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Dug1p Is a Cys-Gly Peptidase of the γ-Glutamyl Cycle of *Saccharomyces cerevisiae* and Represents a Novel Family of Cys-Gly Peptidases

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GSH metabolism in yeast is carried out by the γ-glutamyl cycle as well as by the *DUG* complex. One of the last steps in the γ-glutamyl cycle is the cleavage of Cys-Gly by a peptidase to the constitutent amino acids. *Saccharomyces cerevisiae* extracts carry Cys-Gly dipeptidase activity, but the corresponding gene has not yet been identified. We describe the isolation and characterization of a novel Cys-Gly dipeptidase, encoded by the *DUG1* gene. Dug1p had previously been identified as part of the Dug1p-Dug2p-Dug3p complex that operates as an alternate GSH degradation pathway and has also been suggested to function as a possible di- or tripeptidase based on genetic studies. We show here that Dug1p is a homodimer that can also function in a Dug2-Dug3-independent manner as a dipeptidase with high specificity for Cys-Gly and no activity toward tri- or tetrapeptides *in vitro*. This activity requires zinc or manganese ions. Yeast cells lacking Dug1p (*dug1*Δ) accumulate Cys-Gly. Unlike all other Cys-Gly peptidases, which are members of the metallopeptidase M17, M19, or M1 families, Dug1p is the first to belong to the M20A family. We also show that the Dug1p *Schizosaccharomyces pombe* orthologue functions as the exclusive Cys-Gly peptidase in this organism. The human orthologue CNDP2 also displays Cys-Gly peptidase activity, as seen by complementation of the *dug1*Δ mutant and by biochemical characterization, which revealed a high substrate specificity and affinity for Cys-Gly. The results indicate that the Dug1p family represents a novel class of Cys-Gly dipeptidases.

GSH is a thiol-containing tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) present in almost all eukaryotes (barring a few protozoa) and in a few prokaryotes (1). In the cell, glutathione exists in reduced (GSH) and oxidized (GSSG) forms. Its abundance (in the millimolar range), a relatively low redox potential (−240 mV), and a high stability conferred by the unusual peptidase-resistant γ-glutamyl bond are three of the properties endowing GSH with the attribute of an important cellular redox buffer. GSH also contributes to the scavenging of free radicals and peroxides, the chelation of heavy metals, such as cadmium, the detoxification of xenobiotics, the transport of amino acids, and the regulation of enzyme activities through glutathionylation and serves as a source of sulfur and nitrogen under starvation conditions (2, 3). GSH metabolism is carried out by the γ-glutamyl cycle, which coordinates its biosynthesis, transport, and degradation. The six-step cycle is schematically depicted in Fig. 1 (2).

In *Saccharomyces cerevisiae*, γ-glutamyl cyclotransferase and 5-oxoprolinase activities have not been detected, which has led to the suggestion of the presence of an incomplete, truncated form of the γ-glutamyl cycle (4) made of γ-glutamyl transpeptidase (γGT)[4] and Cys-Gly dipeptidase and only serving a GSH catabolic function. Although γGT and Cys-Gly dipeptidase activities were detected in *S. cerevisiae* cell extracts, only the γGT gene (*ECM38*) has been identified so far. Cys-Gly dipeptidase activity has been identified in humans (5, 6), rats (7–10), pigs (11, 12), *Escherichia coli* (13, 14), and other organisms (15, 16), and most of them belong to the M17 or the M1 and M19 metallopeptidases gene families (17).

S. cerevisiae has an alternative γGT-independent GSH degradation pathway (18) made of the Dug1p, Dug2p, and Dug3p proteins that function together as a complex. Dug1p also seem to carry nonspecific di- and tripeptidase activity, based on genetic studies (19).

We show here that Dug1p is a highly specific Cys-Gly dipeptidase, as is its *Schizosaccharomyces pombe* homologue. We also show that the mammalian orthologue of *DUG1*, CNDP2, can complement the defective utilization of Cys-Gly as sulfur source of an *S. cerevisiae* strain lacking *DUG1* (*dug1*Δ). Moreover, CNDP2 has Cys-Gly dipeptidase activity *in vitro*, with a strong preference for Cys-Gly over all other dipeptides tested. CNDP2 and its homologue CNDP1 are members of the metallopeptidases M20A family and have been known to carry carnosine (β-alanyl-histidine) and carnosine-like (homocarnosine and anserine) peptidase activity (20, 21). This study thus reveals that the metallopeptidase M20A family represents a novel Cys-Gly peptidase family, since only members of the M19, M1, and M17 family were known to carry this function.

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[4] The abbreviations used are: γGT, γ-glutamyl transpeptidase; NTA, nitrilotriacetic acid.

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EXPERIMENTAL PROCEDURES

Materials—All chemicals and reagents were of analytical reagent grade and were procured from different commercial sources. Protein molecular weight markers were purchased from MBI Fermantas. Oligonucleotide primers were synthesized from Biobasic Inc. and Sigma. Peptides (95% purity) were custom synthesized from USV Ltd. Medium components were purchased from Difco. Restriction enzymes, DNA polymerases, and other DNA-modifying enzymes were obtained from New England Biolabs. The DNA sequencing kit (ABI PRISM 310 XL with dye termination cycle sequencing ready reaction kit) was obtained from PerkinElmer Life Sciences. Gel extraction kits, plasmid miniprep columns, and Ni^{2+}-NTA resins were obtained from Qiagen or Sigma. Molecular weight standards for nondenaturing gels and metal ion chelators were obtained from Sigma. Metal salts were obtained from Sigma, SRL, and Merck.

Strains, Media, and Growth Conditions—The *E. coli* strain DH5α was used as a cloning host, and BL21 (pLysS) was used as expression host. Yeast strains used in the study are described in Table 1. *S. cerevisiae* strains were regularly maintained on yeast extract, peptone, and dextrose medium (YPD). Synthetic defined minimal medium contained yeast nitrogen base, ammonium sulfate, dextrose supplemented with methionine, histidine, leucine, lysine, and uracil at 80 mg/liter (as per requirement). *S. pombe* was maintained on yeast extract, dextrose, and supplements as required (YES). Edinburgh minimal medium for *S. pombe* was prepared as described (22). Yeast transformations were carried out by the lithium acetate method, as described for *S. cerevisiae* (23), and using a modified lithium acetate protocol for *S. pombe* (24).

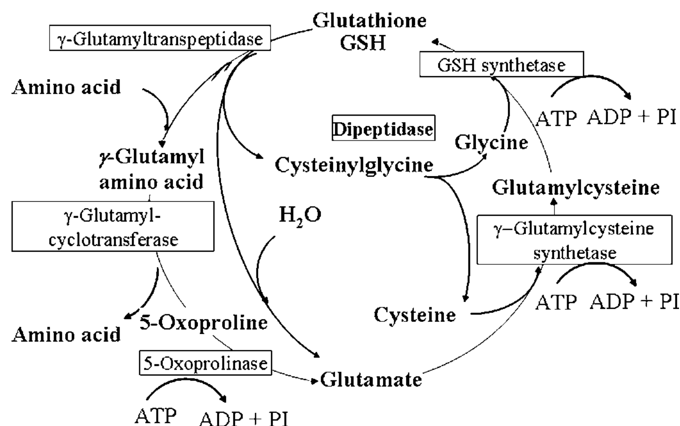

FIGURE 1. **γ-Glutamyl cycle of glutathione metabolism.** γ-Glutamylcysteine synthetase and GSH synthetase carry out the first two steps in glutathione biosynthesis. γ-glutamyltranspeptidase, γ-glutamylcyclotransferase, 5-oxoprolinase, and Cys-Gly dipeptidase are involved in glutathione catabolism. Activities responsible for γ-glutamylcyclotransferase and 5-oxoprolinase have not been detected in *S. cerevisiae*.

