 nm).

Some assays were performed in reaction volumes that were less than 10 µl. To prevent evaporation, these small volume reactions were placed under a mixture of 40% hexadecane and 60% mineral oil in a Teflon block that contained 50 µl-capacity wells.

Care was taken to ensure that for each enzyme, metabolite, or nucleotide determination, product formation was linear with respect to the range of lysate or extract used. For each experiment, several types of reference standards were also analyzed. Standards consisting of either the nucleotide or metabolite of interest, or the product of the enzyme being assayed, were run in parallel with cell lysates or extracts. NAD⁺ or NADPH standards were always added at the cyclizing step in a minus extract or minus lysate control to coincide with levels produced by the experimental reactions. A known amount of malate or 6-phosphogluconate, corresponding to the predicted concentration of pyridine nucleotide produced from the cyclizing reaction, was included as a third reference control. Details of the protocols for each yeast enzyme and metabolite surveyed in this study are presented as Supplemental Material.

To define cell volume, aliquots from each sort of each strain were fixed in 4% glutaraldehyde and embedded in Polybed (Polyscience, Warrington, PA). 70 nm-thick sections were counter-stained with uranyl acetate and lead and viewed under a JOEL ×100 electron microscope. Cell diameters were measured (n = 30 cells/generation/sort), and the average cell volume was defined assuming a spheroidal shape. The volumes (in femtoliters) for generation 0–1 and 7–8 cells were as follows: WT (incubated in YPD containing 2% glucose), 22 ± 6 and 49 ± 7.2, respectively; calorically restricted WT (YPD/0.5% glucose), 21 ± 1.1 and 37 ± 4.8; sip2Δ (YPD/2% glucose), 134 ± 11.4 and 114 ± 10.9; snf4Δ (YPD/2% glucose) 26 ± 2.9 and 57 ± 5.4. In addition, volumes were determined for WT cells at various phases of growth in YPD (2% glucose) as follows: mid-log, 19.6 ± 1.6; early diauxic, 51.3 ± 5.7; late diauxic, 61.5 ± 6.6. These volumes were used to calculate concentrations of various metabolites.

RESULTS

DNA Microarray Analysis of Gene Expression in Aging Yeast Cells—Isogenic WT, sip2Δ, and snf4Δ strains were grown in YPD (2% glucose) and sorted (see Experimental Procedures) into two populations, cells at generation 0–1 and at generation 7–8. RNA was prepared from cells that had been sorted from three independent cultures/strain. Triplicate RNA samples were pooled by strain and generational age. Two cRNA preparations were then independently synthesized from each pooled RNA sample, and each cRNA was used to probe duplicate DNA microarrays representing ~7000 genes from the yeast nuclear and mitochondrial genomes. Age-associated changes in gene expression were defined for each strain based on a comparison of generation 7–8 versus generation 0–1 cells. For a given strain, all transcripts that exhibited age-associated changes of ≥ 2-fold (increased or decreased) in duplicate microarray hybridizations were incorporated into a data base. A total of 95 genes fulfilled the selection criteria in WT cells, 182 genes in sip2Δ cells, and 182 in the snf4Δ strain. Decreases in levels of tRNAs, Ty retro element-derived species, and various snRNAs were prominent features in WT and sip2Δ strains (20 of a total of 59 RNAs with reduced levels in generation 7–8 versus 0–1 WT cells; 53 of 57 RNAs with decreased levels in rapidly aging sip2Δ cells). As longer lived snf4Δ cells progressed from generation 0–1 to 7–8, levels of a number of tRNA, snRNA, and Ty1 species increased.

The yeast genome is highly annotated (genome-www.stanford.edu/Saccharomyces/), with protein functions assigned based on direct assay, sequence homology, or growth phenotype. We used this annotation resource to place the genes exhibiting age-associated changes in expression into ten functional categories as follows: (i) energy production, (ii) energy storage, (iii) lipid/amino acid/sugar/nucleotide metabolism, (iv) DNA replication/repair/chromatin structure/transcription, (v) protein synthesis/modification/degradation/trafficking, (vi) cell cycle/division; (vii) cell signaling, (viii) glucose-regulated genes/proteins, (ix) transporters, and (x) other. Fig. 1 summarizes the representation of genes in the 10 functional categories. A complete list of the annotated entries in each category for each strain is included as Supplemental Material.

