Isolation of an *Ustilago maydis* gene encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductase and expression of a C-terminal-truncated form in *Escherichia coli*

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A gene encoding 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase was isolated from the maize fungal pathogen *Ustilago maydis*. This was accomplished by identifying cDNA and genomic clones that hybridized to an internal fragment of the gene, amplified from *U. maydis* genomic DNA by PCR. The nature of the gene was determined by nucleotide sequence analysis, and by comparing the derived amino acid sequence of the gene with HMG-CoA reductases from yeast, and from other organisms. The hydrophobic nature of the N-terminal region of the deduced protein sequence also supported the view that this gene encoded HMG-CoA reductase. A C-terminal-truncated fragment of the *U. maydis* HMG-CoA reductase gene was shown to be expressed in *Escherichia coli* in a catalytically active form. The expressed protein was also shown to be sensitive to an inhibitor of mammalian HMG-CoA reductase activity.

**Keywords:** basidiomycete, ergosterol biosynthesis, heterologous expression, fungicide, inhibitor target

**INTRODUCTION**

Enzymes of the ergosterol biosynthetic pathway have attracted a great deal of attention in recent years, because inhibitors which interfere with their function have been increasingly successful as agricultural fungicides and as antimycotic drugs (Baldwin, 1989; Marriott, 1990). However, virtually all the commercially important antifungal agents affecting sterol biosynthesis act by inhibiting enzymes that function late in the ergosterol biosynthetic pathway (Kelly *et al.*, 1990), that is, the enzymic steps responsible for the conversion of lanosterol to ergosterol. Established targets for antifungal ergosterol biosynthesis inhibitors include C-14 sterol demethylation, Δ^9→Δ^7 sterol isomerization and Δ^14 sterol reduction (Koller, 1992). Squalene epoxidation, which occurs at an earlier stage in sterol biosynthesis, has also been identified as a potential target for antimycotic drugs (Stutz, 1990). In contrast, no selective inhibitors of the earlier steps prior to squalene epoxidation and cyclization have become available for the control of fungal diseases, in either medical or crop protection applications. This is despite an extensive pharmacological interest in the development of therapeutic drugs, directed towards these enzymes, for lowering blood cholesterol levels (Endo, 1985).

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.34) is an enzyme that functions early in sterol biosynthesis. It catalyses the conversion of (Δ^3)-3-hydroxy-3-methylglutaryl-coenzyme A to (R)-mevalonate. Mevalonate formed by this reaction provides C₅ units, used not only in sterol formation, but also for the synthesis of dolichols, ubiquinone, isopentyladenine, haem A and a diverse array of other isoprenoid metabolites (Panini *et al.*, 1985; Goldstein & Brown, 1990). Isoprene units derived from mevalonate are also required for post-translational modification of some signalling proteins (Sinensky & Lutz, 1992). In higher eukaryotes, HMG-CoA reductase is considered to be the key regulatory enzyme of the sterol biosynthesis pathway (Preiss, 1985). It is subject to a number of complex metabolic regulatory mechanisms including translational and transcriptional control (Osborne, 1991; Nakanishi *et al.*, 1988) and modulation of
enzyme activity by degradation and phosphorylation (Gil et al., 1985; Clarke & Hardie, 1990; Gillespie & Hardie, 1992; Saro et al., 1993). The central role played by this enzyme in regulating sterol biosynthesis, and the importance of normal sterol production for growth, makes HMG-CoA reductase an attractive target for the development of new antifungal agents. This view is supported by the fact that yeast (Saccharomyces cerevisiae) mutants devoid of HMG-CoA reductase activity are unable to grow (Basson et al., 1986). Furthermore, a number of antibiotics have been identified as powerful specific competitive inhibitors of HMG-CoA reductase (Endo, 1985) and at least two of these, compactin and lovastatin, are effective inhibitors of growth in yeasts (Ikeura et al., 1988).