Cloning and Expression of Dug1p—The *DUG1* open reading frame was PCR-amplified from genomic DNA of *S. cerevisiae* strain ABC733 using forward (5′-GGAATTCCATATGTCT-CACTCACTTACTTCCGT-3′) and reverse (5′-CAGCT-GCTCGAGTTCTGGCGATTCAGAGTAAT-3′) primers following standard protocols, digested with NdeI and XhoI restriction enzymes, and ligated to pET23a (+) vector digested by NdeI and XhoI restriction enzymes. The sequence was confirmed by DNA sequencing. Expression of Dug1p was induced at A_{600} = 0.8 by 0.1 mM isopropyl-β-thio-galactopyranoside, and the induction was carried out at 18 °C for 16 h with shaking at 220 rpm.

Purification of Dug1p—*E. coli* cultures induced with isopropyl-β-thio-galactopyranoside were harvested at 6000 × *g* for 5 min, and the pellet was resuspended in lysis buffer (300 mM NaCl, 20 mM imidazole, 0.5 mM dithiothreitol, and 50 mM Tris-HCl, pH 8.0). Cells were lysed by sonication for 5 min. The bulk of the induced protein was found in the soluble fraction. The soluble fraction was recovered by centrifugation at 10,000 × *g* for 20 min. The protein, which had a 6× His tag at the C terminus, was purified using Ni^{2+}-NTA affinity chromatography. Ni^{2+}-NTA columns were washed with water and equilibrated in column equilibration buffer (identical to lysis buffer except that it lacked dithiothreitol). Protein samples were loaded onto the column, and the column was washed with 5 column volumes of the equilibration buffer, 1 column volume of equilibration buffer containing 50 mM imidazole, and the protein was eluted with elution buffer (equilibration buffer containing 150 mM imidazole). The purified protein was dialyzed against the buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0) and further purified by gel filtration chromatography. Briefly, the Ni^{2+}-NTA-eluted Dug1p was loaded onto a gel filtration column (Superdex S200) that was equilibrated with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl. The elution of Dug1p was monitored by running different fractions on SDS-PAGE (25), followed by Coomassie Brilliant Blue R-250 staining.

Enzyme Assay—The peptidase activity was assayed based upon the measurement of L-cysteine liberated during the reaction of the enzyme with cysteine-containing peptides by using the protocol of Gaitonde (26), with minor modifications. The assay was based on the specific reaction of ninhydrin with cysteine under strongly acidic conditions. Under these conditions, the presence of other amino acids, including proline, or other cysteine-containing compounds, such as GSH, GSSG, and cys-

TABLE 1
List of strains used in the study

Strain	Genotype	Source
ABC733 (BY4741, WT)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	J. Boeke
ABC734 (BY4742, WT)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	J. Boeke
ABC1654 (*dug1Δ*)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 yfr044cΔ::kanMX4*	EUROSCARF
ABC1736 (*dug1Δ*)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yfr044cΔ::kanMX4*	EUROSCARF
ABC1730 (*dug2Δ*)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ybr281cΔ::kanMX4*	EUROSCARF
ABC1729 (*dug3Δ*)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ynl191wΔ::kanMX4*	EUROSCARF
ABC1066 (*ecm38Δ*)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ecm38Δ::kanMX4*	EUROSCARF
ABP1948	*S. pombe* h^- *leu1-32 ura4-c190T cys1aΔ*	K. Takegawa
ABP2252	*S. pombe* h^- *leu1-32 ura4-c190T cys1aΔ Spdug1::ura4*$^+$	This study

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tine, does not interfere with the estimations of cysteine (26). Briefly, the standard reaction buffer for Dug1p assay was 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, supplemented with either 20 μM $MnCl_2$ or 200 μM $ZnCl_2$ and purified protein (50 ng to 1 μg). The reaction was initiated by the addition of peptide (typically 2 mM Cys-Gly) in a reaction volume of 150 μl. The reaction was carried out at 30 °C for 15–30 min, terminated with 5% trichloroacetic acid, and centrifuged at 11,000 × *g* for 2 min. To 125 μl of supernatant was added 125 μl of glacial acetic acid and 125 μl of acidic ninhydrin reagent (500 mg of ninhydrin dissolved in 6 ml of acetic acid and 4 ml of concentrated HCl), and the samples were boiled for 10 min in a boiling water bath maintained at 100 °C and cooled to room temperature. To 300 μl of the boiled samples was added 1000 μl of absolute ethanol, and the absorbance was taken at 560 nm. The concentration of the cysteine was measured from a standard curve with known amounts of L-cysteine after subtracting substrate blank. None of the substrates used in the study reacted with ninhydrin. Variations to the above procedures are mentioned under "Results." Activity with tripeptides and tetrapeptides was also assayed by the release of cysteine and was based on the fact that cleavage of these peptides would yield cysteine and, in some cases, Cys-Gly or Gly-Cys, both of which could be cleaved by Dug1p to yield free cysteine. For determination of K_m and V_{max} in the presence of Mn^{2+}, Cys-Gly concentrations ranged between 0.05 and 5.0 mM, and 50 ng of Dug1p was used. For determination of K_m and V_{max} in presence of Zn^{2+}, Cys-Gly concentrations ranged between 0.25 and 5.0 mM, and 100 ng of Dug1p was used. CNDP2 protein assays were performed exactly as for Dug1p but used 50 μM $MnCl_2$ and 50 ng of protein, and the reaction was carried out for 10 min at 30 °C. For the substrate specificity experiments, the assay used for the estimations of relative activity was a single point, end point assay and hence represents the relative activity of Dug1p for different peptides.

Metal Ion Dependence of Dug1p Activity—For inhibition studies with metal ion chelators, 1 μg of Dug1p was incubated with EDTA or 1,10-phenanthroline from a concentration range of 0.1–1 mM for 30 min at 30 °C, and the peptidase activity was measured using 2 mM Cys-Gly at 30 °C for 30 min. For reactivation studies with different metal ions, the peptidase activity of Dug1p was first inhibited by incubating the protein with 10 mM EDTA for 30 min at 30 °C, followed by extensive dialysis against storage buffer (described above). The peptidase activity of the dialyzed protein (1 μg) was measured in the presence of different divalent cations from a concentration range of 2–500 μM, using 2 mM Cys-Gly, and the reaction was carried out at 30 °C for 20 min. The amount of L-cysteine formed was measured as mentioned above. To determine the metal ion concentration required for optimal activity, purified protein (100 ng) was incubated with 2 mM Cys-Gly in the presence of $MnCl_2$ or $ZnCl_2$ from a concentration range of 50 nM to 1 mM; the reaction was carried out at 30 °C for 20 min; and the amount of L-cysteine formed was measured as mentioned above. For determining substrate specificity of Dug1p, the purified protein (50–100 ng) was incubated with a 2 mM concentration of different peptides in the presence of either 20 μM manganese or 200 μM zinc ions; the reaction was carried out at 30 °C for 15 min; and the amount of L-cysteine formed was measured as mentioned above. All of the experiments were repeated three times in duplicates.