For each strain, 25–33% of the affected genes are involved in various aspects of cellular metabolism (energy production, energy storage, lipid/amino acid/sugar/nucleotide metabolism). The total number of WT and snf4Δ cells, a higher percentage of the genes that manifest generational changes in expression in rapidly aging sip2Δ cells encode products involved in energy storage or lipid/amino acid/nucleotide metabolism or are glucose-regulated (the genes themselves and/or their proteins). In contrast, a lower percentage of genes affected in sip2Δ cells are involved in energy production and DNA replication/repair (Fig. 1).

Changes in Expression of Genes Involved in Energy and Glucose Metabolic Pathways—Table I lists well-characterized components of energy and glucose metabolic pathways that fulfilled our selection criteria. The DNA microarrays revealed transcriptional responses in aging WT cells indicative of a loss of Mig1p repression, consistent with our earlier finding that Mig1p undergoes progressive, Snf1p-dependent translocation to the cytoplasm (11). Levels of mRNAs encoding phosphoenolpyruvate carboxykinase (PEPCK; gluconeogenesis) and malate synthase (glyoxylate cycle; see Fig. 2) rise ≥ 2-fold as WT cells age from generation 0–1 to 7–8 (27% of their mean life span). mRNAs derived from non-Mig1p-regulated genes involved in entry into the glyoxylate cycle are also increased (3-oxyacetylthiolase [Pot1p] and acetyl-CoA synthetase [Acs1p]; see Table I).

In sip2Δ cells, transcriptional profiling revealed evidence of more extensive loss of Mig1p repression at generation 7–8 (45% of mean life span) compared with generation-matched WT cells. Levels of mRNAs specifying PEPCK (Pck1p), malate synthase (Mls1p), Hxt2p (glucose transporter), fructose 1,6-biphosphatase (Fbp1p; key regulator of gluconeogenesis), Hap4p (gluconeogenic activator), and maltase (Mal32p) all increased ≥ 2-fold (Table I). A comparison of DNA microarray data sets from young (generation 0–1) WT and sip2Δ cells indicated that the levels of these six mRNAs were not appreciably different. Based on these findings, we concluded that the pronounced loss of Mig1p repression was an age-associated feature of the sip2Δ strain.

Expression of genes not regulated by Mig1p also increased as sip2Δ cells aged. For example, there was a rise in mRNAs encoding glycogen synthase (Gsy1p) and Gac1p. Gac1p is the regulatory subunit of the serine/threonine phosphatase Gcl7p (the Gcl7p-Gac1p holoenzyme is known to promote glycogen accumulation by dephosphorylation of Gsy1p or Gsy2p; see Ref. 19). The increase in levels of Gsy1p and Gac1p mRNAs suggested augmented glycogen accumulation in rapidly aging sip2Δ cells.

Pyruvate decarboxylase (Pdc6p), alcohol dehydrogenase II, acetyl-CoA synthetase (Acs1p), and malate synthase (Mls1p) mRNAs also rose ≥ 2-fold (Table I) as sip2Δ cells aged to generation 8, implying increased activity of the glyoxylate pathway that converts fatty acids and ethanol into glucose.

8 Comparisons of DNA microarray data sets produced from generation 0–1 WT and sip2Δ cells revealed 315 transcripts that exhibit ≥ 2-fold differences in their levels (increased or decreased) in duplicate microarray comparison of the strains (WT = baseline). A total of 181 transcripts fulfilled these criteria when young WT and snf4Δ strains were compared. An annotated list of these genes can be found in the Supplemental Material.
2). The DNA microarray comparisons disclosed no detectable differences in Pdc6p and Mls1p mRNA concentrations in young WT and sip2/H9004 cells, whereas mRNAs encoding alcohol dehydrogenase II and Acs1p were 2–3-fold higher in the sip2/H9004 strain. In contrast, snf4/H9004 cells at generation 7–8 (23% of life span) did not manifest the changes in expression of genes involved in gluconeogenesis and glycogen synthesis that were evident in aging WT or sip2/H9004 cells (Table I). Moreover, Sds22p, encoding another positive regulator of G1c7p (19), underwent age-associated repression, as did genes involved in lipid and ethanol breakdown (e.g. 3-oxoacyl-CoA thiolase and alcohol dehydrogenase II).

