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Metal Ion Resistance in Fungi: Molecular Mechanisms and Their Regulated Expression

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Abstract One stress response in cells is the ability to survive in an environment containing excessive concentrations of metal ions. This paper reviews current knowledge about cellular and molecular mechanisms involved in the response and adaptation of various fungal species to metal stress. Most cells contain a repertoire of mechanisms to maintain metal homeostasis and prevent metal toxicity. Roles played by glutathione, related $(\gamma\text{-EC})_nG$ peptides, metallothionein-like polypeptides, and sulfide ions are discussed. In response to cellular metal stress, the biosynthesis of some of these molecules are metalloregulated via intracellular metal sensors. The identity of the metal sensors and the role of metal ions in the regulation of biosynthesis of metallothionein and $(\gamma\text{-EC})_nG$ peptides are subjects of much current attention and are discussed herein.

Key words: metal resistance, metal tolerance, detoxification, metallothionein, yeast

Certain metal ions such as copper and zinc are essential for normal physiological functioning of living organisms, whereas others such as cadmium and mercury are nonessential and toxic. Even essential metal ions can cause toxicity if the intracellular concentrations of the ions rise above physiologically required levels. The survival of cells depends on their ability to limit the intracellular concentration of essential as well as toxic metal ions. The toxic ions can accumulate within cells due to the inability of the cellular transporters to discriminate between essential and nonessential metal ions that have similar chemical characteristics. The detoxification of metal ions is achieved either by regulating uptake and/or efflux or by intracellular sequestration or compartmentalization (Fig. 1). Metal ion resistance via transport mechanisms is more common among prokaryotes [1,2], whereas sequestration mechanisms are utilized by eukaryotes [3,4]. Compartmentalization of metal ions in subcellular organelles has been described [5], but most attention has been focused on cytoplasmic sequestration. The major molecules involved in intracellular sequestration of metal ions include glutathione (γ-Glu-Cys-Gly), related $(\gamma\text{-Glu-Cys})_nGly$ peptides, and cysteine-rich polypeptides designated metallothioneins (MT). Not all organisms express all these molecules. It is apparent that most cells contain a repertoire of mechanisms for metal resistance, and only some of the mechanisms are currently known. This paper focuses primarily on mechanisms of copper and cadmium detoxification in fungi. General aspects of metabolism and detoxification of metals in both prokaryotes and eukaryotes can be found in recent reviews [6–8].

ROLE OF $(\gamma\text{-GLU-CYS})_n$GLY PEPTIDES IN METAL RESISTANCE

The fission yeast *Schizosaccharomyces pombe* responds to cadmium ions in the culture medium by synthesizing short cysteine-rich peptides, which were first discovered by Murasugi et al. [9]. These authors purified two Cd-binding peptides with identical amino acid composition (Glu_3, Cys_3, and Gly_1) but differed in molecular size, charge properties, and cadmium content [8]. Cadmium ions in the two complexes termed CdBP1 and CdBP2 were bound to thiolate sulfurs of cysteines [9,10]. It was demonstrated that the major difference in the two Cd-binding peptides synthesized in *S. pombe* was the presence of labile sulfide in the more negatively charged component [11]. Determination of the primary sequences of these peptides showed them to be derivatives of glutathione with the structure $(\gamma\text{-EC})_nG$ where n typically varies between 2 and 5 [12–15] (Fig. 2). The peptide linkage in the dipeptide repeat is an isopeptide

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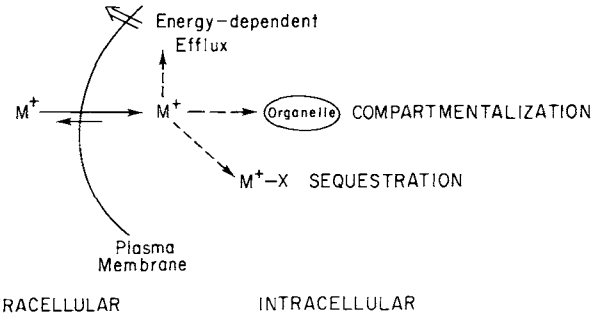

Fig. 1. Metal resistance pathways. The common pathways involved in metal ion resistance involve membrane transport mechanisms in prokaryotes and metal ion sequestration in eukaryotes. Sequestration of metal ions can occur by compartmentalization within an organelle or formation of stable chelates.

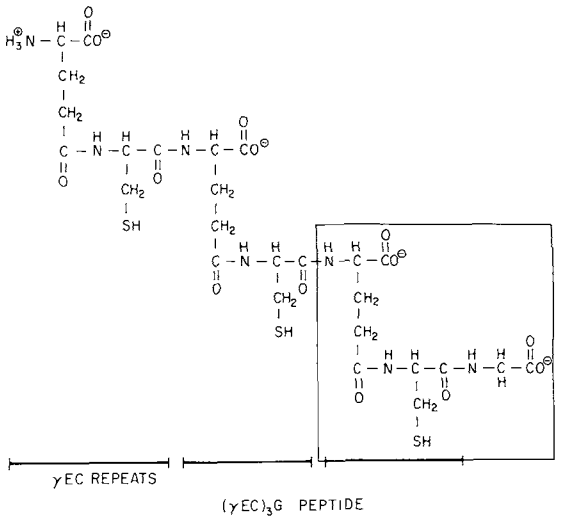

Fig. 2. Structure of a $(\gamma\text{-EC})_3$G peptide showing the isopeptide linkage within the dipeptide repeat. The structure enclosed in the box is the glutathione, and the solid bars near the bottom designate the γ-glutamylcysteine repeats.

bond with the amide bond formed through the γ-carboxylate of glutamic acid (Fig. 2).

