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Growth Inhibition of *Escherichia coli* by Overexpression of Rat Perchloric Acid-soluble Protein

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Summary

Perchloric acid-soluble protein (PSP) and its highly homologous proteins are conserved among plants, archaea, bacteria, and eucarya. Although several functions of PSP have been elucidated in various species, the main role of PSP remains unclear. We previously demonstrated that PSP suppressed cell proliferation in mammalian cell lines. However, to the best of our knowledge, there are no reports on its function in other species. In this study, we investigated cell growth suppression of *E. coli* by rat PSP (rPSP). rPSP-His, GST-rPSP, or a GST expression vector were transformed into *E. coli* DH5α (DE3) cells, and expressions of recombinants were induced by IPTG. All recombinant expressions were detected in the presence of > 0.05 mM IPTG. The growth of transformed *E. coli* cells was suppressed by rPSP-His and GST-rPSP. Suppression ability of rPSP-His was stronger than that of GST-rPSP.

Key words: perchloric acid-soluble protein, *E. coli*, growth

Introduction

Perchloric acid-soluble protein (PSP) was initially isolated from the post-mitochondrial fraction of rat liver [11]. The PSP gene belongs to the YER057c/YjgF family, and all members of this family are highly conserved during evolution. Indeed, PSP and PSP homologous proteins have been reported in various species among plants [7], archaea [8], bacteria [4, 14], and eucarya [2, 10, 13, 15, 17]. Since highly conserved proteins generally have some key cellular functions, it is expected that PSP

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also plays some important roles in organisms. Functions of PSP are specific to eucarya and other species. In mammals, it has been reported that PSP is associated with various functions such as ribonuclease activity [9], fatty acid binding [16], differentiation-dependent cell expression [1], and mitochondrial DNA maintenance [7]. In other studied species including almost all bacteria, PSP was shown to correlate with the isoleucine synthesis pathway [4], and conversion of IMP to AMP and GMP [6]. Therefore, PSP has many functions, but little is known on its main role. Interestingly, there is no report on a common function of PSP among species.

We previously reported that overexpression of PSP decreased cell proliferation in mammalian cell lines [6]. This effect seemed to result from the ribonuclease activity of PSP. If inhibition of cell proliferation depends on a simple mechanism, i.e., digestion of RNA, it is possible that the proliferation inhibitory ability of PSP occurs in other species besides eucarya. In this study, we investigated if overexpression of rat PSP (rPSP) suppressed growth of *Escherichia coli* (*E. coli*). This study aimed to clarify whether there is a common function for PSP between mammalian cells and bacteria.

Materials and Methods

Construction of PSP expression vectors

The pGEX 4T-1 vector (GE healthcare, Tokyo, Japan) was used for glutathione S-transferase (GST) expression. The pGEX 4T-rPSP vector (PSP-GST expression vector) was constructed according to previous protocols [12]. In this study, we newly constructed pET-DEST42-rPSP (rPSP-His expression vector) using GatewayR® (Invitrogen, Tokyo, Japan), according to the manufacturer's instructions. The PSP gene-adapted attB sequence was amplified by Pfx DNA Polymerase (Invitrogen, Tokyo, Japan). The first primer set was 5'- AAAAAGCAGGCTTCGAAGGAGATA GAACCATGTCGTCAATAATCAGA-3' and 5'- AGAAAGCTGGGTCCAGTCCTGCTGTG GTGA-3'. The PCR product-adapted complete attB sequence was cloned into the pDONR201 vector (Invitrogen) to create plasmid pDONR201-PSP. The PSP gene was transferred from the pDONR201-PSP vector to the pET-DEST42 vector (Invitrogen) by LR clonase (Invitorogen, Tokyo, Japan). Each expression vector was purified using the QIAGEN Plasmid MiniKit (Qiagen, Tokyo, Japan).