Together, these transcriptional responses suggest that gluconeogenesis and glucose storage increase as WT yeast cells age, that this metabolic evolution is exaggerated in rapidly aging sip2Δ cells, and that it is attenuated in longer-lived snf4Δ cells. To test this hypothesis directly, we measured glycogen levels, the activities of components of the gluconeogenic, glycolytic, and glyoxylate pathways, plus ATP and NAD⁺ in aging cells from each isogenic strain.

*Biochemical Analysis of Glucose Metabolism in Aging Yeast Cells*—Extracts were prepared from aliquots of the same sorted cell preparations used for the DNA microarray studies. Levels of enzymes and key metabolic intermediates were assayed using classic pyridine nucleotide-based enzyme cycling methods (18). These well established methods are highly sensitive, allowing us to perform a comprehensive analysis of both enzymes and metabolites on a single sample of 10⁶ sorted cells. Glycogen levels rise when WT cells age (Fig. 3). The age-associated increase is significantly greater in sip2Δ cells (p < 0.05), consistent with the observed rise in Gsy1p and Gac1p mRNA levels. In contrast, glycogen concentrations do not change between generations 0–1 and 7–8 in snf4Δ cells and are markedly lower than in generation-matched WT cells (p < 0.05; see Fig. 3).

![Graphical representation of transcriptional responses](image)
 Shift toward Energy Storage in Aging Yeast Cells

Results are presented as the -fold difference in mRNA levels between old versus young cells and represent average values from duplicate GeneChip hybridizations (see “Experimental Procedures”). Gene designations are from the Saccharomyces Genome database (genome-www.stanford.edu/Saccharomyces). NC, no change (average change ±2-fold).

| Gene name | Function | WT | sip2Δ | snf4Δ |
|-----------|----------|----|-------|-------|
| YFR015C   | Glycogen synthase (Gay1p) | NC | 2.7   | NC    |
| YOR175C   | Gac1p (positive regulator of Glc7p activator of Gay) | NC | 2.9   | NC    |
| YKL193C   | Sds22p (positive regulator of Glc7p) | NC | 1.9   | NC    |
| YIL090W   | Glucose isomerase | NC | 2.2   | NC    |
| YLR277C   | Fructose 1,6-bisphosphatase | NC | 3.6   | NC    |
| YKRO97W   | Phosphoenolpyruvate carboxykinase | 2.5 | 2.7   | NC    |
| YOL132C   | Cytosolic malate dehydrogenase | NC | 1.9   | NC    |
| YGR286C   | Biotin synthase | NC | 2.2   | NC    |
| Glycogenesis | Fructose 1,6-bisphosphate and 3-phosphoglycerate levels | | | |
| YOR053C   | Hexokinase I | NC | NC | –2    |
| Glyoxylate cycle | Isocitrate lyase | 2 | NC | NC    |
| YNL117W   | Malate synthase | 2.4 | 4    | NC    |
| Krebs cycle | Mitochondrial malate dehydrogenase | –2 | NC | NC    |
| YKL029C   | | | | |
| Oxidative phosphorylation | | | | |
| YIL11W    | Cytochrome c oxidase chain Vb | NC | NC | 2.7    |
| YDR097W   | Cytochrome c oxidase assembly factor | NC | NC | 2.5    |
| Lipid metabolism | 3-Oxoadyl-CoA thioldase | 2.8 | 7 | –5.1   |
| YIL160C   | Acetyl-CoA synthetase | 2 | 2.7 | –1.9   |
| YAL054C   | 2,4 Dienol-CoA reductase | NC | 1.9 | –2.2   |
| YNL202W   | Carnitine O-acetyltransferase | NC | 2.4 | NC    |
| Ethanol/acetate metabolism | Pyruvate decarboxylase | NC | 10.8 | –5.3   |
| YGR087C   | Transcriptional activator of ADHII | NC | 2.5 | –2    |
| YDR216W   | Alcohol dehydrogenase II (Adh1p) | 2.5 | 7.6 | –2    |
| Other Mig1p-repressed genes | Hap4p (glucokinase activator) | NC | 2.1 | NC    |
| YKL109W   | Malate synthase | 2.4 | 4    | NC    |
| YNL117W   | Hexose transporter 2 (Hxt2p) | NC | 2.7 | NC    |
| YMR011W   | Maltase | NC | 2.1 | NC    |
| YBR299W   | | | | |

a Mig1p-repressed gene.