In eukaryotes, HMG-CoA reductase is an integral membrane glycoprotein of the endoplasmic reticulum. Three distinct regions can be recognized within the protein: a membrane anchor N-terminal domain that contains a number (1–8) of hydrophobic regions which correspond to potential trans-membrane regions; a C-terminal catalytic domain, which extends into the cytoplasm and contains the active site of the enzyme; and a linker region which separates these two domains. In yeast, two structural genes (HMG1 and HMG2) are known to code for HMG-CoA reductase (Basson et al., 1986). In contrast, only a single gene has been found in mammalian genomes (Reynolds et al., 1984), whereas at least three genes, encoding different HMG-CoA reductase isozymes, have been postulated in plants (Bach, 1987). The amino acid sequences of HMG-CoA reductases from different organisms are highly conserved within the C-terminal catalytic domain. In contrast, the N-terminal sequences, in terms of both length and amino acid composition, are diverse (Basson et al., 1988). Nevertheless, despite this lack of homology within the N-terminal region, HMG-CoA reductases from a range of organisms appear to be functionally conserved. For example, HMG-CoA reductases from human, hamster and Arabidopsis thaliana can complement yeast mutants (hmg1, hmg2) lacking HMG-CoA reductase activity (Basson et al., 1988; Learned & Fink, 1989).

The N-terminal membrane-bound domain, although not necessary for catalytic activity, is required for sterol-regulated degradation of the protein (Roitelman et al., 1992) and for membrane proliferation (Jingami et al., 1992; Darnay & Rodwell, 1993; Frimpong et al., 1994). Heterologous expression of HMG-CoA reductase activity in this way, in conjunction with site-directed mutagenesis, has already allowed detailed investigations of catalytically important amino acid residues (Darnay et al., 1992; Darnay & Rodwell, 1993; Wang et al., 1990).

In order to pursue a rational biochemical approach to fungicide design, high yields of pure target enzyme are required to elucidate structure–function relationships, using structural and kinetic data. Recombinant DNA technology offers an attractive route for achieving this goal. In this paper, we report the isolation of an HMG-CoA reductase gene from the basidiomycete fungal pathogen Ustilago maydis, and show that a catalytically active, truncated C-terminal fragment of the protein is expressed in a functional form in E. coli. In addition, we demonstrate that the expressed protein is sensitive to a known inhibitor of mammalian HMG-CoA reductase.

**METHODS**

**Strains, vectors and plasmids.** U. maydis strain 1MI 103761 (Hargreaves & Turner, 1989) was used throughout and was grown on YEPD [1% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) dextrose] medium. E. coli strain LE392 [F r (merA) bidR14 supE44 supF58 lacY1 Yac1 del(15416)6 galK2 galT22 metB1 trpR57] was used for maintaining and screening the genomic library, strain XL1-Blue (recA1 endA1 gmr-438 thi-1 hsdR17 supE44 relA1 lac [F proAB lacPZAM15 Tn10(Tet')]) was used for cDNA library and plasmid manipulations and strain TOP10 (mer-A [mrr·bidRMS·terp-BC] Y80 [NacA15 NacX74 deoR recA1 araD139 (ara, leu)7697 galU galk, rpsL, endA1 supG]) was used in the expression studies. The U. maydis genomic library was constructed in zEMBL3 (Bailey et al., 1994) and the cDNA library was custom synthesized in zZAPII by Clontech Laboratories. Plasmid pUC18 was used for all subcloning and sequencing procedures and pTrcHis C (Invitrogen Corporation) was used for the expression studies, following the manufacturer’s instructions.

**DNA procedures.** Genomic DNA was extracted from exponentially growing cells as described by Keon et al. (1991). PCR amplification was done using a GeneAmp PCR kit (Perkin Elmer). The reactions were cycled 30 times through 94 °C for 2 min, 58 °C for 1 min and 72 °C for 2 min. At the end of the amplification period the products were analysed by electrophoresis through a 1% (w/v) polyacrylamide separating gel and a 4% stacking 0.8 DNA sequencing kit (United States Biochemical). Analysis of DNA sequence data was performed using the University of Wisconsin Genetics Computer Group sequence analysis software package version 7.2. Sequence alignments were derived from a Pearson and Lipman search (Pearson & Lipman, 1988) using the TFASTA program and a word size of 6. The Kyte and Doolittle hydrophathy measurements (Kyte & Doolittle, 1982) were determined using the PepPlot program over a window of 21 amino acid residues.