These peptides have been reported in the yeasts *S. pombe* and *Candida glabrata* and most plant species [see 16 for recent review]. Trivial names such as cadystin [12] and phytochelatin [13] have been used for the $(\gamma\text{-EC})_n$G peptides. None of these names provides accurate description of these peptides, since they can bind metals other than Cd, are not restricted to plants, and are not primary gene products due to the presence of isopeptide bonds. In the absence of a consensus on the nomenclature, this paper designates these peptides as $(\gamma\text{-EC})_n$G peptides.

The importance of the isopeptides in metal resistance was demonstrated by the isolation of mutants of *S. pombe* unable to synthesize $(\gamma\text{-EC})_n$G peptides [17]. The phenotype of these mutants was hypersensitivity to cadmium salts.

Biosynthesis of $(\gamma\text{-EC})_n$G Peptides

The presence of γ-glutamyl bonds in $(\gamma\text{-EC})_n$G peptides indicates that these peptides are not primary gene products. An enzyme isolated from the plant *Silene cucubalus* catalyzed the transfer of the γ-EC dipeptide moiety of glutathione or $(\gamma\text{-EC})_n$G peptides to acceptor glutathione or $(\gamma\text{-EC})_n$G peptides [18]. It is not known whether a similar enzyme is responsible for the synthesis of these peptides in *S. pombe* and *C. glabrata*. The γ-glutamylcysteine dipeptidyl transpeptidase from *Silene cucubalus* was reported to be activated by a variety of metals in the following order: Cd(II), Ag(I), Bi(III), Pb(II), Zn(II), Cu(II), Hg(II), and Au(I) [18]. However, the high-performance liquid chromatography (HPLC) assay used by these authors was not standardized for the separation of $(\gamma\text{-EC})_n$G peptides that contained metals other than Cd(II). It is well established that acidification of peptides/proteins containing Cu(I)-thiolate bonds results in oxidation of thiols, which can be reduced only after removal of Cu(II) [19]. Furthermore, claims of biosynthesis of $(\gamma\text{-EC})_n$G following exposure to zinc salts [13] have not been substantiated by results from other laboratories [14]. If the peptide system was involved in zinc resistance, a mutation in $(\gamma\text{-EC})_n$G peptide formation would confer zinc sensitivity. It would be instructive to determine whether the cadmium-hypersensitive mutants of *S. pombe* are also hypersensitive to other metal ions that have been suggested to induce biosynthesis of $(\gamma\text{-EC})_n$G peptides.

The metalloactivation of the transpeptidase implies that this enzyme functions as an intracellular metal sensor controlling $(\gamma\text{-EC})_n$G pep-

tide formation. There is no structural information available concerning this enzyme, so the structural basis for metalloregulation of catalysis remains unresolved. One intriguing aspect of the $(\gamma\text{-EC})_n$G peptide biosynthesis concerns the specificity in this metalloregulation. The yeast *C. glabrata* synthesizes the isopeptides in response to cadmium salts but not zinc or copper salts [20]. If a transpeptidase is involved in isopeptide formation in yeast, the structural basis of the discrimination between cadmium and zinc ions may be analogous to the differential effects of metal ions on the catalytic activity of metalloenzymes such as carboxypeptidase.

Hayashi and coworkers [21] found mainly $(\gamma\text{-EC})_2$G and $(\gamma\text{-EC})_3$G in *S. pombe,* but stationary-phase cultures of this yeast are capable of synthesizing peptides with n values of up to 6 [13–15]. In addition to this heterogeneity, *S. pombe* produces desGly variants of all the peptides at concentrations that are 10–20% of the parent peptides [15]. These desGly variants are not unique to *S. pombe* but are also found at high concentrations in *C. glabrata* [20] and some plants [22]. Only limited information is available on the time course of biosynthesis of $(\gamma\text{-EC})_n$G peptides. The interpretation of these data is complicated by differences in the assay procedures for $(\gamma\text{-EC})_n$G peptides and in the design of experiments. The synthesis of $(\gamma\text{-EC})_n$G peptides occurs almost without any lag in *S. pombe* cells exposed to cadmium salts with $(\gamma\text{-EC})_2$G appearing prior to $(\gamma\text{-EC})_3$G [9,21]. The concentration of the high-sulfide form of the Cd:peptide complexes increased with time, whereas that of the sulfide-lacking form declined with time [23]. It appeared that the occurrence of the high-sulfide form correlated with the cadmium-stimulated production of sulfide [23]. It was shown in a subsequent study that $(\gamma\text{-EC})_3$G was the major component of the high-sulfide form whereas $(\gamma\text{-EC})_2$G predominated in the low-sulfide form of the peptide complex [21]. It is important to note that sulfide ions stabilize $(\gamma\text{-EC})_n$G peptide complexes and that these sulfide containing complexes tend to incorporate longer peptides [24]. Thus it is obvious that the production of sulfide can alter biological half-lives of $(\gamma\text{-EC})_n$G peptides, favoring recovery of longer peptides. The n value of $(\gamma\text{-EC})_n$G peptides is influenced by the concentration of cadmium sulfate added to the growth medium and the phase of growth [13].

