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Studies on the role of HtpG in the tetrapyrrole biosynthesis pathway of the cyanobacterium *Synechococcus elongatus* PCC 7942

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Abstract

In cyanobacterium *Synechococcus elongatus* PCC 7942, we observed that *htpG*-overexpression caused remarkable growth inhibition. In addition, subcellular fractionation experiments showed that HtpG was localized in the membrane fraction. To understand its function in cyanobacteria, we carried out yeast two-hybrid screening to identify specific proteins interacting with HtpG, and found out, HemE, uroporphyrinogen decarboxylase. When compared to the wild-type strain, the *htpG*-null mutant and -overexpressing strains exhibited higher and lower cytosolic HemE activity, based on the coproporphyrin production, respectively. These results strongly suggest that HtpG is involved in the regulation of tetrapyrrole biosynthesis through interacting with HemE protein.

Keywords: HtpG; Hsp90; HemE; Uroporphyrinogen decarboxylase; Tetrapyrrole; Thylakoid membrane

Hsp90 proteins constitute one of the most conserved Hsp families. In eukaryotes, Hsp90 plays important roles in diverse signal transduction pathways. However, little is known about the roles of HtpG in bacteria, even though this molecular chaperone is highly expressed under various stress conditions. In heterotrophic eubacteria such as *Escherichia coli*, *htpG*-null strains behave like wild-type strains and show no specific phenotype [1]. Interestingly, in cyanobacteria *Synechococcus elongatus* PCC 7942 (previously called *Synechococcus* sp. PCC 7942 [2], and hereafter referred to as *Synechococcus*) and *Synechocystis* sp. PCC 6803, *htpG*-null mutation resulted in heat sensitivity and significant reduction in phycocyanin absorption [3–5]. Moreover, we previously reported that *Synechococcus* HtpG increased in response to various stress such as heat, cold, high light, and oxidation [5–8]. The genome analyses revealed that *htpG* gene is highly conserved in all cyanobacterial strains so far analyzed, but not in all bacteria particularly in Gram-positive low G + C bacteria. These reports suggest that HtpG may have unique physiological roles in cyanobacteria.

In this communication, we suggest a novel function of HtpG in *Synechococcus*. During our comprehensive study of the protein–protein interactions using yeast two-hybrid screening against *Synechococcus* genomic library, a specific interaction between HtpG and HemE (uroporphyrinogen decarboxylase, EC 4.1.1.37) was identified. The possible functions of HtpG in cyanobacteria, in terms of the regulation of HemE and other phenotypes observed in *htpG* mutants, are discussed.

Materials and methods

Bacterial strains and growth conditions. *Synechococcus elongatus* strain PCC 7942 wild-type (NBC100, laboratory stock) and its derivatives were grown photoautotrophically at 30 °C in BG-11 medium under bubbling

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with air and continuous illumination (40 μE/m^2/s) [9]. When necessary, media were supplemented with kanamycin or spectinomycin at final concentration of 10 μg/ml or 40 μg/ml, respectively.

Disruption of htpG. DNA fragments of the upstream (using primers htpG-300f and htpGSp-hr, Table 1) and downstream (primers htpGSp-tf and htpG+300r) sequences of the *htpG* and spectinomycin-resistance gene (primers htpGSp-hf and htpGSp-tr) cassette were amplified by PCR from *Synechococcus* chromosome or pHP45Ω [10]. These fragments were recombined using primers htpG-300f and htpG+300r by a recombinant PCR method [11] and used to transform wild-type NBC100 to spectinomycin resistant. The disruption of *htpG* gene was confirmed by PCR and the resulting strain was named NBC201.