Ferguson Plot Analysis of Molecular Weight of Native Dug1p—For nondenaturing electrophoresis, 0.75-mm-thick acrylamide gels of percentages ranging from 7.5 to 13.0% were made in 375 mM Tris-HCl, pH 8.9. 3 μg of purified protein along with different molecular weight markers all dissolved in 2× loading buffer (20% glycerol, 0.125 M Tris-HCl, pH 8.9, 250 μg of bromphenol blue) were loaded on gels, and the electrophoresis in Laemmli basic buffer (0.05 M Tris-HCl, pH 8.9, glycine 0.38 M) was carried out at 20 mA for 1 h at room temperature. Gels were stained with Coomassie Brilliant Blue R-250. The relative mobility of the molecular weight markers and Dug1p was calculated for different percentage gels and was plotted as a function of the percentage of gel. The slope of the line was taken as the retardation coefficient of the molecular weight of protein, and similarly the retardation coefficient was also calculated for Dug1p. The square roots of the retardation coefficients of the different molecular weight marker proteins were plotted as a function of their log molecular weight to generate a molecular weight standard curve. The molecular weight of Dug1p was calculated by plotting the retardation coefficient of Dug1p on the standard curve.

Disruption of S. pombe DUG1 Homologue—The *DUG1* homologue of *S. pombe* (SPBC1198.08) was PCR-amplified using genomic DNA of *S. pombe* as a template, with forward (5′-GCTCGGATCCATGTCTTTGGATAAGTTATACGAAG-3′) and reverse (5′-GAGCCTCGAGTTAAGGAGAAACAGAAGCCAACTCAT-3′) primers. The amplified PCR product was digested with BamHI and XhoI restriction enzymes and ligated to BamHI-XhoI-digested pTEF416 vector, to yield pTEF-SpDUG1. For disruption of *SpDUG1*, the $ura4^+$ gene was cloned at the EcoRV restriction site of pTEF-SpDUG1 to generate the plasmid pTEF416 spDUG1::$ura4^+$. This resultant plasmid was digested with KpnI and XhoI to release the disruption cassette that was gel-purified and used for transformation in *S. pombe* strain ABP1948 (*cys1a*Δ). The transformants were selected on Edinburgh minimal medium + leucine + cysteine − uracil plates and repatched on the same plates. The disruption of *SpDUG1* was confirmed by PCR using the above mentioned primers.

Cloning of CNDP1 cDNA and CNDP2 cDNA—The human CNDP1 (IMAGE clone ID 8322564) and CNDP2 (IMAGE clone ID 3051369) cDNA clones were obtained from the mammalian gene collection (Invitrogen). CNDP1 cDNA with and without the signal sequence was PCR-amplified from the CNDP1 IMAGE clone using the forward primer (without signal sequence, 5′-TATGGCGAATTCATGCCCCCGGCGCTGTTAGAG-3′; with signal sequence, 5′-TATGGCGAATTCATGGATCCCAAACTCGGGAGA-3′) and reverse primer (5′-ATTGTACTCGAGTTAATGGAGCTGGGCCATCTC-3′) and cloned into pTEF416 at EcoRI and XhoI restriction sites to yield pTEF-CNDP1 and pTEF-ssCNDP1. CNDP2 cDNA was PCR-amplified from the CNDP2 IMAGE clone using the forward primer (5′-TATGGCGAATTCATGGCGGCCCTCACTACCCTG-3′) and reverse primer (5′-ATTGTACTCGAGCTAGTCCTTCAGCTGA-3′) and cloned into pTEF416 vector at EcoRI and XhoI restriction sites to yield pTEF-

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CNDP2. Sequences of the inserts were confirmed by DNA sequencing.

Cloning, Expression, and Purification of CNDP2 Protein—CNDP2 *cDNA* was PCR-amplified from IMAGE clone ID 3051369 using forward (5′-TATGGCCATATGGCGGCCCT-CACTACCCTGTTT-3′) and reverse (5′-ATT GTA CTC GAG GTC CTT CAG CTG GGA GAC-3′) primers following standard protocols, digested with NdeI and XhoI restriction enzymes, and ligated to pET23a (+) vector digested by NdeI and XhoI restriction enzymes. The sequence was confirmed by DNA sequencing. Expression and purification of CNDP2 protein was carried out as mentioned for Dug1p. Briefly, expression of CNDP2 protein was induced at A_{600} = 0.8 by 0.5 mM isopropyl-β-thiogalactopyranoside, and the induction was carried out at 18 °C for 16 h. Induced cultures were harvested and resuspended in lysis buffer (300 mM NaCl, 20 mM imidazole and 50 mM Tris-HCl, pH 8.0) and sonicated. The soluble fraction was recovered by centrifugation. The protein, which had a His_6 tag at the C terminus, was purified using Ni^{2+}-NTA affinity chromatography, as mentioned for purification of Dug1p. The purified protein was dialyzed against the buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0) and further purified by Superdex S200 gel filtration chromatography. The elution of CNDP2 protein was monitored by running different fractions on SDS-PAGE (25) followed by Coomassie Brilliant Blue R-250 staining.

Growth Assays by Dilution Spotting—For dilution spotting assays, the cells were grown for 12–16 h at 30 °C and reinoculated into the desired medium at initial A_{600} = 0.1. Cells were further grown at 30 °C and harvested at A_{600} = 0.6 by centrifugation at 6000 × *g* for 5 min. Cells were washed with water and resuspended in water at A_{600} = 2.0. Serial dilutions of A_{600} = 0.2, 0.02, 0.002, and 0.0002 were made in water, and 5 μl of the each dilution was spotted on the desired plates. Plates were incubated for 3 days (*S. cerevisiae*) or 5 days (*S. pombe*) at 30 °C.

Glutathione Toxicity Assay—The glutathione toxicity of the mutants was measured by the dilution spotting assay. The wild type *S. cerevisiae* strain BY4742 and the *dug1*Δ, *dug2*Δ, *dug3*Δ, and *ecm38*Δ deletion mutants in the same wild type background were transformed with pTEF-HGT1 plasmid and vector plasmid (TEF416) separately. The transformants were grown in minimal selection medium (SD−ura) until stationary phase. 2 × 10^6 cells were taken for each culture, and 10-fold serial dilutions were made. 10 μl of each dilution was spotted on minimal medium plates with various concentrations of glutathione in the micromolar range. The growth was recorded after incubation of plates for 3 days at 30 °C.

Growth Conditions and Metabolite Extraction—The *S. cerevisiae* WT (ABC 734) and *dug1*Δ mutant strains transformed with pTEF-HGT1 plasmid or empty vector were grown in SD selection medium to an A_{600} of 0.3. 100 μM glutathione was added at this *A* value, and then cells were collected at the indicated times (0 min, 15 min, 30 min, 1 h, 2 h, and 4 h) for metabolite extraction. Three independent replicates were made for each sample. The extraction of metabolites was adapted from a procedure described previously (27). Briefly, cells (4–5 × 10^8 cells) were collected by centrifugation (4000 rpm for 3 min at 4 °C), washed once with cold water (4 °C), and centrifuged again (4000 rpm for 3 min at 4 °C). The cells were resuspended in 500 μl of 0.1% perchloric acid (pH 2), boiled (95 °C, 5 min), and centrifuged for 5 min at 4000 rpm at 4 °C. The supernatant containing the metabolites was collected and frozen at −80 °C until further analysis. An aliquot of the unlabeled extract to be analyzed was mixed with an aliquot of a ^{15}N-labeled reference extract, as a source of internal standards. The volume of supernatant was normalized to the number of cells in the initial sample and diluted to 4 × 10^7 cells/ml in water plus 0.1% formic acid.

