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Cadmium Resistance Conferred to Yeast by a Non-metallothionein-encoding Gene of the Earthworm Enchytraeus*

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Anthropogenic pollution of the environment by heavy metals has been recognized as an increasingly threatening hazard for animals, plants, and even human health. Cadmium (Cd), for example, has been utilized eight times more during the last 40 years by mankind than in its entire history; the Cd input into biosphere is estimated to be about 30,000 tons/year (1–3). The situation is deteriorating due to the acid rain that mobilizes the soil-bound Cd thus increasing the bioavailability of Cd (4). Cd is highly toxic because of its strong affinity to purines, pyrimidines, phosphates, porphyrins, and the cysteine and histidine residues of proteins (5, 6). However, organisms are able, at least in part, to cope with the toxic Cd. This is primarily ascribed to metallothioneins (MTs), cysteine-rich 6–7-kDa proteins ubiquitously expressed among eukaryotes as well as in some prokaryotes (7–9). MTs contain an α-domain and a β-domain, each with characteristic sequence arrangements of cysteines. The constitutively expressed MTs are obviously able to detoxify Cd (10). However, their actual primary role does not seem to be the detoxification of Cd. Rather, MTs appear to control cellular zinc distribution, translocation, and availability (11), although, besides other functions, they may also act as general anti-stress factors (12).

Scarce information is available suggesting that, besides MTs, also larger non-MT proteins are involved in Cd detoxification (13). However, the investigation of these non-MTs has been oddly neglected to date. Especially some invertebrates have been reported to contain Cd-binding non-MTs but without any evidence for their role in Cd detoxification or molecular characterization (14–17). We have identified a putative Cd-binding non-MT protein as a cDNA in the earthworm Enchytraeus using differential screening of cDNA library (18, 19). These small oligochaetes worms of high ecological relevance due to their function in soil formation and preservation are capable of surviving in acidic soils highly contaminated with Cd (20). The identified cDNA of Enchytraeus encodes a putative cysteine-rich non-MT 25-kDa protein, termed CRP, with eight tandemly arranged repeats exhibiting a characteristic conserved arrangement of cysteines (19). The crp gene is induced by Cd, and its transcript level positively correlates with Cd accumulation of worms (21). However, Cd inducibility of crp-mRNA does not necessarily mean that the CRP protein is directly involved in Cd detoxification. Indeed, a Cd-inducible mRNA encoding the non-MT CIP2 (cadmium-induced protein) has been recently detected in the fungus Candida sp. (22). However, the only 4 cysteines containing CIP2 is presumably not a Cd-binding protein, and it is not directly involved in Cd detoxification. Rather it is assumed to be involved in coping with the oxidative stress induced by Cd. Here, we provide experimental evidence for the actual role of the non-MT CRP protein in Cd detoxification. Transformation of crp-cDNA into Cd-hypersensitive yeast dramatically increases their Cd resistance, the extent of which is dependent on the number of CRP repeats and on the position of the different cysteines in a given CRP repeat.

EXPERIMENTAL PROCEDURES

Constructs of crp in Yeast Vector pRS425—Yeast expression vector pRS425 (23), kindly provided by Dr. G. Jansen (Institute of Microbiology, Heinrich-Heine University, Düsseldorf, Germany), was modified by inserting a 6× c-Myc tag as N-terminal fusion. Different arrangements of crp repeats were constructed by amplifying corresponding regions of the 1474-bp crp-cDNA (GenBank™ accession number X79344) and inserting the PCR products in the SacI restriction site of pRS425. Constructs of crp repeat 4 were cloned in pRS425 vector without a 6× c-Myc tag.