Construction of htpG-overexpressing strain. Plasmid pTrcAK is a derivative of pTrc99 A/X (GE Healthcare Bio-Sciences Co.) bearing a kanamycin resistance cassette derived from pUC4K (GE). A DNA fragment bearing the *htpG* upstream region (primers htpGup5′Xho and htpGupKm-hr, Table 1) and a kanamycin-resistance gene (primers htpGupKm-hf and km3′) were PCR-amplified from *Synechococcus* chromosome or pUC4K. Both fragments were recombined with the primers htpGup5′Xho and km3′ and cloned into *Xho*I-digested pTrcAK. The full-length *htpG*, amplified using primers htpG5′Nco and htpG3′Bam and digested by *Nco*I and *Bam*HI, was inserted into the aforementioned plasmid. The wild-type NBC100 cells were transformed with the plasmid and selected for kanamycin resistance. PCR analysis confirmed the plasmid integration into *Synechococcus* chromosome at the upstream region of *htpG* and the resulting strain was named as NBC202 (Fig. 1A).

Western blotting analysis. Crude extracts from the *Synechococcus* were prepared as described previously [9]. For the detection of HtpG proteins, rabbit antiserum against HtpG protein was used as the primary antibody [6], and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (GE) was used as the secondary antibody.

Viability assay. The NBC202 cells were grown to the absorbance of 0.1 at 750 nm ($A_{750} = 0.1$) in BG-11 liquid medium. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the culture at the final concentration of 1 mM for *htpG* induction. At appropriate time intervals, aliquots of the culture were serially diluted and plated on solid BG-11 medium. After incubation at 30 °C under light condition for 1 week, colony forming unit (CFU) was determined by counting the number of colonies on the plates.

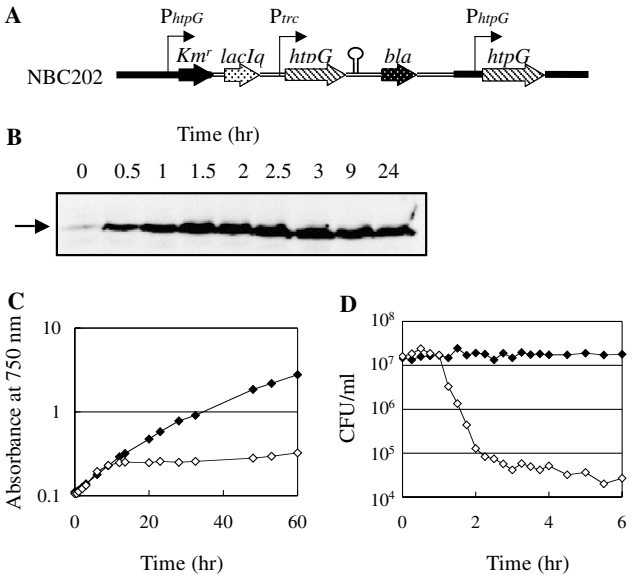

Fig. 1. Overproduction of HtpG protein leads to growth inhibition in NBC202. (A) The *htpG*-overexpressing strain NBC202 was constructed by genomic integration of the plasmid (thin white line) where *htpG* was placed under the control of IPTG-inducible promoter P*trc*. (B) Western blot analysis of HtpG protein. Cells were grown to logarithmic phase at 30 °C and *htpG* was induced by IPTG. Crude extracts were prepared at indicated times after the addition of IPTG, and samples (10 μg protein) were analyzed by Western blotting. (C and D) Growth curve and colony-forming unit (CFU) of NBC202 after HtpG protein induction, respectively. Exponentially growing cells were diluted to A_{750} of 0.1 with fresh medium with (open diamond) or without (filled diamond) IPTG and analyzed.

