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ISOLATION OF MUTANTS OF SCHIZOSACCHAROMYCES POMBE UNABLE TO SYNTHESIZE CADYSTIN, SMALL CADMIUM-BINDING PEPTIDES

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Summary: Schizosaccharomyces pombe synthesize small cadmium-binding peptides cadystin, structure of which is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, in response to cadmium. Mutants unable to synthesize cadystin were found in the mutants hypersensitive to cadmium. Some of them lack activity of either γ-glutamylcysteine synthetase (EC 6.3.2.2) or glutathione synthetase (EC 6.3.2.3), enzyme involved in glutathione biosynthesis. Some mutants have the same activity levels of these enzymes as wild type has. These results indicate that some steps of cadystin biosynthesis are catalyzed by the enzymes catalyzing glutathione biosynthesis.

Cadmium is a highly toxic substance to living organisms. Many organisms have developed defensive mechanisms to this metal. Thioneine (1) found in mammalian cells binds cadmium and diminishes its toxicity in the cell. S. pombe (2,3) and some plants (4,5) synthesize small peptide called cadystin or phytochelatin in response to cadmium. Cadystin forms complexes with cadmium, which are called Cd-BP1 and Cd-BP2 (2). While Cd-BP2 is consists of cadystin and cadmium, Cd-BP1 contains inorganic sulfur in addition to cadystin and cadmium (6).

In order to elucidate the mechanism of cadystin biosynthesis, we tried to isolate the mutants which cannot synthesize cadystin. As cadystin was supposed to be responsible for cadmium resistance in S. pombe (2), it is expected that mutants unable to synthesize cadystin were found in the mutants hypersensitive to cadmium. Since formation of Cd-BP1 or Cd-BP2 from cadystin and cadmium is somehow a self-assembly process (6,7), formation of Cd-BPs in the cell grown in the medium containing cadmium was used to detect synthesis of cadystin.

In this paper, we describe the isolation of the mutants unable to synthesize cadystin and partial characterization of them. We also present the

Abbreviations: EMS, ethylmethanesulfonate; DTT, dithiothreitol.

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evidence that the enzyme catalyzing biosynthesis of glutathione are involved in biosynthesis of cadystin.

Materials and Methods

Strain and culture condition - Schizosaccharomyces pombe HM123 (h^-, leu-1), which is a derivative of L972h^-, was gift from Dr. M. Yamamoto of Institute of Medical Science, University of Tokyo. Cells were grown at 30°C in YPD medium (1% yeast extract, 2% polypeptone, 2% glucose) with vigorous shaking. For solid cultivation, 1.5% agar was added to the medium.

Isolation of mutants hypersensitive to cadmium - Exponentially growing cells were harvested by centrifugation and suspended in 0.1 M Na-phosphate buffer (pH 7.0) at cell density 10^7/ml. They were mutagenized with 5 µl/ml EMS at 30°C for 1 hour. Survival rate of the cells was 30 percent at this condition. EMS treated cells were spread over YPD plate after 1 hour incubation with fresh YPD medium. Colonies appeared after 5 days were tested their sensitivity to cadmium by inoculating them on the YPD plate containing 0.5 mM $CdCl_2$. Clones showed no growth or very poor growth were selected as cadmium hypersensitive mutants. 0.3 percent of the clones showed hypersensitivity to cadmium.

Detection of Cd-BPs by Sephadex G-50 column chromatography - Synthesis of cadystin was induced by administrating $CdCl_2$ (final concentration 0.1 mM) to exponentially growing culture (100 ml). After 17 hours exposure to cadmium cells were harvested by centrifugation and washed twice with buffer A (50 mM Tris-HCl pH 7.6, 100 mM KCl, 1 µM $CdCl_2$). Cells (wet weight 1.3 g) were disrupted by grinding with 2.6 g of quartz sand for 8 minutes and extracted with 2.5 ml of buffer A. Cell lysate was clarified by centrifugation at 13,000 x g for 10 minutes. Clarified cell lysate was applied to the top of Sephadex G-50 column (1 cm x 60 cm) previously equilibrated with buffer A. The column was eluted with the same buffer. Fractions (0.85 ml) were collected and scored optical density at 250 nm. Cadmium concentration of each fraction was determined with Parkin Elmer Model 403 atomic absorption spectrophotometer.