Glucogenic pathway activity increases markedly in rapidly aging, glycogen-rich sip2Δ cells. There is a significant increase in fructose 1,6 bisphosphatase activity and in glucose 6-phosphate (G-6P) and glucose 1-phosphate (G-1P) levels as these cells progress from generation 0 to 8 (Fig. 3). There is not a concomitant generational increase in glycolytic pathway activity; fructose 6-phosphate kinase activity remains unchanged whereas fructose 1,6 bisphosphate and 3-phosphoglycerate levels rise <2-fold (Fig. 3). Moreover, at generation 7–8, the concentrations of these latter two metabolites are significantly lower in sip2Δ compared with WT cells (p < 0.05; see Fig. 3). The suppressed glycolysis encountered in aging sip2Δ cells may be due in part to their marked age-associated elevation in citrate (Fig. 4), a known inhibitor of fructose 6-phosphate kinase.

The biochemical profiling experiments confirmed that snf4Δ cells defer this shift toward glucogenogenesis and glucose storage. For example, hexokinase levels do not rise as cells age from generation 0–1 to 7–8, and the increase in fructose 1,6 bisphosphatase activity is less pronounced than in WT or sip2Δ strains. G-1P concentrations are also significantly lower in the snf4Δ strain compared with WT (p < 0.05 at generation 7–8; see Fig. 3).

* S. cerevisiae contains two known hexokinase genes (HXK1 and HXK2). In WT cells, HXK2 is preferentially expressed during fermentative growth (20). The DNA microarray analysis indicates that Hxk1p mRNA levels fall slightly (2-fold) as snf4Δ cells age from generation 0–1 to 8 whereas Hxk2p mRNA remains unchanged. The fact that there is no significant change in hexokinase activity as snf4Δ cells age (Fig. 3) raises the possibility that Hxk2p is the dominant contributor to this cellular enzymatic activity. Assays that measure enzyme activity in cell extracts may not reflect actual in vivo activity. For example, the substrate concentrations used for all of the in vitro enzyme assays are several fold higher than the K_m. Moreover, enzyme activity in vivo may be greatly affected by cellular levels of inhibitors or activators (e.g. citrate in the case of fructose 6-phosphate kinase). Therefore, whenever possible we addressed these concerns about in vivo activity by measuring cellular levels of enzyme substrates or products. In the case of hexokinase, we were also able to measure hexokinase-catalyzed phosphorylation of glucose in vivo. To do this, sorted generation-matched cells from each strain were incubated for 15 min in 110 mM glucose and 0.1 mM 2-deoxyglucose (2-DG). The trace amount of 2-DG is taken up and metabolized by hexokinase to 2-deoxyglucose 6-phosphate (11). 2-Deoxyglucose 6-phosphate is trapped, because it is a very poor substrate for further enzymatic conversion. The in vivo glucose phosphorylation rate (GPR) can be determined using the equation p(g/d)C(t)/p where p is intracellular 2-deoxyglucose 6-phosphate concentration, g is the intracellular glucose concentration, d is the intracellular 2-DG concentration, C is a constant that estimates the ratio between the velocities of hexokinase with 2-DG and glucose substrates and t is time of incubation with 2-DG (18, 21). The GPR results (Fig. 3) provide direct in vivo evidence for a marked age-associated augmentation of hexokinase activity in sip2Δ cells. Moreover, by considering the GPR in light of the measured concentrations of G-6P, G-1P, glycogen, and 3-phosphoglycerate, we could conclude that there is an in vivo shift toward glucose storage and away from glycolysis as sip2Δ cells age. Comparisons of GPR in aging snf4Δ cells with G-6P, G-1P,
glycogen, and 3-phosphoglycerate concentrations indicate that snf4Δ cells maintain more active glycolysis than sip2Δ cells (Fig. 3).