Unlike the case with *S. pombe* and many plants, peptides longer than $(\gamma\text{-EC})_2$G are rarely detected in *C. glabrata.* However, peptides lacking terminal glycine are present at high levels [20]. The desGly variants of the isopeptides appear to arise from catabolism rather than being a component in the biosynthesis (unpublished observation). The $(\gamma\text{-EC})_2$G peptide appears in *C. glabrata* only after a lag period. In cells cultured in medium containing cadmium salts the initial Cd(II) complex formed appears to be a Cd(II)-glutathione cluster. With time, both sulfide-containing Cd-glutathione complexes and Cd-$(\gamma\text{-EC})_n$G complexes appear.

No information is available on why *C. glabrata* synthesizes predominantly $(\gamma\text{-EC})_2$G peptides whereas *S. pombe* synthesizes peptides with a greater number of repeats. All cells capable of $(\gamma\text{-EC})_n$G peptide formation synthesize a heterogeneous mixture of peptides in response to metal stress. It is unclear whether any selective advantage exists in having a mixture of peptides or in having peptides with a greater number of dipeptide repeats.

Interestingly, *C. glabrata* cells grown in rich medium containing yeast extract and peptone do not synthesize the $(\gamma\text{-EC})_n$G or $(\gamma\text{EC})_n$ peptides [25]. These growth conditions promote sequestration of cadmium ions in complexes comprising glutathione and its desGly derivative and inorganic sulfide [25]. Despite the absence of $(\gamma\text{-EC})_n$G peptides, cells grown in this rich nutrient broth are resistant to cadmium salts.

Structure of Metal: $(\gamma\text{-EC})_n$G Peptide Complexes

Multiple Cd:$(\gamma\text{-EC})_n$G peptide complexes are induced in cultures of *S. pombe* exposed to cadmium salts. The sulfide-containing complexes are discussed below. The sulfide-free complexes were initially designated CdBP2 [21]. It is now clear that CdBP2 consists of two main types of clusters differing in the $(\gamma\text{-EC})_n$G peptide involved. Unique Cd-$(\gamma\text{-EC})_2$G and Cd-$(\gamma\text{-EC})_3$G clusters exist in Cd-treated *S. pombe* [26]. No definitive information is available concerning the molecular weight of these clusters, so their structures remain unresolved. It is clear that the Cd(II) stoichiometries of the two clusters differ. The Cd-$(\gamma\text{-EC})_n$G complex averaged 1.2 mol eq Cd(II) based on the peptide concentration. Since the $(\gamma\text{-EC})_2$G peptide contains only two cysteinyl thiolates, an expected tetrahedral coordination geometry would necessitate either an oligomeric complex with μ-bridging thiolates or nonthiolate ligands.

Copper-binding components from *S. pombe* were shown to be $(\gamma\text{-EC})_n$G peptides of $(\gamma\text{-EC})_2$G

through $(\gamma\text{-EC})_4G$ devoid of sulfide [14]. Luminescence measurements and absorption spectroscopy suggested the presence of Cu(I)-thiolate clusters in these peptides [15]. The quantum yield of emission was analogous to that of Cu-MT from *S. cerevisiae,* suggesting that the isopeptides shielded the Cu-thiolate cluster from solvent interaction. The molecular weight of these clusters was consistent with a structure of a polynuclear Cu-thiolate cluster coated with multiple $(\gamma\text{-EC})_nG$ peptides.

Sulfide-Containing Forms of Cd-$(\gamma\text{-EC})_nG$ Complexes

Sulfide-containing forms of Cd-$(\gamma\text{-EC})_nG$ peptide complexes occur in *S. pombe* and *C. glabrata* [11,23,24]. Considerable information is available on structural aspects of native complexes and those produced by in vitro reconstitution procedures [24]. Studies on both *C. glabrata* and *S. pombe* have shown that sulfide-containing species of Cd-$(\gamma\text{-EC})_nG$ complexes are highly negatively charged and are heterogeneous with respect to cadmium and sulfide contents [20,24]. Elution fractions from Sephadex G-50 showed that fractions with a greater Stokes radius had a higher metal and sulfide content than fractions with smaller Stokes radii [20,24]. Significant changes occurred in the absorption spectrum of fractions across the profile. Reconstitution experiments showed clearly that introduction of sulfide resulted in appearance of near-ultraviolet transitions that were red shifted by increasing the content of sulfide in complexes [24].

