Vol. 188, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1992 Pages 388-394

THE SYNTHESIS OF CADYSTINS, HEAVY METAL CHELATING PEPTIDES, IS INDUCED IN THE FISSION YEAST BY WOUNDS OF THE CELL WALL OR BY INCUBATION WITH CHITOSAN

YUKIMASA HAYASHI[1], SHU-ICHI MORIKAWA[2], MASAO KAWABATA[1] AND YASUO HOTTA[2]

[1]Institute for Developmental Research, Aichi Prefectural Colony, Kasugai, Aichi 480-03, Japan
[2]Institute of Biology, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

Received September 3, 1992

SUMMARY: It has been shown that heavy metal administration induced the synthesis of cadystins, the small metal chelating peptide with the general structure of $(\gamma\text{-glu-cys})_n\text{-gly}$, in the fission yeast and in plants.

Besides heavy metals, wounds to the cell surface or the incubation with chitosan induced the cadystin synthesis in the fission yeast. Under these induction conditions, the membrane permeability of the fission yeast significantly increased suggesting the structural alteration of the membrane. In these induction, the synthesized cadystins formed complexes with the cellular zinc ions together with or without glutathione.

Cadystins were first found as the peptide components of cadmium binding peptides induced in the fission yeast on the exposure to the cadmium ion (1). A few years later, the chemical structure of cadystins were determined as $(\gamma\text{-glutamyl-cysteinyl})_n\text{-glycine}$, n=2,3,4,....(2,3), and abbreviated as cadystins $(\gamma EC)_nG$ (4). Then, these peptides were also found in plants and protozoa, and they were named respectively, such as phytochelatins, or poly(γ-glutamyl-cysteinyl)-glycine, or $(\gamma\text{-glu-cys})_n\text{gly}$ (5-9). These peptides can be induced by other heavy metals, such as zinc, copper, cobalt, lead and mercury (8-10), though the synthesized amounts of cadystins in maximum are less than 10% of that with cadmium (8,10). Like metallothioneins in mammals, cadystins have the function to bind heavy metals to detoxify them, possibly through chelation by thiol groups (11). One of the other functions of cadystins have been assumed to be the more effective scavenger for the active oxygen than glutathione, because the redox potentials of cadystin are higher than that of glutathione (8).

Abbreviations: YPD, yeast extract 1%, polypeptone 2%, dextrose 2%; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography.

0006-291X/92 $4.00

Metallothioneins in mammals are induced not only by the administration of heavy metals but also by glucocorticoid hormones directly, or through a variety of stresses (12,13). In response to the acute stresses, liver specific genes are expressed for the defense including the induction of metallothionein synthesis (13). On the other hand, oligosaccharides derived from the cell surface of the fungi and the plants have been identified that can act as chemical signals in plant tissues to activate a broad spectrum of genes for the defense (14). However, it is not known whether these stress related substances can act as the inducer of cadystin synthesis in the fission yeast or in plants. In the induction of cadystin synthesis in the fission yeast and in plant cells, heavy metals have been known as the inducer. Only our recent paper reported that the anti-fungal agents, tetramethylthiuram disulfide (TMTD) and dimethyldithiocarbonate (DMDTC) worked as the inducer (15), and it was assumed that the target of anti-fungal agents resided in the membrane. To obtain some clues about physiological functions of cadystins besides detoxification and homeostasis of heavy metals, it will be worthy to examine the inducer activity of various materials which affect the structure of the cell membrane.

In the present paper, we report that the physical damage of the cell wall or the incubation with chitosan make the cell membrane permeable and result in the induction of cadystin synthesis in the fission yeast. Although the cellular contents of heavy metals, such as zinc or copper, somewhat increase by these treatments, the increases of these metals are not sufficient to induce and not proportional to the synthesized amounts of cadystins. The direct effect of these treatments is assumed to be the alteration of the membrane structure as indicated by the increase of the membrane permeability.

MATERIALS AND METHODS

Culture--The fission yeast, Schizosaccharomyces pombe L972h$^-$ was grown at 30°C to the early stationary phase in YPD medium as the preculture. The main culture of the experiment started at the early logarithmic phase of growth (3 x 10^6 cells/ml) with 10 ml of YPD medium in 50 ml-flask. To obtain cells with wounded cell-wall, various amounts of acid washed glass beads (0.5 mm diameter) were added to flasks and the culture with rotary shaking (100 rpm) resumed for the indicated time.