Table 1
Oligonucleotide primers used for cloning in this study

Primer	Sequence (5′–3′)[a]
htpG gene disruption	
htpG-300f	GTGGCTCTGCAGGAACGGATTCGGC
htpGSp-hr	*TGATGTTTCCTTA*CCCAAACTAAAGAGTGAC
htpGSp-hf	CTTTAGTTTGGGT*AAGGAAACATCATGAGG*
htpGSp-tr	CCCCTAAACCCT*TTATTTGCCGACTACCTT*
htpGSp-tf	*GTCGGCAAATA*AAGGGTTTAGGGGAACTCTA
htpGsx+300r	GGCAATCGCTCTCAGCCCAAGCCCC
HtpG over-expression	
htpGup5'Xho	*GC<u>CTCGAG</u>*ATCGACACCGCTACCGTCAAAAGC
htpGupKm-hr	*AACACCCCTTCT*ACCCAAACTAAAGAGTG
htpGupKm-hf	TTTAGTTTGGGT*AGAAGGGGTGTTATGAGCC*
km3'	TTAGAAAAACTCATCGAG
htpG5'Nco	*GC<u>CCATGG</u>*CGATTCTAGAGCAAGGC
htpG3'Bam	*GC<u>GGATCC</u>*TCACTGGCGAGTCGTCAACGC
Specificity test of yeast two-hybrid analysis	
htpG-Bam-f	*GC<u>GGATCC</u>*TGGCGATTCTAGAGCAAGGCAAT
htpG-Xho-r	*GC<u>CTCGAG</u>*TCACTGGCGAGTCGTCAACGCCG
htpGN-Xho-r	*GC<u>CTCGAG</u>*TCAGATCGGAACGGGCATGAAGTC
htpGC-Bam-f	*GC<u>GGATCC</u>*TCTATCGCTCGCAACGCGA
hemE-Eco-f	*GC<u>GAATTC</u>*ATGGTCGCGTCGTCTTCGC
hemE-Bam-r	*GC<u>GGATCC</u>*TTAGTGACTCGCTGCCAAGAG

[a] Additional sequences that do not correspond to the sequences of relevant genes are indicated by italics and restriction sites are underlined.

Subcellular fractionation and treatment of the membranes. Forty milligrams of the log-phase cell pellet was subjected to fractionation according to the procedure described previously [12], except for the polysome isolation. Membrane fraction was treated with alkali or urea as previously described [13].

Yeast two-hybrid screening and the specificity test. Yeast two-hybrid analysis for the library screening was carried out according to the method previously described [14–16]. Full-length (primers htpG-Bam-f and htpG-Xho-r, Table 1), the N-terminal (primers htpG-Bam-f and htpGN-Xho-r) or the C-terminal (primers htpGC-Bam-f and htpG-Xho-r) region of *htpG* or the full-length of *hemE* (primers hemE-Eco-f and hemE-Bam-r) was PCR-amplified and cloned into pGBTK, a GAL4 DNA-binding domain fusion vector [17], or pGAD, a GAL4 activation domain fusion vector [15]. The library was prepared by digesting *Synechococcus* genomic DNA with *Aci*I, *Msp*I, *Hin*PII, *Mae*II, *Taq*I, and ligating the products to pGAD plasmids in three different frames to produce three plasmid libraries: pGAD-C1, pGAD-C2, and pGAD-C3. These libraries contained at least 2.7×10^6 clones each. The PJ69-4Aα yeast haploid cell was transformed with all three libraries, and at least 1.3×10^7 prey-containing colonies were harvested and pooled. 1.0×10^8 cells each of the library strains were used for the screening. Positive protein–protein interaction between the bait and a prey clone was detected by the ability of the cells to grow on synthetic complete plates lacking leucine (Leu), tryptophane (Trp), and histidine (His) (SC-LWH), and containing 1 mM 3-aminotriazole, and those lacking Leu, Trp, and adenine (SC-LWA). The pGAD derivatives were extracted from the positive colonies and their insert fragments were sequenced and compared with the cyanobacterial genome database (CYORF: http://cyano.genome.jp/). Specificity test was carried out as described previously

[14,17]. Bait-prey pairs for the specificity test were spotted onto the SC-LWA plate and incubated for 3 days.