Expression of Recombinant PSP proteins

E. coli BL21 (DE3) cells transformed with GST-PSP, PSP-His, or the GST expression vector were grown on LB agar plates in the presence of ampicillin. Some of the clones were grown in LB medium to verify expression of PSP-fused recombinant protein or GST using isopropyl-β-D-thiogalactopyranoside (IPTG). Expressions of recombinant proteins were verified by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, *E. coli* cells transformed with each expression vector were grown to reach an optical density at 600 nm (OD_{600}) of 0.4; and then each recombinant protein was induced by 1 mM IPTG for 4 h. Transformed *E. coli* cells were collected by centrifugation at 3,000 x g for 10 min, and pellets were resuspended in phosphate buffered saline (PBS). Thereafter, pellets were sonicated, and centrifuged at 10,000 x g for 20 min. Supernatants were collected, and protein concentrations in the supernatants were measured using BCA Protein Assay Reagents (Rockford, IL). Thirty μg of proteins for each sample were used for 15% SDS-PAGE. Proteins were stained with coomassie brilliant blue.

Growth rate of E. coli *cells transformed with each expression vector*

Expression vector-transformed *E. coli* cells were inoculated in 4 ml LB medium, and grown until they reached an OD_{600} of approximately 0.4. Then, 0, 0.01, 0.05, 0.1, or 1.0 mM IPTG were added. After 2 h, OD_{600} was measured, and culture medium was diluted to obtain an OD_{600} of 0.025 using LB medium containing each concentration of IPTG (final volume, 25 ml). OD_{600} was measured every hour. The population doubling time [$DT = (t_0 - t_1) \log 2/\log N_1 - \log N_0$] was calculated from the log phase of each growth curve.

Results and Discussion

Overexpression of PSP causes attenuation of cell proliferation in mammalian cell lines [6]. In this study, we investigated whether the inhibitory activity of rPSP was also recognized in *E. coli.* First, we checked expression levels of recombinant proteins in *E. coli* cells that were transformed with rPSP-His, GST-rPSP, and a GST expression vector (Fig. 1). Each expression vector was driven by IPTG. Expected molecular weights of rPSP-His, GST-rPSP, and GST recombinant protein were 18.6 K, 41.0 K, and 26.6 K, respectively. Recombinant GST-rPSP and GST proteins were recognized by single bands, but recombinant PSP-His was detected by 2 bands at about 19 K and 16 K. It was not clear why recombinant rPSP-His showed 2 bands. Although the promoter sequence of rPSP-His expression vector was different from those of GST-rPSP and the GST expression vector, all recombinant proteins were induced in the presence of > 0.05 mM IPTG, but not 0.01 mM IPTG.

E. coli cells transformed with rPSP-His, GST-rPSP, and the GST expression vector showed almost the same growth rate (DT = 0.6 h) compared to untransformed *E. coli* in LB medium in the absence of IPTG (data not shown). Fig. 2 shows growth curves of each transformed *E. coli* in LB medium in the presence of various concentrations of IPTG. Recombinant protein induction and growth inhibitory activities were not recognized in medium containing 0.01 mM IPTG. However, a high dose IPTG (1.0 mM) remarkably suppressed growth of *E. coli* cells that were transformed even with the GST expression vector. We had to compare growth rates among *E. coli* cells transformed with each expression vector under a low concentration of IPTG, because overexpression of recombinant proteins seemed to stress and harm *E. coli* growth. DT of rPSP-His transformed *E. coli*

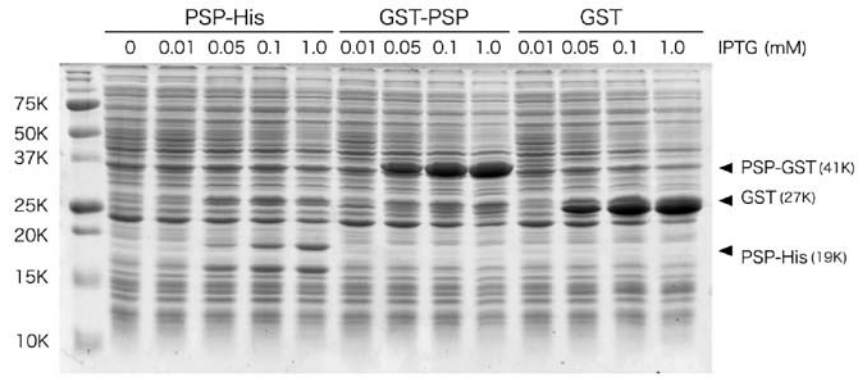

Figure 1. Expression levels of each recombinant protein in *E. coli*. Thirty-mg aliquots of proteins from *E. coli* cells were used for 15% SDS-PAGE. Following electrophoresis, proteins were stained with CBB.