HPLC analysis--At the harvest, cells from 10 ml-culture in 50 ml-flask were washed two times with 0.14M NaCl, then with distilled water, divided in two 1.5 ml-microfuge tubes, and stored frozen at -30°C until use. Cells were vortexed for 2 min with 3 times-weight of acid-washed glass beads (0.3 mm dia.) in the microfuge tubes. Crude cell extract with 0.3 ml of H_2O was centrifuged at 15,000 x g to remove the cell debris. To precipitate proteins TFA was added to the supernatant as 5%, kept at 0°C for 10 min, and the protein precipitate was seperated by centrifugation at 12,000 x g. The resultant supernatant was filtered and applied to reverse phase HPLC (TSK-ODS 80T_M, 0.46 x 25 cm) and analysed by 0-20% acetonitrile linear gradient in 0.05% TFA at flow rate of 1 ml/min. For the determination of cadystin content, the sulfhydryl group (SH) in each 1 ml-fraction was determined by Ellman's method (16). Protein content determination of 5% TFA precipitate was performed by Lowry's method (17).

Amino acid analysis--Besides the analysis of synthesized peptides by HPLC, amino acid constituents of peptide fractions in HPLC were examined to ascertain the cadystin molecules. The respective peptide fractions were combined and lyophilized, oxidized by performic acid, digested by 5.6N HCl for 20 h at 120°C, coupled with phenylisothiocyanate (PTC), and the resulting PTC-amino acids were determined by HPLC (TSK-ODS 80T_M) with the modified elution system of Heinrikson and Meredith (18,19), linear gradient from 100% A [0.05% triethylamine, 0.1M sodium acetate (pH 6.35)/acetonitrile (940:60)] to 100% B [acetonitrile/H_2O (60;40)] in 50 min.

Analysis of metal complexes--For the analysis of metal peptide complexes, cells from 100 ml-culture were ground in a mortar and pestle with 3 times weight of quartz sand, and extracted with 3 volume of A buffer (25 mM Tris-HCl pH 7.5, 100 mM NaCl). The cell extract was centrifuged at 15,000 x g, then at 120,000 x g for 90 min, and the supernatant was applied to Sephadex G50 SF column (1.6 x 65 cm) as described previously (9,20). Four ml-fractions were collected, the absorbance at 250 nm was read, and the zinc or copper content in each fraction was determined by Hitachi Z-7000 polarized Zeeman atomic absorption spectrophotometer. An aliquot of each fraction was applied to HPLC to determine the content of glutathione and cadystins.

Cell permeability by conductivity determination--Acid hydrolyzed and neutralized chitosan was added to the mid-log phase cells grown in YPD medium at 30 °C. The corresponding amounts of NaCl were also added to the respective cultures to make the same salt concentration in the medium with those of containing various amounts of chitosan. Within 15 seconds, the conductivity was determined for the 0-time by the conductivity meter CD-MII (M&S Instrument Co. Ltd.). Then the culture continued and at 5 min interval the conductivity was determined.

RESULTS AND DISCUSSION

Since various stresses to introduce tissue injury and inflammation in mammals induce metallothionein synthesis, we have examined the effect of the wounds to the cell surface of the fission yeast on the induction of cadystin synthesis. To introduce some wounds to the cell wall and/or to the membrane of the fission yeast, glass beads was added to the flask of rotation culture, and after a definite time cells were harvested. Analysis of the acid-soluble fraction of the cell extract by reverse phase HPLC (Fig. 1, a) revealed the appearance of several thiol compounds besides GSH. The elution time of these compounds coincided with those of the standard cadystins (γ EC)$_n$G, n=2 eluted at 16.3 min, n=3 at 23.7 min, and n=4 at 29 min, respectively. These thiol compounds formed complexes with Cd *in vitro*, and the formed complexes were separated on DEAE-Toyopearl 650M column with the same behavior as the standard Cd-cadystin complexes. The separated Cd-thiol complexes were analyzed again in HPLC to purify the thiol compounds. And amino acid analyses of these thiol compounds eluted at 16.3 and 23.7 min indicated that these compounds were consisted of Glu, Cys and Gly at the expected molar ratios of 2:2:1 and 3:3:1, respectively. Then, these thiol compounds were identified as cadystins.

As a function of the added amount of glass beads, the amount of synthesized cadystins increased (Fig. 1,b), but the cell growth was also impaired. The observation under the optical microscope and the viability

