Increased heavy metal sensitivity of *Escherichia coli* producing the expression product of *priA* gene derived from the basidiomycete *Lentinus edodes*

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Abstract

We have previously isolated a developmentally regulated novel gene, *priA*, from the basidiomycete *Lentinus edodes*. The deduced PRIA protein contains the two set of motifs similar to a ‘zinc finger’ typified by transcription factor TFIIIA and the motif of a ‘zinc cluster’ observed in metallothioneins. It also contains a hydrophobic N-terminal sequence. Here *Escherichia coli* cells producing PRIA were found to show a remarkable sensitivity to zinc ion and other heavy metal ions such as nickel and cadmium. Deletion analysis of PRIA revealed that the zinc-binding motifs and the hydrophobic N-terminal sequence are responsible for conferring the heavy metal sensitivity on the host cells. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Zinc-binding motif; Heavy metal sensitivity; *Escherichia coli*; Developmentally regulated gene; *Lentinus edodes*

1. Introduction

The *priA* gene, derived from the basidiomycete *Lentinus edodes* (shiitake), has been shown to be actively transcribed in primordia and immature fruiting bodies [1]. The deduced PRIA protein (258 amino acids), rich in Ser (42 residues), Pro (29 residues) and Thr (27 residues), initiated with a hydrophobic amino acid sequence and terminated with the unique sequence, Cys-Aaa-Aaa-Xaa (where Aaa is an aliphatic amino acid and Xaa is any amino acid), found in various proteins such as fungal mating factors, nuclear lamins, Ras and Ras-related proteins [2]. Polyisoprenylation of Cys residue is known to promote membrane interactions of these proteins. This implies the possibility that the PRIA protein is a membrane-associated protein.

The PRIA protein contained different types of putative zinc-binding motifs (see Fig. 2) [1]. The first two sets of motifs resemble a ‘zinc finger’ typified by TFIIIA [3]. The numbers of the amino acid residues in the loop and between the first two Cys in the PRIA protein, however, are larger than those conserved in TFIIIA. The Phe and Leu conserved in the loop of TFIIIA are absent in that of the PRIA pro-
tein. The second motif looks like a so-called ‘zinc cluster’ observed in metallothioneins [4]. The zinc cluster-like motif was rich in Cys, Asp and Glu. These residues are known to be usual donors for coordinating to zinc ions in zinc metalloenzymes [5]. This led us to attempt the preparation of the PRIA protein in Escherichia coli and analysis of its biochemical properties. During the course of these experiments, we found that E. coli cells producing the recombinant PRIA protein exhibit a remarkable sensitivity to zinc ions and other heavy metal ions.

2. Materials and methods

2.1. Plasmid construction

Full length cDNA of priA (named priAc; 1.3 kb) obtained from the recombinant plasmid pUC19-priAc [1] by digestion with BamHI was inserted into the BamHI site of pMAL-c2 [6], yielding the recombinant plasmid pMAL-c2-priA. The priAc obtained from pUC19-priAc by digestion with EcoRI was ligated to the EcoRI site of pGEX-2TK [7], yielding the recombinant plasmid pGEX-2TK-priA.

A set of pMAL-c2-priA deletion derivatives lacking various parts of the PRIA-encoding region were constructed (see Fig. 2). In these experiments, 5′-protruding ends of restriction fragments were blunted by repair DNA synthesis using Klenow fragment of E. coli DNA polymerase I and 3′-protruding ends were blunted by the 3′ → 5′ exonuclease activity and then the polymerizing activity of T4 DNA polymerase. The nucleotide sequence of the fusion junction of recombinant plasmid DNA was confirmed by sequencing [8]. The priAc 0.7-kb NheI–EcoRI fragment was blunt and inserted in-frame into PstI-cleaved and blunt pMAL-c2, yielding the plasmid pMAL-c2-priA(80−777). The priAc 0.27-kb ApaLI–EcoRI fragment was blunt and inserted in-frame into XbaI-cleaved and blunt pMAL-c2, yielding pMAL-c2-priA(509−777). The priAc 94-bp EcoRI-FokI fragment was blunt and ligated to the BamHI linker, and then inserted in-frame into the BamHI site of pMAL-c2-priA(509−777), yielding pMAL-c2-priA(1−91, 509−777). The priAc 0.48-kb MfeI–MfeI fragment was inserted in-frame between PstI and XbaI sites of pMAL-c2, yielding pMAL-c2-priA(80−568). Plasmid pMAL-c2-priA was digested with MfeI and PstI, blunted and recircularized, yielding pMAL-c2-priA(1−568).