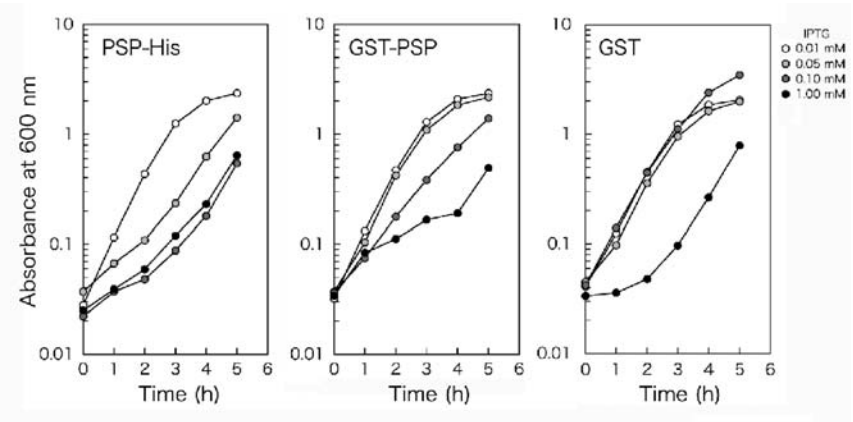

Figure 2. Growth curves of transformed *E. coli* cells under the induction of recombinant proteins. Impurity of medium (OD_{600}) was analyzed. Transformed *E. coli* cells were cultured in LB medium and various concentrations of IPTG were added. After 2 h, *E. coli* cells were diluted to reach an OD_{600} of 0.025, and cultured in medium containing various concentrations of IPTG. OD_{600} was measured every hour.

was 1.1 h in the presence of 0.05 mM IPTG, and growth rate was 1.8 times delayed. DT of GST-rPSP- or GST-transformed *E. coli* cells did not change in the presence of 0.05 mM IPTG. In the presence of 0.1 mM IPTG, differences were clearer. Growth suppression occurred in rPSP-His- and GST-PSP-transformed *E. coli* cells, and DTs were 1.6 h and 0.85 h, respectively. Growth suppression by rPSP-His was stronger than that by GST-rPSP.

It was suggested that the growth inhibitory activity of PSP came from its ribonuclease activity [5]. However, the activity site of PSP remains unclear. GST-rPSP suppressed growth of *E. coli* less than PSP-His. We speculate that the GST-tag interrupts the activity site of PSP. PSP constructs form homotrimers [3, 18]. Homotrimer formation of PSP might correlate with the growth inhibitory activity of PSP. We confirmed that recombinant rPSP protein suppressed growth of *E. coli* as well as mammalian cells. This effect of PSP is the first common function between mammalian cell lines and bacteria. In this study, we investigated the effects of rat PSP protein on growth of *E. coli*, but *E. coli* cells also contain the PSP homologous protein YjgF protein (AAN83763), that has 47.3% similarity to rPSP. It is possible that YjgF correlates with the growth of *E. coli*, but we need to investigate whether YjgF protein suppresses growth. Additionally, the method using *E. coli* in the present study for the growth inhibitory activity of PSP was convenient to unveil the active site of PSP with a high throughput.

References

[1] Asagi, K., Oka, T., Arao, K., Suzuki, I., Thakur, MK., Izumi, K., Natori, Y.: Purification, characterization and differentiation-dependent expression of a perchloric acid soluble protein from rat kidney. Nephron, 79, 80-90 (1998)

[2] Ceciliani, F., Faotto, L., Negri, A., Colombo, I., Berra, B., Bartorelli, A., Ronchi, S.: The primary structure of UK114 tumor antigen. FEBS Lett., 393, 147-150 (1996)

[3] Deriu, D., Briand, C., Mistiniene, E., Naktinis, V., Grutter, MG.: Structure and oligomeric state of the mammalian tumour-associated antigen UK114. Acta Crystallogr. D Biol.