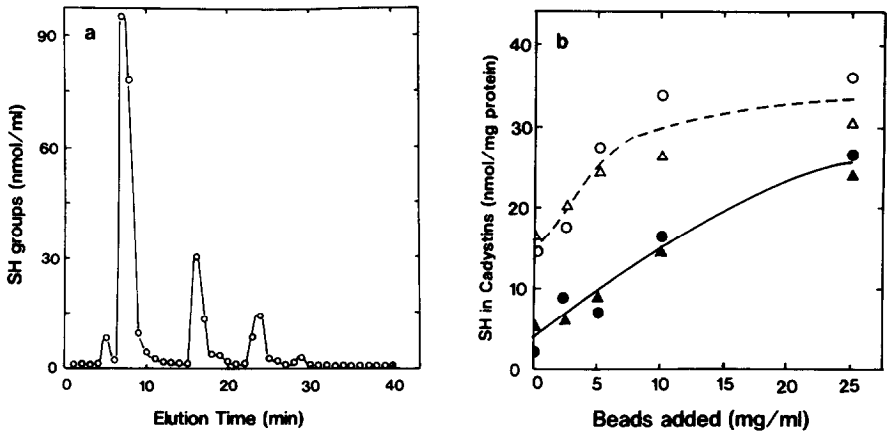

Fig. 1. The induction of cadystin synthesis in the fission yeast by adding glass beads to the medium. The fission yeast growing at the log-phase was added with the various amounts of glass beads and incubated in a rotary shaker for 12 h. (a) The cell extract was analyzed for the content of cadystins by HPLC as described. (b) Cadystin synthesis as a function of added amounts of glass beads, without addition of the zinc ion (closed circles and triangles) or with simultaneous addition of the zinc ion at 2 mM (open circles and triangles). Circles and triangles indicate the independent series of experiments.

check revealed that the damaged cells and the dead cells increased depending on the added amount of glass beads. Addition of 2 mM $ZnCl_2$ to the medium markedly reduced the cell death and somewhat recovered the growth rate (Fig. 1, b). Changing the pH to acidic at the start of the culture increased the induced synthesis of cadystin by glass beads (data not shown), suggesting the lower pH may be recognized as the stress or may induce a certain alteration of the cell surface structure.

It is well known that wounds to the cell wall of plants result in the release of oligosaccharides from the cell wall, and that these saccharides work as the inducers to synthesize a wide spectrum of defensive materials in plants (14). When the lower pH together with the physical damage of the cell surface could be recognized as the stress, oligosaccharides might be released as the chemical signal and the cadystin synthesis was induced as one of the protective responses of the cell.

Polycations, such as polylysine and chitosan, were known to alter the membrane structure and increase the membrane permeability of the yeast (21, 22). In our present experiments, the conductivity of the medium changed about 5% within 30 min after the addition of chitosan (0.05 mg/ml) possibly by the result of permeability increase, though the effect was markedly reduced by the presence of various cations in the medium (22). As shown in Table I, polycations at the concentration to increase the membrane permeability were effective to induce the cadystin synthesis with or without Zn^{2+}.

Table I. Induction of cadystin synthesis by various inducers[a]

Additions[b]	Metals[c]	Incbation time (h)	GSH (SH n mol/mg protein)	cadystins
None	-	10	105.3	3.0
	Zn	10	138.4	15.8
	Cd	10	122.4	292.6
	Cu	20	95.3	11.0
Polylysine	-	15	68.0	16.4
	Zn	15	87.7	23.5
Chitosan	-	15	157.1	18.5
	Zn	15	193.5	24.5
Chitin	-	15	101.4	20.7
	Zn	15	125.7	23.5
Chitosan hydrolysate[d]	-	15	147.6	35.6
	Zn	15	167.2	45.9

[a] The culture started at 3.1×10^6 cells/ml of YPD. [b] 0.025 mg/ml medium for polylysine, 0.05 mg for chitin and chitosan. [c] $ZnCl_2$ 2 mM, $CdCl_2$ 0.1 mM, $CuSO_4$ 2.5 mM. [d] Hydrolysis in 1 N HCl for 2 h at 100°C.

Although Zn and Cu contents in the cadystin synthesis-induced cell by wounds or by chitosan increased about 1.5 - 2 times of that of the control, these were about one-tenth of those in the respective heavy metal-induced cells. When cadystin synthesis is induced by the heavy metals, multiples of cadystins form the metal complexes with those heavy metals (1, 9, 20). In the induction by wounds or by chitosan, the cell extract was analyzed for the presence of metal-cadystin complexes by gel filtration as described previously (9, 20). In the eluted fractions, two significant zinc peaks were observed (Fig.2). One sharp peak in the high molecular weight region and one broad peak in the low molecular weight region (Fig. 2). Between two main Zn peaks, small insignificant zinc peaks were seen (fraction 17-26), to which the UV absorbing broad peak of chitosan oligomers corresponded. On the other hand, only one significant copper peak was detected in the high molecular weight region (data not shown). These results indicated that the synthesized cadystins in the induction by wounds or chitosan exsisted as the zinc-complexes in the cell. Glutathione and cadystin species were determined in all fractions of this gel filtration. As seen in Fig.2, most of cadystins were found in fraction 28-33, centered at fraction 31 (Mr 1,200) and glutathione was found in fraction 30-35, centered at fraction 33 (Mr 800). Thus, the broad zinc peak in the low molecular weight region was consisted of, either both the zinc-cadystin complexes and the zinc-glutathione complex, or various forms of the zinc complex with the mixture of cadystins and glutathione. The ratios of n=3/n=2 in cadystins $(\gamma EC)_nG$ were 2.2 in fraction 28 and 0.1 in fraction 33, indicating the longer peptide made the larger complex with zinc ions.

