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PCR-Cloning of Cadmium-Inducible Peptides in the Barnacle, *Megabalanus volcano*

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ABSTRACT—A 340 bp DNA fragment was amplified from barnacle (*Megabalanus volcano*) cDNA by polymerase chain reaction using primers designed based on the amino acid sequences of barnacle cadmium-inducible peptides CdIP1 and CdIP2. The whole sequence was determined by rapid amplification of cDNA ends method. The cDNA contained an open reading frame encoding 71 amino acid residues and the sequences for CdIP1 and CdIP2 were found to be located in the center of this coding region. Although CdIP1 and CdIP2 had been detected only in the cadmium-exposed barnacles, their mRNA was present both in cadmium-exposed barnacles and in unexposed barnacles. These results suggest that posttranslational proteolytic processing may be induced in the presence of cadmium.

Key words: barnacle, cadmium, peptide, PCR, proteolytic processing

INTRODUCTION

Marine organisms are continuously exposed to various metals, some of which are essential and some of which are toxic to organisms. Mechanisms of utilization or detoxification of metals in marine organisms have not been clarified yet, but metals are bound by some ligands such as low-molecular-weight metal-binding proteins (Hamer, 1986). In the case of cadmium, one of the well-known low-molecular-mass cadmium-binding and -inducible proteins is metallothionein (MT), which is thought to be concerned with detoxification of cadmium (Hamer, 1986). On the other hand, many non-MT-like cadmium-binding/inducible proteins which are characterized by low cysteine content have also been found in marine organisms, such as molluscan *Crassostrea virginica* (Ridlington and Fowler, 1979), *Buccinum tenuissimum* (Dohi *et al*., 1983), *Nassarius reticulatus* (Andersen *et al*., 1989), annelidan *Nereis diversicolor* (Nejmeddine *et al*., 1988) and *Allolobophora caliginosa* (Nejmeddine *et al*., 1992). Most of non-MT-like proteins were found as cadmium-binding proteins and thought to be cadmium-inducible. Molecular weights of MTs and other non-MT-like proteins have been reported to be 5-20 kDa, especially in the range of 6-10 kDa.

Previously we reported two unique cadmium-inducible peptides CdIP1 and CdIP2 in barnacle, *Megabalanus volcano* (Togi *et al*., 1998a,b). They were detected only in cadmium-exposed *M. volcano*, but not in unexposed ones. CdIP1 and CdIP2 are composed of 15 and 19 amino acids and their molecular weights are 1703 and 2004, respectively. Their amino acid sequences are nearly identical; CdIP2 has an additional four amino acids in the amino-terminus of CdIP1. They are rich in glutamic acid and thought to be a kind of non-MT-like cadmium-inducible proteins.

In the present study, we report the cDNA sequence of *M.volcano* CdIP and site-specificity of expression of CdIP (CdIP1 and CdIP2) mRNA, and suggest the possibility of posttranslational proteolytic processing for production of CdIP.

MATERIALS AND METHODS

RNA extraction and cDNA synthesis

Barnacles, *Megabalanus volcano*, were collected from Izu (Japan) and were exposed to 200 μg of Cd/l of sea water for 4 weeks at room temperature. Total RNA was extracted from the internal organs by a total RNA separator kit (CLONTECH Laboratories, USA), and mRNA was purified from the total RNA using oligo(dT) beads, Oligotex^{TM}-dT30 Super (Japan Synthetic Rubber, Japan). Double-stranded cDNA was synthesized from the mRNA by a cDNA synthesis module (Amersham Pharmacia Biotech, Sweden).

