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Perchloric Acid-soluble Protein Binds Hypoxhantine Phosphoribosyl Transferase, but not Affect its Enzymatic Activity

Hiroaki KANOUCHI[1], Shigenobu TONE[2], Masaaki OKABE[1],
Mitsuharu MATSUMOTO[3], Tatsuzo OKA[1]*
([1] *Laboratory of Veterinary Pathobiology*)
([2] *Department of Biochemistry, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan*)
([3] *Laboratory of Veterinary Anatomy*)
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Summary

Perchloric Acid-soluble Protein (PSP) is highly conserved protein in the course of evolution. There was no report about PSP associated protein, although various functions of PSP have been reported. We previously revealed a candidate gene for PSP binding protein, *hypoxanthine-guanine phosphoribosyltransferase (HPRT)* was identified by two hybrid screening system. Using glutathione S-transferase (GST)-pull down method and immunoprecipitation method, we concluded that PSP and HPRT could directly bind. We examined whether PSP affected HPRT enzymatic activity in vitro or not. As a result, PSP showed no effect its activity. The meaning of PSP and HPRT binding has not been addressed.

Key words: Perchloric acid-soluble protein, hypoxanthine-guanine phosphoribosyltransferase, yeast two hybrid screening

Introduction

Perchloric acid-soluble protein (PSP) was purified from rat cytosol as stable in 5% perchloric acid [10]. PSP homologous proteins were found in numerous species of animal [1, 2, 8, 10, 14, 16], bacteria [13], archea [6], and plant [5]. The PSP protein is constructed 144 amino acids, and there is unknown functional domain in the primary structure. However, there are many reports about various functions of PSP such as ribonuclease activity [7], fatty acid binding activity [15], mitochondrial DNA maintenance activity [12], and association of isoleucine synthesis [4]. We speculated that PSP had co-factors or PSP was a co-factor with any proteins, and PSP functions were influenced by its associated proteins. Recently, we tried to identify PSP associated proteins using yeast two hybrid screening system. As a result, some genes were detected. One of them was a *hypoxanthine-guanine phosphoribosyltransferase (HPRT).* The HPRT primarily functions to salvage purines from degraded DNA to renewed purine synthesis [11]. In this role, it acts as a catalyst in the

* Correspondence to: T. OKA (Laboratory of Veterinary Pathobiology, Kagoshima University)
Tel and Fax: +81-99-285-8714; E-mail: toka@agri.kagoshima-u.ac.jp

reaction between guanine and phosphoribosyl pyrophosphate (PRPP) to form GMP. In this study, it was evaluated whether PSP binds to HPRT protein. It has been reported that *Yabj*, which is PSP homologous protein in *Bacillus subtilis*, located immediately downstream from *purR* in the operon [13]. PurR is a suppressor the purA operon, which encodes genes of purine de novo synthesis. Since PurR activity was disturbed by null of *Yabj*, Yabj seemed to relate purine metabolism. In this experiment, we examined whether PSP binds HPRT and relates to HPRT enzymatic activity or not.

Materials and Methods

Constructions of expression vectors

The cloning of the PSP cDNA and the construction of PSP-GST vector and GFP-PSP vector have been described previously [3]. HPRT PSP cDNA was isolated from RLN-10 cells by two steps of polymerase chain reaction (PCR) using specific primers for Gateway system and platinium Pfx DNA polymerase (Invitrogen, Tokyo, Japan). The first step of PCR was performed using a set of primers, 5'- AAAAAGCAGGCTTCGAAGGAGATAGAACCATGTCGACCCTCAGTCCCA GCGT-3' and 5'-AGAAAGCTGGGTCGGCTTTGTACTTGGCTTTTCCACT-3'. In the second step, a product of the first PCR was amplified using 5'-GGGGACAAGTTTGTACAAAAAA GCAGGCT-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'. These PCR products were cloned into the Gateway entry vector and then transferred to pDEST vector expressing His tag-fused protein according to the manufacturer's instructions (Invitrogen, Tokyo, Japan).

