Isolation and Characterization of Mevinolin Resistant Mutants of *Saccharomyces cerevisiae*

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Mutants of *Saccharomyces cerevisiae* resistant to mevinolin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme-A (HMGCoA) reductase (EC 1.1.1.34) were isolated and one mutant (MV71) was extensively characterized. While growth of resistant strains in the presence of mevinolin was greater than that of the wild-type strain, mevinolin at the concentration used still inhibited growth. Diploids produced by mutant/wild-type matings showed levels of mevinolin resistance which indicated incomplete dominance. Sterol synthesis in the presence of mevinolin was inhibited in strain MV71 but to a lesser degree than seen in the wild-type strain. All mevinolin resistant mutants also demonstrated a slight resistance to the antibiotic nystatin. The subcellular location of HMGCoA reductase activity in MV71 and the wild-type strain were determined and it was shown that yeast HMGCoA reductase is not regulated by a dephosphorylation mechanism as has been shown for mammalian reductases. *In vivo* and *in vitro* studies of strain MV71 and the wild-type indicated that mevinolin resistance did not result in changes in HMGCoA reductase activity as has been demonstrated in mammalian systems. Based on growth data, sterol analysis, and the lack of detection of HMGCoA reductase activity differences between strain MV71 and the wild-type, mevinolin resistance is concluded to result possibly from a mutation in *HMG2*, one of the two functional yeast HMGCoA reductase genes, which accounts for a minor (up to 17%) amount of total cellular reductase activity.

INTRODUCTION

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme-A (HMGCoA) reductase (EC 1.1.1.34) has been extensively studied due to the fact that it is the rate-limiting enzyme in mammalian sterol biosynthesis (Brown & Goldstein, 1980; Chin *et al.*, 1982). Mevalonate, the product of the reaction catalysed by this enzyme, functions not only as a sterol precursor but also as a precursor of ubiquinone, dolichols, and isopentenyl tRNA. Thus, HMGCoA reductase plays an important role in a variety of essential cellular metabolic activities.

Mevinolin is an unmethylated derivative of compactin; both of these compounds have been shown to be competitive inhibitors of HMGCoA reductase (Alberts *et al.*, 1980; Brown *et al.*, 1978; Endo *et al.*, 1976; Kaneko *et al.*, 1978). Resistance to these compounds has been described in several mammalian cell lines and has been shown to involve several mechanisms, most of which result in increased reductase activity. In a baby hamster kidney cell line compactin resistance has been shown to result in a 100-fold increase in HMGCoA reductase synthesis (Hardeman *et al.*, 1983). Subsequent investigation (Hardeman *et al.*, 1984) indicated that the increased synthesis was due to increased HMGCoA reductase mRNA synthesis. Several

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Abbreviation: HMGCoA, 3-hydroxy-3-methylglutaryl-coenzyme A.

compactin resistant cell lines have been shown to exhibit increased HMGCoA reductase activity as a result of amplification of the reductase gene (Luskey *et al.*, 1983; Skalnik *et al.*, 1985). In rat hepatocyte cell lines mevinolin has been shown to decrease the rate of HMGCoA reductase degradation (Edwards *et al.*, 1983*a, b*). A similar mechanism of resistance has also been reported for Chinese hamster ovary cell lines (Sinensky & Logel, 1983).

The HMGCoA reductase from *Saccharomyces cerevisiae* is similar in several ways to its mammalian counterpart. It shows inhibition by the end-product sterol, ergosterol (Bard & Downing, 1981), is sensitive to compactin (Nakamura & Abeles, 1985), and is the rate-limiting enzyme in ergosterol biosynthesis (Kawaguchi, 1970; Quain & Haslam, 1979). In contrast to the mammalian enzyme, the yeast enzyme is encoded by two functional genes (Basson *et al.*, 1986). The presence of an active form of either gene is sufficient to permit normal growth, thus explaining the inability to isolate non-functional reductase mutants in this organism (Servouse *et al.*, 1984).