Polymerase chain reaction (PCR) and sequencing of the PCR product

From the amino acid sequence of CdIP2, a degenerate oligonucleotide primer P-1 was designed and synthesized (Japan Bio Service, Japan): 5'-GT(AGCT)GG(AGCT)GA(AG)AT(ACT)GA(AG)-AA(AG)(AC)G-3' (see Fig. 1). The reaction mixture (20 μl) for PCR

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contained 2.5 ng of the template cDNA, 0.6 μg of the mixed primer P-1, 0.6 μg of anchored oligo(dT) $_{25}$ (a mixed population of three primers, dT_{25} with dA, dC or dG at the 3' end), 200 μM dNTPs and 0.5 units of recombinant *Taq* DNA polymerase in the PCR buffer (TaKaRa, Japan). The reaction was carried out for 1 min at 94 ˚C, 1 min at 42°C, and 2 min at 75°C and was repeated for 30 cycles, and the final step at 75°C was extended to 10 min, by using a Mini Cycler^{TM} (MJ Research, USA).

The PCR product was fractionated in a 3% agarose gel (Nusieve 3:1; FMC Bioproducts, USA) and purified by glass-milk/sodium iodine method using a GENE CLEAN^{TM} kit (BIO101, USA). The DNA fragment was then subcloned to pT7Blue(R) T-vector (Novagen, USA) and introduced into *Escherichia coli* JM109. The plasmid DNA was purified with a Plasmid Mini kit (QIAGEN, Germany), and the cloned DNA was sequenced with a 373 DNA sequencer (Applied Biosystems, USA) using a Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase FS (Perkin Elmer, USA).

5'-RACE (Rapid Amplification of cDNA Ends) and 3'-RACE

The whole cDNA sequence was obtained through 5'- and 3'-RACE reactions (Frohman *et al.*, 1988). Primers 5RACEAP-1, 5RACEAP-2 and 5RACEAP-3 were used for 5'-RACE, and 3RACEP-1 and 3RACEP-2 were used for 3'-RACE (see Fig. 1). Following the reverse transcription reaction using an RT-PCR kit (STRATAGENE, USA), PCRs were performed twice. The reaction was carried out for 1 min at 94°C, 1 min at 50°C, and 2 min at 75°C and was repeated for 30 cycles, and the final step at 75°C was extended to 10 min. The second PCR products were separated in a 3% agarose gel and subcloned to pT7Blue(R) T-vector and introduced into *E. coli* JM109. The cloned DNAs were sequenced as mentioned above.

To confirm the cDNA sequence of CdIP, PCR was performed using primer P-11 and the oligo(dT)$_{25}$ (see Fig. 1). The reaction was carried out for 1 min at 94°C, 1 min at 42°C, and 2 min at 75°C and was repeated for 30 cycles, and the final step at 75°C was extended to 10 min. The sequence was determined as mentioned above.