Mass Spectrometry Experiments—The intracellular Cys-Gly levels in wild type and *dug1*Δ cells were determined by using a method initially developed for metabolomic applications. It is a fast and reliable tool that has been successfully applied earlier to investigate the metabolic response of *S. cerevisiae* to cadmium exposure (28). Typical yeast mass spectra contain a few hundred analytically relevant *m/z* signals. The Cys-Gly signal was extracted from these mass spectra and reliably identified due to accurate mass measurements in the Orbitrap, with errors of less than 5 ppm.

The procedure has been adapted from Madalinski *et al.* (28). Briefly, the analyses were performed by flow injection analysis by using an Accela pump and an Accela autosampler from Thermo Fisher Scientific (San Jose, CA). Each direct introduction analysis was carried out by injecting 20 μl of sample at a flow rate of 100 μl/min of a mobile phase consisting of acetonitrile/water (1:1, v/v) plus 0.1% formic acid.

The mass spectrometer was an LTQ-Orbitrap Discovery (Thermo Fisher Scientific) fitted with an electrospray source. Mass spectra were recorded in the positive ion mode from 80 up to 800 Thomson units using a mass resolution of 30,000 full width at half-maximum at 400 Thomson units in the Orbitrap analyzer. The electrospray voltage was set to 5 kV, the capillary voltage to 8 V, and the tube lens offset to 65 V. The sheath and auxiliary gas flows (both nitrogen) were optimized at 35 and 8 (arbitrary units), and the drying gas temperature was set to 275 °C.

Data Processing—All data were processed using the Qualbrowser of the Xcalibur software version 2.0.7 (Thermo- Fisher Scientific). Signals at *m/z* 179.0485, 149.0575, and 311.0822, corresponding to Cys-Gly, [^{15}N]glutamate, and [^{15}N]glutathione, respectively, and their corresponding peak areas were extracted from the mass spectra. The two latter ions were used as internal references, in order to check for the stability of the signal and the accurate mass measurements (below 5 ppm) during the experiment.

RESULTS

Recombinant Dug1p Is a Homodimer—To investigate Dug1p peptidase activity, a C-terminally His-tagged version of this protein was purified from *E. coli* extracts as described under "Experimental Procedures." Purified Dug1p migrated as a highly pure 52-kDa species on SDS-PAGE, although two minor bands that co-purified with Dug1p could be seen when the protein load on the gel was upscaled, even after the gel filtration step, which suggested that they represent Dug1p degradation products (data not shown).

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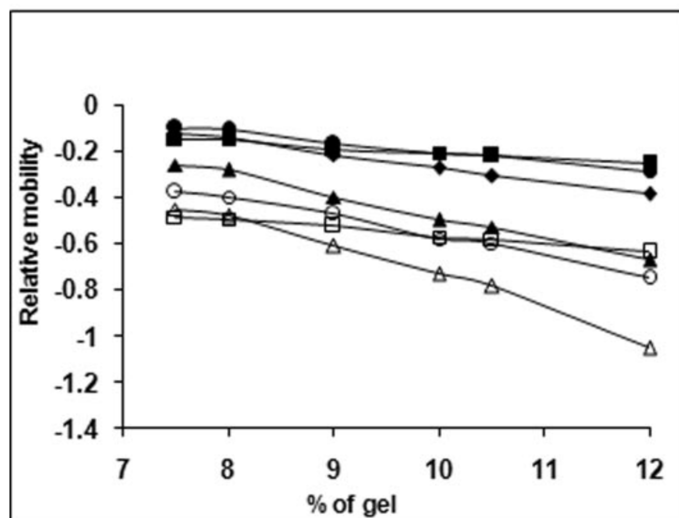