Since mevinolin has potential as a cholesterol-lowering agent as well as an antifungal agent, we decided to assess the characteristics of mevinolin resistance in *S. cerevisiae*. In this report we describe the isolation of such mutants and demonstrate that they do not arise as the result of increased HMGCoA reductase activity.

METHODS

Chemicals. Mevinolin was the generous gift of A. Alberts of Merck Sharp & Dohme Research Laboratories. It was used as a 20 mg ml⁻¹ stock solution, which was prepared by hydrolysing the drug in ethanolic NaOH (15%, v/v, ethanol, 0.25% w/v, NaOH) at 60 °C for 1 h and then filter sterilizing. Ergosterol was added from a 0.2% (w/v) stock solution in 50% (v/v) Tween 80 and 47.5% (v/v) ethanol.

Organisms, mutant selection, and media. The wild-type strain of *S. cerevisiae*, A184D, has been described by Molzhan & Woods (1972). Mutagenesis was done using ethyl methane sulphonate (50% kill). Surviving cells were plated on yeast minimal medium (0.67%, w/v, Difco yeast nitrogen base, 2%, w/v, glucose and 2%, w/v, agar) containing 600–1000 µg mevinolin ml⁻¹ and incubated at 30 °C. Yeast complete medium consisted of 1% (w/v) Difco yeast extract, 2% (w/v) Difco Bacto peptone and 2% (w/v) glucose (YEP/glucose). Yeast complete medium containing a non-fermentable energy source was identical to YEP/glucose with the exception that 3% (v/v) glycerol replaced the glucose. Diploids were isolated by micromanipulation after crossing the appropriate strains of opposite mating type.

Determination of MIC values. Approximately 10⁵ cells were added to YEP/glucose solid medium (kept molten at 47 °C), which contained varying concentrations of mevinolin. The concentration of mevinolin which prevented any appearance of growth after 24 h of incubation at 30 °C was designated the MIC.

Determination of growth yields. Flasks containing YEP/glucose were inoculated with cells to a final concentration of 10⁵ cells ml⁻¹. Media supplemented with mevinolin and mevinolin plus ergosterol were prepared by adding mevinolin stock solution to a final concentration of 400 µg ml⁻¹ and ergosterol stock solution to a final concentration of 5 × 10⁻⁵ M. Control solutions without mevinolin or ergosterol were added to control media. After 48 h of growth at 30 °C on a rotary shaker, cells were harvested and dry weights were determined following 48 h in an 80 °C oven.

Sterol analysis. Cells were grown to exponential phase (100 Klett units) or stationary phase (1000 Klett units) as determined using a Klett–Summerson colorimeter with a no. 66 red filter (640–700 nm), at 30 °C in yeast complete medium containing 5% (w/v) glucose with mevinolin at a concentration of 200 µg ml⁻¹ or with no added mevinolin. Extraction of non-saponifiable fractions and quantification of sterols has been described by Downing *et al.* (1980). GLC analyses were done with a Hewlett–Packard 5710A gas chromatograph using a 1% SE-30 column. Column temperature was 240 °C and nitrogen carrier gas flow rate was 45 ml min⁻¹. Sterols were quantified against an ergosterol standard.

Cell fractionation. Exponential phase cells were centrifuged, washed twice in 0.1 M-phosphate buffer (pH 7.5), recentrifuged, and suspended in buffer A (25 mM-imidazole/HCl, pH 7.2, 5 mM-EGTA, 200 mM-sucrose, 50 µM-leupeptin and 5 mM-DTT) to which 1.5 vols of acid-washed glass beads (0.4 mm) were added. Cells were lysed by vortexing and the lysate was centrifuged at 1000 g for 6 min to clear. The supernatant was then centrifuged at 16 000 g for 6 min to yield the crude mitochondrial fraction. The 16 000 g supernatant was ultracentrifuged at 140 000 g for 60 min to obtain the microsomal and cytosolic fractions. Both the mitochondrial and microsomal precipitates were suspended in 4 ml of buffer B (50 mM-imidazole/HCl, pH 7.2, 1 mM-EDTA, 1 mM-EGTA, 20 µM-leupeptin and 5 mM-DTT) and ultracentrifuged at 140 000 g to obtain the washed mitochondrial and microsomal fractions, which were resuspended in 0.5 ml buffer B.