**Protein procedures and determination of enzyme activity.** SDS-PAGE was performed according to Laemmli (1970), using a 12% (w/v) polyacrylamide separating gel and a 4% stacking
U. maydis HMG-CoA reductase

Amplification of an internal DNA fragment of a U. maydis HMG-CoA reductase gene

Two sets of 20-mer oligonucleotides (5'-GGA GCT TGT/C TGT GAG/A AAT GTG and 3'-CTG TTC TTT GGT/A CGA/G CGG/A TA) were synthesized, using the standard eukaryotic nuclear gene codon assignments, that corresponded to two conserved regions within HMG-CoA reductase proteins derived from other organisms. The amino acid sequences encoded by these regions were Gly-Ala-Cys-Cys-Glu-Asn-Val and Asp-Lys-Lys-Pro-Ala-Ala-Ile, respectively, and were located approximately 170 residues apart within the catalytic domain of the protein. Amplification of a corresponding DNA fragment of the U. maydis gene was accomplished by PCR, using these primers and U. maydis genomic DNA as a template. A single amplified DNA product (approx. 500 bp) was obtained, as judged by agarose gel electrophoresis. The size of this product corresponded to an expected size of 517–523 bp. The nature of the amplified product was confirmed by Southern hybridization to restriction enzyme digests of cloned HMG-CoA reductase genes from S. cerevisiae (HMGl) in pJR59, A. thaliana in pUCHMG2 and Syrian hamster in pDC56. Strong hybridization signals, corresponding to DNA fragments containing the C-terminal catalytic domains of the genes, were obtained from all the plasmids (data not shown). These results indicated that the PCR product amplified from U. maydis genomic DNA corresponded to a region within the catalytic domain of a U. maydis HMG-CoA reductase gene.

Isolation and characterization of cDNA clones derived from a U. maydis HMG-CoA reductase gene

The PCR-amplified U. maydis DNA fragment was employed as a homologous probe to identify cDNA clones from a U. maydis AZAPII library. Approximately 20000 recombinant clones were screened. Two positive plaques (λ31B and λ41B) were isolated and purified, and

RESULTS

Amplification of an internal DNA fragment of a U. maydis HMG-CoA reductase gene

For extraction of HMG-CoA reductase activity, IPTG-induced bacterial cells from a 50 ml culture were pelleted by centrifugation at 8000 g for 1 min then resuspended in 5 ml 20 mM sodium phosphate buffer, pH 7.8, containing 10 μM β-mercaptoethanol, 0.25 % (v/v) Tween 20, 10 μM phenylmethyl-sulphonamide and 0.1 mg lysozyme ml⁻¹. Cells were subjected to three cycles of freezing in liquid nitrogen and thawing at 37 °C. Between each cycle the cells were sonicated for 20 s at 0 °C. The extract was then centrifuged at 100000 g for 30 min to remove cell debris. HMG-CoA reductase activity was determined spectrophotometrically by following the oxidation of NADPH (Frimpong et al., 1993). The reaction mixture was maintained at 37 °C and contained 200 μM sodium phosphate buffer, pH 7.5, 10 μM β-mercaptoethanol and 0.14 μM NADPH in a total volume of 1 ml. The reaction was initiated by the addition of 10 μl 0.3 mM HMG-CoA. In some experiments the inhibitory action of compound WL165748 was tested. This compound (Fig. 1) is a substituted dihydroxy-alkyl-indole derivative described in a patent (WO 84/02131) by the pharmaceutical company Sandoz as an inhibitor of mammalian HMG-CoA reductase.

Fig. 1. Structure of WL165748.
Isolation and sequence of the *U. maydis* HMG-CoA reductase gene

In order to isolate a DNA fragment containing a full-length *U. maydis* HMG-CoA reductase gene, the amplified PCR product was used to screen a *U. maydis* lambda library (approx. 40,000 clones) by plaque hybridization. Three positive clones (lambda3L4, lambda4L3, and lambda6L3) were identified, after hybridization at a high stringency. Digestion of DNA isolated from the three genomic lambda clones, with the restriction enzymes HindIII and SalI, and Southern hybridization analysis using the cDNA clones p31B and p41B as probes, identified common DNA fragments in clones lambda3L4, lambda4L3, and lambda6L3 which hybridized to both cDNA clones (data not shown). A 6 kb HindIII fragment and a 60 kb SalI fragment, derived from lambda3L4, were chosen for further restriction enzyme analysis and subcloned into pUC18 to form pUMHMG1A and pUMHMG1B, respectively (Fig. 3). The relevant regions of the DNA inserts in these plasmids were then sequenced on both strands, by extension of specific oligonucleotides as illustrated in Fig. 3. The nucleotide and deduced amino acid sequence of the gene are shown in Fig. 4. The sequence starts 292 bp upstream of an initiation codon (ATG) and continues...
within the N-terminal region are numbered. Three alternative translation initiation sites were identified at amino acid positions 27, 34 and 45. The hydropathy plot of the 603 bp beyond a stop codon (TAG). A continuous open reading frame begins at position 1 and terminates at position 3495. Three alternative translation initiation sites were identified at amino acid positions 27, 34 and 45.