Northern blot analysis

M. volcano total RNAs were extracted by a total RNA separator kit (CLONTECH Laboratories) from unexposed barnacles and

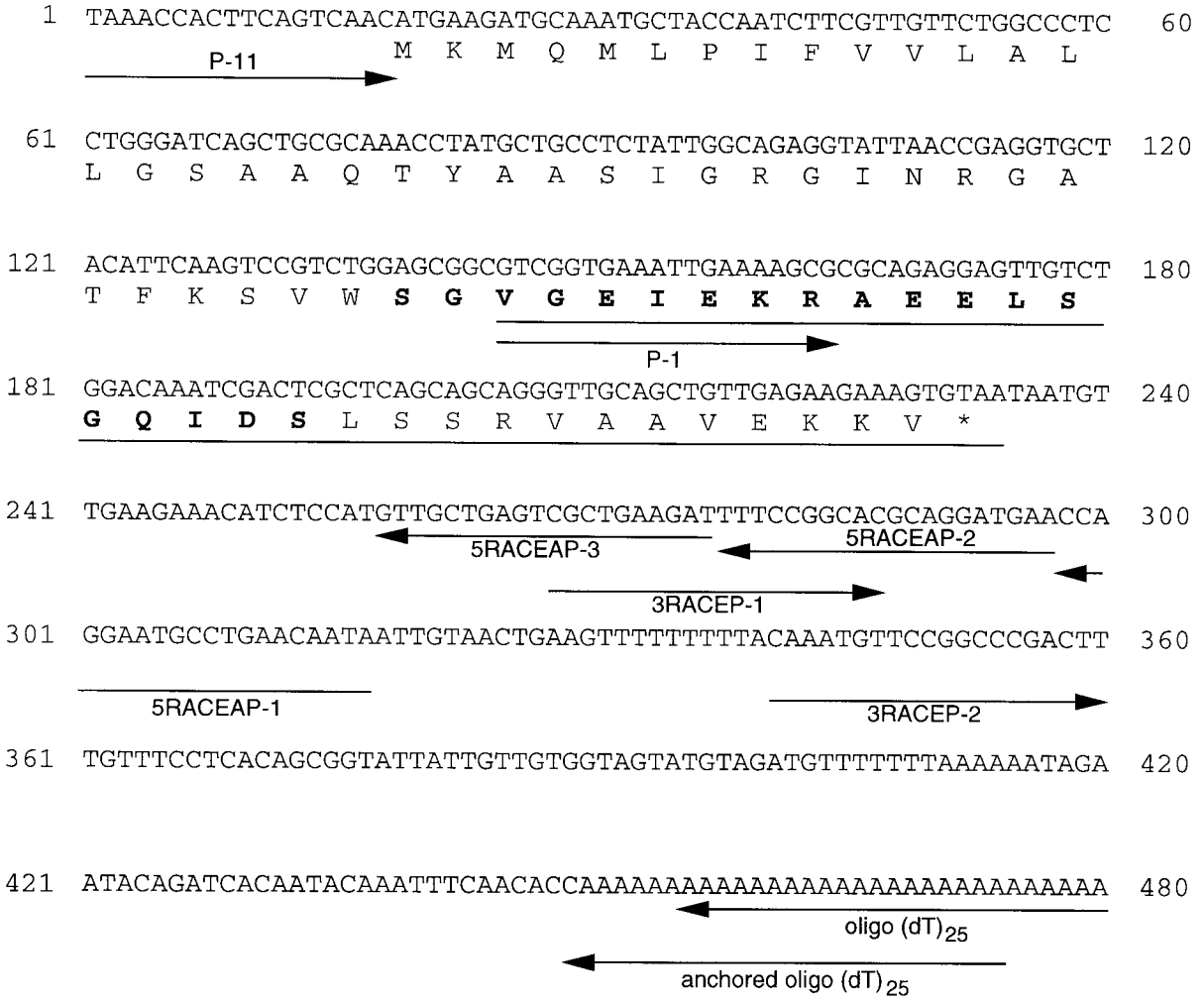


Fig. 1. The cDNA sequence of CdIP and the deduced amino acid sequence. The cDNA sequence of CdIP determined by RACE method and the deduced amino acid sequence are shown. The CdIP amino acid sequence is written in boldface. Arrows show primers used for the first PCR and RACE method: P-1 and anchored oligo(dT)$_{25}$ were used for the first PCR. 5RACEAP-1, 5RACEAP-2 and 5RACEAP-3 were used for 5'-RACE, and 3RACEP-1 and 3RACEP-2 were used for 3'-RACE. P-11 and oligo(dT)$_{25}$ were used for the final PCR. A probe for northern blotting is underlined.

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barnacles exposed to 200 μg of Cd/l of sea water for 1 and 5 days. All preparations were performed separately for each of five individual barnacles. Twenty micrograms of each total RNA was fractionated in a 3% denaturing agarose gel containing formaldehyde and subsequently transferred onto Hybond^{TM} N^+ Nucleic acid transfer membrane (Amersham Pharmacia Biotech). A probe was designed from the sequence of the first PCR product and was prepared by PCR (see Fig. 1). The probe was ^{32}P-labeled by using a Random Primer DNA labeling kit Ver. 2 (TaKaRa) and hybridized to the membrane for 14 hr at 50°C. Blots were finally washed with 0.2 x SSC for 60 min at 60°C. Signals were detected by a FUJIX BAS 1000 (FUJI PHOTO FILM, Japan) after exposure to the imaging plate BAS-IIIs (FUJI PHOTO FILM).