HMGC α reductase assay. Reductase activity was determined using the radiochemical/TLC procedure described by Ingebritsen & Gibson (1981) with the modification that [^3H]mevalonolactone internal standard was added after termination of the incubations. The total reductase activity of the microsomes was determined by preincubating microsomal samples with protein phosphatase. One unit (U) of reductase activity was defined as that required for the formation of 1 nmol of mevalonate min^{-1} . Protein concentration was determined by the method of Lowry.

Rat liver HMGC α reductase. This was prepared as previously reported (Parker *et al.*, 1984).

In vitro inhibition of HMGC α reductase by mevinolin. Microsome samples (approx. 45 μg protein for yeast and 85 μg protein for rat liver) were preincubated with varying concentrations of mevinolin for 10 min at 37 °C. The samples were then assayed for HMGC α reductase activity.

Nystatin resistance. Cells were grown overnight at 30 °C in YEP/glucose. Appropriate volumes were transferred to 20 ml fresh medium in 250 ml nephelometer flasks to yield starting concentrations of 8–15 Klett units (absorbance of approximately 0.04). Nystatin was added from a stock solution (4 mg ml^{-1} in dimethylformamide). Dimethylformamide alone did not affect growth at the concentrations used in this study. Growth was monitored with a Klett–Summerson colorimeter and mean generation times were calculated. Percentage inhibitions were then calculated from these data.

RESULTS AND DISCUSSION

Mutant isolation and determination of mevinolin resistance

Mutagenesis and selection for mevinolin resistance resulted in the isolation of approximately 200 colonies from two experiments. The resistant strains were found to be resistant to the same concentration of mevinolin on yeast complete medium containing either glucose or glycerol. Thus, resistance was confirmed in both fermentative and respiratory metabolism.

Table 1 shows growth yields of four mevinolin resistant strains and the wild-type parental strain on YEP/glucose and the same medium supplemented with mevinolin (400 μg ml^{-1}) either alone or plus ergosterol (5×10^{-5} M). MV71 was clearly the most resistant strain, with a cell yield of 53.2% of that of the control, as compared to the wild-type where the yield was only 1.6% of the control. Supplementation with ergosterol increased yields, ranging from 71 to 88% of the control levels for the mutant strains but only 18% of the control levels for the wild-type. These data indicate that mevinolin still had a significant inhibitory effect on the reductase enzyme of the mutant strains since their growth yields were reduced by 47 to 75% in the presence of mevinolin. Ergosterol supplementation did not completely restore normal growth yields, presumably due to the reduced levels of mevalonate, which serves as a precursor of other essential end products such as ubiquinone, isopentenyl tRNA, and dolichols. An alternative explanation for the inability of ergosterol supplementation to confer wild-type growth would be that ergosterol uptake is incomplete in these strains. It was also noted that growth yields for the mutant strains were less than those obtained for the wild-type, when grown in control medium. This effect is understandable since mevinolin is a competitive inhibitor of HMGC α reductase, and a mutation conferring resistance might easily modify reductase activity or its regulation. Wild-type cells were concluded to be producing very low levels of mevalonate in the presence of mevinolin. This was based on the observation that ergosterol supplementation increased the

Table 1. *Growth yields of wild-type and resistant strains in YEP/glucose medium containing mevinolin or mevinolin plus ergosterol*

The values shown are the means $\pm$ SD for three independent determinations; the numbers in parentheses indicate the percentage of the control yield.