Site-directed Mutagenesis—This was performed with the “Altered Sites in Vitro Mutagenesis” system (Promega, Madison, WI). In brief, PCR-generated fragments of the complete coding region of the crp-cDNA (753 bp) and the crp repeat 4 (93 bp) were cloned in the SacI site of vector pALTER-1. Oligonucleotides used for mutation of the nine cysteines of repeat 4 and the conserved residues at the repeat junctions 4/5 and 6/7, respectively, were as follows: Cys4 (5′-GAGTCGACAATG-GCTCCTGTGTT); Cys5 (5′-AACAATGTGCTCCAGTGGTTCAGGA);
The MT of the free living nematode region comprising repeats 2 play the highest identities of more than 50% to the same CRP similar to CRP. Only the much smaller analysis does not reveal the existence of any other protein consensus sequence of the CRP repeats (Fig. 1 in Cys-Cys and Cys-Cys segments as it is shown for the repeat CRP regions 4 to CRP repeats 6, whereas the human and the mouse MT-III show highest identities of about 37% to CRP repeats 2 and 3. The multiple sequence alignment in Fig. 1B shows that there is a conspicuous conserved distribution of the cysteines in the CRP repeats and in the different MTs, besides some other amino acids such as glycine, lysine, and serine.

For expression in yeast, diverse crp constructs were generated by PCR (Fig. 2A). All crp constructs, with the exception of crp-r4, were N-terminally tagged with a 8-kDa 6x-Myc by cloning in yeast expression vector pRS425 under the control of a MET25 promoter (23). The crp-r4 construct was cloned in pRS425 without a Myc tag to avoid possible steric hindrance by the Myc polypeptide. All constructs were transfected in C. elegans Saccharomyces cerevisiae strain DTY167 (30), which harbors an inactivated gene for the yeast Cd factor (ycf1), normally required for Cd resistance (36). The crp constructs are expressed in approximately equal amounts in yeast (Fig. 2B). The expression rate is low, because the Myc polypeptides are not detectable in Cmono-stained SDS gels but only by Western blot analysis using anti-Myc antibody. Confocal laser scanning microscopy reveals that the 25-kDa CRP is exclusively localized in the cytoplasm but not in the vacuole or nuclei of yeast cells (Fig. 2C).

In order to investigate the possible role of CRP in mediating Cd resistance, the DTY167 cells expressing the 25-kDa CRP (strain designated as DTY167-CRP), the isogenic wild-type DTY165 cells, and the Cd-hypersensitive DTY167-pRS425 cells transformed with empty vector were exposed for 72 h to Cd2+ concentrations up to 500 μM (Fig. 3A). The DTY167-CRP cells do not only restore the Cd resistance but even exhibit a dramatically increased Cd resistance in comparison with wild-type DTY165 cells. At 100 μM Cd2+, DTY167-CRP reaches about 90% of growth that can be observed for control cultures without Cd. By contrast, wild-type DTY165 cells only reach a level of about 60% at 100 μM Cd2+. There is no growth observed for DTY165 at 300 μM Cd2+ and higher concentrations. However, strain DTY167-CRP even tolerates 500 μM Cd2+, because it still reaches 20% of growth level of the untreated control (Fig. 3A). The hypersensitive DTY167-pRS425 cells are unable to grow at the used Cd2+ concentrations.

The levels of Cd resistance restored in DTY167 positively correlate with the increasing number of CRP repeats (Fig. 3B). All transformants expressing CRP repeats, except for DTY167-CRP-r4, show comparable growth at 100 μM Cd2+ after 72 h. Deletion of the N-terminal region and repeat 1 (DTY167-CRPΔr1) has no significant influence on the Cd resistance compared with DTY167-CRP (Fig. 3B). The five repeats expressing transformants DTY167-CRP-r12345 and DTY167-CRP-r12678 have identical growth rates, but both display much lower resistance at 300 μM Cd2+ and higher Cd2+ concentrations compared with DTY167-CRP. Growth of these strains stops at 400 μM Cd2+. A further reduced Cd resistance is observed for those yeasts expressing only three repeats such as DTY167-CRP (Fig. 3C). The five repeats expressing transformants DTY167-CRP-r345 and DTY167-CRP-r678 have equivalent levels of Cd resistance, although the three repeats expressed in the cells are different. Growth of both strains is completely inhibited at 300 μM Cd2+. DTY167-CRP-r4 cells still exhibit a slight Cd resistance at 100 μM Cd2+, because they
reach 15% of the growth of the untreated control cells. Depending on the number of expressed repeats, generation times span between 3.17±0.23 h for DTY167-CRP and 4.60±0.64 h for DTY167-CRP-r4 at 40 μM Cd^{2+}, whereas hypersensitive DTY167-pRS425 cells double only after 13.65±1.73 h at this Cd concentration.