HemE activity assay. For the measurement of HemE activity, NBC100, NBC201, and NBC202 cells were grown in liquid BG-11 medium to $A_{750} = 0.5–1.0$. In the case of NBC202, IPTG was added to the final concentration of 1 mM for HtpG induction. The cells were harvested by centrifugation and resuspended in 50 mM sodium phosphate buffer (pH 6.8). The cells were disrupted by two passages through a chilled French pressure cell at 66 MPa (700 kg/cm^2), and soluble fraction was isolated by centrifugation. Five milligram protein equivalent of the soluble cell extracts was mixed with 2.5 nmol uroporphyrinogen-III, which was freshly prepared from uroporphyrin-III dihydrochloride (Frontier Co., UT) using sodium amalgam. After incubation for 1 h under the dark, the reaction was stopped by addition of acetic acid and acetonitrile. The supernatant of samples was oxidized for 10 min under the woods lamp. Coproporphyrin-III, the oxidized form of coproporphyrinogen-III, was subjected to HPLC on a C18 column (PEGASIL ODS, 5 μm particle size, 4.6 × 150 mm; Sensyu Scientific Co., Japan) at the flow rate of 1 ml/min in solvent (30% acetonitrile, 0.1 M ammonium acetate, pH 5.16) and monitored by fluorescence (λ_{ex} 404 nm) [18]. Coproporphyrin was identified and quantified using authentic standards (Frontier).

Results

Effect of htpG-overexpression on growth of cyanobacteria

In order to overexpress the *htpG* gene constitutively, the gene placed under the control of the IPTG inducible promoter was introduced into *Synechococcus*. Western blot analysis of the strain NBC202 using anti-HtpG antiserum showed marked increase in the level of HtpG after induction with 1 mM IPTG (Fig. 1A and B). Maximal expression of the exogenous HtpG was detected one hour after the induction. As shown in Fig. 1C, the strain also exhibited severe growth deficiency about 12 h after the addition of IPTG and greatly reduced survival rate starting an hour after induction (Fig. 1D). These results indicate that excess HtpG protein is lethal in cyanobacteria.

Subcellular localization of each HtpG protein

To determine the localization of HtpG protein in the cells, wild-type NBC100, *htpG*-disruptant NBC201, and *htpG*-overexpressing NBC202 cells were fractionated into cytosol and membrane factions by centrifugation, and Western blotting analysis with anti-HtpG antiserum was carried out for each fraction (Fig. 2A). Although HtpG does not contain a predicted membrane-spanning domain, the HtpG protein was detected in the membrane fraction of wild-type cells as well as the cytosol fraction, whereas in NBC202, it appears that excess HtpG protein was released from the membrane to the cytosol (Fig. 2A). Moreover, when the membrane fraction of NBC100 was extracted with 20 mM Hepes–NaOH at pH 8.0 or 0.2 M Na_2CO_3 at pH 11, a substantial amount of HtpG protein in the membrane was recovered in the soluble fraction (Fig. 2B, lanes 5 and 7), and after treatment with 6 M urea, all the HtpG protein was recovered in the soluble fraction (Fig. 2B, lane 9). These results suggest that HtpG associates with membrane as a peripheral protein.

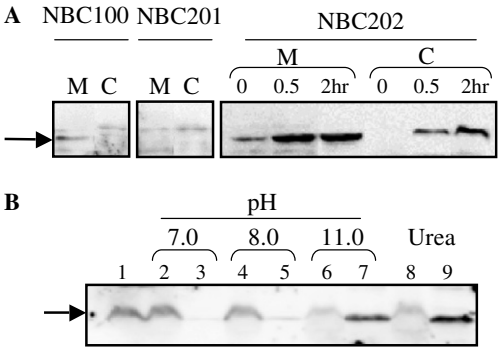

Fig. 2. Subcellular localization of HtpG protein in *Synechococcus* and its mutant strains. (A) Cells of NBC100, NBC201, and NBC202 were subfractionated into membrane (M) and cytosol (C). Aliquots (10 μg protein) were analyzed for the distribution of the HtpG proteins by Western blotting. (B) Extractability of the membrane-bound HtpG protein, determined as previously described [13]. Membrane fractions of NBC100 (10 μg protein, lane 1) were treated with 20 mM Hepes–NaOH (pH 7.0, lanes 2 and 3, pH 8.0, lanes 4 and 5) or 0.2 M Na_2CO_3 (pH 11, lanes 6 and 7) or 6 M urea (lanes 8 and 9). Peripheral membrane proteins were recovered in the supernatant (lanes 3, 5, 7, and 9) and membrane-associated proteins were precipitated as membrane pellets (lanes 2, 4, 6, and 8).