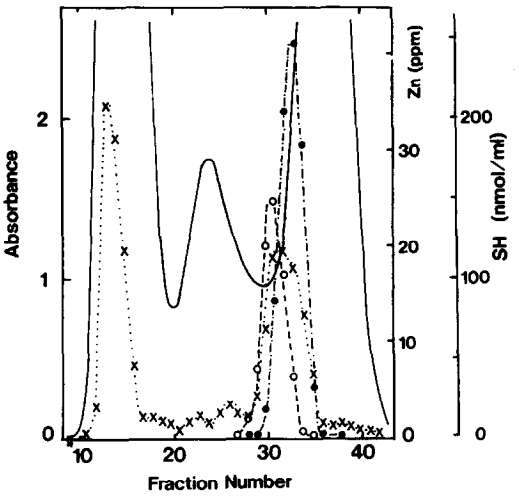

Fig. 2. Analysis of the metal-cadystin complexes. The cell extract obtained from the fission yeast treated for 15 h by hydrolyzed chitosan (0.05 mg/ml) was applied to Sephadex G50 SF column. Four-ml fractions were collected, absorbance at 250 nm (——) was read, zinc content (...x...) in each fraction was determined in an atomic absorption spectro-photometer and an aliquot of each fraction was analyzed by HPLC for the content of GSH (-.-●-.-) and cadystins (--o--).

There must be some physiological roles for cadystins synthesized in response to alteration of the membrane structure. One possible role could be the detoxification of superoxide or hydroxyl radicals produced by the infiltration of foreign compounds, since this infiltration may be reflected by the change of the membrane structure. Or, as it is suggested in the physiological function for metallothioneins (24), cadystins may also play an important role in the essential metal flux like a stimulus to enhance the uptake of zinc and increase the cellular level of zinc, which may be required for the defense reaction.

REFERENCES

1. Murasugi, A., Wada, C.,and Hayashi, Y.(1981) J. Biochem. 90,1571-1574
2. Kondo, N., Isobe, M., Imai, K., Goto, T., Murasugi, A., and Hayashi, Y. (1983) Tetrahedron Lett. 24,925-928
3. Kondo, N., Imai, K., Isobe, M., Goto, T., Murasugi, A., Nakagawa, C.W. and Hayashi, Y.(1984) Tetrahedron Lett. 25,3869-3872
4. Grill, E., Winnacker, E.-L., and Zenk, M.H.(1985) Science 230,674-676
5. Robinson, N.J., and Jackson, P.J.(1986) Physiol. Plant 67,499-506
6. Steffens, J.C., Hunt, D.F., and Williams, B.G.(1986) J. Biol. Chem. 261,13879-13882
7. Weber, D.N., Shaw, C.F.III., and Petering, D.H.(1987) J. Biol. Chem. 262,6962-6964
8. Murasugi, A.(1982) Ph.D. Thesis, Nagoya Univ., Nagoya, Japan
9. Hayashi. Y., Nakagawa, C.W., and Murasugi, A.(1986) Envir. Hlth. Perspect. 65,13-19

10. Grill, E., Winnacker, E.L., and Zenk, M.H.(1987) Proc. Natl. Acad. Sci. U.S.A. 84,439-443
11. Karin, M.& Hershman, H.R.(1979) Science 204,176-177
12. Oh, S.H., Deagen, J.T., Whanger, P.D., and Weswig, P.H.(1978) Am. J. Physiol. 234,E282-E285
13. Karin, M.(1985) Cell 41,9-10
14. Ryan, C.A.(1988) Biochem. 27,8879-8883
15. Mutoh, N., Kawabata, M., and Hayashi, Y.(1991) Biochem. Biophys. Res. Comm. 176,1068-1073
16. Ellman, G.L.(1959) Arch. Biochem. Biophys. 82,70-77
17. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J.(1951) J. Biol. Chem. 193,265-275
18. Tarr, G.E.(1981) Anal. Biochem. 136,27-32
19. Heinrikson, R.L., and Meredith, S.C.(1984) Anal. Biochem. 136,65-74
20. Hayashi, Y., Nakagawa, C.W., Uyakul, D., Imai, K., Isobe, M., and Goto, T. (1989) Biochem. Cell Biol. 66,285-295
21. Yphatis, D.A., Dainko, J.L., and Schlenk, F. (1967) J. Bacteriol. 94, 1509-1515
22. Jaspers, H.T.A., Cristianse, K., and von Steveninck, J.(1975) Biochem. Biophys. Res. Comm. 65,1431-1439
23. Young, O.H., Kohle, H., and Kauss, H.(1982) Plant Physiol. 70,1449-1454
24. Bremner, I.(1987) Experientia Suppl. 52,81-107