To analyze expression of CdIP mRNA in each internal organ, total RNAs were prepared from three barnacles' internal organs: the muscle and the epidermis, the cirri, the stomach and the intestine, the sperm reservoir, the ovary, and the egg mass in the mantle cavity, respectively. Ten micrograms of each total RNA was fractionated by denaturing agarose electrophoresis and transferred onto Hybond^{TM} N^+ Nucleic acid transfer membrane. Hybridization was performed as described above.

RESULTS AND DISCUSSION

The cDNA sequence of CdIP

A 340 bp DNA fragment was amplified from *M. volcano* cDNA by PCR using primers anchored oligo(dT) $_{25}$ and P-1 designed based on the amino acid sequence of CdIP2. After sequencing of the 340 bp DNA, 5'- and 3'-RACE was performed to determine the cDNA sequence of the upstream and the downstream region. Following determination of the sequences of the RACE products, PCR was performed using primers P-11 and the oligo(dT)$_{25}$ (see Fig. 1) to amplify the entire cDNA of CdIP. The plasmids of 6 clones were sequenced and their sequences were all identical. The determined CdIP cDNA sequence and the deduced amino acid sequence are shown in Fig. 1. No genes homologous with CdIP were found on the DDBJ database.

The cDNA contained an open reading frame encoding 71 amino acid residues. As shown in Fig. 2 (a), the deduced amino acid sequence of the precursor protein seemed to consist of 4 regions: putative signal peptide region (Von Heijne, 1986), PEP1 (peptide1) region (24 amino acid residues), CdIP region and PEP2 (peptide2) region (12 amino acid residues). CdIP was located in the center of the coding region. The calculated isoelectric point and the hydrophilicity of each region (except for the putative signal peptide) are shown in Figs. 2 (a) and (b), respectively. They suggest a different characteristic of CdIP from other regions. The calculated isoelectric point of CdIP is acidic, while those of the other regions are basic. The region of CdIP is more hydrophilic than those of PEP1 and PEP2. The secondary structure prediction of proCdIP (the peptide containing PEP1,