Crystallogr., 59, 1676-1678 (2003)

[4] Enos-Berlage, JL., Langendorf, MJ., Downs, DM.: Complex metabolic phenotypes caused by a mutation in yjgF, encoding a member of the highly conserved YER057c/YjgF family of proteins. J. Bacteriol., 180, 6519-6528 (1998)

[5] Kanouchi, H., Oka, T., Asagi, K., Tachibana, H., Yamada, K.: Expression and cellular distribution of perchloric acid-soluble protein is dependent on the cell-proliferating states of NRK-52E cells. Cell. Mol. Life Sci., 57, 1103-1108 (2000)

[6] Kanouchi, H., Tachibana, H., Oka, T., Yamada, K.: Recombinant expression of perchloric acid-soluble protein reduces cell proliferation. Cell. Mol. Life Sci., 58, 1340-1343 (2001)

[7] Leitner-Dagan, Y., Ovadis, M., Zuker, A., Shklarman, E., Ohad, I., Tzfira, T., Vainstein, A.: CHRD, a plant member of the evolutionarily conserved YjgF family, influences photosynthesis and chromoplastogenesis. Planta, (2006) in press

[8] Miyakawa, T., Hatano, KI., Lee, WC., Kato, Y., Sawano, Y., Yumoto, F., Nagata, K., Tanokura, M.: Crystallization and preliminary X-ray analysis of the YjgF/YER057c/UK114-family protein ST0811 from Sulfolobus tokodaii strain 7. Acta Crystallograph. Sect F Struct. Biol. Cryst. Commun., 61, 828-830 (2005)

[9] Morishita, R., Kawagoshi, A., Sawasaki, T., Madin, K., Ogasawara, T., Oka, T., Endo, Y.: Ribonuclease activity of rat liver perchloric acid-soluble protein, a potent inhibitor of protein synthesis. J. Biol. Chem., 274, 20688-20692 (1999)

[10] Nordin, H., Matsumoto, M., Suzuki, K., Kaneki, K., Natori, Y., Kishi, K., Oka, T.: Purification, characterization and developmental expression of chick (Gallus domesticus) liver PSP protein. Comp. Biochem. Physiol. B Biochem. Mol. Biol., 128, 135-143 (2001)

[11] Oka, T., Tsuji, H., Noda, C., Sakai, K., Hong, YM., Suzuki, I., Munoz, S., Natori, Y.: Isolation and characterization of a novel perchloric acid-soluble protein inhibiting cell-free protein synthesis. J. Biol. Chem., 270, 30060-30067 (1995)

[12] Oka, T., Nishimoto, Y., Sasagawa, T., Kanouchi, H., Kawasaki, Y., Natori, Y.: Production of functional rat liver PSP protein in Escherichia coli. Cell. Mol. Life Sci., 55, 131-134 (1999)

[13] Oxelmark, E., Marchini, A., Malanchi, I., Magherini, F., Jaquet, L., Hajibagheri, MA., Blight, KJ., Jauniaux, JC., Tommasino, M.: Mmf1p, a novel yeast mitochondrial protein conserved throughout evolution and involved in maintenance of the mitochondrial genome. Mol. Cell. Biol., 20, 7784-7797 (2000)

[14] Rappu, P., Shin, BS., Zalkin, H., Mantsala, P.: A role for a highly conserved protein of unknown function in regulation of Bacillus subtilis purA by the purine repressor. J. Bacteriol., 181, 3810-3815 (1999)

[15] Samuel, SJ., Tzung, SP., Cohen, SA.: Hrp12, a novel heat-responsive, tissue-specific, phosphorylated protein isolated from mouse liver. Hepatology, 25, 1213-1222 (1997)

[16] Sasagawa, T., Oka, T., Tokumura, A., Nishimoto, Y., Munoz, S., Kuwahata, M., Okita, M., Tsuji, H., Natori, Y.: Analysis of the fatty acid components in a perchloric acid-soluble protein. Biochim. Biophys. Acta, 1437, 317-324 (1999)

[17] Schmiedeknecht, G., Kerkhoff, C., Orso, E., Stohr, J., Aslanidis, C., Nagy, GM., Knuechel, R., Schmitz, G.: Isolation and characterization of a 14.5-kDa trichloroacetic-acid-soluble translational inhibitor protein from human monocytes that is upregulated upon cellular differentiation. Eur. J. Biochem., 242, 339-351 (1996)

[18] Sinha, S., Rappu, P., Lange, SC., Mantsala, P., Zalkin, H., Smith, JL.: Crystal structure of Bacillus subtilis YabJ, a purine regulatory protein and member of the highly conserved YjgF

family. Proc. Natl. Acad. Sci. U. S. A., 96, 13074-13079 (1999)