Distinct roles of processes modulated by histone deacetylases Rpd3p, Hda1p, and Sir2p in life extension by caloric restriction in yeast

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Abstract

Caloric restriction has been demonstrated to extend life span and postpone aging in a variety of species. The recent extension of the caloric restriction paradigm to yeast places the emphasis of the search for the longevity effectors at the cellular level. To narrow the range of potential effectors of the caloric restriction response, we have examined the effects of the histone deacetylases Rpd3p, Hda1p, and Sir2p, which have distinguishable but partially overlapping influences on global patterns of gene expression, on the life extension afforded by caloric restriction. Deletion of the *RPD3* gene extended life span, and there was no additive effect of caloric restriction. Deletion of *HDA1* had no effect of its own on longevity but acted synergistically with caloric restriction to increase life span. *SIR2* deletion shortened life span but did not prevent extension of life span by caloric restriction. The results suggest that Rpd3p affects both processes that play an obligate and those that play a synergistic role in life extension by caloric restriction, while Hda1p and Sir2p affect processes that are not the obligate longevityeffectors of caloric restriction but instead synergize with them, although in opposite directions. From the known patterns of gene expression elicited by *rpd3D*, *hda1D*, and *sir2D*, we propose that the major longevity effectors of caloric restriction in yeast involve carbohydrate/energy metabolism and mitochondrial function. © 2002 Elsevier Science Inc. All rights reserved.

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1. Introduction

Caloric restriction is a proven method for extending life span and postponing the manifestations of aging in mammals, where it elicits a multitude of changes at the systemic, cellular, and molecular levels (Masoro, 1995; Weindruch and Sohal, 1997). These changes include alterations in the global patterns of gene expression (Cao et al., 2001; Han et al., 2000; Kayo et al., 2001; Lee et al., 1999; Lee et al., 2000). Calorie restricted animals exhibit many physiologic changes, including an early drop in blood glucose levels (Masoro et al., 1992; Cartee and Dean, 1994), a decrease in circulating insulin and an increase in insulin receptor levels (Pahlavani et al., 1994). These metabolic aspects and the absence of reduction in metabolic rate (Weindruch et al., 1986; McCarter and McGee, 1989) suggest that caloric restriction does not alter the intensity of fuel use, but rather its characteristics (Masoro, 1995; Masoro et al., 1992).

Attempts at identification of the longevity effectors...
of the caloric restriction response have entailed whole genome scans of gene expression in mice during aging and caloric restriction (Cao et al., 2001; Han et al., 2000; Lee et al., 1999; Lee et al., 2000). It is not clear which of the large number of alterations in gene activity were primary responses to caloric restriction and which constituted secondary effects. It is also difficult to ascertain which of these changes contribute to extended longevity and postponed senescence. Indeed, it is not clear whether there are specific changes in gene expression that affect life span, or whether it is the prevention of age changes in gene expression, in general, that mediates the caloric restriction effect on aging. This is not a trivial issue, because it has been suggested that gene dysregulation is a cause of aging (Jazwinski, 1996).

An intervention resembling caloric restriction has been demonstrated in the yeast *Saccharomyces cerevisiae* (Jiang et al., 2000). This metabolic mechanism for life extension was shown to be distinct from the retrograde response, another pathway for life extension that alters cell metabolism (Jiang et al., 2000; Kirchman et al., 1999). Evidence suggesting that caloric restriction in yeast operates through the Sir2p NAD-dependent histone deacetylase (Lin et al., 2000) provides a mechanism by which this nutritional manipulation might work, at least in yeast. As in mammals, the identity of the signaling pathways and the longevity effectors induced by caloric restriction has yet to be elucidated in yeast.

Genes that alter chromatin-dependent gene expression, also known as transcriptional silencing have been shown to affect yeast life span (Kim et al., 1999; Imai et al., 2000). The genes studied encode the trichostatin-sensitive histone deacetylases Rpd3p and Hda1p (Struhl, 1998), and the Sir2p histone deacetylase (Tanner et al., 2000). These histone deacetylases affect global patterns of gene expression in yeast, as might be expected for proteins that modulated the silencing of chromatin. However, it is only recently that the varied panorama of their effects on gene expression has been investigated (Wyrick et al., 1999; Hughes et al., 2000; Bernstein et al., 2000). These gene expression effects need not be all the product of the direct action of the histone deacetylase (Bedalov et al., 2001).