Biophysical studies on sulfide forms of Cd-$(\gamma\text{-EC})_nG$ peptides from both *S. pombe* and *C. glabrata* showed that these complexes formed novel nanometer-scale quantum cadmium sulfide crystallites [27]. The size of the crystallites and therefore the Stokes radius of a particle are dictated by the magnitude of the sulfide production by the cells. Transmission electron microscopy and powder X-ray diffraction showed that *C. glabrata* complexes were nearly monodisperse particles with diameters of ~20 Å. A mixture of particles varying in particle size is typically seen in *S. pombe.* It appears that the maximal diameter of the CdS particles formed with $(\gamma\text{-EC})_nG$ peptides is near 20 Å.

Sulfide-containing Cd-$(\gamma\text{-EC})_nG$ peptide complexes are chemically more stable than Cd:peptide complexes devoid of sulfide. The chemical and presumably biological stability of sulfide-containing Cd-$(\gamma\text{-EC})_nG$ peptides makes them more suitable for detoxification of cadmium [24,28]. Mutants of *S. pombe* unable to form the sulfide-containing Cd:peptide complexes were shown by Mutoh and Hayashi [17] to be cadmium hypersensitive.

METALLOTHIONEIN IN COPPER RESISTANCE IN *S. CEREVISIAE*

The phenomenon of copper adaptation in laboratory strains of *Saccharomyces* was known to early Japanese workers [29]. Some of these copper-resistant strains produced excess sulfide, but a clear correlation between the production of sulfide and levels of copper resistance could not be obtained [29]. Genetic studies showed that high levels of copper resistance were attributed to a locus called *CUP1* [30–32]. The cloning and characterization of this locus demonstrated amplification at the locus and a direct correlation between levels of copper resistance and degree of amplification [cf. 33–35]. The *CUP1* locus contains two open reading frames, one of which codes for a cysteine-rich polypeptide analogous to the well characterized animals MTs [34,35]. The second open reading frame appears to encode an unknown protein that is not involved in metal resistance.

Copper-binding proteins with an amino acid composition similar to that of MT had previously been isolated from *S. cerevisiae* cells grown in medium containing copper sulfate [36,37]. Amino acid sequencing of the purified copper-binding protein from *S. cerevisiae* showed that this protein was a processed product of the *CUP1* locus [19]. The processing involved the proteolytic cleavage of eight N-terminal amino acids [19,38]. The significance of this proteolytic cleavage is not yet understood.

Metal-Binding Characteristics of *S. cerevisiae* MT

Like all other copper-containing MTs, *S. cerevisiae* MT binds the metal as Cu(I). Eight Cu(I) ions are bound to this yeast MT exclusively through cysteinyl thiolates [19]. Three sulfur atoms coordinate each Cu(I) ion [39]. The in vitro reconstitution of apoMT with increasing equivalents of Cu(I) near neutral pH leads to formation of a metal-thiolate cluster in an all or none fashion [40]. Preliminary nuclear magnetic resonance (NMR) data on Ag_8MT suggest that *S. cerevisiae* MT may enfold a single polynuclear metal-binding cluster [41]. The structure

of CuMT is of significance as the only detailed structural information on metallothionein is on mammalian Cd,ZnMT. The structural distinction between CuMT and CdMT remains to be resolved. *S. cerevisiae* is an ideal system for structural studies on metallothionein in that 1) the CuMT is quite stable and easily purified, 2) the native CuMT and CdMT can be obtained from one species, and 3) the CuMT is a model system for the Cu-trans-acting factor (see below).

The contributions made by different cysteinyl residues along polypeptide chain in formation of the metal-thiolate cluster(s) have been studied in detail using several mutant MT molecules [40,43]. These mutants were of two types: 1) truncated versions of the protein and 2) mutant molecules in which pairs of cysteinyl residues were converted to serines. These mutant proteins were expressed using plasmids in an *S. cerevisiae* strain from which the genomic *CUP1* locus was deleted by insertion mutagenesis [40,43]. The deletion or conversion of the carboxyl-terminal pair of cysteines did not influence metal-binding characteristics of the protein or copper resistance of the host [40,43]. Progressive deletion of carboxyl-terminal residues resulted in mutant proteins that exhibited decreased stability and reduced metal-binding ability and did not protect the host against copper toxicity [40]. Mutation of pairs of cysteinyl residues to serines showed that the stability of mutant proteins was dependent on which pairs were converted [40]. These results suggest considerable variations in the contributions made by different cysteinyl residues along the polypeptide chain towards formation of polynuclear metal-thiolate cluster(s) [40]. The carboxyl-terminal mutant CuMTs may be interesting molecules for structural analysis to determine the type of adaptation in tertiary conformation to yield stable Cu:thiolate clusters. The driving force for structure in metallothioneins is metal: thiolate coordination; thus multiple conformers are possible when specific cysteinyl residues are replaced.