The glyoxylate cycle allows acetate (from ethanol) and acetyl-CoA (from β-oxidation of fatty acids) to be metabolized to oxaloacetate and enter the gluconeogenic pathway. Fumarate is produced exclusively in the tricarboxylic acid cycle, whereas malate and citrate are generated by both the tricarboxylic acid and glyoxylate cycles (Fig. 2). Thus, an age-associated rise in the ratio of cellular malate to fumarate (or citrate to fumarate) without an accompanying change in the malate to citrate ratio can be taken as an indicator of increased glyoxylate cycle activity. The measured ratios of these glyoxylate cycle intermediates (Fig. 4D) provided additional evidence for a shift toward gluconeogenesis in aging WT cells, its augmentation in sip2Δ cells, and its attenuation in snf4Δ cells.

The Effects of Aging on ATP and NAD⁺ Levels—ATP rises markedly as sip2Δ cells undergo rapid aging, whereas ATP in generation 7–8 snf4Δ cells is significantly lower than in generation-matched WT or sip2Δ cells (p < 0.05; see Fig. 5A). Calorically restricted WT cells have lower ATP at generations 0–1 and 7–8 than those fed a normal glucose diet (p < 0.05; see Fig. 5A).

Sir2p (silent information regulator 2) is a NAD-dependent histone deacetylase that transfers an acetyl group from histone to a NAD⁺ breakdown product to produce the novel product O-acetyl-ADP-ribose (22, 23). Genetic studies have indicated that caloric restriction-mediated life span extension in yeast requires SIR2 plus NPT1, a nicotinic acid phosphoribosyltransferase involved in regeneration of NAD⁺ (10). These findings led to a proposal that caloric restriction prolongs life span through augmentation of NAD⁺ levels, activation of Sir2p, and increased silencing of chromatin. Surprisingly, we found that there is a statistically significant age-associated increase in NAD⁺ levels in rapidly aging sip2Δ cells (p < 0.05; see Fig. 5B). The increase in NAD⁺ in rapidly aging sip2Δ cells may be explained, in part, by reduced glycolysis (i.e., NAD⁺ is reduced to NADH during active glycolysis; see Fig. 2). NAD⁺ levels decrease in calorically restricted WT and normally fed snf4Δ cells as they age (p < 0.05), but the absolute levels of NAD⁺ are

---

**FIG. 2. Outline of cellular glucose metabolism.** Nomenclature is provided for yeast enzymes catalyzing selected steps in glucose metabolism that were identified from DNA microarray analysis of aging cells and/or assayed using microanalytic biochemical methods.

---

Shift toward Energy Storage in Aging Yeast Cells

---

36005
not significantly different from generation-matched, normally fed WT cells (Fig. 5B).

Rapidly aging sip2Δ cells exhibit evidence of decreased silencing, as judged by progressive sterility, redistribution of Sir complexes from telomeres to the nucleolus, and increased recombination at rDNA loci (11). The marked increase in NAD+/H11001 concentrations as sip2Δ cells rapidly age points to the difficulty of directly correlating Sir2p activity with total cellular NAD+/H11001 pool size and stresses the importance of identifying factors that regulate exposure of the histone deacetylase to NAD+/H11001.

Similarities between Aging sip2Δ Cells and WT Cells Entering the Diauxic Transition—As WT cells proceed from log phase through the diauxic transition toward stationary phase, they accumulate glycogen as a form of stored energy. Glycogen levels subsequently drop as a function of time in stationary phase. WT cells that recover from stationary phase show signs of accelerated aging (24).

The pronounced enhancement of glucose storage in rapidly aging sip2Δ cells is reminiscent of the response of WT yeast to the nutrient deprivation they encounter as they transition from log phase growth to stationary phase. This prompted us to explore whether the biochemical profile of aging sip2Δ cells phenocopies aspects of “starving” diauxic phase WT yeast. Indeed, our microanalytic biochemical assays disclosed that the diauxic response includes increased glucose storage (with elevations in G-6P, G-1P, and glycogen), augmented gluconeogenesis (increased fructose 1,6 bisphosphatase), and reduced glycolysis (decreased fructose 6-phosphate kinase) (see Figs. 3 and 4).