Estimation of glutathione content in cell lysate - Exponentially growing culture (20 ml) was harvested by centrifugation. Cells were suspended in 0.25 ml of 10 mM Na-phosphate buffer pH 7.0 - 1 mM EDTA. They were disrupted by vortexing (30 seconds x 4 with intermittent coolings) with 0.3 g of glass beads in 1.5 ml polypropyrene sampling tube. Cell debris and glass beads were removed by centrifugation at 13,000 x g for 10 minutes. Cell lysate was deproteinized by addition of equal volume of 2 M $HClO_4$ - 4 mM EDTA. After neutralization with 2 N KOH, glutathione content was estimated enzymatically by using glutathione S transferase (EC 2.5.1.18) as described by Asaoka and Takahashi (8). Protein content in cell lysate was determined by the method of Lowry et al. (9) using bovine serum albumin as a standard.

Determination of activities of enzymes - Cell extract was prepared as described above for estimation of glutathione content except that cells were suspended in 50 mM Tris-HCl pH 8.2 - 120 mM KCl - 20 mM $(CH_3COO)_2Mg$. Activity of glutathione synthetase was assayed as follows. Reaction mixture (0.2 ml) contains 100 mM Tris-HCl pH 8.2, 50 mM KCl, 20 mM $MgCl_2$, 2 mM EDTA, 5 mM creatine phosphate, 1 unit creatinephosphokinase, 10 mM ATP, 5 mM γ-glutamylcysteine, 1 mM DTT, 10 µCi [^{3}H]glycine (20 Ci/mmole) and cell lysate (250 µg protein). After incubation at 30°C for 30 minutes, reaction was stopped by adding 1 ml of ice cold 20 mM acetic acid - 1 mM DTT. They were applied to the top of Dowex 1 column (0.9 cm x 3 cm) equilibrated with 20 mM acetic acid - 1 mM DTT. After washed with 20 ml of the same solution to elute unreacted glycine, glutathione was eluted by 1.5 M ammonium acetate - 1 mM DTT. More than 80 percent of radioactive materials eluted in this fraction is glutathione (C. Wada-Nakagawa, unpublished observation). Activity of γ-glutamylcysteine synthetase was assayed by the same method as that for glutathione synthetase except that [^{3}H]cysteine and sodium glutamate were

added as substrates in place of [^{3}H]glycine and γ-glutamylcysteine. γ-glutamylcysteine was also eluted by 1.5 M ammonium acetate - 1 mM DTT. Buthionine sulfoximine, a specific inhibitor of γ-glutamylcysteine synthetase (10), added to the reaction of wild type lysate reduced the radioactivity eluted in this fraction to the level found in the reaction without cell lysate. This indicates that most of radioactive materials eluted in this fraction was the reaction product γ-glutamylcysteine.

Chemicals - EMS and cadmium chloride were purchased from Nakarai Chemicals LTD. Glutathione S transferase and buthionine sulfoximine were obtained from Sigma. Glutathione and γ-glutamylcysteine were from Kojin Co. LTD. Radioactive materials were from Amersham.

