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Metal-specific synthesis of two metallothioneins and γ-glutamyl peptides in *Candida glabrata*

(phytochelatin/cadystin/metal detoxification)

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ABSTRACT **Cellular resistance to heavy metal cytotoxicity in most species is mediated by the binding of metal ions either to a cysteine-rich polypeptide in the metallothionein family or to short cysteine-containing γ-glutamyl peptides. One of these metal binding systems has been found in most organisms studied. However, the yeast *Candida (Torulopsis) glabrata* expresses both metallothionein and the γ-glutamyl peptides for metal detoxification, and each system is regulated in a metal-specific manner. Exposure of *C. glabrata* to copper salts stimulates formation of two metallothionein-like polypeptides with a cysteine content of 30 mol% and the repeated sequence Cys-Xaa-Cys. The cells synthesize γ-glutamyl peptides upon exposure to cadmium salts. Penta- and tetrapeptides that form a cadmium–thiolate cluster in a peptide oligomer containing labile sulfur are synthesized.**

Cells regulate the intracellular concentrations of metal ions that are potentially cytotoxic by limiting the concentrations of free ions. Two widely distributed metal-binding molecules contribute to cellular resistance to metal toxicity. Animal species synthesize a cysteine-rich polypeptide, metallothionein (MT), in response to copper, cadmium, and zinc ion stress (1–3). In plants cysteine-containing γ-glutamyl peptides (sometimes designated phytochelatin) supplant MT as the major metal-detoxification mechanism (4–6). Diverse organisms within the kingdom Fungi have evolved to express either MT or the γ-glutamyl peptide system (7–9). To our knowledge, no species has yet been identified that has both systems responsive to metal ion stress. Both MT and γ-glutamyl peptides are capable of binding a variety of heavy metal ions, but not all metal salts actively induce formation of the polypeptide or peptide. Some species, such as *Saccharomyces cerevisiae* and *Neurospora crassa*, synthesize MT only in response to copper salts, leaving the cells susceptible to cadmium-induced cytotoxicity (10, 11). *Schizosaccharomyces pombe* synthesizes γ-glutamyl peptides in response to copper and cadmium (12). Organisms that regulate their particular detoxification system in a metal-specific fashion may still require other mechanisms to handle certain metal ions.

Candida glabrata (also known as *Torulopsis glabrata*) (13) is capable of growth in minimal medium containing copper and cadmium salts. A copper-inducible component distinct from the MT-like polypeptide found in *Saccharomyces cerevisiae* and *Candida albicans* was hypothesized for *C. glabrata* (14). In this report we demonstrate that *C. glabrata* expresses both MT and γ-glutamyl peptides and the biosynthesis of each is regulated in a metal-specific manner.

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METHODS

Purification of Copper-Binding Proteins. *C. glabrata* strain 67 was kindly supplied by T. Butt (Smith Kline & French). The yeast cells were grown on a synthetic complete minimal medium containing 0.125% yeast nitrogen base, 0.02% glucose, and a mixture of uracil and amino acids. $CuSO_4$ was added to the medium at time zero at a final concentration of 0.5 mM. The cells were harvested after seven or eight doublings. Cell extracts prepared as described (12) were chromatographed on a Sephadex G-75 column equilibrated with 10 mM Tris Cl (pH 7.4) containing 0.1% 2-mercaptoethanol. The main copper-containing peak was fractionated on Whatman DE-52 ion-exchange resin with a linear gradient of KCl from 0 to 0.5 M in the equilibration buffer. Two copper-binding components were individually pooled and further purified on a Sephadex G-50 column (100 × 2.5 cm) in nitrogen-saturated 10 mM Tris Cl (pH 7.4).

Purification of Cadmium-Binding Components. Yeast cells were grown in a minimal medium containing 1 mM $CdSO_4$. The cell extract was initially separated on a column of Sephadex G-75 equilibrated with 10 mM Tris Cl (pH 8) followed by anion-exchange and Sephadex G-50 chromatography.

Reverse-Phase HPLC Separation of Isoforms of Cadmium-Binding Peptide. Purified cadmium-binding peptides were further resolved into isoforms by using reverse-phase HPLC on an Altex C_8 column (1 × 25 cm). The acidified peptide solution was applied to the HPLC column equilibrated with 0.1% trifluoroacetic acid. Isoforms were separated with a linear gradient of 0–60% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid.