**DISCUSSION**

Combining DNA microarray-based transcriptional profiling with well established, but no longer commonly used, pyridine nucleotide-based microanalytic biochemical methods has provided a view of aging in *S. cerevisiae* as a metabolic evolution toward enhanced gluconeogenesis and augmented energy storage.

The age-associated changes in glucose metabolism observed in *S. cerevisiae* may be conserved over the course of metazoan evolution. As noted in the Introduction, genetic studies in
model organisms have implied that changes in glucose and energy metabolism can alter life span. For example, in Caenorhabditis elegans, increased longevity is produced by loss-of-function mutations affecting daf-2, encoding a protein with homology to the insulin receptor, or the age-1 phosphatidylinositol-3-kinase that functions immediately downstream of daf-2 in a signal transduction cascade (reviewed in Ref. 3). An inactivating mutation in the Drosophila Indy gene, specifying a protein with sequence similarities to mammalian cotransporters involved in uptake of di- and tricarboxylic intermediates generated by the tricarboxylic acid cycle, doubles life span (4). In C. elegans a null allele of Ctk-1, encoding a protein with homology to an activator of gluconeogenesis in S. cerevisiae (Cat5p), leads to life span extension (1, 2), a finding consistent with the correlation between reduced gluconeogenesis and increased life span we observed in yeast. Lee et al. (17) performed a DNA microarray-based survey of changes in gene expression in aging mouse skeletal muscle. A review of their published data set provides circumstantial evidence for an age-associated decrease in glycolysis (i.e. ≥2-fold drop in the levels of glucose 6-phosphate isomerase and α-enolase mRNAs) and an increase in glyogen synthesis (decrease in mRNA encoding IPP-2, an inhibitor of glycogen synthesis). These age-associated decreases in isomerase, enolase, and IPP-2 mRNAs were forestalled when mice were subjected to chronic caloric restriction (17). We have found that a prominent feature of the metabolic evolution of aging yeast cells is activation of the Snf1p kinase pathway. Evidence for this activation includes the following. First, Mig1p, a known substrate of Snf1p, undergoes relocalization from the nucleus to the cytoplasm as cells age. This correlates with the derepression of Mig1p-repressed genes defined by DNA microarray profiling of gene expression. sip2Δ cells undergo accelerated aging, precocious relocation of Mig1p to the cytoplasm, and more pervasive derepression of Mig1p repressed genes. Inhibiting Snf1p activation by removing Snf4p delays Mig1p derepression and extends life span. Second, the biochemical responses of aging cells are entirely consistent with increased Snf1p kinase activity. The observed age-associated increase in glycogen accumulation correlates with the known Snf1p activation of Gac1p-Glc7p (activator of glycogen synthases; see Ref. 25). Our finding that fructose 1,6-bisphosphatase activity rises in aging yeast cells is consistent with previous reports that Snf1p phosphorylation of another downstream transcription factor, Sip4p, activates the gene encoding this key regulator of gluconeogenesis (FBP1; see Ref. 26). Snf1p activation has also been shown to inhibit fatty acid biosynthesis through its phosphorylation of acetyl-CoA carboxylase (12). Our biochemical profiling experiments provide evidence for increased fatty acid degradation in aging yeast cells, as manifested by increased glyoxylate cycle activity. The interaction between Snf1p and its activator, Snf4p, appears to be promoted as cellular glucose levels fall (27). Remarkably, the age-associated increases in Snf1p kinase activity, gluconeogenesis, and glucose storage occur in absence of any decrease in cellular glucose concentrations (Fig. 3). This raises the as yet unanswered question of why these cells are responding as if they are glucose-deprived.