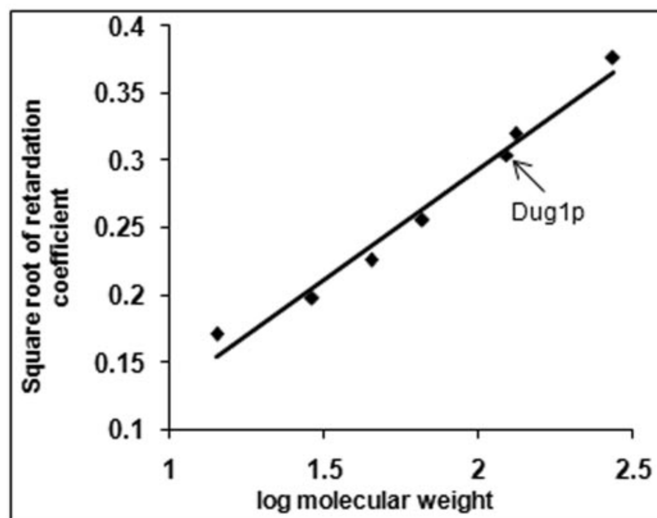

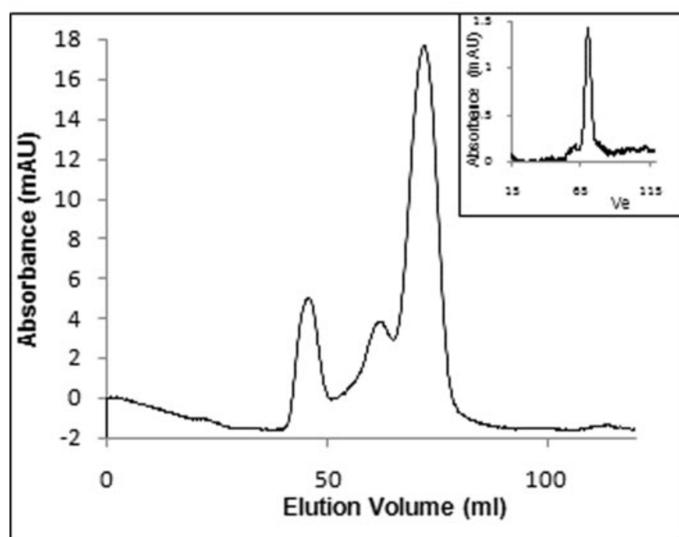

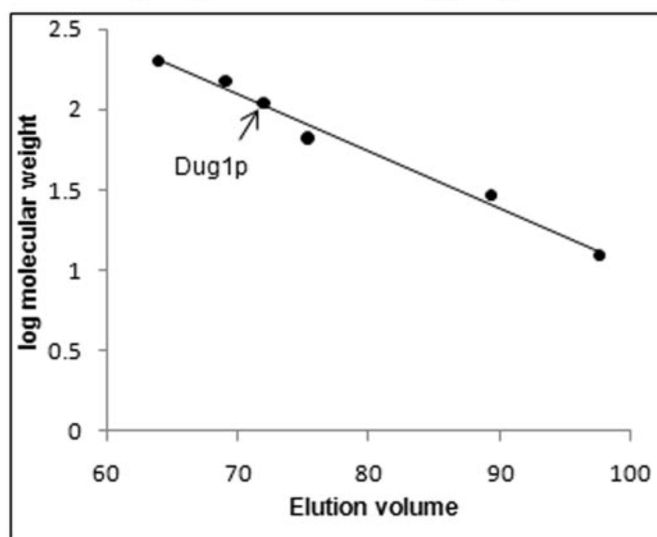

FIGURE 2. **Dug1p dimeric status as determined by Ferguson plot analysis and gel filtration chromatography.** *A*, Ferguson plot analysis. *A1*, relative mobility of standard proteins and Dug1p under nondenaturing gel conditions in different percentage acrylamide gels. Purified Dug1p (3 μg) and molecular weight standards were run on different percentage nondenaturing polyacrylamide gels, and the relative mobility of the proteins was plotted as a function of the percentage of the gel. *A2*, the retardation coefficients for each of the molecular weight marker proteins and Dug1p were determined from the slope of their respective lines. The molecular masses of the marker proteins are as follows: α-lactalbumin from bovine milk (14.2 kDa) (■), carbonic anhydrase from bovine red blood cells (29 kDa) (□), chicken egg white albumin (45 kDa)(●), bovine serum albumin (66 kDa)(♦), bovine serum albumin dimer (132 kDa) (▲), Jack bean urease (272 kDa)(△), and Dug1p (○). *B*, gel filtration analysis. Square roots of the retardation coefficients are plotted as the function of log molecular weights of standard proteins to form a molecular weight standard curve. The molecular weight of Dug1p is calculated by plotting its retardation coefficient (*circled* on the *plot*). *B1*, Ni^{2+}-NTA-purified Dug1p was dialyzed against 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and loaded on the Superdex S200 column. The *inset* shows the profile of the dimer peak reloaded on the column. *B2*, molecular mass standard graph (cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa)).

Dug1p strongly interacts with itself, as shown by co-immunoprecipitation and by the yeast two-hybrid assay (19). Since homodimerization is a known feature of M20A family peptidases (29), we checked the oligomeric status of our protein preparation. On nondenaturing PAGE, Dug1p migrated as a 113.9 ± 1.9-kDa species, as established by Ferguson plot analysis (30) (Fig. 2*A*), consistent with the size of an homodimer. Gel filtration chromatography on a Superdex S200 column also showed a predominant elution peak at 119.6 ± 3.6 kDa but no monomeric species. A higher oligomeric eluting in the void volume was also observed (Fig. 2*B*), which presumably corresponds to a protein aggregate, since it was not seen after gel filtration of the dimeric form (Fig. 2*B*, *inset*). The protein homodimer obtained by gel filtration was subsequently used for biochemical analyses.

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Dug1p Requires Zn^{2+} or Mn^{2+} for Optimal Peptidase Activity—Purified Dug1p displayed peptidase activity from 25 to 40 °C with a maximum at 30 °C and at pH 6.0–9.0, with a maximum at pH 8.0 (data not shown). Since Dug1p was predicted to be member of the metallopeptidase M20A family, we investigated its metal dependence. Metal chelation in the presence of EDTA or 1,10-phenanthroline indeed inhibited Dug1p Cys-Gly dipeptidase activity. EDTA at 1.0 μM led to about 50% loss of activity. 1,10-phenanthroline was less potent at low concentrations (1–10 μM). However, at 1 mM, both metal chelators completely inhibited enzyme activity, clearly indicating a divalent metal ion requirement for Dug1p activity (data not shown).

M20A and other metallopeptidases have different metal ion requirements. We thus tested Dug1p activity in the presence of different metals after thoroughly removing all of its metal content by EDTA treatment followed by extensive dialysis. Only Zn^{2+} or Mn^{2+} was able to restore apo-Dug1p Cys-Gly peptidase activity (data not shown) and not Cd^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+}, and Ca^{2+} when tested at concentrations of 2 μM up to 500 μM (data not shown). Adding Zn^{2+} or Mn^{2+} also increased the activity of purified Dug1p, indicating loss of protein-bound metal during purification. Mn^{2+} (10-fold increase) was more potent than Zn^{2+} (6-fold increase) in boosting Dug1p peptidase activity (data not shown).

Dug1p Is a Highly Specific Cys-Gly Dipeptidase—A previous study showed that Dug1p, but not Dug2p or Dug3p, was required for growth of *S. cerevisiae* in the presence of dipeptides (Cys-Gly) and tripeptides (Glu-Cys-Gly) as the sole sulfur source, which suggested that this enzyme could be a nonspecific di-/tripeptidase (19). We thus examined the Dug1p peptidase activity toward different di-, tri-, and tetrapeptides, all containing a cysteine residue, since the assay relies upon the measurement of released free cysteine. Experiments were performed in the presence of either Zn^{2+} or Mn^{2+}. With Zn^{2+}, Dug1p had a strong preference for the Cys-Gly dipeptide but also acted, albeit less potently, on Ala-Cys (73% of the activity seen with Cys-Gly). Phe-Cys, Cys-Phe, Cys-Asp, Gly-Cys, Ser-Cys, Leu-Cys, and Cys-Leu were very inefficiently cleaved (10–30%), whereas Cys-Ala, Asp-Cys, Cys-Ser, Lys-Cys, Cys-Lys, and Cys-Pro could not be degraded. With Mn^{2+}, all measured activities were higher but with the same high preference for Cys-Gly (Table 2). Phe-Cys cleavage was significantly increased by Mn^{2+} but still remained lower than the Cys-Gly cleavage. No activity of Dug1p toward tripeptides (Cys-Gly-Gly, Gly-Cys-Gly, and Glu-Cys-Gly) and a tetrapeptide (Gly-Gly-Cys-Gly) could be recorded. These results clearly indicate that Dug1p is a dipeptidase with high specificity toward Cys-Gly.

Kinetic Parameters—In the presence of Mn^{2+}, Dug1p Cys-Gly dipeptidase had a K_m value measured at 0.4 ± 0.1 mM and a K_{cat} at 86.5 ± 7.4 s^{-1}, whereas in the presence of Zn^{2+}, the K_m was measured at 0.8 ± 0.1 mM and the K_{cat} at 76.9 ± 5.1 s^{-1}.

DUG1 Deletion Leads to Cys-Gly Accumulation and Enhanced GSH Toxicity—Intracellular GSH is turned over minimally in yeast cells (18). Exogenous glutathione during sulfur limitation is degraded very efficiently by the Dug pathway; however, the intermediates of this pathway are not known. A yeast strain lacking Dug1p is unable to use Cys-Gly as the sole sulfur source (19), clearly indicating that Dug1p acts as a Cys-Gly dipeptidase *in vivo*. To determine the impact of a *dug1*Δ deletion *in vivo*, we therefore exploited the phenomenon of GSH toxicity, where it was observed that overproduction of the glutathione transporter, Hgt1p (31), leads to glutathione toxicity above 10–15 μM GSH levels present in growth medium (32). Different mutants of GSH degradation were exposed to the above conditions. We observed that *dug1*Δ but not *ecm38*Δ, *dug2*Δ, or *dug3*Δ deletion made the cells very sensitive to even low concentrations (2.5 μM) of GSH. Thus, only the *dug1*Δ mutant showed enhanced toxicity at low concentrations of GSH. However, at a higher concentration of GSH (12.5 μM or above), all of the mutants show toxicity similar to that of the wild type strain (Fig. 3).