Other recombinant plasmids used for a control were constructed as follows. The priB cDNA 2.3-kb fragment containing the entire coding sequences of the PRIB and 3′ untranslated sequence [9] was inserted into the BamHI site of pMAL-c2, yielding pMAL-c2-priB. The mfbA cDNA 1.3-kb fragment [10] was inserted into the BamHI site of pMAL-c2, yielding pMAL-c2-mfbA(1746−3031). The priB cDNA 0.55-kb fragment was inserted into the EcoRI site of pGEX-2TK, yielding pGEX-2TK-priB(1−552).

2.2. Other methods

pMAL-c2- and pGEX-2TK-series plasmids were introduced into E. coli DH5α (supE44 ΔlacU169(80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1) [11] and BL21 (hsdS gal(λ, clts857 indI Sam7 nin5 lacUV5-T7gene1)) [11], respectively. E. coli transformation was carried out according to the method of Inoue et al. [12]. pMAL-c2- and pGEX-2TK-series plasmids used for protein expression, all carry the tac promoter which is induced using the lactose analogue isopropyl-1-thio-β-D-galactopyranoside (IPTG). Transformed E. coli strains were cultured in LB medium containing 100 µg ml−1 of ampicillin at 37°C for 1.5−2 h. To the cultures IPTG (0.1 mM) and heavy metal ions (the indicated concentrations) were added and cultured at 37°C for the indicated periods. Growth rate was analyzed by monitoring the increase in absorbance at 600 nm. For preparation of E. coli protein extracts, cultures of transformed strains at 37°C for 2 h were induced by IPTG (0.5 mM) for 2.5 h and the cells were harvested by centrifugation. The cell pellets were washed three times with ice-cold PBS and disrupted with a sonicator. The protein extracts (30 µg each) were resolved by 0.1% SDS-10% polyacrylamide gel electrophoresis (PAGE) and subsequently stained with Coomassie brilliant blue.
Fig. 1. Production of the PRIA protein and other proteins (A) and PRIA deletion derivatives (B) in E. coli by the fusion/expression vectors. A: DH5α[pMAL-c2] (lanes 1 and 2), DH5α[pMAL-c2-priA] (lane 3), DH5α[pMAL-c2-priB] (lane 4) and DH5α[pMAL-c2-mfbA(1746–3031)] (lane 5) and BL21[pGEX-2TK] (lanes 6 and 7), BL21[pGEX-2TK-priA] (lane 8) and BL21[pGEX-2TK-priB(1–552)] (lane 9) were cultivated in the absence (lanes 1 and 6) or presence (lanes 2–5, 7–9) of IPTG. B: DH5α[pMAL-c2] (lane 1), DH5α[pMAL-c2-priA] (lane 2), DH5α[pMAL-c2-priA(80–777)] (lane 3), DH5α[pMAL-c2-priA(1–568)] (lane 4), DH5α[pMAL-c2-priA(1–568)] (lane 5), DH5α[pMAL-c2-priA(80–568)] (lane 6) and DH5α[pMAL-c2-priA(509–777)] (lane 7) were cultivated in the presence of IPTG. In both panels, the protein extracts (30 μg each) prepared from the plasmid-harboring E. coli strains were resolved by SDS-PAGE and subsequently stained with Coomassie brilliant blue. Markers are shown in lane M. Asterisks on the right of the proteins show each of the fusion proteins.
3. Results and discussion

3.1. Production in E. coli of PRIA protein

The *L. edodes priA* gene product was expressed in *E. coli* using maltose-binding protein (MBP) fusion vector pMAL-c2 [6] and glutathione S-transferase (GST) fusion vector pGEX-2TK [7]. *E. coli* DH5α cells carrying the plasmid pMAL-c2-priA (named DH5α[pMAL-c2-priA]) and *E. coli* BL21 cells carrying the plasmid pGEX-2TK-priA (named BL21[pGEX-2TK-priA]) produced MBP-PRIA (70 kDa) and GST-PRIA (54 kDa), respectively, upon induction of the cultures by IPTG (Fig. 1A).

