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NOTE

Molecular Characterization of a Cystathionine Beta-Synthase Gene, *CBS1*, in *Magnaporthe grisea*

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***CBS1* from *Magnaporthe grisea* is a structural and functional homolog of the cystathionine β-synthase (CBS) gene from *Saccharomyces cerevisiae*. Our studies indicated that *M. grisea* can utilize homocysteine and methionine through a CBS-independent pathway. The results also revealed responses of *M. grisea* to homocysteine that are reminiscent of human homocystinuria.**

In the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*, inorganic sulfur is assimilated directly into either homocysteine (Fig. 1, enzyme 5) or cysteine (Fig. 1, enzyme 6) (15). The transsulfuration pathways allow the interconversion of homocysteine and cysteine, with the intermediary formation of cystathionine (Fig. 1) (15). Cystathionine β-synthase (CBS) catalyzes the formation of cystathionine from homocysteine and serine (Fig. 1, enzyme 1). Cysteine is synthesized from cystathionine in a reaction catalyzed by cystathionine γ-lyase (Fig. 1, enzyme 2). There is only one existing transsulfuration pathway in mammals, i.e., from homocysteine to cysteine (6). In *A. nidulans*, *N. crassa*, and the yeast *Saccharomyces cerevisiae*, an opposite transsulfuration pathway is present, allowing the conversion of cysteine to homocysteine (Fig. 1, enzymes 3 and 4) (5, 15). In addition, there is no evidence that enzymes 3 and 4 can catalyze the reverse reaction from homocysteine to cysteine. CBS has been conserved in eukaryotic evolution (12) and is directly involved in the removal of homocysteine from the methionine cycle. In humans, CBS deficiency results in an elevated level of circulating homocysteine (homocystinuria), which is a risk factor for a number of neurological defects and vascular diseases (17). This disorder is commonly caused by recessive mutations in the human CBS gene (17).

A genome-wide effort (7) has been initiated to study gene functions in *Magnaporthe grisea*, a filamentous fungus that causes diseases in rice and other cereal crops (18). As part of this effort, a cosmid clone from an *M. grisea* (strain Guy11) (13) genomic library (7) was shotgun sequenced as described previously (8). BLASTX searches (1) of the sequence against the National Center for Biotechnology Information nonredundant protein database (27 June 2001) identified a putative gene, *CBS1*, encoding a CBS-like protein. The coding sequence (GenBank accession number AF422799) is interrupted by an intron of 71 bp, a finding which was confirmed by comparison to a cDNA sequence.

The deduced gene product of *CBS1* shares extensive homology with CBS proteins from *S. cerevisiae* (46% identity) and humans (45% identity) (Fig. 2). CBS is a pyridoxal phosphate (PLP)-dependent enzyme (10). In human CBS, Lys119 is the PLP binding residue (11), and this residue is conserved in *M. grisea* and *S. cerevisiae* (Fig. 2). Similarly, the human CBS domain, comprising residues 417 to 470 (2), can be identified in the *S. cerevisiae* and *M. grisea* proteins (Fig. 2) by hidden Markov model searches against the Pfam database (P score = 1.8×10^{-14}; 18 February 2002). CBS domains are also present in a wide range of unrelated proteins (2). The region containing the human CBS domain is involved in regulation by *S*-adenosyl-L-methionine (9). The Cys52 and His65 residues that axially coordinate the iron in the heme group of human CBS (16) are not conserved in *M. grisea* and *S. cerevisiae* (Fig. 2). In fact, *S. cerevisiae* CBS was recently found to be a nonheme protein (10, 14). It is therefore likely that the *M. grisea* enzyme does not contain a heme group, either. In *S. cerevisiae*, the biosynthesis of cysteine occurs exclusively through the CBS pathway, and CBS null mutants are cysteine auxotrophs (5).