Metalloregulation of the Expression of *CUP1*

The *CUP1* locus in all strains of *S. cerevisiae* except 301N [42] is transcriptionally regulated by copper and silver but not cadmium ions [33,44]. Importantly, the *CUP1* locus in *S. cerevisiae* strain 301N responds to cadmium ions [42]. Recent studies from different laboratories have provided insights into the mechanism(s) involved in the regulation of *CUP1* by copper ions [44–50]. It has been demonstrated that an increase in the intracellular concentration of copper leads to activation of a DNA-binding protein, specifically a transcription factor, which in turn causes accelerated transcription of the *CUP1* locus, metallothionein, by binding to certain DNA segments in the promoter region of the gene. The accelerated synthesis of MT results in the sequestration of copper ions, eventually shutting off the activation of the transcription factor and consequently the activation of the *CUP1* locus. This process thus constitutes a self-regulating loop (Fig. 3). The Cu-transcription factor (*ACE1* or *CUP2*) is a nuclear protein [51], so the copper-activation of the factor must involve presentation of copper ions to the nucleus. There is no information on whether this presentation involves transport or diffusion through nuclear pores.

The copper-activated transcription factor that regulates expression of the *CUP1* locus was identified by mutagenesis studies and has been designated *ACE1* [44,45] or *CUP2* [46]. It is now agreed that the *ACE1* and *CUP2* loci are identical [49,50]. As was mentioned above, strains of *S. cerevisiae* containing multiple copies of the *CUP1* gene grow normally in medium containing high concentrations of copper sulfate. Conversion of such a strain to a copper-sensitive phenotype by mutagenesis with a point mutagen such as ethanemethanesulfonate could in principle result from mutation in a gene the product of which trans-activates *CUP1*. This strategy was successful in identification of the mutants *cup2* and *ace1* [45,46]. The cloning and characterization of the *ACE1* gene showed that the trans-acting factor is a 24 kd protein consisting of a positively charged amino-terminal domain and a negatively charged carboxyl-terminal domain [44]. It was likely that the amino-terminal domain acted as a copper ion sensor, since it contained several cysteinyl residues that were arranged in Cys-X-Cys sequences. Such sequences are expected to form polynuclear metal clusters as has been demonstrated in well characterized MTs [3,4]. The net positive charge would likely confer DNA-binding activity on this domain. The metal-binding and DNA-binding activities of this domain have been confirmed by gel-retardation and limited proteolysis assays [44,48]. It is clear that the binding of copper as Cu(I) activates *ACE1* for specific DNA binding by inducing as yet undetermined change in the tertiary fold of this protein.

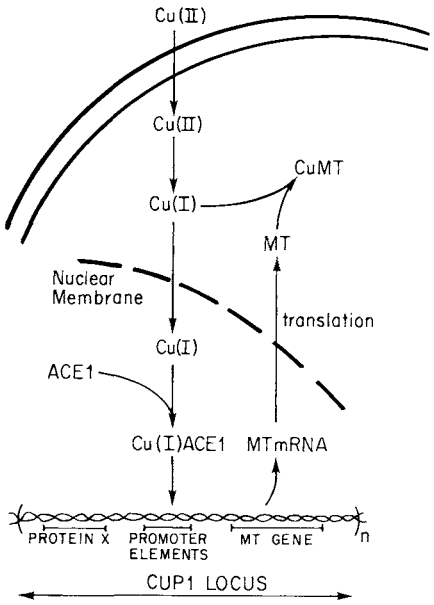

Fig. 3. Metalloregulation of the *CUP1* locus in *Saccharomyces cerevisiae*. The Cu-*ACE1* metalloprotein complex interacts with upstream sequences from the *CUP1* coding sequences and facilitates transcription of the metallothionein (MT) gene within the *CUP1* locus. Translation of the MT mRNA yields MT protein that buffers the cytosolic copper ion concentration.

ACE1 contains the same number of cysteines as does MT, the gene that it regulates. The structure of the Cu-metallothionein may therefore serve as a structural model for the Cu-*ACE1* molecule. Metallothionein is devoid of structure in the apo state. Copper binding induces a tertiary fold in metallothionein that is distinct from the conformation induced by Cd(II) ions [43]. CuMT forms a Cu_8S_{12} cluster, whereas yeast CdMT has a Cd_4S_{12} stoichiometry. The Cu(I) specificity of the *ACE1* molecule may arise from a related Cu(I)-thiolate polynuclear cluster. The presence of the Cu(I) in Cu-*ACE1* has been deduced from 1) gel-retardation assays showing that the addition of Cu(I) chelators significantly reduced DNA-binding activity of *ACE1*, 2) isolation of *ACE1* as a Cu-protein complex, and 3) the luminescence observed with the purified protein characteristic of solvent-excluded Cu(I) clusters [40]. Accurate estimates of the Cu(I)-binding stoichiometry and the elucidation of the nature of metal-thiolate clusters await further study. These studies will advance our knowledge about the Cu(I)-induced conformational switch in *ACE1* and the resulting interactions between this protein and upstream activating sequences in the promoter of *CUP1*.