3.2. Susceptibility to zinc ion and other heavy metal ions of *E. coli* cells producing PRIA protein

*E. coli* DH5α[pMAL-c2-priA] cells were cultivated in LB liquid medium containing 1 mM ZnSO₄ and 0.1 mM IPTG. As a control, we cultivated DH5α[pMAL-c2], DH5α[pMAL-c2-priB] and DH5α[pMAL-c2-mfbA(1746–3031)] producing MBP(-LacZα), MBP-PRIB and MBP-MFBA(582–1006), respectively (Fig. 1A). The *priB* and *mfbA* genes have been isolated from *L. edodes* [9,10]. The PRIB protein (565 amino acids) is a transcription factor with a Zn(II)₂Cys₆ zinc cluster DNA-binding motif [9]. The MFBA(582–1006) peptide is the fragment (425 amino acids) of MFBA protein (2157 amino acids) and contains a cell-surface attachment-promoting RGD motif [10]. As shown in Table 1, the growth of DH5α[pMAL-c2-priA] was remarkably slow when compared with those of DH5α[pMAL-c2], DH5α[pMAL-c2-priB] and DH5α[pMAL-c2-mfbA(1746–3031)]. If DH5α[pMAL-c2-priA] was cultivated in the absence of ZnSO₄ and the presence or absence of IPTG, its growth was similar to those of the three other strains cultivated under the same conditions. The DH5α[pMAL-c2-priA] producing the MBP-PRIA protein had a doubling time of 122 min in the absence of ZnSO₄ (and presence of 0.1 mM IPTG) and 290 min in the presence of 1 mM ZnSO₄ (and presence of 0.1 mM IPTG). The growth rate was calculated by division of 122 by 290 to be 42%. In the same manner, the growth rates of DH5α[pMAL-c2], DH5α[pMAL-c2-priB] and DH5α[pMAL-c2-mfbA(1746–3031)] were calculated to be 85%, 75% and 87%, respectively. These results suggest that the susceptibility to zinc ion of *E. coli* DH5α cells markedly increased by expressing the PRIA protein. The effect of PRIA production was also tested for *E. coli* BL21 (Table 1). The growth of BL21[pGEX-2TK-priA] in the presence of 1 mM ZnSO₄ and 0.1 mM IPTG was significantly slow as compared with those of BL21[pGEX-2TK] and BL21[pGEX-2TK-priB(1–552)]. The *priB*(1–552) encodes the amino acid sequences containing the

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Fig. 2. Zinc sensitivity of *E. coli* strains producing recombinant PRIA protein and its deletion derivatives. The percentage growth rates of *E. coli* strains were calculated as described in the note to Table 1. The numbers in parentheses of the proteins and plasmids are the positions of amino acid residues and nucleotide residues, respectively.
Zn(II)2Cys6 zinc cluster DNA-binding domain of PRIB protein [9]. Analogously to the case of DH5α, the growth rates of BL21[pGEX-2TK-priA], BL21[pGEX-2TK] and BL21[pGEX-2TK-priB(1–552)] were calculated to be 5%, 86% and 77%, respectively. The zinc sensitivity of BL21 cells producing PRIA protein was significantly higher than that of DH5α cells producing it. This seems to be due to the difference in the level of PRIA production between the two strains (see Fig. 1). The zinc sensitivity of the E. coli cells producing PRIB (or its deletion) protein was slightly higher than that of the E. coli cells containing the vector alone or producing MFBA fragment. We will discuss this point later.

Furthermore, the effects of nickel and cadmium ions on the growth of E. coli DH5α[pMAL-c2-priA] were examined. Upon induction by IPTG, the growth rate of DH5α[pMAL-c2-priA] in the presence of 1.5 mM NiSO4 or 0.5 mM CdCl2 markedly decreased to almost the same level as that in the presence of 1 mM ZnSO4 (data not shown).