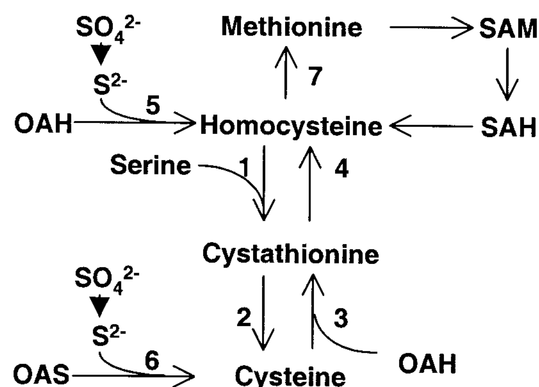

FIG. 1. Transsulfuration pathways in the filamentous fungi *A. nidulans* and *N. crassa*. OAH, *O*-acetyl-homoserine; OAS, *O*-acetyl-serine; SAM, *S*-adenosyl-methionine; SAH, *S*-adenosyl-homocysteine. Enzymes: 1, CBS; 2, cystathionine γ-lyase; 3, cystathionine γ-synthase; 4, cystathionine β-lyase; 5, homocysteine synthase; 6, CYS; 7, methionine synthase.

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Mg  ------------------------------------------------------------
Sc  ------------------------------------------------------------
Hs  MPSETPQAEVGPTGCPHRSGPHSAKGSLEKGSPEDKEAKEPLWIRPDAPSRCTWQLGRPA 60
                                                                ↓
Mg  --MAANTMAARGAQSVTESATDLIGNTPLVRLNKIPQSLGIKAQVFGKLELMNTGGSVKD 58
Sc  ------MAKSEQQTDSRHNVIDLVGNTPLIALKKLPKALGIKPQIYAKLELYNPGGSIKD 54
Hs  SESPHHHTAPAKSPKILPDILKKIGDTPMVRINKIGKKFGLKCELLAKCEFFNAGGSVKD 120
              .     .    .   . :*:**:: ::*: : :*:* :: .* *: *.***:**

Mg  RIALRMIEEAEKEGRIKPG-DTLIEPTSGNTGIGLALVGAVKGYKTIITLPEKMSAEKVS 117
Sc  RIAKSMVE-AEASGRIHPSRSTLIEPTSGNTGIGLALIGAIKGYRTIITWPEKMSNEKVS 113
Hs  RISLRMIEDAERDGTLKPG-DTIIEPTSGNTGIGLALAAAVRGYRCIIVMPEKMSSEKVD 179
    **:   *:* ** .* ::*. .*:*************** .*::**: **. ***** ***.

Mg  VLKALGATIIRTPTQAAWDAPESHIGVAKRLLKEIPNSHILDQYSNPNNPLAHEHGTAEE 177
Sc  VLKALGAEIIRTPTVAAWDSPESHIGVAKKLEKEIPGAVILDQYNNMMTPEAHYFGTGRE 173
Hs  VLRALGAEIVRTPTNARFDSPESHVGVAWRLKNEIPNSHILDQYRNASNPLAHYDTTADE 239
    **:**** *:**** * :*:****:*** :* :***.: ***** *   .* **    *. *

Mg  IWAQTGG-----KITAVVAGAGTGGTISGLARGLRKHSKTVKVIAADPIGSILALPESLN 232
Sc  IQRQLEDLNLFDNLRAVVAGAGTGGTISGISKYLKEQNDKIQIVGADPFGSILAQPENLN 233
Hs  ILQQCDG-----KLDMLVASVGTGGTITGIARKLKEKCPGCRIIGVDPEGSILAEPEELN 294
    *  *  .      ::  :**..******:*::: *:::      :::..** ***** **.**

Mg  DNGVNEPYKVEGIGYDFIPDVLDRELVDKWYKTEDRESFMYARRLIAEEGLLVGGSSGSA 292
Sc  KTDITD-YKVEGIGYDFVPQVLDRKLIDVWYKTDDKPSFKYARQLISNEGVLVGGSSGSA 292
Hs  QTEQTT-YEVEGIGYDFIPTVLDRTVVDKWFKSNDEEAFTFARMLIAQEGLLCGGSAGST 353
    ..  .   *:*********:* **** ::* *:*::*. :* :** **::**:* ***:**:

Mg  MAAMVRAVKDL-NLGEDDVVVVVLPDSIRSYLSKFADDDWLAANDLLLPTPDSSVNGDTL 351
Sc  FTAVVKYCEDHPELTEDDVIVAIFPDSIRSYLTKFVDDEWLKKNNLWDDDVLARFDSSKL 352
Hs  VAVAVKAGQEL--LQEGQRCVVILPDSVRNYMTKFLSDRWMLQKGFLKEEDLTEKKPW-- 409
    .:. *:  ::    * *.:  *.::***:*.*::** .* *:  :.:        :  .

Mg  DSPAVAEAQALHKSRARRASNPYGGATVRSLRLKPVTSVLADSKCSEAIETMRDKGFDQL 411
Sc  EASTTKYADV------------FGNATVKDLHLKPVVSVKETAKVTDVIKILKDNGFDQL 400
Hs  ---------------------WWHLRVQELSLSAPLTLVLPTITCGHTIEILREKGFDQA 447
                          :    *:.* *..  :*    .   ..*: ::::****

Mg  PVVRD-NKLVGLVTLGNMLSYVSRGRVKTNSPVSDVMFDFGRLDEVITDPRDVEGAAKMS 470
Sc  PVLTEYGKLSGLVTLSELLRKLSINNSNNDNTIKVEYLDVKKLNNF--N--DVSSYNENK 456
Hs  PVVDEAGVILGMVTLGNMLSSLLAGKVQPSDQVGKVIYKQFKQIRS----------TDTL 497
    **: : . : *:***.::*  :  .. : .. :     .  :  .             .

Mg  TAHGRKKRNFVEITMNTPLDVLSKFLEWNSAAIVTEKSGEVSKPVAVVTKVDLLTWMMKE 530
Sc  SG---KK-KFIKFDENSKLSDLNRFFEENSSAVITDG----LKPIHIVTKMDLLSYLA-- 506
Hs  GT----------LSHILEMDHFALVVHEQIQYHSTGKSSQRQMVFGVVTAIDLLNFEAAQ 547
                :     :. :  ... :     *           . :** :***.:

Mg  LKA-- 533
Sc  -----
Hs  ERDQK 552
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FIG. 2. Amino acid alignment of *M. grisea* CBS1 (Mg) with *S. cerevisiae* CBS (Sc) (GenBank accession number AAC37401) and human CBS (Hs) (GenBank accession number A55760). Sequences were aligned by using the Clustal W program and Lasergene software (DNASTAR, Madison, Wis.). Gaps in the alignment are indicated by dashes. Asterisks indicate identical residues in all sequences. Colons indicate conservative substitutions. Dots indicate semiconserved substitutions. Lys119 in human CBS is involved in PLP binding and is conserved in *M. grisea* and *S. cerevisiae* (arrow). The Cys52 and His65 residues that axially coordinate the iron in the heme group in human CBS are doubly underlined. The CBS domains in the C termini of the sequences are underlined.