GST-pull down method

GST-PSP and GST were purified according to the method of Oka et al [9]. [^{35}S]-labeled HPRT was obtained using an in vitro transcription translation system (Promega, Tokyo, Japan). [^{35}S]-labeled HPRT was mixed with GST-PSP containing 0.5% Triton X-100 and added glutathione-sepharose beads (GE healthcare, Tokyo, Japan), to which purified GST-PSP had been pre-adsorbed. After incubation at 4℃ for 2 h, the beads were washed four times with phosphate buffered saline (PBS) containing 0.5% Triton X-100 and the bound proteins were released by boiling in SDS sample buffer. Purified GST adsorbed on glutathione-sepharose beads was used as a negative control. The bound proteins were separated by SDS-PAGE and visualized using a FLA-2000 (Fuji Film, Tokyo, Japan).

Immunoprecipitation

Immunoprepicitation using anti-GFP or anti-His antibody against cell extracts of RLN-10 cells transfected with GFP-PSP and HPRT-His expression vector. RLN-10 cells were cultured in DMEM (Sigma, Tokyo, Japan) supplemented with 10% fetal bovine serum at 37℃ in 5% CO_2. The GFP-PSP and HPRT-His vectors were co-transfected to RLN-10 cells using the Lipofectmine (Invitrogen, Tokyo, Japan) according to the manufacturer's protocol. At 48 h after transfection, the cells were washed twice with PBS and lysed in ice-cold lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA and 0.1% Tween-20). After incubating the cell lysates for 15 min at 4℃, insoluble material was removed by centrifugation at 15,000 x g for 30 min. The supernatants were diluted with an equal volume of lysis buffer without Tween-20 and mixed with 50 μl of Ni agarose beads. The mixtures were incubated for 2 h on a turning wheel. The beads were washed four times with washing buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA and 0.1% Tween-20) and the bound material was eluted by boiling in SDS-PAGE sample buffer. Protein samples were

resolved by SDS-PAGE and transferred to nitrocellulose membranes. The resulting membranes were blocked for 1 h in PBS containing 5% skim milk and incubated with anti-GFP antibodies (Sigma, Tokyo, Japan, used at 1:1000 dilution) at 4℃ for overnight. After washing three times in TBST (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20), the membranes were further incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG (GE healthcare, Tokyo, Japan, used at 1:3000 dilution) at room temperature. After washing three times in TBST, the blots were incubated with ECL plus Reagent (GE healthcare, Tokyo, Japan) and the protein bands were visualized by LAS-1000 system.

HPRT enzymatic activity

HPRT enzymatic activity was measured according to the previously reported method [11]. Sixty two ng of recombinant HPRT-His was added into the HPRT assay buffer (125 mM Tris-HCl pH 7.8, 1.2 mM $MgCl_2$, 5 mM DTT, 2.5 mM 5-phospho-D-ribose 1-diphosphate (PRPP), 0.15 mM hypoxanthine, 3.7 x 10^6 cpm hypoxanthine, 0.1 mg/ml BSA). Various concentration of PSP was added the reaction mixtures. Reaction mixtures were incubated at 37℃ for 30 min, then added ice cold 50 mM Tris-HCl pH 8.0 to stop the reaction. All sample applied to 1 ml DEAE-cellulose, and the column were washed with 5 ml of 50 mM Tris-HCl pH 8.0. The synthesized nucleotide was eluted with 10 ml of 1N HCl, collected directly into a scintillation vial, and counted with 10 ml of scintillation fluid.

Results and Discussion

HPRT was identified as PSP associated protein using yeast two hybrid screening method. First, we confirmed whether PSP and HPRT directly bound by GST-pull down method. Each recombinant GST-PSP and GST was expressed in *E-coli*, and purified using Glutathione Sepharose columns. Radiolabeled-HPRT-His protein was synthesized using reticulocyte lysate system with [^{35}S]-methionine. The lysate was mixed with GST-PSP or GST. These mixtures were applied to GST

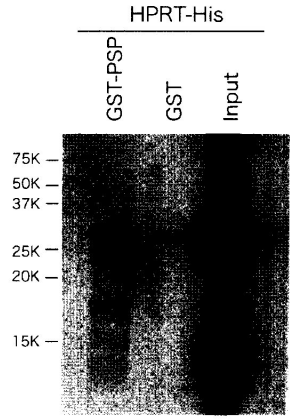

Figure 1. Binding of GST-PSP and HPRT-His confirmed using GST-pull down method. The HPRT-His protein was synthesized in the total 10 μl of rabbit reticulocyte lysate cell-free system containing of [^{35}S]-methionine. A half of synthesized products were incubated with purified GST-PSP or GST, and then glutathione-sepharose beads were added into the mixtures. Bounded fractions were applied to SDS-PAGE and the radio labeled HPRT-His protein was detected by FLA-2000.