Amino Acid Analysis and Sequence Analysis. Amino acid analyses on purified copper- and cadmium-binding proteins/peptides were performed as described (12). Cysteine was determined as cysteic acid after performic acid oxidation of the samples. Amino-terminal sequences of copper-binding polypeptides were determined by sequential Edman degradations on metal-free and alkylated protein samples (7). The removal of copper ions from polypeptide I was accomplished by proton displacement by using techniques described earlier (15). This procedure was not effective with polypeptide II as the protein precipitated upon acidification. However, removal of copper was accomplished by boiling the protein with a solution of 0.1 M EDTA in 6 M guanidinium hydrochloride containing 0.1 M Tris (pH 8.6). The metal-free polypeptides were reduced and alkylated (7) before sequence analysis. Proteolysis of the alkylated protein (40 nmol) was carried out with chymotrypsin (8 μg) in 1.0 ml of 0.2 M *N*-ethylmorpholine (pH 8.2) at 37°C. The lyophilized peptides were dissolved in 0.1% trifluoroacetic acid before fraction-

Abbreviation: MT, metallothionein.

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ation on C_8 reverse-phase HPLC with linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid.

Determination of the Structure of the Cadmium-Binding Peptides. The presence of amino-terminal γ-glutamyl bonds in the cadmium-binding peptide was determined by digesting the peptide with γ-glutamyl transpeptidase (16). HPLC-purified peptides (10–20 nmol) were incubated with 1 unit of the enzyme in 1.0 ml 100 mM Tris Cl (pH 8) at 37°C for 15–18 hr. The carboxyl-terminal residue was determined by digesting the peptide with carboxypeptidase P (Sigma). Peptide (5–10 nmol) was incubated with 0.5–1 μg of enzyme in 0.2 ml of 0.2 M acetate (pH 4) at 37°C for 18 hr. Peptide (5–10 nmol) was also treated sequentially with carboxypeptidase P and γ-glutamyl transpeptidase. Peptide digested with carboxypeptidase P was adjusted to pH 8 with 2 M Tris base before incubation with γ-glutamyl transpeptidase. Enzyme/substrate molar ratios were similar to those described above. The reaction was finally terminated by the addition of HCl. Amino acids released in each incubation were quantified by amino acid analysis.

Assays. Absorption spectra of the samples were recorded on a double-beam Cary model 219 recording spectrophotometer. Luminescence measurements on the copper-binding proteins were conducted on a Perkin–Elmer model 650-10S fluorimeter with a band-pass filter. Emission spectra were recorded after excitation of the sample at 280 nm. Sulfide analysis was performed by using the methylene blue assay described by King and Morris (17). The procedure was standardized by iodine titration by using 0.100 M iodine standard solution purchased from Ricca Chemical (Arlington, TX).

RESULTS

C. glabrata is capable of growth in minimal medium containing high concentrations of copper and cadmium salts. Growth is not inhibited by 50% until cadmium and copper concentrations of 0.9 and >1 mM, respectively, are reached. We sought to determine if metal-sequestering macromolecules contributed to the observed resistance. Fractionation of the clarified cell extract prepared from cells grown in copper-supplemented medium on Sephadex G-75 gel filtration yielded a single copper-containing component. This copper-containing eluent was resolved into an asymmetric copper elution profile on anion-exchange chromatography (Fig. 1*A*). The eluent was divided into three pools for subsequent chromatography on Sephadex G-50. Pools I and II eluted in fractions with an apparent polypeptide molecular weight of 7000. Amino acid analysis of the two copper-containing components revealed a cysteine content of nearly 30 mol%, as would be typical for known mammalian MTs (Table 1). The molecules differed significantly in their content of threonine, glycine, alanine, and lysine. Pool III was found to contain polypeptides identical to the polypeptide in pool II but in aggregated form. Homogeneity was verified by obtaining an identical amino acid composition of molecules eluting on C_8 reverse-phase HPLC. Sequential Edman degradations yielded amino-terminal sequences of each polypeptide (Fig. 2). Polypeptide I contains two Cys-Xaa-Cys sequences in a repeating sequence of Cys-Xaa-Cys-Pro-Asn (Xaa refers to an unspecified residue). The Cys-Xaa-Cys motif is typical of MT polypeptides. The amino-terminal segment of polypeptide II revealed the Cys-Xaa-Cys motif and significant homology to MT-I in the positions of cysteine residues. Edman degradation of a chymotryptic fragment of polypeptide II showed two Cys-Xaa-Cys sequences and the adjacent repeating pentapeptide sequence Cys-Gln-Thr-Cys-Lys.