In order to investigate the importance of the individual Cys residues within a given CRP repeat for Cd resistance, Cys→
Ser replacements were introduced in crp-r4 by oligonucleotide-directed site-specific mutagenesis. The nine mutated crp-r4 constructs cloned in vector pRS425 were transformed in hypersensitive strain DTY167 and exposed to 100 μM Cd^{2+} for 72 h. All Cys mutants reveal a dramatic decrease in Cd resistance compared with wild-type CRP-r4 expressing DTY167. Incidentally, the latter cells exhibit different growth depending on the used experimental conditions (cf. Figs. 3 and 4). The extent of the decreased Cd resistance depends solely on the mutated Cys position in crp-r4 (Fig. 4). Cys^3, Cys^14, Cys^22, and Cys^23 are obviously of particular importance, because Cd resistance of the corresponding mutants decreases almost to the level of hypersensitive DTY167-pRS425 cells. Cys^9 and Cys^18 mutants confer a slightly better resistance since reaching about 10% of the growth of DTY167-CRP-r4. Mutations of Cys^1, Cys^7, or Cys^27 are less influential on Cd resistance than the other Cys positions, as the growth of these mutants is reduced by only 60% compared with DTY167-CRP-r4. Moreover, determination of generation times at a sub-lethal concentration of 30 μM Cd^{2+} confirms the relevance of Cys^3. Mutation of that position at least doubles the generation time to 7.9 ± 0.4 h, whereas the generation times of the other mutants span between 3.26 ± 0.08 h (Cys^27) and 3.78 ± 0.28 h (Cys^22). However, wild-type DTY167-CRP-r4 has a doubling time of 3.08 ± 0.1 h at the same Cd concentration.

Besides the conserved Cys residues in the CRP repeats, there are also other conserved amino acids such as Gly, Pro, Val, and Asp at the repeat junctions (Fig. 5A). In order to investigate their role in functioning of CRP in Cd resistance, oligonucleotide-directed site-specific mutagenesis was used to generate amino acid replacements at two repeat junctions in the 25-kDa CRP (Fig. 5A). A Pro^{136} → Leu replacement was introduced in repeat 5 at the junction between repeat 4 → 5, and the mutated cDNA, cloned in pRS425, was expressed in strain DTY167 (designated as DTY167-CRPm4/5). Furthermore, a mutant CRP with an Asp^{196} → Asn replacement in repeat 6 at repeat junction 6 → 7 was constructed (DTY167-CRPm5/6). Comparative determination of the generation times at sub-lethal 40 μM Cd^{2+} and the growth in increasing Cd^{2+} concentrations up to...
Meyer flasks containing 100 Cys replacements were grown in 20 ml of SD medium in 100-ml Erlenmeyer flasks. DTY167 cells transformed with empty pRS425 plasmid or with wild-type crp-r4 were used as controls. Numbers indicate the position of each Cys within repeat 4.

750 μM reveal no significant difference in Cd resistance among native DTY167-CRP, DTY167-CRPm4/5, and DTY167-CRPm6/7 (Fig. 5B).

**Discussion**

Our data provide evidence that, besides MTs, also larger non-MT cysteine-rich proteins are able to detoxify Cd. Indeed, the non-MT 25-kDa CRP protein of the terrestrial earthworm *Enchytraeus* mediates Cd resistance to the Cd-hypersensitive yeast strain DTY167 when transformed by crp. Remarkably, Cd resistance is not only restored but rather dramatically increased in comparison to isogenic wild-type yeast.