The presence of a Cu(I)-thiolate polynuclear cluster in Cu-*ACE1* analogous to the cluster in CuMT may confer the basis for metallospecificity in the regulation of the *CUP1* locus. Cu(I) confers a distinct conformation on yeast MT compared with Cd(II). The only metal ion that yields a metal cluster in MT comparable to the Cu(I) cluster is Ag(I). Ag(I) ions also bind to *ACE1* and activate the trans-acting factor for DNA binding. Thus it is conceivable that the metalloregulation of *CUP1* is based on metal clusters with trigonal metal ion coordination. Cadmium may not induce MT gene expression in that Cd-thiolate clusters usually involve tetrahedral Cd(II) coordination.

Strain 301N is a significant model system for studies on the question of how metal ion specificity is achieved in metalloregulation. The basis for the Cd(II) regulation of *CUP1* gene expression in strain 301N is unclear. The *ACE1* molecule in most laboratory strains of *S. cerevisiae* can bind Cd(II) ions, but the cadmium complex is not competent in regulating *CUP1* expression. It is conceivable that the Cd(II)-*ACE1* complex in 301N is effective in enhancing MT gene expression by virtue of an altered *ACE1* molecule or an altered MT promoter sequence.

Amplification at the *CUP1* Locus

As was mentioned above, the level of copper resistance in *S. cerevisiae* is directly related to

the copy number of the gene at the *CUP1* locus. In some strains, MT genes are amplified by duplication of chromosome VIII, which carries the *CUP1* locus [cf. 33]. The MT genes are tandemly arranged at this locus so that digestion of genomic DNA with a restriction enzyme having a single site in the repeating unit produces fragments of the length of the repeating unit [31–34]. This length is about 2 kb in most laboratory strains of *S. cerevisiae,* although variations in the length of the repeating unit have been observed in some industrial strains of this yeast [32]. In these industrial strains, multiple *CUP1* loci can also exist in the genome. Adaptation of low-copy-number strains to increasing concentration of copper sulfate in medium leads to appreciable increases in the copy number, but no amplification has been reported in strains that contain only one copy of the *CUP1* gene [33,52]. Genetic analysis suggests that amplification at the *CUP1* locus takes place via nonreciprocal recombination or gene conversion [33,53]. According to this model, a single strand of the repeating unit on one chromatid is symmetrically transferred to the other chromatid leading to loop formation with the single strand. Subsequent repair of the single stranded loop will increase the copy number. A reverse mechanism involving degradation of loop can lead to deamplification. Mammalian amplicons generally contain autonomously replicating sequences and some models of amplification propose autonomous replication of amplified DNA sequences [54]. It is not known if the *S. cerevisiae* MT gene has any autonomously replicating sequences.

Most *S. cerevisiae* strains are not cadmium resistant, in that the *CUP1* locus is not Cd(II) regulated. Industrial strains of *S. cerevisiae* are known to exhibit cadmium resistance without the involvement of metallothionein, but the basis of resistance is not resolved. Potential mechanisms may involve reduced uptake through plasma membrane transport systems, facilitated efflux, compartmentalization of cadmium ions in vacuoles, or adsorption on cell wall. Elucidation of additional metal resistance pathways in yeast may yield insight on normal mechanisms used by cells to maintain homeostasis of essential metal ions.

MULTIPLE METAL RESISTANCE PATHWAYS IN *C. glabrata*

It had been known for some time that clinical isolates of *C. glabrata* and *C. albicans* exhibit significantly high levels of resistance to both copper and cadmium salts, although the molecular basis of this resistance was not known [33]. Recent studies on *C. glabrata* have revealed that this yeast detoxifies cadmium and copper salts by different mechanisms [20]. As was mentioned above, cadmium salts stimulate the production of $(\gamma\text{-EC})_nG$ peptides, whereas copper salts induce the synthesis of a family of metallothioneins (MT). This yeast is of further interest in that it shows a facile adaptation to enhanced copper resistance.

Wild-type strains including clinical isolates of *C. glabrata* exhibit marked resistance to copper sulfate [55]. Copper was found to be bound to two polypeptides classified as MTs based on their amino acid composition and limited sequence analysis [20]. The two MTs were designated MT-I and MT-II, respectively, based on their elution from an ion-exchange column. Subsequent studies revealed a multigene family of MTs in this yeast [55,56]. This family comprises two subfamilies; the MT-I subfamily contains a single member, whereas the MT-II subfamily consists of at least two members. The principal MT molecules, MT-I and MT-II, are 62 and 51 amino acid polypeptides, respectively [55]. The *C. glabrata* MTs exhibit very limited sequence homology with each other or with any known MTs. However, Cys-X-Cys sequence motifs typical of mammalian MTs are present in *C. glabrata* MTs. Native MT-II contains 16 cysteines and binds about 10 mol eq of copper ions [55]. The ultraviolet (UV) and luminescence spectroscopy indicate the presence of Cu(I)-thiolate clusters shielded from solvent environment [55]. Preliminary studies involving limited proteolysis of MT-I containing subsaturating amounts of Cu(I) or Cd(II) have identified two metal-binding domains in this protein (unpublished data).