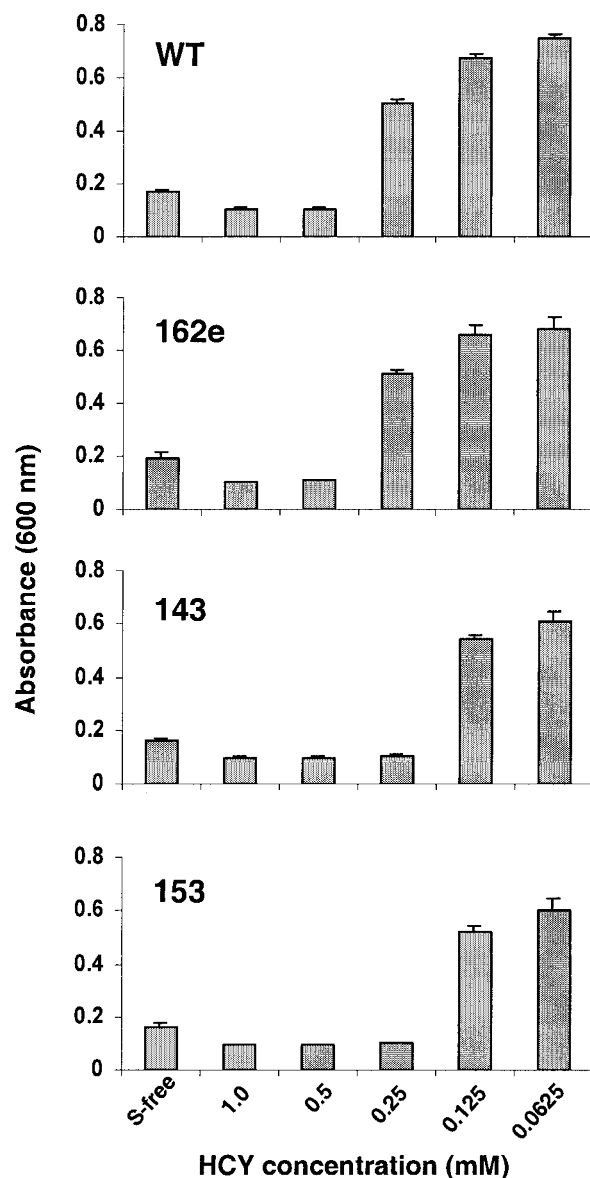

FIG. 3. Inhibition of growth of *M. grisea* strains by homocysteine. The fungal strains were grown in MM containing different concentrations of homocysteine (HCY) as a sole sulfur source. Mycelial growth was monitored by measuring the absorbance at 600 nm 7 days after inoculation. Growth inhibition is reflected by an absorbance value lower than that obtained in sulfur-free (S-free) medium. The WT strain and an ectopic transformant (162e) were inhibited at ≥0.5 mM homocysteine. *cbs1* mutants 143 and 153 showed increased sensitivity to homocysteine. Data are reported as means (columns) and standard deviations (error bars).

We demonstrated that the introduction of an expression plasmid containing *M. grisea CBS1* rescued the growth defect of a CBS-deficient *S. cerevisiae* strain in the absence of cysteine (data not shown). These findings indicate that *M. grisea CBS1* is a structural and functional homolog of the *S. cerevisiae* CBS gene.

CBS1 is a single-copy gene in *M. grisea*, as revealed by genomic Southern analysis (data not shown). We performed in silico hybridization (TBLASTN searches) (1) of the CBS1 amino acid sequence against our internal *M. grisea* unigene database and the complete *N. crassa* genome database (version 2; Whitehead Institute, MIT Center for Genome Research; www-genome.wi.nit.edu/annotation/fungi/neurospora). There was no evidence of a second gene encoding CBS in either filamentous fungus. The closest matches from both databases were sequences encoding cysteine synthase (CYS)-like proteins. CBS and CYS are related proteins, and both require PLP as a cofactor. The amino acid sequence identity between *M. grisea* CBS1 and *A. nidulans* CYS (GenBank accession number P50867, the only annotated filamentous fungal CYS in National Center for Biotechnology Information nr as of 18 October 2001) is 38%. However, CYS proteins are considerably shorter (~300 amino acids) than CBS proteins (>500 amino acids) (5). There are no indications that CYS proteins from different species exhibit CBS activities. Based on these findings, we conclude that *CBS1* is the only gene encoding CBS in *M. grisea*.