Results

S. pombe is relatively resistant to cadmium and grows in the medium containing 2 mM $CdCl_2$ (2). Mutants which grow very poorly on the plate containing 0.5 mM $CdCl_2$ were isolated by EMS mutagenesis. Fig. 1 shows growth curves for wild type and three cadmium hypersensitive mutants with or without

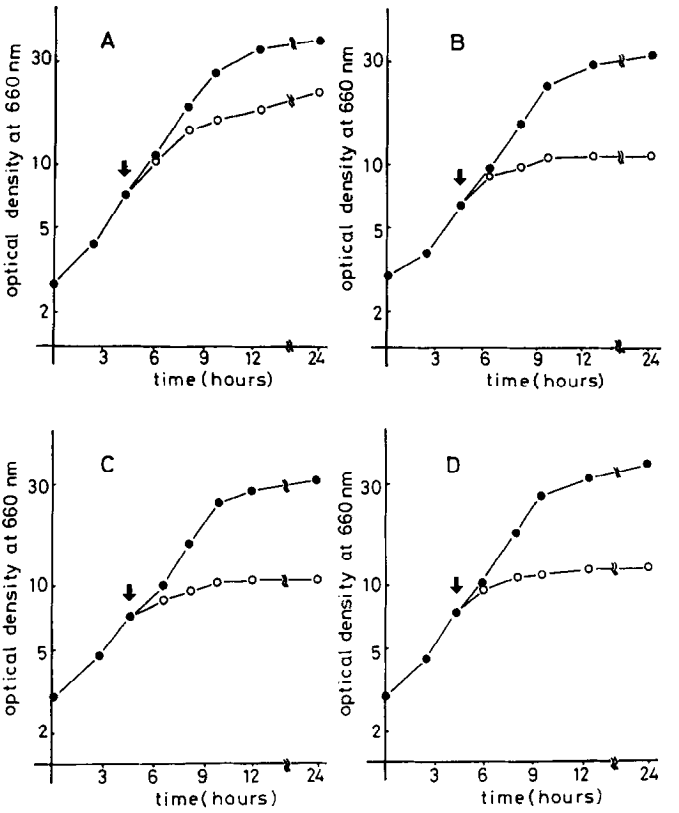

Fig. 1. Growth of the wild type HM123 (A) and mutants MN101 (B), MN120 (C) and MN138 (D), with (○) and without (●) cadmium. Early stationary culture of each strain was diluted 10 times and was grown at 30°C in YPD medium. Cadmium was added at the time indicated by an arrow. Portions of each culture were taken at the time indicated and the optical density at 660 nm was determined.

$CdCl_2$. HM123 (wild type) showed slight inhibition of growth in the presence of 0.5 mM $CdCl_2$. Growth of the mutants was severely inhibited at this condition. These mutants showed no growth defect in the absence of cadmium except for MN70 (data not shown), which grew slowly in the medium without cadmium and showed severe growth defect in the medium containing cadmium.

Wild type strain synthesize cadmium-peptides complexes called Cd-BP1 and Cd-BP2 in the medium containing cadmium (2). These compounds are supposed to be responsible for the cadmium-resistance in S. pombe. In order to see whether the mutants make Cd-BPs or not, cell lysates of the mutants were analyzed by Sephadex G-50 column. Cadmium chloride concentration of 0.1 mM was used to induce Cd-BPs because these mutants grow at least 1.5 generations at this concentration of cadmium. Fig. 2A shows elution profile of Sephadex G-50 column chromatography of wild type lysate after 1.5 generations of growth in the presence of 0.1 mM $CdCl_2$. Two peaks of optical density at 250 nm and cadmium content were seen in the fractions 30 to 47. The first peak appeared in the fractions 30 to 38 is the peak of Cd-BP1. The second peak appeared in the fractions 39 to 47 represents Cd-BP2. Fig. 2B, C, D show three typical elution patterns of mutants' lysates. Fig. 2B shows the first type of the