However, none of these sites could be differentiated on the basis of the nucleotide sequences surrounding initiation codons optimal for translation initiation in fungi (Ballance, 1991; Gurr et al., 1987). Unkles, 1992). Neither did the location of a putative CAAT motif, at positions -240 to -236, allow discrimination between these initiation sites. Comparison of the nucleotide sequence of the genomic DNA with those of clones p31B and p41B revealed that the cDNA clones were identical to the nucleotide sequences 161-52358 and 2446-3572, respectively, within the U. maydis gene. The positions of p31B and p41B within the gene sequence are underlined in Fig. 4.

Gap alignment of the deduced amino acid sequence of the 62.3% similarity (45.4% identity) and 55% similarity (36.7% identity) respectively. Several stretches of conserved amino acids, also present in HMG-CoA reductases from other unrelated organisms, were identified within the C-terminus catalytic domain (Fig. 5). The hydropathy plot of the 8 peaks of hydrophobicity within the N-terminus domain (Fig. 6). A similar number of hydrophobic regions have been identified in the S. cerevisiae HMG1 and HMG2 proteins, and seven of these have been recognized as membrane-spanning domains (Sengstag et al., 1990).

Southern analysis of SaII- and HindIII-digested U. maydis genomic DNA, using the amplified PCR product as a probe, resulted in hybridization patterns which were consistent with there being two related HRIG-CoA reductase genes in the U. maydis genome (Fig. 7). Both SaII and HindIII digestion resulted in two distinct hybridization bands; only one (a 6.0 kb SaII fragment and
Fig. 8. SDS-PAGE analysis of total protein from E. coli cells containing either pTrcHis C (lane 1) or pUMHMG2 (lanes 2 and 3). Proteins in lanes 1 and 3 were extracted after incubation of cells with IPTG at 37°C for 3 h. Proteins in lane 2 were extracted from non-induced cells. The arrow marks the position of the new protein produced in induced cells containing pUMHMG2.

Expression of a C-terminal-truncated U. maydis HMG-CoA reductase protein in E. coli

A 2.7 kb PstI–HindIII DNA fragment containing the C-terminal of the U. maydis HMG-CoA reductase gene was inserted, in the correct reading frame, into the prokaryotic expression vector pTrcHis C to form pUMHMG2. This DNA fragment coded for amino acids 655–1165, and therefore constituted a truncated form of the U. maydis HMG-CoA reductase, consisting of only the cytosolic catalytic domain of the protein. E. coli cells containing pUMHMG2 synthesized a prominent protein of about 62 kDa, as judged by SDS-PAGE, 3 h after induction with 1 mM IPTG (Fig. 8). This size was in close agreement with the expected size of the truncated HMG-CoA reductase (61 kDa). No synthesis of the protein was observed in non-induced cells or in IPTG-induced cells containing pTrcHis C alone. In addition, extracts of induced cells transformed with pUMHMG2 were found to contain HMG-CoA reductase activity. No activity was detected in non-induced cells. The apparent $K_m$ for HMG-CoA in induced-cell lysates, as calculated from a reciprocal plot of initial velocity rates versus substrate concentration, was 11.6 μM. HMG-CoA reductase activity in bacterial cell lysates was inhibited by WL165748, which is a potent inhibitor of mammalian and fungal HMG-CoA reductases (T. E. May, unpublished results), with an $K_i$ value of 5.4 nM.

DISCUSSION

As reported for S. cerevisiae (Basson et al., 1986) and other fungi (Burmester & Czempinski, 1994), U. maydis appears, as judged by Southern analysis, to contain two structural genes coding for HMG-CoA reductase. In this study, we describe the isolation and identification of one of these genes. The deduced amino acid sequence of the U. maydis HMG-CoA reductase gene exhibited greater overall similarity to the S. cerevisiae HMG1 gene product than to the product of the HMG2 gene. However, similar identity to the products of both genes was observed within the C-terminal region (52.4% identity over 546 amino acids for HMG1 and 58.1% identity over 480 amino acids for HMG2). It could, therefore, be argued that the U. maydis gene isolated here is more likely to be equivalent to the S. cerevisiae HMG1 gene than to the HMG2 gene. This conclusion could be confirmed by overexpressing the U. maydis gene in S. cerevisiae and determining the effect of its overexpression on the assembly of 'karmellae'. These are a proliferation of stacked membrane pairs, surrounding the nucleus, that are induced by overexpression of the HMG1 gene, but not by overexpression of the HMG2 gene (Wright et al., 1988).