Amplification of MT-II Gene in *C. glabrata*

MT-II-specific oligonucleotide probes hybridized to several DNA fragments on Southern blot analysis of *C. glabrata* genomic DNA [55,56]. A comparison of Southern blot analyses of different wild-type strains showed differences in hybridization intensity of a 1.1 kb EcoRI or a 1.25 kb BamHI band depending on the strain used. No variations were seen in the intensities of other MT-II bands [56]. More recent studies show that this 1.1 kb band is absent from one of the wild-type strains. It was established that

these differences in hybridization intensity were due to amplification of one MT-II gene [56]. Southern blot analyses using restriction enzymes with unique sites in the amplified gene showed tandem arrangement of amplified units [56]. Of the five wild-type strains tested, only one strain had a single copy, whereas other strains had three to eight copies of the MT-II gene. No amplification was detected in the MT-I locus or the other MT-II hybridizable bands.

Highly copper-resistant strains of *C. glabrata* can be obtained by growing cells in medium containing increasing concentrations of copper sulfate [56]. The concentration of copper sulfate required to inhibit growth by 50% was ~1 mM for most wild-type strains and it increased to ~7 mM in the most tolerant strain of *C. glabrata.* Southern blot analyses demonstrated selective amplification of the 1.25 kb MT-II gene in all the resistant strains [56]. There was evidence suggesting duplication of the chromosome carrying the MT-II gene in one of the resistant strains [56]. MT-I and the other MT-II genes did not show any amplification in any of these strains. Recent experiments show that copper adaptation of a wild-type strain carrying a single copy of the 1.25 kb MT-II gene did not result in tandem amplification of the gene, although amplification by duplication of chromosome was suggested (unpublished observations). As was discussed previously, amplification has never been observed at the *CUP1* locus in strains of *S. cerevisiae* that carry a single copy of this gene [33]. These observations are consistent with a mechanism of amplification that involves nonreciprocal recombination [33]. It has been shown that the 1.25 kb MT-II repeating unit contains autonomously replicating sequence(s) (unpublished data), so amplification may conceivably proceed via independent replication of amplicons.

As was mentioned above, the level of copper resistance in *S. cerevisiae* is directly related to the copy number of *CUP1* locus [33]. In *C. glabrata,* no difference in copper resistance was observed when the copy number of MT-II increased from one to nine, although very high levels of amplification did result in considerable increase in copper resistance [56]. It is possible that mechanisms other than MT production are involved in detoxification of copper in *C. glabrata.* Alternatively, only a few copies of the MT-II gene are functional at the amplified locus.

Induction of MTs in *C. glabrata*

Northern analysis of the total cellular RNA isolated from *C. glabrata* cells grown in medium containing copper sulfate shows that the levels of MT-I mRNA are always lower than those of MT-II mRNA [55]. Similar results are obtained at the protein level in that the amount of MT-II produced is far greater than MT-I [20]. Since MT-II gene generally occurs in multiple copies, it appeared that differences in the induction/accumulation of the two proteins were related to gene dosage [55]. This inference is not supported by the analysis of a strain that carries a single copy each of MT-I and the principal MT-II gene, since this strain too produced far greater amounts of MT-II than MT-I (unpublished data). Although the mechanism(s) of induction of MTs by copper ions in *C. glabrata* is not understood, by analogy with *S. cerevisiae* [46], it appears likely that the induction is mediated via a cellular factor that is activated by copper ions and in turn activates transcription by binding to cis-acting sequences. Differences in the inducibility of MT-I and MT-II may be related to variations in activability of cis-acting sequences, as has previously been observed in some human MT isoforms [57]. However, other factors such as stability of mRNAs and of proteins may also contribute to the overall accumulation of these MTs.

C. glabrata cells grown in medium containing copper sulfate also accumulate black deposits identified as copper sulfide (unpublished data). Preliminary experiments indicate that accumulation of sulfide depends on the concentration of copper sulfate in medium (unpublished data). High concentration of the salt appear inhibitory to sulfide accumulation. Sulfide production does not confer any significant resistance to *C. glabrata* cells, although some contribution of sulfide in protecting *S. cerevisiae* against copper toxicity has been suggested [29]. Plasmids involved in production of sulfide have been described in *Mycobacterium scrofulaceum* [58]. The accumulation of copper sulfide in this bacterium also does not influence its copper-resistance.