To further demonstrate the *in vivo* enzymatic function of Dug1p, we measured intracellular Cys-Gly levels in wild type and *dug1*Δ mutant cells during exponential growth. Cys-Gly could indeed be measured at a significant level in *dug1*Δ mutant

TABLE 2
Activity of Dug1p toward different peptide substrates

Peptide substrates were used at 2 mM concentration. Zn^{2+} and Mn^{2+} were used at final concentrations of 200 and 20 μM, respectively. The relative activity assays were single point, end point assays. Values are mean ± S.E., and all of the experiments were repeated three times in duplicates. ND, activity was not detected under the experimental conditions.

Substrate	Relative activity	
	Zinc	Manganese
Cys-Gly	100.0 ± 2.0	100.0 ± 1.1
Ala-Cys	73.6 ± 2.1	75.4 ± 0.9
Cys-Asp	32.4 ± 0.5	29.9 ± 1.0
Cys-Phe	31.1 ± 0.5	11.6 ± 0.1
Gly-Cys	21.3 ± 0.6	3.6 ± 0.3
Ser-Cys	15.8 ± 0.5	31.9 ± 1.4
Cys-Leu	13.3 ± 0.6	26.1 ± 0.5
Leu-Cys	13.1 ± 0.3	29.3 ± 0.4
Phe-Cys	10.5 ± 0.5	48.53 ± 1.7
Cys-Ala	ND	ND
Cys-Ser	ND	ND
Cys-Lys	ND	ND
Cys-Pro	ND	ND
Lys-Cys	ND	ND
Asp-Cys	ND	ND
Cys-Gly-Gly	ND	ND
Gly-Cys-Gly	ND	ND
Glu-Cys-Gly	ND	ND
Gly-Gly-Cys-Gly	ND	ND
γ-Glu-Cys-Gly	ND	ND

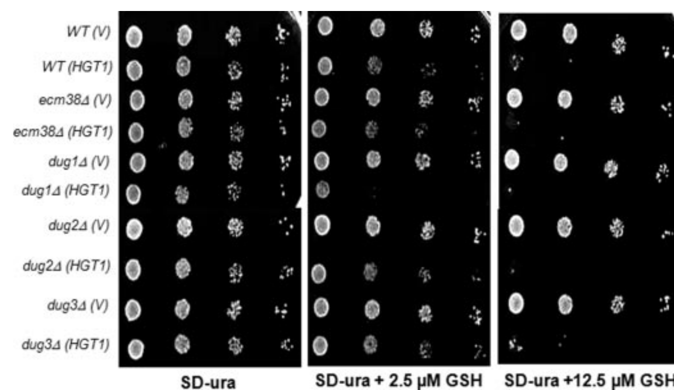

FIGURE 3. **Dug1Δ cells overexpressing Hgt1p exhibit increased glutathione toxicity.** WT (BY4742), *dug1*Δ, *dug2*Δ, *dug3*Δ, and *ecm38*Δ cells were transformed with control vector or HGT1 under the TEF promoter. The individual transformants were grown overnight in SD medium, and 10-fold serial dilutions were made (2 × 10^6, 2 × 10^5, 2 × 10^4, and 2 × 10^3 cells/ml). 10 μl of each dilution was spotted onto SD plates containing different concentrations of GSH. The plates were grown for 3 days at 30 °C.

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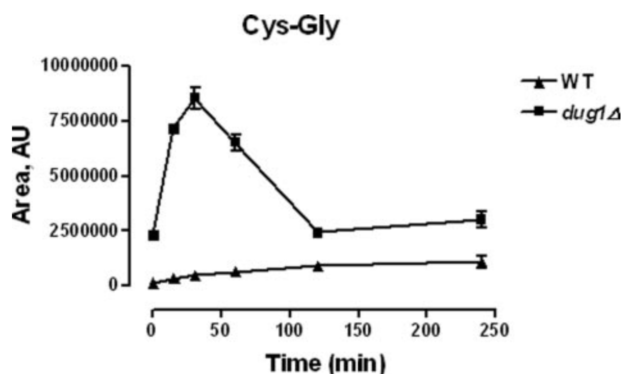

FIGURE 4. ***In vivo* detection of Cys-Gly using the FIA/ESI-LTQ/Orbitrap procedure.** WT and *dug1*Δ cells were grown on SD + 100 μM GSH and were collected after different time intervals (0, 15, 30, 60, 120, and 240 min) for metabolite extraction. The data for each time point are from three independent cultures each of WT and *dug1*Δ. The values on the *y* axis are arbitrary units (*AU*) that correspond to the area of the Cys-Gly peaks.

cells but was totally absent in wild type cells (Fig. 4). Further, adding GSH (100 μM) in the growth medium resulted in a more than 3-fold increase in the Cys-Gly intracellular concentration in *dug1*Δ mutant cells but not in wild type. Such an increase was not seen in wild-type cells. Surprisingly, in *dug1*Δ, Cys-Gly returned to the level seen prior to adding GSH, after 2 h. Cys-Gly efflux out of the cell cannot account for such decrease, since no Cys-Gly could be measured in the supernatant of these cells (data not shown). This observed decrease of Cys-Gly might be related to mixed disulfide formation between the dipeptide and proteins. Another less efficient route of Cys-Gly degradation can be ruled out based on our previous data that indicated that the *dug1*Δ mutant cannot use Cys-Gly as sole sulfur source and therefore cannot degrade this dipeptide. In summary, the exclusive detection of Cys-Gly in *dug1*Δ and its transient accumulation in the presence of exogenous GSH clearly indicates an essential role of Dug1p in the normal metabolism of Cys-Gly.

The S. pombe Homologue of Dug1p Has Cys-Gly Dipeptidase Activity in Vivo—In addition to putative members of the M17 peptidase family (SPAC13A11.05, 42% identity with bovine M17 Cys-Gly peptidase), which encompass the majority of Cys-Gly peptidases from bacteria to mammals, *S. pombe* carries a putative M20A peptidase (SPBC1198.08). *S. pombe* SPBC1198.08 open reading frame (named *SpDUG1* hereafter) encodes a protein that has 54% identity and 71% similarity with *S. cerevisiae* Dug1p. We thus checked whether the *SpDUG1* gene product had Cys-Gly peptidase activity, using as a tool the auxotrophy for cysteine of the *cys1a*Δ strain, lacking cysteine synthase (33, 34). *SpDUG1* was deleted in a *cys1a*Δ background strain. The resulting double null strain (*cys1a*Δ*spdug1*Δ) was unable to grow in the presence of Cys-Gly as a unique source of cysteine (Fig. 5). These data indicate that the *S. pombe* Dug1p homologue is also a Cys-Gly peptidase in *S. pombe* and that the putative M17 peptidase family members do not carry this activity.