The two polypeptides were isolated as copper-binding proteins with copper contents between 10 and 13 mol per mol of protein for each. The molecules exhibited electronic transitions in the near ultraviolet similar to the typical charge-transfer transitions seen with copper-binding MTs (Fig. 3*A*). The copper-binding proteins were luminescent with an uncorrected emission maxima near 575 nm after excitation in the ultraviolet range (Fig. 3*B*). The luminescence is characteristic of Cu(I)–thiolate coordination in an environment shielded from solvent interactions (18). The

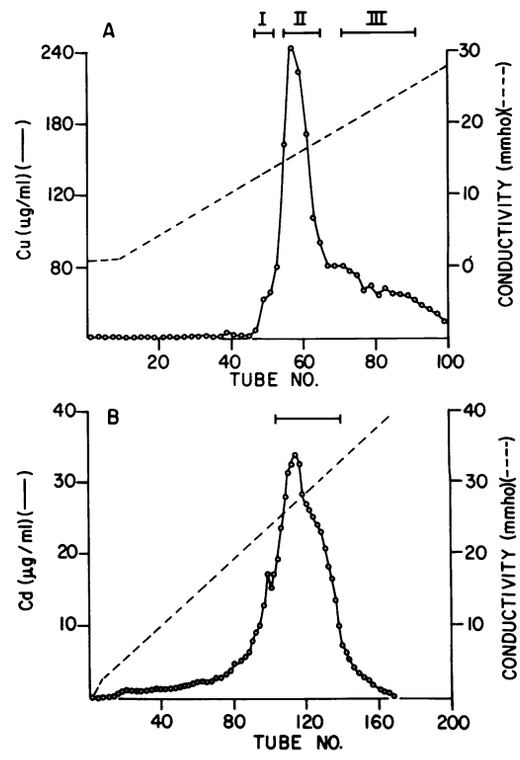

FIG. 1. (*A*) Ion-exchange chromatography of Cu–MT proteins from *C. glabrata*. Fractions from Sephadex G-75 containing the main copper-containing peak were chromatographed on DEAE-cellulose (2.5 × 5 cm) equilibrated with 10 mM Tris Cl (pH 7.4) containing 0.1% 2-mercaptoethanol with a gradient of 0–0.5 M KCl. The horizontal bars indicate fractions that were mixed to create pools I, II, and III for subsequent chromatography on Sephadex G-50. (*B*) Ion-exchange purification of the cadmium-binding peptide from *C. glabrata* under the same conditions above except 2-mercaptoethanol was omitted from the buffer at pH 8. The horizontal bar indicates fractions that were mixed for further purification on Sephadex G-50.

Table 1. Amino acid composition of MT-I and MT-II from *C. glabrata*

Amino acid	Residues, no. per molecule	
	MT-I	MT-II
Asx	6	7
Thr	0	4
Ser	6	6
Pro	2	2
Glx	6	7
Gly	10	0
Ala	2	5
Cys	18	16
Val	0	1
Tyr	0	1
His	2	1
Lys	6	3

Values are based on a 24-hr hydrolysis; cysteine was determined as cysteic acid after performic acid oxidation.

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                    5                  10                  15
MT-I   Ala-Asn-Asp-Cys-Lys-Cys-Pro-Asn-Gly-Cys-Ser-Cys-Pro-Asn-Cys-Ala-X - X - X

MT-II  Pro-Glu-Gln-Val-Asn-Cys-Gln-Tyr-Asp-Cys-His-Cys-Ala-Asn-Cys-Ala-Cys-Glu-Asn

        X+1              X+5                X+10                X+15
MT-II  -Ser-Ala-Ser-Asn-Glu-Cys-Ser-Cys-Gln-Thr-Cys-Lys-Cys-Gln-Thr-Cys-Lys-Cys
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FIG. 2. Amino-terminal sequences of MT-I and MT-II peptides. X+5, X+10, and X+15 refer to positions of an internal peptide.

high cysteine content of the two polypeptides, repeating Cys-Xaa-Cys motifs, and Cu(I)–thiolate electronic transitions are salient features of copper-binding MTs, so we designate the *C. glabrata* copper-binding polypeptides as Cu–MT-I and Cu–MT-II. The MT-II polypeptide was present in marked excess over MT-I in cells grown with copper salts in the medium. No evidence of either polypeptide was seen in cultures grown in the presence of cadmium salts.