The CRP protein is unique to date, i.e., data bank analysis does not reveal any other similar non-MT protein in any other organism. The best fitting alignments can be obtained with MT of different sources. MT of other earthworms such as *Lumbricus* (37) or *Eisenia* (38) display the best identities of about 50–53% to distinct regions of CRP, and even the more distant and diverse MT types of mouse and human exhibit identities of 37–44%. Besides the conserved cysteines, both MT and CRP contain still other conserved amino acids such as lysines, glycines, and serines. This structural similarity suggests a role of CRP in Cd detoxification similar to that of MT. Accordingly, mammalian MTs has been shown to mediate Cd resistance in yeast (39).

In yeast, Cd resistance is normally regulated by complex mechanisms. These involve GSH, the GSH-derived phytochelatins, and diverse membrane transporters (40). The hypersensitive strain we used in our study harbors an inactive ycf1 (yeast cadmium factor) gene (30). The ATP-binding cassette YCF1 protein is localized in the vacuolar membrane and serves as a pump, which transports Cd as a glutathione S-conjugate into the yeast vacuole (36, 41). A similar pump mechanism is mediated in yeast by the transporter MRPI, the human multidrug-associated protein (42). CRP does not simply replace the lost function of YCF1 in Cd resistance of yeast, but rather CRP works by a different mechanism. This view is substantiated by our finding that CRP is uniformly distributed among the cytoplasm and is not detectable in the vacuolar membrane, although the CRP contains a putative transmembrane domain at the N terminus (19).

Currently, two mechanisms of MT in Cd detoxification are considered as follows: (i) chelation of Cd through coordinate covalent bonds to –SH groups of cysteines and/or (ii) scavenging of free radicals originating during Cd-induced stress (8). CRP presumably functions by the same mechanisms. This view is supported by our finding that (i) Cd resistance of yeasts increases with increasing numbers of expressed CRP repeats and that (ii) mutations of distinct cysteines in a given CRP repeat result in a dramatic decrease or even loss of Cd resistance. However, our data also indicate differences in the mode of action of CRP and MT in Cd detoxification in yeast. First, each cysteine of a given CRP repeat is important for Cd resistance. Mutations of the cysteines in the CRP repeat at positions 1, 7, 9, and 27 result in a dramatic reduction of Cd resistance by at least 60%, whereas mutations in Cys3, Cys14, Cys18, Cys22, and Cys27 even result in a complete loss of the capability of mediating Cd resistance. By contrast, mammalian MTs expressed in yeast contain at least some cysteines without any effect on Cd resistance at all (43).

**Fig. 4. Importance of individual Cys residues in CRP repeat 4 for Cd resistance.** DTY167 cells expressing crp-r4 mutants with the Cys replacements were grown in 20 ml of SD medium in 100-ml Erlenmeyer flasks containing 100 μM Cd²⁺. A₅₀₀ was determined after growth for 72 h. DTY167 cells transformed with empty pRS425 plasmid or with wild-type crp-r4 were used as controls. Numbers indicate the position of each Cys within repeat 4.

**Fig. 5. Contribution of CRP structure to Cd resistance.** A, amino acid sequence at repeat junctions 4/5, 5/6, 6/7 with replacements of Pro⁰ → Leu and Asp⁰ → Asn. B, DTY167 cells expressing the CRP mutants CRP-m6/7 (○), CRP-m4/5 (□), as well as the wild-type CRP (○) were inoculated at different Cd concentrations and grown under the conditions described in legend to Fig. 4. A₅₀₀ was determined after growth for 72 h.
Collectively, our data indicate (i) that the non-MT CRP protein is able to detoxify Cd and (ii) that this capability is dependent on the availability of –SH groups similar to MTs. Although our results in yeast cannot yet be considered as applying to the situation in the earthworm *Enchytraeus* without further studies, the differences in the regulation of expression of the *crp* and *mt* genes reported to date suggest different actual physiological roles of both proteins.

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