CBS1 was deleted from *M. grisea* by replacement with a

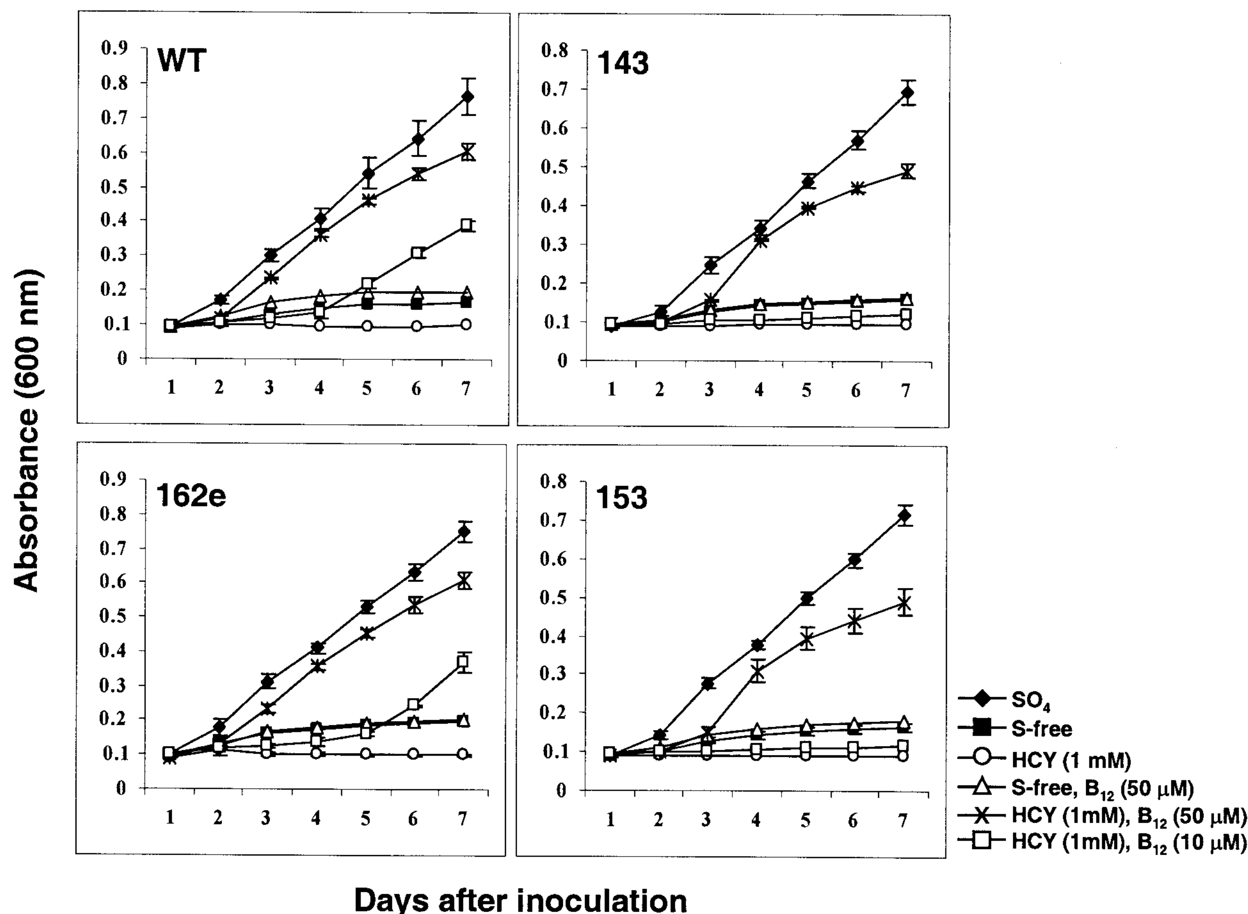

FIG. 4. Effect of vitamin B_{12} on homocysteine sensitivity in *M. grisea* strains. Vitamin B_{12} was added to MM containing homocysteine (HCY) as the sole sulfur source. Complete inhibition of growth was observed for all strains growing in the presence of 1 mM homocysteine. Mycelial growth was restored in all strains when 50 μM vitamin B_{12} was added to the medium. At 10 μM vitamin B_{12}, the growth of the WT strain and an ectopic transformant (162e) was delayed and partially restored. *cbs1* mutants 143 and 153, which are hypersensitive to homocysteine, did not respond to vitamin B_{12} at this concentration. Note that vitamin B_{12} added to sulfur-free (S-free) medium did not support growth. Fungal strains growing in MM containing inorganic sulfate (SO_4) as the sole sulfur source were used as positive controls. Data are reported as means (as indicated) and standard deviations (error bars).