Interaction of HemE with the N-terminal region of HtpG

To elucidate specific target protein(s) of HtpG, we adopted a yeast two-hybrid screening. We constructed *Synechococcus* genomic library for the yeast two-hybrid analysis, as described in Materials and methods, and we also cloned the full-length, the N-terminal (1–209 a.a.) regions of the *htpG* gene into pGBTK to obtain DNA-binding domain fusions. A large number of independent positive clones were obtained with the N-terminal HtpG fragment. After sequencing a number of clones, we identified and categorized them into fragments derived from four genes. Among them, only annotated gene was *hemE*, a highly conserved gene from bacteria to human [19]. The fragments obtained in prey clones of HemE were sorted into two groups derived from basically the same region containing two α helices (Fig. 3A). The interaction was also confirmed by the specificity test (Fig. 3B). Interaction between the N-terminal domain of HtpG and the HemE-M (163–208 a.a.) region was detected even when the bait and prey constructs were switched. Moreover, the specific interaction between C-terminal regions of *Synechococcus* HtpG (HtpG-C, 344–639 a.a.) suggests that cyanobacterial HtpG also forms a homodimer. In fact, isolated *Synechococcus* HtpG forms a homodimer (Ishikawa, Y. and Nakamoto, H., unpublished observation).

HtpG exerts a negative effect on the HemE activity

To investigate the possible role of HtpG on HemE activity, the enzymatic activity in the cell extracts was assayed. HemE is located at the first branching point of

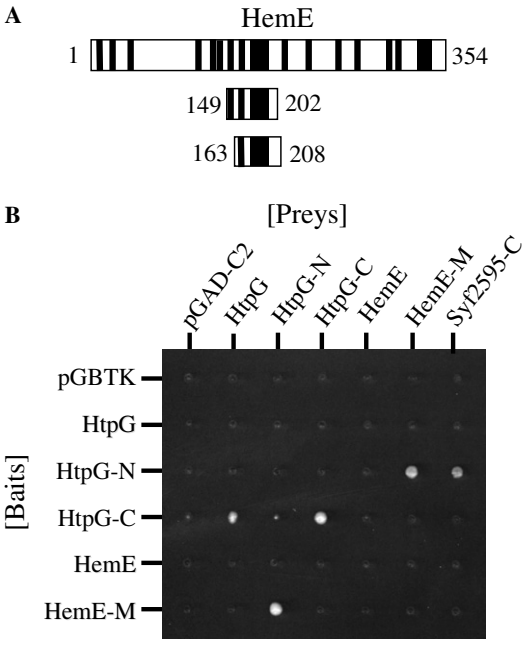

Fig. 3. Specific interaction between HemE and N-terminal domain of HtpG. (A) The HemE fragments contained in two independent prey clones isolated by yeast two-hybrid screening with HtpG-N (1–209 a.a.) as a bait protein are schematically represented along with the full-length HemE showing α helixes (shaded box) predicted by NNPREDICT (http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html). The numbers correspond to amino acid residues of the wild-type protein. (B) Specificity test using the yeast two-hybrid analysis. Diploid strains were constructed by mating PJ69-4Aa cells harboring pGBTK or its derivatives (Baits) with PJ69-4Aα cells harboring pGAD-C2 or its derivatives (Preys), in which each derivative plasmid contained the HtpG region (HtpG: full-length; HtpG-N: 1–209 a.a.; HtpG-C: 344–639 a.a.) or the HemE region (HemE: full-length; HemE-M: 163–208 a.a.) or the Syf2595 region (Syf2595-C: 186–476 a.a.).

the ubiquitous tetrapyrrole biosynthetic pathway, as illustrated in Fig. 4A. Decarboxylation of uroporphyrinogen-III by HemE leads to the biosynthesis of hemes, chlorophylls, and phycocyanin, whereas its C-methylation by uroporphyrinogen-III methyltransferase (CobA) initiates the synthesis of vitamin B_{12} and siroheme. Uroporphyrin-III and Coproporphyrin-III are metabolic byproducts, resulted from oxidation of Uroporphyrinogen-III and Coproporphyrinogen-III, respectively [20].