The deduced amino acid sequence of the U. maydis HMG-CoA reductase gene within the C-terminal domain exhibited several stretches of striking homology to HMG-CoA reductases from a wide range of other organisms, indicating that these amino acid residues are functionally conserved and may be associated with substrate and/or cofactor binding sites or catalytic activity. Amino acid residues which have already been shown to be important for catalysis in HMG-CoA reductases from P. mervaloni and hamster include the histidine residue at amino acid position 1093 (Darnay et al., 1992; Darnay & Rodwell, 1993), and the glutamate and aspartate residues at positions 786 and 995, respectively (Wang et al., 1990; Frimpong & Rodwell, 1994). Of particular interest, with respect to the regulation of HMG-CoA reductase activity, is the serine residue at position 1099. This residue is located close to the catalytically important histidine residue. In eukaryotic HMG-CoA reductase, the serine residue at this position is known to be phosphorylated by an AMP-activated protein kinase, resulting in a loss of catalytic activity (Clarke & Hardie, 1990; Gillespie & Hardie, 1992; Sato et al., 1993). This suggests that the low activity of phosphorylated HMG-CoA reductase might be due to ionic and/or steric hindrance at the active site of the enzyme (Darnay & Rodwell, 1993). Interestingly, neither of the S. cerevisiae HMG-CoA reductases contains a serine residue at this position in the protein.

The N-terminal region of the U. maydis HMG-CoA
reductase gene contained eight putative hydrophobic domains, as deduced from Kyte and Doolittle hydrophathy plots. A similar number of hydrophobic regions have been identified in the N-terminal domain of the *S. cerevisiae* HMG-CoA reductases (Basson et al., 1988). However, only seven of these eight hydrophobic regions have been recognized as possible transmembrane domains (Sengstag et al., 1990). In contrast, eight membrane-spanning domains have been implicated in higher eukaryote HMG-CoA reductases (Roitelman et al., 1992; Olender & Simoni, 1992). The membrane-spanning region, besides anchoring the protein to the endoplasmic reticulum, also serves a number of other functions. For example, in mammalian cells, the membrane-bound portion of the protein is required for sterol-regulated proteolytic degradation of the enzyme (Gil et al., 1985). The N-terminal region of the protein is also important, in both *S. cerevisiae* and mammalian cells, for triggering increased membrane synthesis (Anderson et al., 1983; Wright et al., 1988). Structural similarities between the membrane-associated domain of HMG-CoA reductases and other eukaryotic transmembrane proteins that interact with heterotrimeric GTP-binding proteins have been noted (Sengstag et al., 1990), and this similarity could imply that HMG-CoA reductase has a role in information transfer within the cell.

The hydrophobic N-terminal domain of HMG-CoA reductases is not necessary for the catalytic activity of the enzyme. Indeed, soluble preparations of the enzyme from tissues often contain a proteolytically-derived fragment of the native protein (Edwards & Fogelman, 1985) and this property of the enzyme has been exploited to gain high levels of expression of active HMG-CoA reductases in heterologous systems (Darnay & Rodwell, 1993; Ferrec et al., 1990; Frimppong et al., 1993). A C-terminal fragment of the *U. maydis* HMG-CoA reductase was similarly catalytically active when expressed in *E. coli* in the present study. The *Km* value calculated for this recombinant protein was similar to the value obtained for HMG-CoA isolated from rat liver (*Km* = 9.1 μM; Endo, 1985). In addition, the truncated form of the protein was shown to be sensitive to an inhibitor of mammalian HMG-CoA reductases.

Expression of a catalytically active form of the *U. maydis* HMG-CoA reductase in *E. coli* now provides a reliable source of active enzyme in quantities necessary for more detailed kinetic and inhibitor studies. In addition, the availability of a regular supply of recombinant enzyme, in conjunction with a simple spectrophotometric assay, should enable automated procedures to be developed for routine *in vitro* screening of potential inhibitors of fungal HMG-CoA reductases.

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