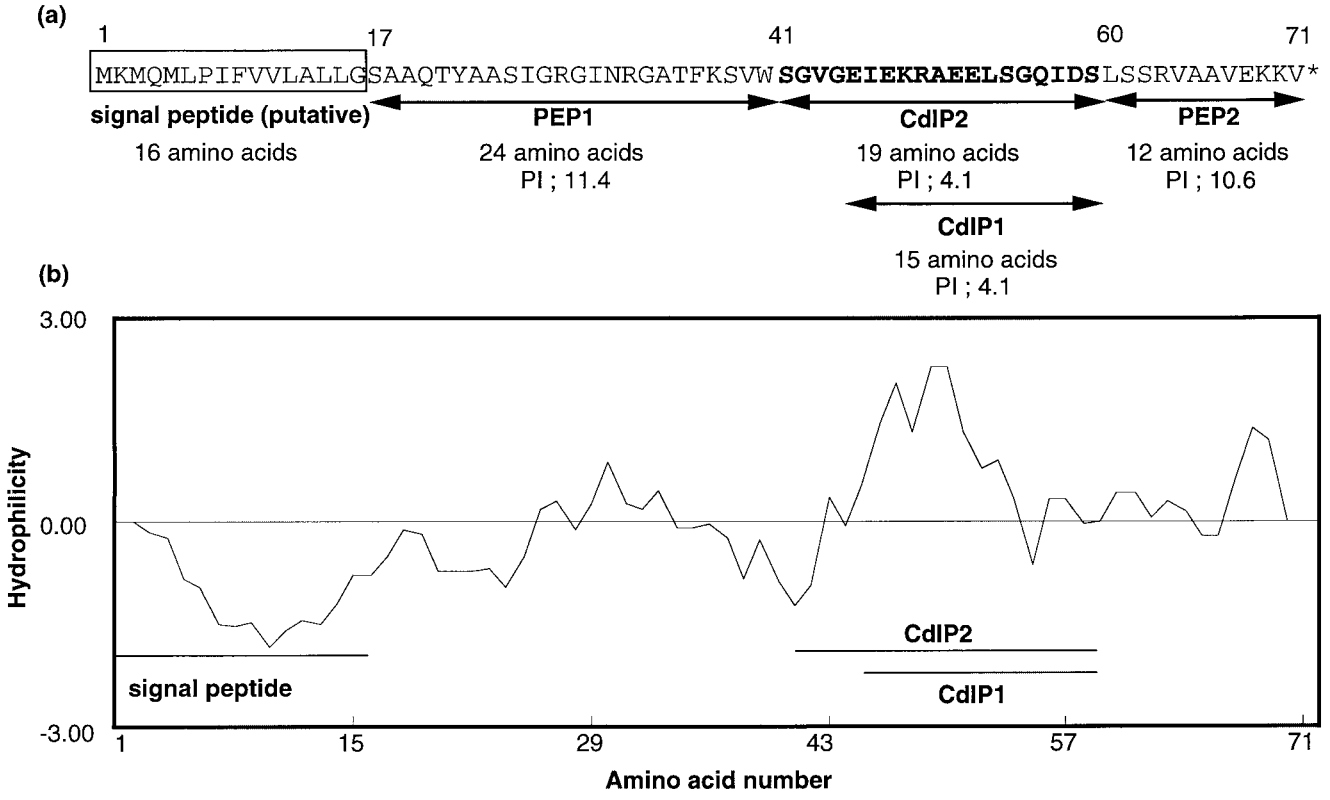


Fig. 2. The isoelectric point and the hydropathy profile of CdIP. (a) The deduced amino acid sequence from the cDNA sequence of CdIP is shown. It can be divided into 4 regions: signal peptide, PEP1, CdIP and PEP2. The isoelectric point of each region (except for the putative signal peptide) is also given. (b) Hydropathy profile of the deduced amino acid sequence is depicted. The hydrophilicity was calculated based on parameters in Hopp and Woods' method (Hopp and Woods, 1981).

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CdIP and PEP2) by Chou-Fasman's method (Chou and Fasman, 1978) suggested α-helical structure.

After cleavage of the signal peptide, CdIP may be released from proCdIP by proteolytic processing, which is often seen in other bioactive peptides. The predicted digestion sites for processing of proCdIP were Trp-Ser and Ser-Leu. Further study is required to confirm the processing.

Expression of CdIP mRNA

To investigate whether CdIP mRNA was cadmium-inducible or not, northern blot analysis was performed. Total RNAs were extracted from cadmium-exposed and unexposed *M. volcano*. As shown in Fig. 3, a single positive band could be detected both in cadmium-exposed and in unexposed *M. volcano* total RNA. This result showed that CdIP mRNA was present both in cadmium-exposed and in unexposed *M. volcano*, and that the expression was constitutive. We reported previously that CdIP1 and CdIP2 are cadmium-inducible because they were not detected in unexposed but only in cadmium-exposed *M. volcano* (Togi *et al.*, 1998). These results suggest that posttranslational proteolytic processing may occur in the presence of cadmium.

Expression of CdIP mRNA in each internal organ

To analyze expression of CdIP mRNA in each internal organ, northern blot analysis was performed for total RNA isolated from each internal organ of *M. volcano* (Fig. 4). The signal was detected in the muscle and epidermis, cirri, stomach and intestine, and sperm reservoir, but not in the ovary nor the egg mass in the mantle cavity. The signal was the strongest in sperm reservoir.

It has been reported that MTs and non-MT-like cadmium-binding/inducible proteins were found in the liver, kidney, intestine, pancreas, spleen, duodenum, placenta, brain, heart and testis in vertebrates, and also in the liver, kidney, hepatopancreas, gills, mantle and soft tissue in invertebrates (Hamer, 1986). Neither MT nor non-MT-like cadmium-binding/inducible protein has been found in the ovary. The fact that the site-specificity of CdIP is similar to those of MTs or non-MT-like cadmium-binding/inducible proteins may indicate that CdIP has functions like them.