Strain	Cell yield (mg dry wt ml^{-1})		
	Mevinolin	Mevinolin + ergosterol	Control
A184D	0.18 $\pm$ 0.05 (1.6)	1.98 $\pm$ 0.73 (17.9)	11.04 $\pm$ 0.04
MV1	2.98 $\pm$ 0.05 (30.7)	7.30 $\pm$ 0.80 (75.2)	9.71 $\pm$ 0.70
MV2	2.26 $\pm$ 0.17 (24.7)	7.35 $\pm$ 0.55 (80.2)	9.16 $\pm$ 0.45
MV7	2.50 $\pm$ 0.10 (33.5)	5.35 $\pm$ 0.73 (71.7)	7.46 $\pm$ 0.31
MV71	4.48 $\pm$ 0.30 (53.2)	7.43 $\pm$ 0.23 (88.2)	8.42 $\pm$ 0.17

Table 2. *Growth yields of mutant/wild-type diploids in YEP/glucose medium containing mevinolin*

The values shown are the means $\pm$ SD for three independent determinations except for the MV1/A184D diploid, where the value was derived from six independent determinations.

Strain	Cell yield (mg dry wt ml ⁻¹)	
	Mevinolin	Control
MV1/A184D	1.06 $\pm$ 0.50	10.37 $\pm$ 0.25
MV2/A184D	1.68 $\pm$ 0.46	9.37 $\pm$ 0.27
MV7/A184D	2.21 $\pm$ 0.40	9.20 $\pm$ 0.45
MV71/A184D	2.94 $\pm$ 0.34	9.76 $\pm$ 0.48
A184D/A184D	0.13 $\pm$ 0.04	10.29 $\pm$ 0.24

Table 3. *Total sterol accumulation in strains A184D and MV71 grown to late exponential or late stationary phase in the presence or absence of mevinolin*

The values shown are expressed as mg total sterols per 100 mg dry wt of cells and are the means of two independent determinations (individual values are given in parentheses), except for the exponential phase cells grown in the absence of mevinolin, which are the means $\pm$ SD of four independent determinations.

Growth phase	Strain	Additions to media	Percentage sterol content
Exponential	A184D	—	1.58 $\pm$ 0.08
	MV71	—	1.31 $\pm$ 0.14
	A184D	Mevinolin	0.21 (0.20, 0.22)
	MV71	Mevinolin	0.34 (0.32, 0.35)
Stationary	A184D	—	2.19 (2.11, 2.27)
	MV71	—	2.11 (2.04, 2.18)
	A184D	Mevinolin	0.27 (0.25, 0.29)
	MV71	Mevinolin	0.34 (0.33, 0.35)

growth yield from 1.6 to 17.9% of the control. This would not occur without trace amounts of mevalonate for synthesis of the other essential end products. A similar observation has been reported in mammalian systems (Kaneko *et al.*, 1978).

Table 2 shows the growth yields of diploids produced by matings between each of the mutant strains and the wild-type strain. Growth yields on the control medium of mutant/wild-type diploids resulted in values within 10% of the value for the wild-type diploid. In the presence of mevinolin, the mutant/wild-type diploids showed incomplete dominance. All values were significantly greater than that of the wild-type diploid but somewhat less than that for the haploid mutants (Table 1).

Since MV71 demonstrated the greatest level of mevinolin resistance, further analyses of this strain and the wild-type strain were initiated in order to characterize more fully the nature of the mevinolin-resistance mutation. The MIC values for the wild-type and MV71 strains were 200 μ g ml⁻¹ and >800 μ g ml⁻¹ respectively. The value for MV71 was an estimate since mevinolin was insoluble at higher concentrations. MV71 was crossed to A184D and diploids were isolated and sporulated. Tetrad analysis (seven tetrads) gave 2:2 segregation for resistance:sensitivity on YEP/glucose medium containing 1000 μ g mevinolin ml⁻¹, indicating mutation at a single gene locus.

Sterol analysis

The sterol levels accumulated by MV71 and the wild-type, A184D, were examined to assess the effects of the HMGCoA reductase mutation on sterol synthesis in the presence and absence of mevinolin. Table 3 shows the results of this analysis in exponential and stationary phase cultures of both strains. As was reflected in the growth yield studies (Table 1) exponential

Table 4. Growth characteristics of wild-type and mevinolin resistant strains in the presence of nystatin

The values shown are the means $\pm$ SD of three to eight independent determinations; the numbers in parentheses are the percentage inhibitions as compared to the control value.