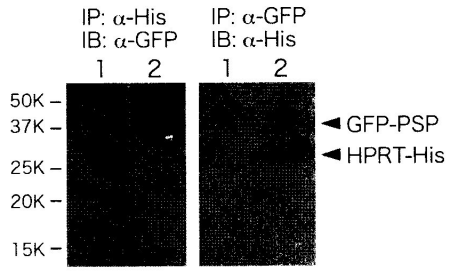

Figure 2. Binding of PSP-GFP and HPRT-His confirmed using co-immunopurification. PSP-GFP and HPRT-His expression vectors were introduced into RLN-10 cells. After 24 h, cell lysates were prepared and 150 μg of protein was used for immunoprecipitation. Lane 1 and lane 2 are cell lysates from mock transfected cells and expression vectors transfected cells, respectively. Anti-GFP or anti-His antibodies were used for immunoprecipitation and immunoblotting. Molecular weight of GFP-PSP and HPRT-His are 44K and 29K, respectively.

pull-down assay. In the figure 1, the lane of GST-PSP shows major two bands about 28,000 and 24,000 daltons. Since calculated molecular weight of HPRT-His was 28,735 dalton, therefore, 28,000 band was seemed to be HPRT-His protein. Input was loaded same amounts cell extract without Glutathione Sepharose columns. The intensity of 28,000 band pull-downed with GST-PSP was higher than that with GST. But, HPRT-His was also pull-downed with GST. So we checked the binding of PSP and HPRT by another method. GFP-PSP and HPRT-His expression vectors were co-transfected into RLN-10 cells, and then immunoprecipitated with anti-GFP or anti-His antibody. Each immunoprecipitated fractions was subjected to immunoblot analysis using anti-His or anti-GFP antibody (Figure 2). As a result, His-HPRT and GFP-PSP were detected in the bound fraction immunoprecipitated with anti-GFP or anti-His antibody, respectively. The both unbound fractions did not show any specific binding. From these results, it was suggested that PSP and HPRT can directly bound in the mammalian cells.

We confirmed PSP and HPRT binding so we evaluated whether PSP affect enzymatic activity of HPRT. The enzymatic activity of HPRT was evaluated conversion of [^{3}H]-hypoxanthine to [^{3}H]-IMP. Converted [^{3}H]-IMP were dose-dependently increased by HPRT (figure 3 left). The

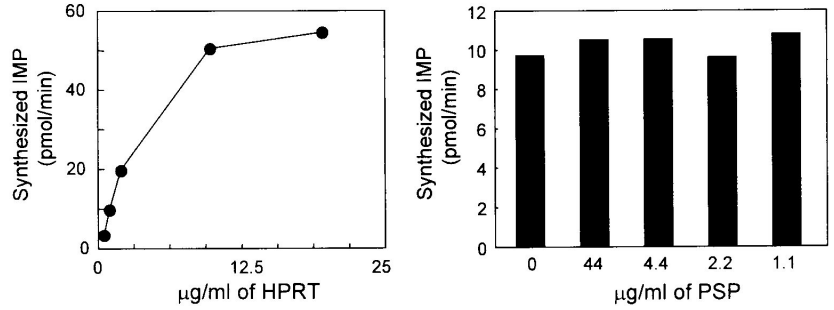

Figure 3. Assay of HPRT enzyme activity. HPRT enzyme activity was shown as synthesized IMP calculated from radio activity of synthesized IMP by HPRT. Left panel, synthesized IMP was dependent on HPRT concentration (0.5, 1.0, 2.0, 10.0, and 20.0 μg/ml) Right panel, amounts of synthesized IMP by 1.7 μg/ml of HPRT was not effected by recombinant PSP.

reaction solution containing 1.7 μg/ml of HPRT was added various concentration of PSP. Unfortunately, the enzymatic activity of HPRT did not affected by any concentration of PSP (figure 3 right).

We firstly identified PSP associated proteins using yeast two hybrid screening method. HPRT was one of them a candidate of PSP binding protein. We confirmed to bind to PSP directly by two different methods. In bacteria, PSP homologous protein Yabj is concerned with de novo purine synthesis [13]. We expected that PSP also related to purine metabolism in mammalian cells. However, PSP could not influence HPRT enzymatic activity. The meaning of PSP and HPRT binding has not been addressed.

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