Human Carnosinase-like Dipeptidase CNDP2 Has Cys-Gly Dipeptidase Activity in Vivo—Mammals have two Dug1p homologues, carnosinase (CNDP1) and carnosinase-like dipeptidase (CNDP2) (20, 21). CNDP1 protein has 44% identity and 62% similarity and CNDP2 protein has 54% identity and 64% similarity with *S. cerevisiae* Dug1p. CNDP1 protein is a secreted peptidase primarily found in the brain, liver, and plasma and has a substrate preference for carnosine (β-alanyl-histidine) and carnosine-like dipeptides (homocarnosine and anserine). CNDP2 is intracellular and ubiquitously expressed and has a lesser activity toward carnosine and carnosine-like dipeptides compared with CNDP1 and a preference for other dipeptides (21). Reverse BLAST "best hit" analysis reveals that CNDP2 protein is likely to be the true orthologue of Dug1p (data not shown).

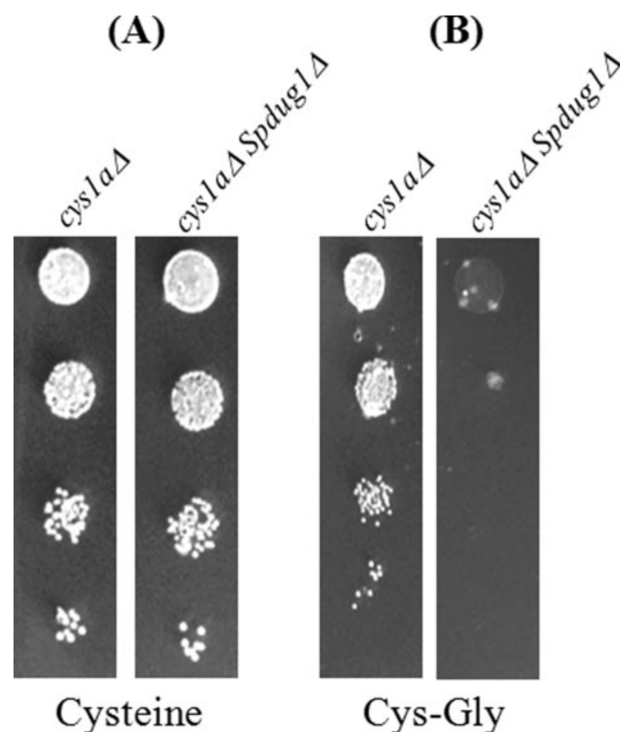

FIGURE 5. **SpDug1p is required for Cys-Gly utilization in S. *pombe*.** The *S. pombe cys1a*Δ and *cys1a*Δ *Spdug1*Δ strains were grown in minimal supplemented medium (containing leucine and uracil), and cells were harvested, washed, and resuspended to A_{600} = 2. Serial dilutions of A_{600} = 0.2, 0.02, 0.002, and 0.0002 were made, and 5 μl of each dilution was spotted on minimal medium + cysteine (*A*) and minimal medium + Cys-Gly (*B*). Plates were incubated at 30 °C for 5 days.

Genes encoding CNDP1 (both with and without a signal sequence) and CNDP2 were cloned into yeast expression vectors downstream of the TEF promoter and expressed in the *S. cerevisiae dug1*Δ strain. The CNDP2, but not the CNDP1, expression vector was able to rescue the *dug1*Δ defect (Fig. 6) in the utilization of Cys-Gly as sole sulfur source, thus indicating that CNDP2 can function as a Cys-Gly dipeptidase *in vivo*.

CNDP2 Is a Cys-Gly Dipeptidase—To further characterize the CNDP2 Cys-Gly dipeptidase activity, a His-tagged version of this protein was purified from *E. coli* extracts by Ni^{2+}-NTA affinity chromatography, followed by gel filtration on a Superdex S200 column. The purified protein was 95% pure as attested by Coomassie Blue staining after SDS-PAGE separation (data not shown). Purified CNDP2 protein existed as a dimer in solution, and its activity was Mn^{2+}-dependent, as reported earlier (21). We next tested CNDP2 substrate specificity. CNDP2 had a strong preference for Cys-Gly but was also active toward other peptides although with relatively lesser activity (Table 3). How-

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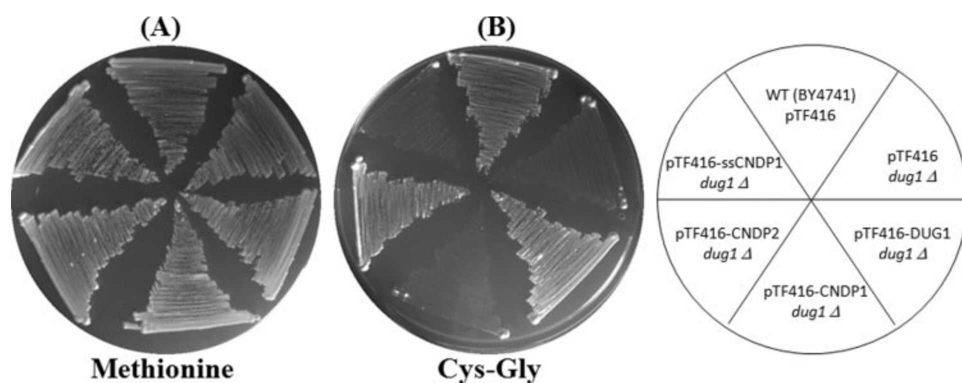

FIGURE 6. **Complementation of *dug1*Δ phenotype by CNDP1 and CNDP2.** pTEF-*CDNP1*, pTEF-*ssCNDP1*, and pTEF-*CNDP2* vectors were transformed into the *dug1*Δ strain of *S. cerevisiae*, the transformants were grown in the minimal supplemented medium, and cells were harvested, washed, and resuspended to A_{600} = 2.0. Serial dilutions of A_{600} = 0.2, 0.02, 0.002, and 0.0002 were made, and 5 μl of each dilution was spotted on minimal medium having methionine (*A*) or Cys-Gly (*B*). Plates were incubated at 30 °C for 2 days in the case of methionine and 4 days in the case of Cys-Gly.

TABLE 3
Activity of CNDP2 protein toward different peptide substrates

Peptide substrates were used at 2 mM concentration. Mn^{2+} was used at final concentrations of 50 μM. The relative activity assays were single point, end point assays. Values are mean ± S.E., and all of the experiments were repeated three times in duplicates. ND, activity was not detected under the experimantal conditions.

Substrate	Relative activity (manganese)
Cys-Gly	100.2 ± 3.9
Ala-Cys	50.8 ± 1.4
Ser-Cys	27.4 ± 1.2
Cys-Phe	22.6 ± 0.7
Gly-Cys	17.8 ± 1.1
Leu-Cys	17.1 ± 0.6
Phe-Cys	16.9 ± 0.3
Cys-Leu	12.9 + 0.4
Cys-Asp	ND
Cys-Ala	ND
Cys-Ser	ND
Cys-Lys	ND
Cys-Pro	ND
Lys-Cys	ND
Asp-Cys	ND
Cys-Gly-Gly	ND
Gly-Cys-Gly	ND
Glu-Cys-Gly	ND
Gly-Gly-Cys-Gly	ND

ever, CNDP2 could not cleave many dipeptides used in the study and the tripeptides Cys-Gly-Gly, Gly-Cys-Gly, and Glu-Cys-Gly and tetrapeptide Gly-Gly-Cys-Gly (Table 3). Previous kinetics studies have provided a K_m of CNDP2 for carnosine of 15 mM (21). Kinetics measures of the CNDP2 Cys-Gly dipeptidase activity in the presence of Mn^{2+} gave a K_m estimate of 0.6 ± 0.1 mM for Cys-Gly, and a K_{cat} of 247.8 ± 30.3 s^{-1}. Taken together, these data strongly indicate that CNDP2 is a Cys-Gly dipeptidase.