Strain	Mean generation time (min)			
	0	Nystatin (mg l ⁻¹):		
		0.25	0.50	1.0
A184D	94.9 $\pm$ 11.1	107.3 $\pm$ 9.4 (12.5)	113.6 $\pm$ 9.9 (16.4)	174.8 $\pm$ 34.8 (45.1)
MV1	103.7 $\pm$ 8.2	114.8 $\pm$ 8.9 (9.7)	114.6 $\pm$ 7.6 (9.5)	143.3 $\pm$ 13.7 (27.6)
MV2	100.0 $\pm$ 16.1	109.3 $\pm$ 9.5 (8.5)	114.3 $\pm$ 9.3 (12.5)	157.7 $\pm$ 22.1 (36.6)
MV7	96.9 $\pm$ 5.1	102.5 $\pm$ 7.5 (5.5)	107.5 $\pm$ 7.2 (9.9)	122.0 $\pm$ 13.1 (20.6)
MV71	101.8 $\pm$ 10.9	99.0 $\pm$ 10.8 (0)	115.0 $\pm$ 9.5 (11.5)	128.9 $\pm$ 20.5 (20.5)

cultures of MV71 produced less total sterol than did wild-type cultures when grown under control conditions, again indicating a less efficient reductase in MV71. The addition of mevinolin resulted in a significant inhibition of sterol synthesis in both strains. The sterol accumulation value of the wild-type was only 0.21% of total cell dry weight. This value may be the minimal level of sterol needed to support growth. Taylor & Parks (1981) have demonstrated that yeast mutants requiring ergosterol as a growth factor accumulate up to 0.2% of their dry weight as sterol. Although strain MV71 also showed a sharp decline in sterol synthesis in the presence of mevinolin (0.34% of total cell dry weight), significantly, the accumulation was still 60% above the minimal value seen in the wild-type. The sterol profiles of both strains grown in control medium were qualitatively similar. Both strains accumulated zymosterol, ergosterol and an ergostadiene precursor (ergosta-5,7-dienol and/or ergosta-7,22-dienol; Lees *et al.*, 1984). The relative amounts of the individual sterols were proportional in the two strains.

Nystatin resistance of mevinolin resistant mutants

Since the strain MV71 contained slightly less ergosterol than the wild-type in control medium it was of interest to determine whether these strains could be distinguished experimentally on the basis of nystatin resistance. Previous work with other ergosterol-producing strains of *S. cerevisiae* (Lees *et al.*, 1984) containing varying levels of ergosterol showed that such strains displayed different sensitivities to nystatin, a fungicidal polyene antibiotic that interacts with ergosterol (Kinsky, 1970). In addition, nystatin sensitivity was considered an important parameter in the event that mevinolin is used as an antifungal agent and resistance appears clinically. Table 4 shows the inhibitory effects of nystatin on four mevinolin resistant strains and on the wild-type strain. The sensitivity level of the wild-type was similar to that reported for other wild-type strains of *S. cerevisiae* (Lees *et al.*, 1984). The inhibition levels shown for the mutant strains, especially those at a nystatin concentration of 1 mg l⁻¹, indicate that they all have a slight resistance to the antibiotic.

HMGCoA reductase assays

In order to determine whether mevinolin resistance was due to an increase in HMGCoA reductase activity, microsomal reductase activities from late exponential phase cultures of wild-type and MV71 were assayed. For cultures grown in the absence of mevinolin, the mean reductase activities resulting from three independent preparations of enzyme were 13.3 U mg⁻¹ and 15.9 U mg⁻¹ for MV71 and the wild-type, respectively. Since there was no significant difference under control conditions, the assay was repeated with cultures grown in the presence of mevinolin. Activity values of 8.67 U mg⁻¹ and 8.24 U mg⁻¹ for MV71 and the wild-type, respectively, were obtained, again indicating no significant difference in enzyme activity.