Here, we have examined whether the histone deacetylases affect the response of yeast cells to caloric restriction, using life span as the assay. The results suggest that there may be significant overlap between caloric restriction and the suites of genes whose expression is modulated by these deacetylases, and they are consistent with the notion that caloric restriction operates by eliciting specific changes in gene expression rather than by a generalized effect on gene dysregulation that may accumulate with age.

2. Materials and methods

2.1. Yeast strains and growth conditions

The *S. cerevisiae* strain YPK9 (MATa, ade2-101 ochre, his3Δ-200, leu2Δ-1, lys2-801 amber, trp1Δ-63, ura3-52) was used in this study (Kirchman et al., 1999). Deletions of the genes RPD3, HDA1, and SIR2 in YPK9 have been described (Kim et al., 1999). Modified, chemically defined (CM) medium used (Jiang et al., 2000). Caloric restriction was imposed by reduction of either glucose levels (from 2 to 0.1%) or nonessential amino acids levels (from 100 to 0% of that normally present), leaving the required supplements constant (Jiang et al., 2000). The nonessential amino acids in the medium were arginine, aspartate, glutamate, methionine, phenylalanine, serine, threonine, tyrosine, and valine. YPG medium contained 2% peptone, 1% yeast extract, and 2% glycerol. Cells were cultured at 30°C.

2.2. Life span determination

The yeast life span is measured by the number of times individual cells divide, not by calendar time (Mortimer and Johnston, 1959; Müller et al., 1980). Yeast were pre-cultured on YPG medium to eliminate petites, which lack fully functional mitochondria. Life spans were determined as described previously (Jiang et al., 2000), starting with 35 to 40 viable cells, and expressed in generations. The significance of differences in life spans was assessed using the Mann–Whitney test. All differences noted were significant at \( p < 0.001 \) and were reproducible.
3. Results

3.1. Rpd3p and caloric restriction

Rpd3p and glucose appear to operate on the same processes in affecting life span, except that Rpd3p is more effective. The deletion of RPD3 on its own markedly extended life span (Kim et al., 1999) (Fig. 1(A)). The maximum life extension through the lowering of glucose concentration in this growth medium is seen at 0.1% (Jiang et al., 2000). However, the life extension on 0.1% glucose was not as great as afforded by deletion of RPD3 (Fig. 1(A)). Combining the reduction of glucose to 0.1% with the gene deletion gave no greater life extension than the RPD3 deletion alone (Fig. 1(A)). This suggests that glucose restriction and RPD3 deletion impact common pathways or processes, with RPD3 deletion being more effective in extending life span. It does not necessarily mean that they actually reside in the same pathway, although they could.

Life extension in yeast can also be effected by reducing the amino acid content of the growth medium, with the greatest increase in longevity seen when the nonessential amino acids are totally absent (Jiang et al., 2000). The elimination of amino acids resulted in a pronounced extension of life span, greater than that found by lowering glucose (Fig. 1(B)). A combination of these two nutritional manipulations resulted in no further increase in longevity over that observed on elimination of amino acids (Fig. 1(B)). It seems surprising that combining RPD3 deletion with the elimination of nonessential amino acids did not affect longevity in the same way when it was coupled with restriction of glucose, because glucose and amino acids appear to exert their influence on life span via a common final pathway. The results suggest that Rpd3p inhibits processes that extend longevity, which may be common to both glucose and amino acid limitation, but the absence of the protein otherwise creates a requirement for amino acids in the absence of glucose.

3.2. Hda1p and caloric restriction

In contrast to the independent effect of Rpd3p on longevity, Hda1p can only cooperate with glucose limitation, but cannot act independently. Deletion of
HDA1 on its own did not affect life span (Fig. 2). Interestingly, the effects of HDA1 deletion and growth on 0.1% glucose were synergistic, resulting in an even greater increase in longevity than by lowering glucose alone (Fig. 2(A)).