DISCUSSION

In the present work, we have characterized Dug1p as a dipeptidase responsible for the Cys-Gly dipeptidase activity of *S. cerevisiae*. Dug1p was previously identified as a component, along with Dug2p and Dug3p, of an alternative pathway of GSH degradation (19) and based on genetics was suggested to have at least two distinct functions in the cell. The first function of Dug1p was identified as part of the Dug1p-Dug2p-Dug3p complex (the Dug complex) that degrades GSH, and its second function was that of a putative di-/tripeptidase, independent of Dug2p and Dug3p. In the present study, we have investigated the second function of Dug1p. Purified recombinant Dug1p appeared as a 52-kDa species that was present as a homodimer in solution and functioned as a dipeptidase. Furthermore, Dug1p dipeptidase activity was highly specific for the Cys-Gly dipeptide. We propose that Dug1p is the elusive Cys-Gly dipeptidase that is part of the *S. cerevisiae* γ-glutamyl cycle, which has surprisingly eluded discovery so far. It is intriguing that both Dug1p functions converge toward GSH metabolism: the alternative γ-GT-independent GSH degradation pathway and the last step of the γ-glutamyl cycle.

Dug1p is the unique *S. cerevisiae* Cys-Gly dipeptidase, based on the inability of *dug1*Δ to utilize Cys-Gly as the sole source of sulfur. Since Dug1p is a member of the M20A metallopeptidase family, this adds a new twist to the already confusing picture of Cys-Gly peptidases, which usually belong to other subfamilies of metallopeptidase (17). Since Dug1p shows very high specificity for Cys-Gly as compared with other dipeptides used in the study, it seems exceedingly likely that Cys-Gly is the physiologically relevant substrate of the Dug1p peptidase. This is also supported by the mass spectral studies, where Cys-Gly was detectable in *dug1*Δ cells. Hitherto, all other Cys-Gly peptidases from either mammals or bacteria have been found to belong to the M17 family of leucine aminopeptidases (11, 14, 15), although there is one report of a member of the M1 family displaying Cys-Gly peptidase activity (10) (although specificity toward other substrates was not examined). Another isolated report suggested that members of the M19 family of membrane-associated dipeptidases may also be involved in Cys-Gly cleavage (7). These families, despite belonging to the larger family of metallopeptidases, show little similarity to each other. In fact, members of the M17 family are not found in *S. cerevisiae*, which possibly explains why the Cys-Gly peptidase of *S. cerevisiae* has eluded identification till now.

The M20A metallopeptidases, homologues of the Dug1p Cys-Gly peptidase, are found across a wide variety of organisms. This raises the immediate question as to whether the homologues in other organisms have a specificity for the Cys-Gly dipeptide. We have explored the possibility that Dug1p homologues in other organisms that contain not only Dug1p homologues but also an M17 homologue might still function as a Cys-Gly peptidase by examining the role of the *S. pombe* Dug1p. The investigation of strains bearing a deletion in this *DUG1* homologue of *S. pombe* have revealed that the *S. pombe* Dug1p homologue is likely to be the sole Cys-Gly peptidase even in *S. pombe* despite the presence of homologues of the M17 family in fission yeast.

This study also demonstrates that the human orthologue of Dug1p, CNDP2, functions as a Cys-Gly peptidase. CNDP1 and

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CNDP2 were both initially described as carnosinases. Subsequent studies, however, revealed that although CNDP1 protein was likely to be a carnosinase, since it preferentially cleaves carnosine and carnosine-like dipeptides, CNDP2 does not show the preference for carnosine but cleaves other dipeptides. CNDP2 has since been named as a carnosinase-like nonspecific dipeptidase (21) The precise function of CNDP2 still has not been defined. The ubiquitous presence of this peptidase in all tissues, like glutathione, and the ability to cleave Cys-Gly seem to indicate that its true role might be as a Cys-Gly peptidase of the γ-glutamyl cycle. In the present study, we clearly demonstrated that the CNDP2 protein shows a very high preference for Cys-Gly over all other dipeptides tested, and the turnover of Cys-Gly by CNDP2 protein was even better than that of Dug1p. Moreover, the affinity of CNDP2 protein for Cys-Gly as seen from the K_m (0.6 mM for Cys-Gly) is far higher than for carnosine (K_m = 15 mM) (21). These data clearly suggests that the CNDP2 protein may be a major Cys-Gly dipeptidase in mammalian tissues and is an important enzyme for GSH metabolism in different cell types in the body. The Dug1p M20A family thus represents a novel family of Cys-Gly dipeptidases.

Since the earlier genetic and physiological data had shown that *dug1Δ* strains could not utilize either Cys-Gly or the tripeptide Glu-Cys-Gly as a source of sulfur, they had suggested that Dug1p might function as both a dipeptidase as well as a tripeptidase (19). However, substrate specificity studies with the purified protein clearly show that it is a dipeptidase without having any activity toward tripeptides or tetrapeptides. Therefore, the dependence of Glu-Cys-Gly-like tripeptides on Dug1p for growth indicates that an aminopeptidase-like activity must exist that acts on tripeptides releasing Cys-Gly, which is then acted on by Dug1p. It would be interesting to identify if there might be a specific aminopeptidase responsible for the initial cleavage of Glu-Cys-Gly to Cys-Gly in yeast or if there might be redundant activities in the cell carrying out this function.

In attempting to investigating the *in vivo* effects of the loss of the Dug1p Cys-Gly peptidase, enhanced toxicity was observed in *dug1Δ* strains bearing an overexpressed glutathione transporter, Hgt1p. Although overexpression of this transporter leads to acute glutathione toxicity (at 12.5 μM GSH and above), the *dug1Δ* deletions were supersensitive even at 2.5 μM GSH. This supersensitivity was not seen in strains carrying deletions of the other genes encoding proteins of the Dug complex (*DUG2* or *DUG3*) or in strains lacking the γ-glutamyl transpeptidase (encoded by *ECM38*) and suggests that the supersensitivity might be due to the accumulation of Cys-Gly in strains lacking Dug1p. Cys-Gly has been reported to have a pK_a value of 6.5 and proposed to exist mainly as a disassociated, negatively charged species (Cys-Gly$^-$) at physiological pH. This Cys-Gly$^-$ has a high tendency to undergo redox reactions and also plays a role in signaling. In the presence of Fe^{3+} ions, Cys-Gly$^-$ can be oxidized to the thiyl radical (Cys-Gly$^•$), which can lead to thionylation of proteins and peroxidation of lipids in the plasma membrane (35–38). This might be a possible explanation of the supersensitivity of *dug1Δ* strains.

In conclusion, we have identified the Cys-Gly peptidase of *S. cerevisiae* as being encoded by the *DUG1* gene, an enzyme activity that was detected in *S. cerevisiae* over 2 decades ago but whose gene has eluded identification. Furthermore, we have also shown that the human orthologue of Dug1p, CNDP2, also functions as a Cys-Gly peptidase. It is hoped that the identification of the Cys-Gly peptidase would not only enable understanding of the Cys-Gly peptidases of other organisms better but would also enable the study of their role in glutathione metabolism both by the *DUG* complex and γ-glutamyl cycle and in signaling mediated by the Cys-Gly dipeptide.

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