We compared cytosolic HemE activity of *htpG*-null or -overexpressing strain with that of wild-type by measuring the level of coproporphyrin production as described in Materials and methods. Compared to the wild-type NBC100, the deletion strain NBC201 exhibited 2.4-fold increase in HemE activity (Fig. 4B). In the *htpG*-overexpressing strain NBC202, on the other hand, the activity was assayed using the lysates prepared 0, 30, and 60 min after the HtpG induction. As shown in Fig. 4C, HemE activity was decreased two- to three-fold within 60 min after the induction. After this period, cells exhibited a marked drop in the survival rate (Fig. 1D).

Discussion

In this report, we present data strongly supporting the idea that HtpG plays an important physiological role in cyanobacteria. We revealed that excess HtpG protein causes deleterious effect on growth of cyanobacteria (Fig. 1C and D), although marked growth inhibition has not been observed in *E. coli* [21]. These lead us to strongly suggest that HtpG exerts an important physiological role in cyanobacteria. Our results suggest that the HtpG protein is located in membrane fraction as a peripheral protein (Fig. 2A and B). Similarly, HSP90C, which is the plastidic protein in *Chlamydomonas reinhardtii*, is localized in both stroma and thylakoid membrane fractions [22]. We also found the interaction of a putative membrane protein Syf2595 with the N-terminal domain of HtpG using yeast two-hybrid screening (Fig. 3B). Therefore, HSP90/HtpG family could play important role(s) on the surface of membrane.

Our yeast two-hybrid analysis revealed that HtpG interacts with HemE via the N-terminal region (Fig. 3B). In *E. coli*, the N-terminal region of HtpG, which contains ADP/ATP-binding site, is involved in substrate binding, as in the case of yeast HSP90 [23,24]. It would be interesting to examine whether cyanobacterial HtpG has ATPase activity and the interaction between the N-terminal domain and HemE depends on ATP. The crystal structures of HemE derived from human and tobacco have been determined [19,25], and it has been reported that dimerization of HemE is an important step for its enzymatic function [19]. The HtpG-interacting region of HemE contains two α helices (Fig. 3A), which are located within the dimer interface, suggesting that HtpG modulates HemE dimerization. The HemE enzymatic assay in the *htpG*-null and -overexpressing strains suggests that HtpG negatively regulates HemE activity (Fig. 4B and C). We assume that HemE is a cytosolic protein in cyanobacteria, since its enzymatic activity was not detected in the membrane fraction (data not shown). Although HtpG protein is suggested to localize in the membrane, cytosolic HemE activity could be inhibited by excess HtpG accumulated in cytosol in the HtpG-overexpressing strain NBC202 (Figs. 2A and 4C).

HtpG amount is increased in response to oxidative stress conditions in cyanobacteria [7,8]. Protoporphyrin IX, derived from the coproporphyrinogen-III (Fig. 4A), is the most-reactive photosensitizer of the tetrapyrrole biosynthetic pathway that upon excitation may lead to reactive oxygen species. Therefore, accumulation of protoporphyrin IX is damaging to the cell and levels of these intermediates need to be tightly regulated [26]. HtpG might be involved in fine-tuning of protoporphyrin IX synthesis through regulation of the HemE activity under stress conditions. In fact, *htpG*-null strain slightly exhibited high light sensitivity [7], which could involve the accumulation of protoporphyrin IX caused by the up-regulation of the HemE activity (Fig. 4B). In *htpG*-overexpressing strain NBC202, the reduction of HemE activity, which elicits