To determine whether induction of the two MT polypeptides conferred resistance to growth inhibition by cadmium salts, we purified the cadmium-binding component from *C. glabrata* by using a strategy similar to that outlined for the Cu–MT molecules. A single cadmium-containing component was observed in the elution from anion-exchange chromatography (Fig. 1*B*). Subsequent chromatography on Sephadex G-50 yielded a cadmium-containing component eluting with an apparent molecular weight of 8000 or 4000 in elution buffers containing 0 and 0.2 M KCl, respectively. The amino acid composition of this component contained only glutamic acid, cysteine, and glycine in the molar ratio 2.4:2.2:1. The composition was consistent with that of γ-glutamyl peptides. The general structure of the γ-glutamyl peptide $(\gamma\text{-Glu-Cys})_n$-Gly has the repeated Glu-Cys dipeptide unit in isopeptide linkage (4–6, 9). The peptide also occurs in glycine-deficient forms (23). Resolution of the γ-glutamyl peptides on reverse-phase HPLC revealed two peptides (Fig. 4). The enzyme γ-glutamyl transpeptidase is effective in hydrolyzing amino-terminal γ-glutamyl bonds (16), the efficiency of cleavage of the γ-glutamate bond in glutathione averages 90% (12). Incubation of the HPLC-purified cadmium-containing components with γ-glutamyl transpeptidase confirmed the presence of an amino-terminal γ-glutamyl peptide bond in both peptides (Table 2). Half of the total glutamic acid content was released in both peptides. Peptide in the second HPLC peak was found to be devoid of the carboxyl-terminal glycine by carboxypeptidase P digestions. Carboxypeptidase P is known to cleave the α-peptide bonds linking the repeating dipeptide units in addition to cleavage of the terminal glycine residue (19). Samples predigested with carboxypeptidase P and subsequently incubated with γ-glutamyl transpeptidase had a total release of glutamic acid (Table 2). The γ-Glu-Cys dipeptide unit observed on amino acid analysis after carboxypeptidase P treatment of the sample was abolished by γ-glutamyl transpeptidase treatment. These data are consistent with two γ-glutamyl bonds in the peptide with the susceptibility of one bond to γ-glutamyl transpeptidase cleavage being dependent on carboxypeptidase P incubations.

The cadmium–γ-glutamyl peptide complex appears oligomeric in that the complex containing tetrapeptides and pentapeptides elutes with an apparent molecular weight of 4000 in high salt. The dependence of the Stroke's radius on ionic strength is presumably due to charge repulsion between glutamyl carboxylates. The peptide stoichiometry per com-

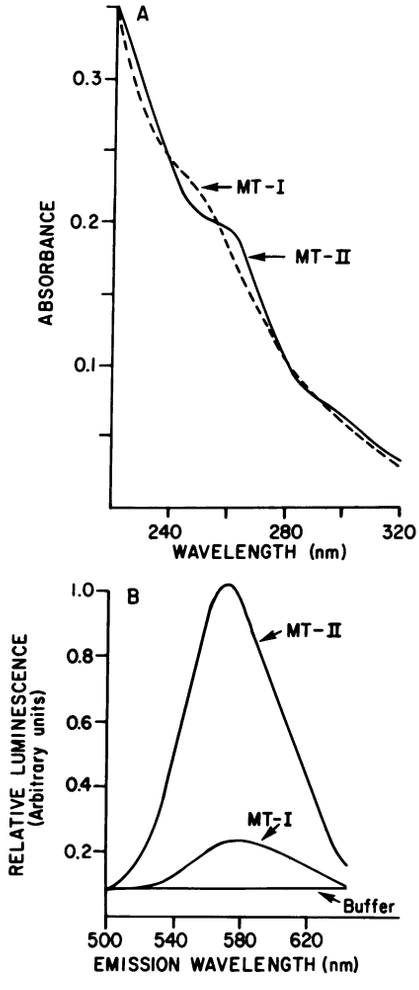

FIG. 3. UV absorption spectra (*A*) and luminescence emission spectra (*B*) of Cu–MT-I and –MT-II. Each of the protein samples contained 15.7 nmol of copper.