Since yeast mitochondria have been reported to contain HMGCoA reductase activity (Trocha & Sprinson, 1976) the relative subcellular distributions of the reductase from the wild-type and MV71 strains were examined. The data presented in Table 5 show that the relative

Table 5. *Subcellular distribution of HMGCoA reductase activity in strains MV71 and A184D*

Fraction	HMGCoA reductase activity [U (mg protein) ⁻¹]	
	Strain A184D	Strain MV71
Cytosol	0.63	1.22
Mitochondria	14.7	13.0
Microsomes	21.1	19.4
Phosphatase-treated microsomes	21.2	20.5

Table 6. *Inhibition of MV71 and A184D microsomal HMGCoA reductase activity by mevinolin in vitro*

Source of microsomes	Mevinolin concn (nM)	HMGCoA reductase activity (mU per assay)	Percentage inhibition
Rat liver	0	809	
	30	144	82
	100	60	93
A184D (No mevinolin)	0	420	
	30	128	77
	100	49	91
A184D (+ mevinolin)*	0	330	
	30	87	60
	100	46	79
MV71 (No mevinolin)	0	557	
	30	95	77
	100	33	92
MV71 (+ mevinolin)*	0	212	
	30	98	70
	100	46	86

* Mevinolin (200 µg ml⁻¹) present in growth medium.

amounts of reductase activity in the various subcellular locations were identical in both strains. In addition, in order to determine whether the reductases in the wild-type and MV71 strains varied in the proportion of active and inactive forms, microsomes from both strains were activated by dephosphorylation (Ingebritson & Gibson, 1981) prior to assay. The phosphatase-treated microsomes showed no increase in reductase activity over the controls, indicating that the reductases from both strains were fully activated in control preparations. This confirms the observation of H. J. Harwood and V. W. Rodwell (personal communication) that yeast HMGCoA reductase is not regulated by a dephosphorylation mechanism, which is in contrast to the mammalian enzyme.

Finally, the effect of mevinolin on *in vitro* inhibition of microsomal reductase was determined. Reductase activity from MV71 grown in the presence or absence of mevinolin was inhibited to approximately the same degree as the enzyme activity from the wild-type strain grown under the same conditions (Table 6). The same level of inhibition was also noted in a preparation of rat liver HMGCoA reductase.

Resistance to mevinolin in strain MV71 of *S. cerevisiae* clearly results from a mechanism unrelated to an increase in reductase activity. Whether strain MV71 is altered in other aspects of sterol biosynthesis regulation such as up-regulation of HMGCoA synthase is not known (Luskey *et al.*, 1983). It is possible that the plasma membrane of MV71 is less permeable to mevinolin than the membrane of the wild-type strain. Tsujita *et al.* (1986) have reported that in certain rat tissues a hydroxylated compactin derivative, CS-514, was excluded from cellular uptake while compactin itself entered freely. The possibility also exists for mevinolin exclusion from specific

subcellular compartments which contain reductase. However, the permeability mechanisms to explain resistance become less plausible in view of the partial dominance shown in the mutant/wild-type diploids (Table 2). An alternative explanation, which is consistent with the observations of Basson *et al.* (1986) that *S. cerevisiae* shows normal growth when either one of the two reductase genes is non-functional, is that mevinolin resistance is the result of a mutation in one of the two genes. From the data presented here, the likely candidate would be the gene *HMG2*, whose gene product accounts for only a maximum of 17% of total cellular reductase activity. These investigators demonstrated that a strain in which the *HMG2* gene is disrupted contained 17% of wild-type reductase activity whereas a strain in which the *HMG1* gene was disrupted contained slightly higher activity (9%) than the wild-type. A mutation conferring mevinolin resistance in *HMG2* would be expected to result in a strain with near normal growth characteristics in the absence of mevinolin and a significantly reduced growth in the presence of mevinolin, since at least 83% of the total reductase activity provided by *HMG1* would be mevinolin sensitive. Mevinolin resistance due to an alteration in *HMG2* would also be expected to show incomplete dominance in diploids and, in the presence of mevinolin, reduced amounts of sterol would be produced, again due to the prevalence of the *HMG1* gene product. Data consistent with these expectations are presented in Tables 1, 2, and 3. The inability to detect *in vivo* and *in vitro* differences in reductase activity in MV71 and wild-type strains is likely to be due to the fact that the resistant form of the enzyme present in strain MV71 is a minor contributor to the total reductase activity of the cell.

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