modified hygromycin phosophotransferase gene (4) as described previously (21). The null (*cbs1*) mutants were found to retain virulence on rice (data not shown). In addition, the *cbs1* mutants were not auxotrophic and were able to utilize inorganic sulfate, cysteine, cystathionine, homocysteine, or methionine as a sole sulfur source (data not shown). In the absence of inorganic sulfur sources, the pathway through CBS (Fig. 1) is the only known route for cysteine biosynthesis in filamentous fungi and other microbes (16) (Kegg metabolic pathways) (http://www.genome.ad.jp/kegg/metabolism.html) (8 January 2002). Thus, homocysteine and methionine can be utilized via a CBS-independent pathway in *M. grisea cbs1* mutants. Similarly, *Schizosaccharomyces pombe*, which lacks CBS naturally, is able to convert methionine to cysteine (3). Alternatively, homocysteine or methionine may be degraded through unknown pathways and the resulting sulfide ion may be assimilated by *M. grisea*.

Our growth studies revealed that homocysteine is toxic to *M. grisea*. Spore suspensions were inoculated into minimal medium (MM) (18) containing different concentrations of homocysteine as described previously (7). Fungal growth was monitored as an increase in absorbance at 600 nm. Complete inhibition of growth was observed when the wild-type (WT) strain was grown in homocysteine at a concentration of 0.5 mM or higher (Fig. 3). The *cbs1* mutants were hypersensitive to exogenous homocysteine. For example, the growth of the *cbs1* mutants was completely inhibited at a concentration of 0.25 mM (Fig. 3). Inhibitory effects on growth were not evident with cysteine, cystathionine, or methionine at all the tested concentrations (data not shown). Toxicity of homocysteine for fungal growth has not been described elsewhere. However, humans with homocystinuria have been known to develop different clinical phenotypes caused by elevated levels of circulating homocysteine (17). This disorder is most frequently a consequence of CBS deficiencies. It is possible that *M. grisea* and human CBS proteins share a common physiological function as a detoxification mechanism for homocysteine.

Vitamin or coenzyme treatments of homocystinuria patients serve to enhance pathways that remove excess circulating homocysteine (19). Interestingly, we demonstrated that the addi-

tion of vitamin B_{12} relieved the toxicity of homocysteine for *M. grisea*. As shown in Fig. 4, supplementation with vitamin B_{12} at 50 μM allowed both the WT and *cbs1* mutant strains to grow in MM containing 1 mM homocysteine. The vitamin B_{12} response appeared to be concentration dependent. Thus, the growth of WT and ectopic strains in the presence of homocysteine was moderately restored when 10 μM vitamin B_{12} was supplied (Fig. 4). The growth of the *cbs1* mutants, which were more sensitive to homocysteine, remained inhibited at 10 μM vitamin B_{12} (Fig. 4). In the human body, homocysteine either can be remethylated to methionine by methionine synthase or can undergo transsulfuration reactions via CBS to form cysteine (6). The remethylation of homocysteine to methionine by methionine synthase is dependent on vitamin B_{12}, betaine, and folate, while the CBS-catalyzed reaction requires vitamin B_6 as a coenzyme (20). In *M. grisea*, methionine synthase (Fig. 1, enzyme 7) activities likely were enhanced with vitamin B_{12} supplementation to remove homocysteine.

In conclusion, our studies of *CBS1* in *M. grisea* indicate that homocysteine and methionine can be utilized by the fungus through pathways that are independent of CBS. In addition, our results reveal similarities between *M. grisea* and humans with regard to sensitivities to homocysteine and responsiveness to vitamin B_{12} supplementation. The fungus may be exploited as a system to screen for therapeutic agents to relieve homocysteine toxicity.

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