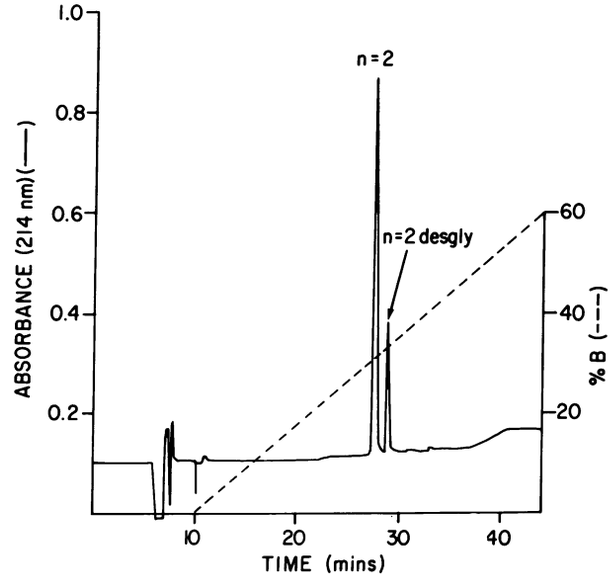

FIG. 4. HPLC separation of the size isomers of the cadmium–γ-glutamyl peptide complex. The purified peptide complex was fractionated by C_8 reverse-phase HPLC column with a gradient of 0–60% (vol/vol) acetonitrile (%B) in 0.1% trifluoroacetic acid.

Table 2. γ-Glutamyl transpeptidase and carboxypeptidase P digestion of cadmium-binding peptides from *C. glabrata*

Enzyme treatment	Amino acid released, mol%	
	Glutamic acid	Glycine
CPP alone	0 (0)	86 (0)
γ-GT alone	48.3 (46.4)	0 (0)
CPP + γ-GT	104 (100)	90 (0)

γ-Glutamyl transpeptidase (γ-GT) and carboxypeptidase P(CPP) digestions of HPLC purified cadmium-binding peptides from *C. glabrata*. Values of mol% amino acid released are for HPLC peaks I and II (in parentheses).

plex is unclear since no rigorous molecular weight of the complex is known. The oligomeric complex is presumably a shell of peptide subunits enclosing an internal metal–thiolate cluster.

Charge transfer transitions characteristic of Cd–S coordination are observed in the cadmium–γ-glutamyl peptide complex (Fig. 5). A separate transition due to acid-labile sulfur is observed in the region of 270–320 nm. The content of sulfide in the complex varies between 0.3 and 1.6 mol per mol of peptide. Similar variability is seen in the sulfide content of the cadmium–γ-glutamyl peptide complex from *Schizosaccharomyces pombe* and this range is due in part to growth conditions (20, 21). The absorbance maximum of the sulfide-dependent transition was dependent on the sulfide content of the complex. Ultraviolet absorption spectra of Sephadex G-50 elution fractions of a high-sulfide (1.6 mol per mol of peptide) isolate of the cadmium–γ-glutamyl peptide complex showed that leading (higher Stokes' radius)-edge fractions had a peak at 318 nm whereas trailing-edge fractions had absorption bands at lower wavelengths. Quantitation of the sulfide concentration in these fractions revealed sulfide levels of 2 mol per mol of peptide at the leading edge and 1 mol/mol at the trailing edge. The Cd(II) content of the complex varied with the sulfide content, higher sulfide levels correlating with higher Cd(II) levels. Binding stoichiometries

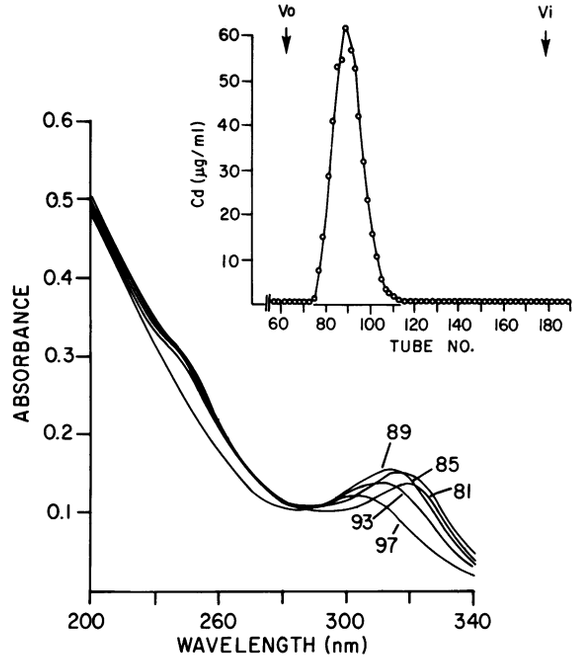

FIG. 5. Ultraviolet absorption spectra of a high-sulfide form of cadmium–γ-glutamyl peptide complex eluted from Sephadex G-50. (*Inset*) Sephadex G-50 elution profile. The various fractions were diluted to a cadmium ion concentration of 35.5 nmol/ml.

for Cd(II) ranging from 1.6 to 5 mol per mol peptide were observed. One result of this heterogeneity is variance in the Stokes' radius of the complex–higher sulfide levels increase the Stokes' radius of the complex. Isolates with sulfide levels below 0.3 did not exhibit any obvious sulfide-dependent electronic transitions in the ultraviolet.