HDA1 deletion had no effect on the life extension provided by elimination of nonessential amino acids (Fig. 2(B)). Indeed, no further life extension occurred in an hda1Δ strain when glucose was restricted in the

Fig. 2. Effects of caloric restriction and HDA1 in determining life span. All of the life span determinations were performed in the same experiment, and thus the control is the same. They are shown in two panels for legibility. (A) Effect of glucose limitation and hda1Δ. Reduction of glucose levels increased mean life span from 13.3 (†) to 21.2 (B) generations (p ≤ 0.0001). Deletion of HDA1 had no effect on mean life span (12.3 generations) (□) (p = 0.13); however, it synergistically increased mean life span on 0.1% glucose (27.5 generations) (◇) (p ≤ 0.0001). (B) Effect of amino acid limitation and hda1Δ. Elimination of nonessential amino acids increased mean life span from 13.3 (†) to 24.4 (◇) generations (p ≤ 0.0001). Deletion of HDA1 had no further effect on mean life span (27.5 generations) (■) (p = 0.17). Combination of glucose reduction and elimination of amino acids increased mean life span to 26.4 generations (◇) (p ≤ 0.0001), and hda1Δ had no effect on this, resulting in a mean life span of 26.9 generations (△) (p = 0.74). Each life span determination was initiated with 35 viable cells.

HDA1 on its own did not affect life span (Fig. 2). Interestingly, the effects of HDA1 deletion and growth on 0.1% glucose were synergistic, resulting in an even greater increase in longevity than by lowering glucose alone (Fig. 2(A)).

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Fig. 3. Effects of caloric restriction and SIR2 in determination of life span. (A) Effect of glucose limitation and sir2Δ. Reduction of glucose levels increased mean life span from 12.5 (†) to 16.6 (◇) generations (p < 0.0005). Deletion of SIR2 did not prevent life extension by glucose limitation. It reduced mean life span on 2% glucose to 6.7 generations (■) (p = 0.0001) and on 0.1% glucose to 9.1 generations (◇) (p ≤ 0.0001), but the increase in life span of the sir2Δ on 0.1% as compared to 2% glucose was significant (p < 0.001). The effects of sir2Δ and caloric restriction on longevity were additive, with an approximate 54% decrease in mean life span caused by deletion of SIR2 at either glucose concentration. Each life span determination was initiated with 40 viable cells. (B) Effect of amino acid limitation and sir2Δ. Elimination of nonessential amino acids increased mean life span from 13.1 (†) to 26.5 (◇) generations (p ≤ 0.0001). Deletion of SIR2 partially prevented this. It reduced mean life span to 11.0 (◇) generations (p ≤ 0.0001) in the absence of the nonessential amino acids, distinct from the reduction to 8.2 generations (■) (p = 0.0001) in their presence. Each life span determination was initiated with 35 viable cells.

HDA1 deletion had no effect on the life extension provided by elimination of nonessential amino acids (Fig. 2(B)). Indeed, no further life extension occurred in an hda1Δ strain when glucose was restricted in the
absence of nonessential amino acids (Fig. 2(B)). Unlike RPD3 deletion, the increase in longevity due to HDAI deletion on glucose limitation is not mitigated by elimination of amino acids from the growth medium (Fig. 2). Given overall similarity in the overlap of the effects of Rpd3p and Hdalp and nutrient limitation, the lack of a requirement for amino acids in the absence of Hdalp and glucose is curious. This difference suggests lack of overlap in some of the processes under the control of Rpd3p and Hdalp, particularly those affecting amino acid signaling and/or utilization.

3.3. Sir2p and caloric restriction

In contrast to Rpd3p and Hdalp, Sir2p acts on processes that affect life span, which are distinguishable from those on which caloric restriction operates. Deletion of SIR2 shortened life span (Fig. 3). This negative effect on longevity was surmounted by restriction of glucose in the growth medium (Fig. 3(A)). The effects of SIR2 deletion and glucose limitation were strictly additive, indicating no overlap between the processes affected by these manipulations. This suggests that Sir2p and caloric restriction act on distinguishable processes and do not lie in the same pathway for life extension.