### 3.3. The domain(s) of PRIA protein responsible for conferring the zinc sensitivity on E. coli

To analyze the domain(s) of PRIA protein responsible for conferring the zinc sensitivity on E. coli, a set of pMAL-c2-priA deletion derivatives lacking various parts of the PRIA-coding region (see Fig. 2) were constructed and introduced into DH5α. The growth of DH5α[pMAL-c2-priA(1–91, 509–777)] producing MBP-PRIA(1–30, 171–258) protein (Fig. 1B) and DH5α[pMAL-2c-priA(1–568)] producing MBP-PRIA(1–189) protein (Fig. 1B) was significantly inhibited in the presence of 1 mM ZnSO4 (Fig. 2). However, the growth of DH5α[pMAL-c2-priA(80–777)] producing MBP-PRIA(28–258) protein (Fig. 1B) and DH5α[pMAL-c2-priA(80–568)] producing MBP-PRIA(28–189) protein (Fig. 1B) was partly inhibited in the presence of ZnSO4 (Fig. 2); the growth rates of these strains were similar to that of the (MBP-PRIB)-producing strain (see Table 1). The DH5α[pMAL-c2-priA(509–777)] producing MBP-PRIA(171–258) protein (Fig. 1B) exhibited the similar growth rate to that of DH5α[pMAL-c2] in the presence of ZnSO4 (Fig. 2 and Table 1). These data suggest that both the hydrophobic N-terminal region and either the zinc finger-like motifs or the zinc cluster-like motif are required for conferring the zinc sensitivity on E. coli DH5α. The growth rate of the DH5α strain producing intact PRIA protein was clearly higher than those of the DH5α strains producing the PRIA fragments with the hydrophobic N-terminal region and either one of the zinc-binding motifs. Although we have no data explaining these results, it is possible to consider that the tertiary structures of intact PRIA protein and PRIA fragments are different from each other and the structures of the latter function more harmfully to E. coli cells.

### Table 1

Zinc susceptibility of E. coli strains producing various recombinant proteins

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Protein</th>
<th>Doubling time (min)</th>
<th>Growth rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(~IPTG and ~ZnSO4) (a)</td>
<td>(+IPTG and ~ZnSO4) (b)</td>
<td>(+IPTG and ZnSO4) (c)</td>
</tr>
<tr>
<td>pMAL-c2-priA</td>
<td>MBP-PRIA</td>
<td>68</td>
<td>122</td>
</tr>
<tr>
<td>pMAL-c2</td>
<td>MBP(-LacZo)</td>
<td>73</td>
<td>102</td>
</tr>
<tr>
<td>pMAL-c2-priB</td>
<td>MBP-PRIB</td>
<td>73</td>
<td>109</td>
</tr>
<tr>
<td>pMAL-c2-mfBA(1746–3031)</td>
<td>MBP-MFBA(582–1006)</td>
<td>69</td>
<td>95</td>
</tr>
<tr>
<td>pGEX-2TK-priA</td>
<td>GST-PRIA</td>
<td>75</td>
<td>175</td>
</tr>
<tr>
<td>pGEX-2TK</td>
<td>GST</td>
<td>68</td>
<td>137</td>
</tr>
<tr>
<td>pGEX-2TK-priB(1–552)</td>
<td>GST-PRIB(1–184)</td>
<td>70</td>
<td>155</td>
</tr>
</tbody>
</table>

Various E. coli strains were cultivated under the indicated (a, b and c) conditions. Three independent cultivations were done for each of the strains. The doubling times were determined for the exponential cultures of various strains under the indicated conditions. The percentage growth rates were calculated by division of the doubling times determined under condition b by those determined under condition c.
marked sensitivity to not only zinc ion but nickel and cadmium ions. The hydrophobic N-terminal region and zinc-binding motif of PRIA protein are shown to be necessary for its harmful effect on E. coli. PRIB protein also possesses the zinc cluster DNA-binding motif Zn(II)2Cys6. However, it conferred only a slight inhibitory effect on E. coli. This may probably be due to the absence of a noticeable hydrophobic region in PRIB. There is the possibility that interaction of the hydrophobic N-terminal region of PRIA with substance(s) of the inner membrane of E. coli is required for an expression of the significant toxic effect of PRIA. In E. coli, metal resistance often involves an exclusion of the metal ions from the cells [13,14]. The PRIA protein presumably interferes with this exclusion process. Previous papers reported that small metal-binding proteins, metallothioneins, which confer resistance to heavy metal ions on eukaryotic cells, also endow E. coli with the resistance to heavy metal ions [15,16]. Metallothioneins are considered to decrease the concentration of free toxic metal ions via their zinc cluster domain. To solve the problem why PRIA protein exhibits the toxic function in E. coli, a more detailed analysis is necessary.

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References