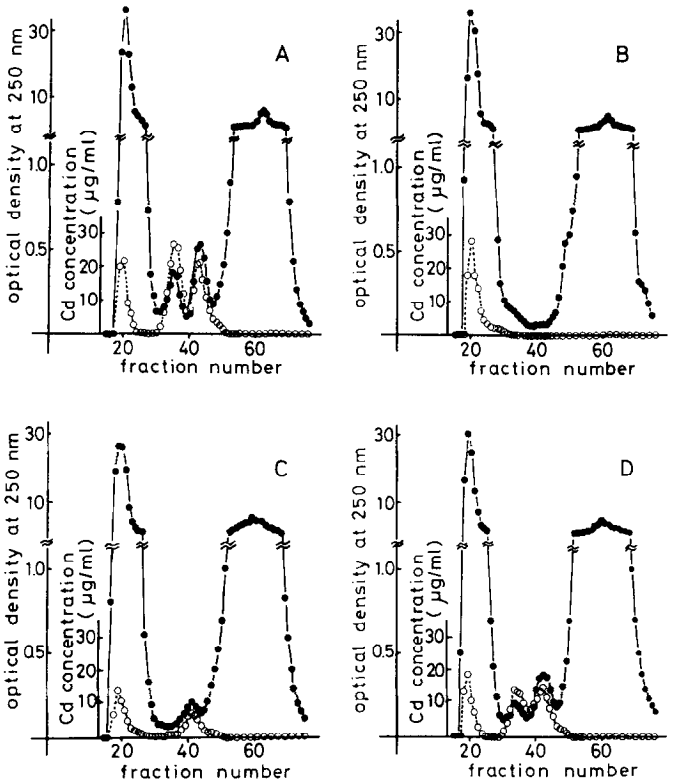

Fig. 2. Elution profiles of cell lysate from Sephadex G-50 Column. Cell lysates of wild type HM123 (A) and mutants MN101 (B), MN181 (C) and MN77 (D) were analyzed by Sephadex G-50 column as described in materials and methods. (●) optical density at 250 nm. (○) cadmium concentration.

mutants, which has neither Cd-BP1 nor Cd-BP2. Fig. 2C shows the second type of the mutants, which has Cd-BP2 but lacks Cd-BP1. The third type of the mutants is shown in Fig. 2D, which has both Cd-BP1 and Cd-BP2. The peak of cadmium detected in the void volume was probably cadmium nonspecifically bound to protein. Table 1 summarizes the results of Sephadex G-50 analyses of cell lysates of all the mutants. Seven mutants, MN52, MN55, MN65, MN70, MN72, MN101 and MN120, belong to the first type (type 1 mutant). Two strains, MN138 and MN181 belong to the second type (type 2 mutant). Other mutants are the third type (type 3 mutant).

Cd-BPs are complexes of small peptides cadystin, whose structure is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n=2 and 3) (11), and cadmium. In the structure of cadystin,

Table 1

Glutathione and Cd-BPs contents of wild type and mutants

strain	glutathione[a] (μmole/mg protein)	Cd-BPs[b] (optical density at 250 nm)	
		Cd-BP1	Cd-BP2
HM123 (wild type)	0.23	1.23	2.18
MN52	0.04	0	0
MN54	0.15	0.44	1.29
MN55	0.05	0	0
MN58	0.39	0.30	1.08
MN60	0.20	1.01	1.18
MN65	0.03	0	0
MN68	0.22	0.96	0.64
MN69	0.27	1.23	0.97
MN70	0.31	0	0
MN72	0.19	0	0
MN77	0.30	1.10	2.62
MN92	0.19	1.92	0.87
MN94	0.34	0.86	2.67
MN96	0.43	2.04	2.73
MN101	0.04	0	0
MN120	0.03	0	0
MN138	0.23	0	4.06
MN139	0.22	0.75	2.82
MN148	0.24	0.65	0.78
MN181	0.23	0	0.97

[a]Glutathione content was determined as described in materials and methods.
[b]Cell lysate (150 units of optical density at 250 nm) of each strain was analyzed by Sephadex G-50 column chromatography as described in materials and methods. Cd-BPs content was estimated by summing up optical density at 250 nm of fractions of the peak correspond to each substance.