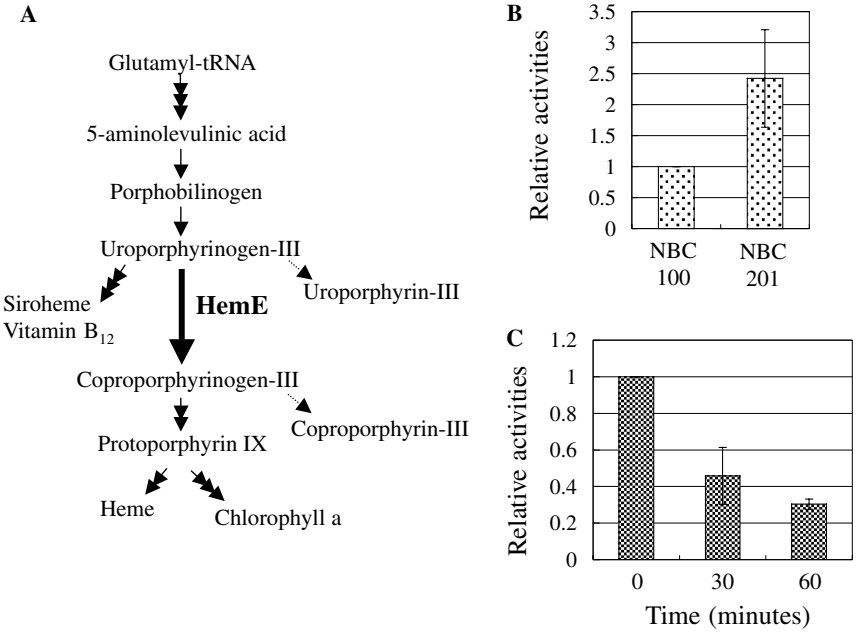

Fig. 4. The effect of HemE enzymatic activity by HtpG. (A) Metabolic pathway of tetrapyrrole biosynthesis. (B) Comparison of HemE enzyme activities in cell lysate of *htpG*-null strain NBC201 with that of wild-type strain NBC100. The activity of the strain NBC201 was expressed as the ratio to that of wild-type. Experiments were carried out four times and standard error was indicated by a vertical bar. (C) Effect of *htpG*-overexpression on the HemE activity. Cell lysates of NBC202 were prepared 0, 30, and 60 min after the induction of HtpG. HemE enzyme activities were shown as relative values to that of non-induced (time 0) cells. Experiments were performed three times.

synthetic arrest of metabolic pathways downstream of uroporphyrinogen-III, could be one of the causes of growth inhibition (Figs. 1C and 4C). Indeed, the absorption of chlorophyll and phycocyanin was decreased in NBC202 after HtpG induction (data not shown). In tetrapyrrole biosynthesis pathway, two pathways branch from Uroporphyrinogen-III (Fig. 4A). HemE leads to heme and chlorophyll synthesis, whereas CobA leads to siroheme and vitamin B_{12} synthesis. Although our data suggest that HtpG negatively regulates HemE, the effect of HtpG on CobA activity is not known yet. An important question, which needs to be addressed in the future, is whether HtpG is also involved in siroheme and vitamin B_{12} biosynthesis.

Involvement of the HSP90/HtpG family in the tetrapyrrole metabolism has also been reported in plants. In *Arabidopsis thaliana*, chlorate resistant 88 (*cr88*) mutant, carrying a point mutation in the C-terminus of Hsp90 targeted to the chloroplast stroma, displays a delay in greening process [27]. In cotyledons and young leaves of the mutant, plastids are less developed than those of the wild-type. The development of plastids is regulated by the plastid signal, which mediates communication between plastids and nucleus [20], and it has been proposed that intermediates of tetrapyrrole biosynthesis participated in the plastid signal [28]. These reports suggest that plastid HSP90 is involved in the plastid signal. The modulation of HemE by HtpG might be a critical finding for understanding not only a novel function of HtpG, but also the regulation system of tetrapyrrole metabolism in phototrophs.

Acknowledgments

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