Sulfide acts to enhance the effectiveness of the γ-glutamyl peptide system of Cd(II) detoxification and may also act in a secondary role to increase metal tolerance through precipitation of cadmium ions extracellularly as CdS. In 24 hr sulfide ions accumulated in the growth medium at concentrations up to 0.2 mM (<0.01 mM at time 0). Ultracentrifugation of the medium (100,000 × *g*) resulted in the sedimentation of a cadmium–sulfide colloid containing equimolar amounts of Cd(II) and sulfide. The presence of cadmium salts in the growth medium stimulated the generation of sulfide ions severalfold. This was demonstrated in experiments in which the gas evolved from culture during growth and after acidification to 6 M HCl was collected in a zinc acetate trap and subsequently analyzed for sulfide.

DISCUSSION

C. glabrata synthesizes two distinct MT polypeptides in a copper-dependent manner and γ-glutamyl peptides in a cadmium-dependent manner. To our knowledge, this is the first organism reported to express these two widespread metal-detoxification molecules. The MT polypeptides in *C. glabrata* exhibit repeats of the Cys-Xaa-Cys sequence motif common in mammalian MT and also coordinates copper ions in Cu(I)–thiolate clusters. The cysteine content of the two molecules of 30 mol% is comparable with that for mammalian MT (32 mol%) and MT molecules from *Saccharomyces cerevisiae* (22 mol%) and *Neurospora crassa* (28 mol%). Unlike fungal MT, the mammalian MT is inducible by cadmium salts and binds maximally 7 mol equivalents of Cd(II) (1). The synthesis of mammalian MT is transcriptionally regulated by metal ions in addition to a variety of other effectors (1). MT polypeptides from the three fungal sources mentioned above bind Cd(II) only under *in vitro* reconstitution procedures. Cadmium salts are not capable of stimulating transcriptional expression of MT mRNA (14). The specificity of induction of fungal MT by copper salts may suggest that MT evolved initially for copper-specific functions and only later in Animalia did cadmium- and zinc-related functions arise.

The cadmium–γ-glutamyl peptide complex expressed in *C. glabrata* is similar to the γ-glutamyl peptides synthesized in plants and *Schizosaccharomyces pombe* (4–6). The *C. glabrata* peptides are distinct in that only $n = 2$ and $n = 2$ des-Gly peptides are formed. Peptides of $n = 3$ and $n = 4$ are most common in *Schizosaccharomyces pombe* and plants (12, 22). Des-Gly-peptide variants of these peptides occur in *Schizosaccharomyces pombe* but account for <20% of the parent peptides (23). The metal–thiolate cluster is formed with an oligomer of γ-glutamyl peptides and includes labile sulfur. The complex appears to consist of three to five peptides with a minimum of one sulfide ion. Higher quantities of sulfide can be accommodated within the cluster to yield a complex with an increased Stokes' radius (20). No information is available concerning the structure of the metal–thiolate center or the mode of sulfide binding. It is clear that sulfide ions participate in metal coordination as the electronic transitions in the ultraviolet are dependent on the metal bound (20).

The presence of γ-glutamyl bonds in the peptide suggest that synthesis is enzymatic rather than ribosomal. The mechanism of synthesis has not been elucidated. The synthesis must be regulated by molecules that bind only specific metal ions. It is not known whether metal ions directly regulate the enzymatic activity of proteins involved in γ-

glutamyl peptide synthesis or transcriptionally regulate expression of the necessary enzymes. In either mechanism, a metal-regulatory molecule exists. A competition may exist between the two types of regulatory molecules in *C. glabrata* for a particular metal ion. The affinity and/or coordination preference of the molecules may dictate whether MT or γ-glutamyl peptides are synthesized. There is precedence in mammalian MT for differential coordination of copper and cadmium ions, so it is conceivable that regulation of the *C. glabrata* pathways is also dependent on specific coordination complexes in the regulatory molecules. *C. glabrata* seems a promising organism for the study of the cellular regulation mechanisms of these common metal detoxification systems.

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