In the present study, the cDNA sequence of CdIP was

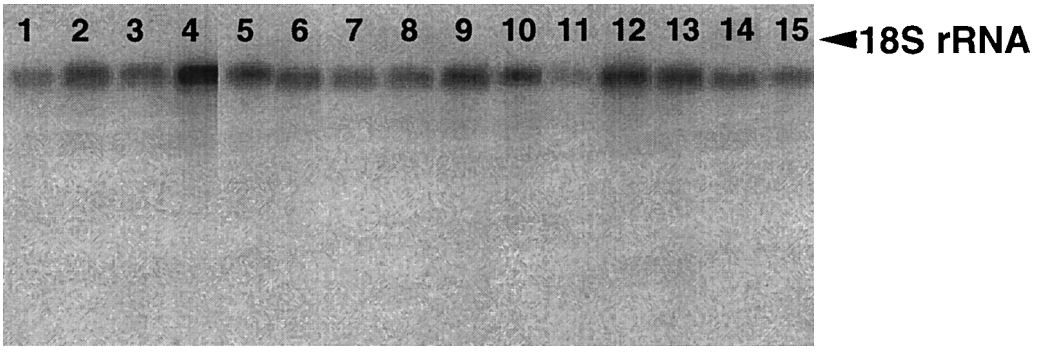
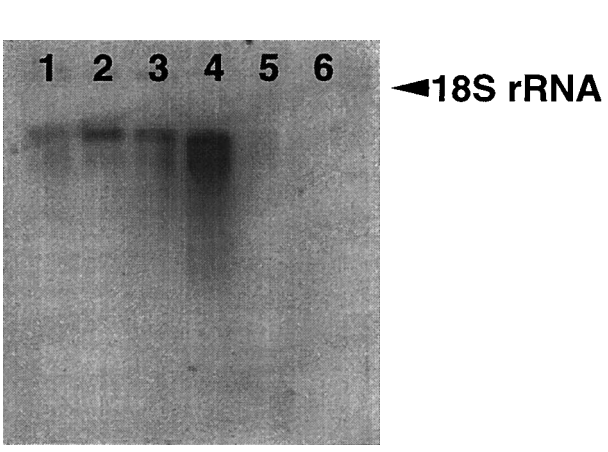


Fig. 3. Northern blot analysis of cadmium-exposed and unexposed *M. volcano*. Lanes 1–5, total RNAs isolated from unexposed *M. volcano*; Lanes 6–10, total RNAs isolated from *M. volcano* exposed to cadmium for 1 day; Lanes 11–15, total RNAs isolated from *M. volcano* exposed to cadmium for 5 days. An arrowhead shows the 18S rRNA.



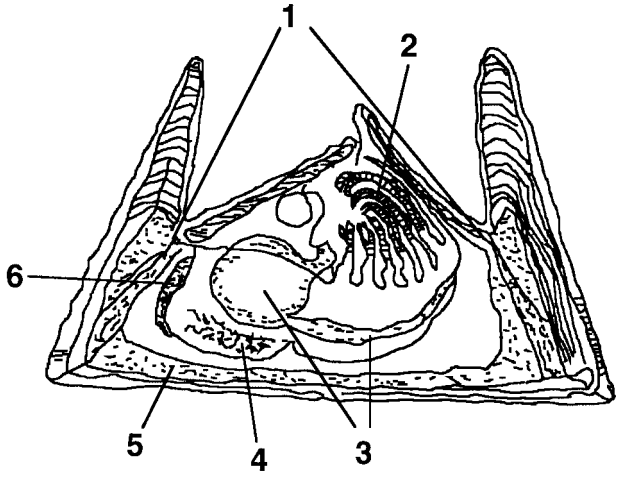


Fig. 4. Northern blot analysis of each internal organ of *M. volcano*. Lanes: 1, the muscle and the epidermis; 2, the cirri; 3, the stomach and the intestine; 4, the sperm reservoir; 5, the ovary; 6, the egg mass in the mantle cavity. An arrowhead shows the 18S rRNA.

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determined. The deduced amino acid sequence and northern blot analysis suggest that CdIP may be subjected to proteolytic processing in the presence of cadmium. Further studies are necessary to prove this hypothesis. Detection of a precursor of CdIP (proCdIP) and processed peptides (PEP1 and PEP2) and analysis of their localization are being tried using antibodies.

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