Unlike its additive effect to glucose limitation, deletion of SIR2 prevented the realization of the full benefits for longevity of the elimination of the nonessential amino acids (Fig. 3(B)). However, the deletion of this gene did not completely reverse the life extension observed by eliminating these amino acids. This suggests that the processes affected by SIR2 and by abolishing the availability of nonessential amino acids may overlap partially, but not completely.

4. Discussion

We have demonstrated that the modulation of global patterns of gene expression that is known to occur when the silencing status of yeast cells is manipulated shares features in common with the caloric restriction phenomenon, as evidenced by alterations in life span. Based on studies in mammals, caloric restriction causes broad changes in gene expression profiles (Cao et al., 2001; Han et al., 2000; Kayo et al., 2001; Lee et al., 1999; Lee et al., 2000). Thus, the overlap we observe between the effects on life span of nutrient manipulation and histone deacetylase genes are likely to result from common, specific changes in gene expression and in the processes in which these genes participate.

The effects on life span of the three histone deacetylases examined here differ. Similarly, the relationship between glucose and amino acid limitation and Rpd3p, Hdalp, and Sir2p are distinguishable. Rpd3p has a strong effect of its own in limiting yeast longevity. Removal of the protein has as large an effect on longevity as any form of nutrient manipulation, and the effects overlap completely (Fig. 1). Elimination of Hdalp, on the other hand, has a synergistic effect to glucose limitation in extending life span (Fig. 2). This synergy is quantitatively equivalent to the effects of eliminating the nonessential amino acids.

There remains the divergent effect of elimination of amino acid on life extension evinced by the rpd3Δ and the hda1Δ when glucose is limiting. The hda1Δ strain may be saved from the negative effects of elimination of amino acids by the induction of amino acid biosynthesis genes (Hughes et al., 2000; Bernstein et al., 2000). The negative effect may occur in the rpd3Δ strain, because these genes are repressed (Hughes et al., 2000; Bernstein et al., 2000). The effect of elimination of amino acids becomes apparent in this strain on lowering glucose. We suggest that this is due to the overwhelming requirement for Krebs cycle activity to retain energy production through oxidative phosphorylation, at low glucose levels. Under these circumstances, it may not be as easy for the cell to withdraw Krebs cycle intermediates for amino acid biosynthesis as it is at high glucose levels, at which glycolysis/fermentation can function to produce energy through substrate level phosphorylation. In addition, the rpd3Δ strain may not be able to activate the glyoxylate pathway because of repression of RTG3 (Hughes et al., 2000; Bernstein et al., 2000; Jia et al., 1997), further contributing to the proposed amino acid-biosynthetic predicament of the rpd3Δ strain by elimination of this pathway as an alternate source of biosynthetic intermediates. Confirmation of the model proposed
here will require the determination of key enzyme activities and metabolite levels in the cell. The effect of Sir2p on caloric restriction is complex (Fig. 3). It is clear that elimination of Sir2p shortens life span. However, deletion of this gene does not prevent life extension obtained by limiting glucose availability. On the other hand, it does partially prevent the increase in life span observed on eliminating nonessential amino acids from the growth medium. This is surprising, because glucose and amino acid restriction appear to impinge upon the same final pathway for extended longevity. There is an apparent inconsistency here. The major systematic impact of deletion of SIR2 appears to be the upregulation of many amino acid biosynthesis genes, especially basic and sulfur-containing amino acids (Wyrick et al., 1999; Hughes et al., 2000; Bernstein et al., 2000). It is difficult to ascribe life span curtailing to this effect, however, because similar effects occur in hda1Δ strains (Hughes et al., 2000; Bernstein et al., 2000) and are compatible with life extension (Fig. 2). Rather, the deleterious effect on life span of deletion of SIR2 is consistent with the loss of transcriptional silencing at the ribosomal DNA locus (Kim et al., 1999).

Our results conflict with an earlier conclusion that caloric restriction in yeast operates through a pathway whose downstream mediator is the Sir2p (Lin et al., 2000). We searched directly for an interaction between SIR2 and glucose restriction, and found none. These results were not peculiar to the particular yeast strain used (data not shown). In the previous study, an interaction between the Ras2p pathway and Sir2p was detected; however, evidence for a direct link with caloric restriction was not provided. The involvement of the Ras2p pathway in yeast longevity is well-established (Sun et al., 1994). The partial suppression by SIR2 deletion of the life extension obtained by elimination of nonessential amino acids is not consistent with the involvement of Sir2p as a mediator in this pathway of caloric restriction either.