Table 2

Activities of γ-glutamylcysteine synthetase and glutathione synthetase of mutants and wild type

strain	γ-glutamylcysteine synthetase	glutathione synthetase
HM123 (wild type)	9.1	0.51
MN52	0.1	0.53
MN55	0.1	0.53
MN65	0.1	0.60
MN70	5.4	0.60
MN72	8.2	0.79
MN101	7.7	0.03
MN120	0.1	0.40

Activities of enzymes were determined as described in materials and methods.
Values denote nmole/mg protein/minute of ^{3}H incorporation into the fraction eluted from Dowex 1 column by 1.5 M ammonium acetate - 1 mM DTT.

n=1 represents glutathione. This suggests that the enzymes catalyzing biosynthesis of glutathione are involved in biosynthesis of cadystin. Suppose it is correct, mutants deficient in biosynthesis of glutathione might not synthesize cadystin. Glutathione content of the mutants was determined. Table 1 shows that strains MN52, MN55, MN65, MN101 and MN120 contained less than 20 percent of glutathione compared with wild type. All of these mutants belonged to the type 1 mutant and lacked cadystin. In the type 1 mutants, two strains MN70 and MN72 contained normal level of glutathione. Activities of γ-glutamylcysteine synthetase and glutathione synthetase, enzymes involved in glutathione biosynthesis, of these mutants were assayed (Table 2). Strains MN52, MN55, MN65 and MN120 lacked activity of γ-glutamylcysteine synthetase and strain MN101 lacked activity of glutathione synthetase. Two strains MN70 and MN72, which contained normal levels of glutathione, showed the same activity levels of both enzymes as wild type did.

Discussion

Mutants of S. pombe unable to synthesize cadystin were found in the mutants hypersensitive to cadmium. This result supports the hypothesis that synthesis of cadystin is responsible for cadmium-resistance in S. pombe.

Mutants that contained Cd-BP2 but lacked Cd-BP1 were also found in the cadmium hypersensitive mutants. This suggests that Cd-BP2 alone is not sufficient for full resistance to cadmium in S. pombe. This is consistent with the findings that cadmium is bound tighter in Cd-BP1 than in Cd-BP2 (12).

Though biosynthetic pathway of cadystin is not known, the structural similarity of cadystin to glutathione suggests that the enzymes involved in glutathione biosynthesis are working to synthesize cadystin. Biosynthesis of glutathione from its component amino acids is accomplished in two steps. First, a peptide bond is formed between the γ-carboxyl group of glutamic acid and the amino group of cysteine by γ-glutamylcysteine synthetase. Then the second peptide bond is formed between the product γ-glutamylcysteine and glycine by glutathione synthetase. In plant, buthionine sulfoximine, a potent inhibitor of γ-glutamylcysteine synthetase (10), inhibits biosynthesis of cadystin (5). Our results that the mutant deficient in glutathione synthetase as well as the mutants deficient in γ-glutamylcysteine synthetase were not able to synthesize cadystin indicate that both γ-glutamylcysteine synthetase and glutathione synthetase are required to synthesize cadystin. Two mutants showed normal activities of these enzymes but lacked cadystin. This means that the enzymes catalyzing glutathione biosynthesis are not enough to synthesize cadystin. Enzyme(s) deficient in these mutants might be the enzyme(s) responsible for induction of cadystin in response to cadmium, though it is possible that glutathione is required to induce the enzyme(s) involved in cadystin biosynthesis.

Characterization of type 2 and type 3 mutants was not performed. As Cd-BP1 contains inorganic sulfur (6), type 2 mutants might be the mutants deficient in sulfur metabolism. Type 3 mutants might be the mutants which altered some vital enzymes, such as DNA polymerase or RNA polymerase, to hypersensitive to cadmium or they might be the leaky mutants of type 1 or type 2 mutants.

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