METAL RESISTANCE IN OTHER FUNGI

Wild-type strains of *C. albicans* grow to confluence in medium containing millimolar concentrations of copper or cadmium salts (unpublished data). Both high- and low-molecular-weight Cd-binding complexes were present in *C. albicans*

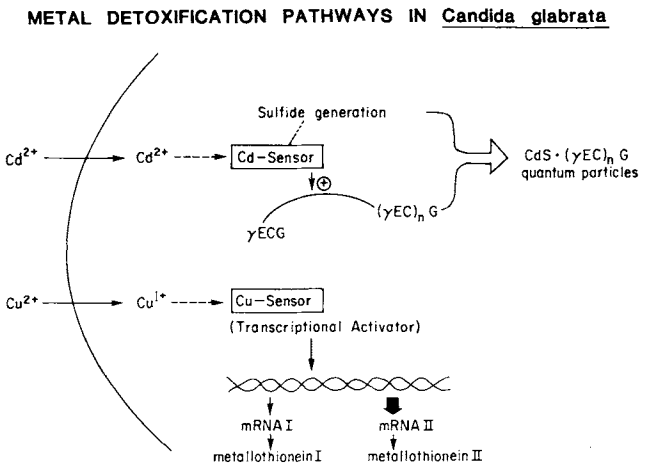

Fig. 4. Dual metal resistance pathways in *Candida glabrata*. Cadmium salts trigger the biosynthesis of $(\gamma\text{-EC})_nG$ peptides from glutathione. Cd-$(\gamma\text{-EC})_nG$ peptide complexes incorporate sulfide ions to form quantum CdS crystallites. The enzyme involved in $(\gamma\text{-EC})_nG$ peptide biosynthesis appears to be the cellular Cd(II) sensor regulating Cd resistance. Copper salts induce the biosynthesis of a family of MT molecules via an intracellular sensor analogous to the *ACE1* transcriptional activator from *S. cerevisiae*. Only minimal amounts of the MTI molecule are synthesized relative to MTII(s).

cells grown in the presence of cadmium sulfate (unpublished data). These components have not been identified, although sulfide was detected in high-molecular-weight fraction. There have been reports suggesting that the *CUP1* DNA probes hybridize with genomic DNA from *C. albicans* [33]. However, we were not able to reproduce these results. Nevertheless, we have detected the presence of a copper-binding low-molecular-weight protein in this yeast that luminesced upon UV irradiation, implying the presence of Cu(I)-thiolate clusters.

Among molds, *Neurospora crassa* and *Agaricus bisporus* have been studied extensively for their response to copper salts in the growth environment [59–64]. The response of *N. crassa* to copper salts in the growth medium depends on the phase of growth. Logarithmically growing cells bind most of the accumulated copper to a high-molecular-weight fraction, whereas ~10% of the copper accumulated by the cells in stationary phase was bound to a low-molecular-weight cysteine-rich protein [59]. This protein, comprising only 25 amino acids, was classified as MT because of the presence of Cys-X-Cys motifs and limited homology with the N-terminal sequence of animal MTs [59]. The *N. crassa* MT exhibits a high copper to cysteine ratio as this protein contains seven cysteinyl residues and binds six Cu(I) ions. Mammalian MTs containing 20 cysteinyl residues bind only 12 Cu ions [3,4]. The presence of Cu(I)-thiolate clusters in *N. crassa* MT has been inferred from luminescence measurements and X-ray absorption spectroscopy [60,61], although the cluster structure is yet to be elucidated. The MT gene is regulated only by copper salts [59–61,63]. The gene has no homology to promoter sequences of other MTs. Gene expression probably involves a copper-activated trans-acting factor in analogy with the *S. cerevisiae* system.

CONCLUSIONS

Fungi invoke a variety of pathways to respond to increased concentration of metal ions in intracellular milieu. Different species vary in the repertoire of mechanisms they employ for detoxification of metal ions, and this may, in part, determine the threshold of concentration at which a given metal ion is toxic to a particular cell. The cascade of detoxification/homeostatic reactions begins with the sensing of an increased concentration of a given metal ion by a biosensor. This initial sensing response is followed by synthesis of a detoxifying molecule in the case of sequestration mechanisms. Metallothioneins and $(\gamma\text{-EC})_nG$ peptides constitute the two most widely used detoxification molecules. Glutathione appears to provide the first line of defense and may be the only detoxifying molecules under certain conditions. The production of inorganic sulfide does not appear to protect

cells against acute toxicity but may be of significance in increasing cell mass during stationary periods of growth. The biosensor sensing increased intracellular concentration of copper in *S. cerevisiae* has been identified as a Cu(I)-activated protein that up-regulates the expression of MT genes by a trans-acting mechanism. Increased accumulation of MT diminishes the cellular concentration of free copper ions in the cell, thereby reducing the concentration of active biosensor (Cu-trans-acting factor). Future research should address the details of this self-regulating loop in *S. cerevisiae* and identify similar mechanisms in other fungi so as to evolve a global model of homeostasis/detoxification of copper ions. The other major biosensor sensing increased metal ion concentrations, especially Cd, is the enzyme responsible for synthesis of $(\gamma\text{-EC})_nG$ peptides. Efforts should be directed towards identification of such enzymes in *C. glabrata* and *S. pombe,* although some plant studies indicate this enzyme to be a γ-glutamyl transpeptidase. Synthesis of $(\gamma\text{-EC})_nG$ peptides also constitutes a self-regulating loop provided that the synthesizing enzyme is directly activated by free metal ions.

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