The AMPK pathway is activated in response to various stresses, including glucose deprivation, heat shock, hypoxia, and exercise (12). Once activated, AMPK phosphorylates some of the same downstream targets that are recognized by Snf1p, including acetyl-CoA carboxylase and glycogen synthase (12). Moreover, both Snf1p and AMPK are activated by phosphorylation. Although the kinase that phosphorylates Snf1p has yet to be identified, it can be activated in vitro by the mammalian AMPK-kinase kinase (12). Together, these observations suggest that it may be informative to assess the effects of aging on the activity of the AMPK pathway in various mammalian species.

In summary, our studies indicate that Snf1p activation is not only a new marker of aging but may also be one of its mediators. A progressive shift toward energy storage, rather than energy expenditure, could have important consequences to myriad aspects of cellular function. The idea that we age because we become more “conservative” should be testable in genetically manipulatable multicellular eukaryotes and in humans, using the same transcriptional and biochemical profiling methods employed for S. cerevisiae.

Acknowledgments—We thank Lisa Roberts for assistance with electron microscopy and Thalia Farazi and Kaveh Ashrafi for helpful discussions.

REFERENCES
1. Ewbank, J. J., Barnes, T. M., Lakowski, B., Lussier, M., Bussey, H., and Hekimi, S. (1997) Science 275, 980–983
2. Arking, R. (1996) Biology of Aging, 2nd Ed., pp. 417–420, Sinauer Associates, Sunderland, MA
3. Guarente, L., and Kenyon, C. (2000) Nature 408, 255–262
4. Rogina, B., Reenan, R. A., Nilsen, S. P., and Helfand, S. L. (2000) Science 290, 2137–2140
5. Vanfleteren, J. R., and De Vreese, A. (1995) FASEB J. 9, 1355–1361
6. Wolkow, C. A., Kimura, K. D., Lee, M. S., and Ruvkun, G. (2000) Science 290, 147–150
7. Muller, J. (1971) Arch. Microbiol. 77, 20–25
8. Kennedy, B. K., Austrinio, N. R., Jr., Zhang, J., and Guarente, L. (1995) Cell 80, 485–496
9. Smeal, T., Claus, J., Kennedy, B., Cole, F., and Guarente, L. (1996) Cell 84, 633–642
10. Lin, S. J., Defossez, P. A., and Guarente, L. (2000) Science 289, 2126–2128
11. Ashrafi, K., Lin, S. S., Manchester, J. K., and Gordon, J. I. (2000) Genes Dev. 14, 1872–1885
12. Hardie, D. G., Carling, D., and Carlson, M. (1998) Annu. Rev. Biochem. 67, 821–855
13. Klein, C. J., Olson, L., and Nielsen, J. (1998) Microbiology 144, 13–24
14. Devi, M. J., and Johnston, M. (1999) Curr. Biol. 9, 1231–1241
15. Jiang, J. C., Jaruga, E., Repnevskaya, M. V., and Jazwinski, S. M. (2000) FASEB J. 14, 2135–2137
16. Lakowski, B., and Hekimi, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13091–13096
17. Lee, C. K., Klipp, R. G., Weintraud, R., and Prolla, T. A. (1999) Science 283, 1390–1393
18. Passonneau, J. V., and Lowry, O. H. (1993) Enzymatic Analysis: A Practical Guide, Humana Press, Totowa, NJ
19. Ramaswamy, N. T., Li, L., Khalil, M., and Cannon, J. P. (1996) Genetics 149, 57–72
20. Herrero, P., Galindez, J., Ruiz, N., Martinez-Campa, C., and Moreno, P. (1995) Yeast 11, 137–144
21. McDougal, D. B., Ferrendelli, J. A., Yip, V., Pasateri, M. E., Carter, J. G., Chi, M. M., Norris, B., Manchester, J., and Lowry, O. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1357–1361
22. Tanner, K. G., Landry, J., Sternglanz, R., and Denu, J. M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 14178–14182
23. Tanny, J. C., and Moazed, D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 415–420
24. Ashrafi, K., Sinclair, D., Gordon, J. I., and Guarente, L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9100–9105
25. Francois, J., and Parrou, J. L. (2001) FEMS Microbiol. Rev. 25, 125–145
26. Vincent, O., and Carlson, M. (1998) EMBO J. 17, 7002–7008
27. Lesage, P., Yang, X., and Carlson, M. (1996) Mol. Cell. Biol. 16, 1921–1928