We propose a model to explain the dilemma arising from the apparent inconsistency of the effects of Sir2p on life extension by glucose and amino acid restriction. This model also integrates the roles of Rpd3p and Hdalp (Fig. 4). In this model, caloric restriction and the three histone deacetylases modulate the expression of genes that affect the life span of yeast cells. Elimination of nonessential amino acids from the growth medium or deletion of RPD3 potentiates...
an array of processes that extend life span. A subset of these processes are ones that limitation of glucose availability also induces. The induction of these glucose-regulated processes is absolutely essential for the increased longevity afforded by caloric restriction. The additional processes affected by Rpd3p and amino acids do not in and of themselves extend life span, but they can further augment the effect of the glucose-regulated processes. As suggested earlier, the reduction in life span observed in an rpd3Δ strain under amino acid and glucose limiting condition may be the result of an intracellular amino acid supply problem. Deletion of HDA1 is proposed to potentiate that set of processes that is uniquely under the control of amino acids and Rpd3p but not glucose, in this model. In this way, a synergistic effect of glucose limitation and HDA1 deletion is observed. Finally, deletion of SIR2 blocks at least some of the same processes that HDA1 deletion potentiates to affect life span. The life span curtailing effect of SIR2 deletion is the result of loss of silencing at the ribosomal DNA locus.

The obvious question now concerns the identity of the processes controlled by the histone deacetylases and by caloric restriction, which can be modulated to extend life span. Some clues can be derived from the global patterns of gene expression induced by deletions of RPD3, HDA1, and SIR2 (Wyrick et al., 1999; Hughes et al., 2000; Bernstein et al., 2000), based on the ways these deletions affect the caloric restriction response. It appears from a comparison of these gene expression profiles that extension of life span by caloric restriction in yeast could be associated with a remodeling of carbohydrate/energy metabolism and mitochondrial function. The induction of HXX1, CIT1 and SDH1 on caloric restriction (unpublished data) is consistent with this. This conclusion awaits verification through not only an analysis of gene expression changes induced by caloric restriction in yeast, but also more importantly it requires the determination of the metabolic changes that occur on caloric restriction to alter cell physiology in a way that extends longevity.

This study does not provide evidence for a direct role of histone deacetylases as mediators of signaling pathways that are triggered by caloric restriction and affect longevity. Nevertheless, the results are consistent with such a role for Rpd3p, which is generally required for regulating silencing (Sun and Hampsey, 1999), and remain noncommittal for Hda1p. However, the data argue against this role for Sir2p. It is likely that this protein modulates some of the processes that determine life span independently of the caloric restriction response. On the other hand, Rpd3p may be part of a caloric restriction pathway, given the possibility that it interacts with the TOR kinase pathway, which responds to nutrients, through the rapamycin-binding proteins (Arevalo-Rodriguez et al., 2000). Clearly, the Snf1 protein kinase fuel gauge (Hardie et al., 1998) acts in concert with the histone acetyltransferase Gcn5p to regulate transcription (Lo et al., 2001), suggesting that nutritional signals can fine-tune the transcriptional status of chromatin. The Snf1p has been implicated in determining yeast life span (Ashrafi et al., 2000).

Histone deacetylases have been shown to modulate life span in Drosophila as well (Kang et al., 2002). Their effects on transcription and thus cell physiology may be mediated either through alterations of chromatin structure or directly through the modification of transcription factors (Prives and Manley, 2001). Alternatively, their effects on life span could be at least in part secondary, as a result of compensatory changes to the alterations in gene expression that they induce. With knowledge of the mutual effects of caloric restriction and histone deacetylases in modulating life span, it should be possible to place some boundaries on the processes and pathways that are elicited by caloric restriction and perform as the longevity effectors. The approach of combining analysis of genetic with environmental manipulations and examination of partially overlapping gene expression profiles should prove helpful in identifying the effectors of other complex responses.

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