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Research Article

Recombinant expression of perchloric acid-soluble protein reduces cell proliferation

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Abstract. Perchloric acid-soluble protein (PSP) may play an important role in the regulation of cellular physiological functions because it has been highly conserved throughout evolution; however, this role has not been well elucidated. In previous reports, we suggested that PSP regulates cell proliferation. In this study, we examined the effect of PSP expression on proliferation of the normal rat kidney cell line NRK-52E, the rat hepatocyte cell line RLN-10, and the rat hepatoma cell line dRLh-84. Cells transfected with pcDNA-sense-PSP (pcDNA-S-PSP) over-expressed PSP mRNA and protein, and cell proliferation of the transfected cells was suppressed compared with that of cells transfected with pcDNA-empty (pcDNA-E). Cell viability of pcDNA-S-PSP-transfected cells was similar to that of pcDNA-E-transfected cells. Thus, over-expression of PSP suppresses cell proliferation without any influence on cell viability. These findings are the first to report an inhibitory activity of PSP on cell proliferation.

Key words. Perchloric acid-soluble protein; p14.5; protein synthesis inhibitor; cell proliferation inhibitor.

Perchloric acid-soluble protein (PSP) was first isolated from rat liver [1]. PSP homologous proteins (p14.5s) were then purified from mice [2], goat [3], and human [4]. The nucleotide sequences of the cDNA encoding p14.5s are highly similar to that of a new family (YER057c/YJGF) of small proteins that are conserved from prokaryotes to eukaryotes. The high conservation of p14.5s suggests that they play an important role in cellular physiological functions. Previous reports suggested that p14.5 proteins inhibit cell-free protein synthesis [5, 6], fatty acid-binding activity [7] and calpain activator [8], but their critical role has not yet been elucidated.

Previous studies showed that the expression of p14.5 proteins was related to cellular differentiation [4]. Human p14.5 mRNA was weakly expressed in freshly isolated monocytes, but was significantly upregulated when these monocytes were subjected to differentiation. Immunohistochemical study of an immature rat kidney showed that the expression of PSP increased from fetal day 17 to the 4th postnatal week, and remained at almost the same level until the 7th postnatal week [5]. Moreover, we reported that the expression of PSP was lower in the proliferating than in the stationary phase during the cell growth of rat NRK-52 kidney cells [5]. We also found that when the expression of PSP in cultured cells is high, cellular protein synthesis is low [5]. Thus, the expression of PSP seems to be related to cell proliferation and differentiation. Recently, we constructed a PSP expression vector that was

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useful for producing functional rat PSP in *Escherichia coli* [8]. In this study, we reconstructed the PSP expression vector for use in a mammalian expression system and examined the effect of PSP over-expression on cell proliferation.

Materials and methods

Construction of PSP expression vector. The nucleotide sequence encoding PSP was amplified by PCR using the cDNA from NRK-52E cells as a template. The primers for PCR were 5′-cgagcggccgcatgtcgtcaataatcagaaaa-3′ and 5′-cgagcggccgcttacagtcctgctgtggtgaa-3′. The PCR fragment was inserted into the pUC19 vector (pUC19-PSP). The nucleotide sequence of pUC19-PSP was determined and was identified as sense (S) or antisense (AS) PSP. These DNAs were digested by the restriction enzymes *Eco*RI and *Xba*I. The new plasmid DNAs, pcDNA-AS-PSP and pcDNA-S-PSP, were constructed by ligating with *Eco*RI- and *Xba*I-digested pcDNA3.1 (Invitrogen, San Diego, Calif.).

Cell culture and transfection

NRK-52E cells were obtained from the American Type Tissue Culture Collection (Manassas, Va.). Rat hepatocyte RLN-10 cells (JCRB0415) and rat hepatoma dRLh-84 cells (JCRB0410) were obtained from the Human Science Research Resources Bank (Osaka, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (Life Tech, Rockville, Md.) supplemented with nonessential amino acids (ICN, Costa, Calif.), 50 µg/ml streptomycin, 50 units/ml penicillin, and 5% fetal bovine serum. Cells were replated before reaching confluence and the medium was changed every 3 days. pcDNA-AS-PSP, pcDNA-S-PSP, and pcDNA-empty (pcDNA-E) were transiently transfected into NRK-52E, RLN-10, and dRLh-84 cells using the electroporation method, and the cells were seeded at an initial density of 1.3×10^5 living cells/6-cm dish. Living cells were counted at 17, 40, and 68 h after the inoculation.

Immunoblotting

NRK-52E, RLN-10, and dRLh-84 cells were homogenized in homogenizing buffer containing 25 mM KCl, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), 0.2 U/ml aprotinin, and 0.2 mM PMSF using a Potter-Elvehjem-type homogenizer. The protein concentration was determined with the BCA protein assay kit (Pierce, Rock Ford, IL, USA). Immunoblotting was performed according to the method described previously [6]. Twenty micrograms of homogenate protein was electrophoresed on 15% SDS-PAGE, and then transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany) and immunoblotted with anti-PSP serum (1:3000).

Quantitative RT-PCR

Quantitation of PSP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was performed using RT-PCR as described previously [5]. Total cellular RNA was obtained using TRIzol (Life Technologies, Rockville, Md.) from transfected NRK-52E cells, and cDNA was synthesized from 0.2 µg of total RNA using a commercial kit (Amersham, Little Chalfont, UK), according to the manufacturer's instructions. PSP and GAPDH cDNA were amplified by PCR using specific primers (PSP sense primer: 5′-gccattggtgcctacagcca-3′, PSP antisense primer: 5′-aaaggcccctggacag-3′, GAPDH sense primer: 5′-gatgctggtgctgagtatgtc-3′; GAPDH antisense primer: 5′-gtggtgcaggatgcattgctg-3′). Reaction aliquots included 1 µM of each primer, 200 µM of deoxy-NTP mixtures, 1.5 mM MgCl, 10 × buffer, and 1 unit of Taq DNA polymerase (Fermentas, Vilnus, Lithuania). The PCR condition was as follows: denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. The PCR cycles for quantification were performed as described previously; the number of cycles was 15 and 20 for PSP and GAPDH, respectively [5]. Amplified DNA was applied to gel electrophoresis on 2% agarose gels and transferred onto nylon transfer membranes (Hybound-N^+; Amersham). Blots were visualized with fluorescein-labeled locus-specific probes (PSP: 5′-tgagattctgaaggctgcag-3′, GAPDH: 5′-ggtgtgaaccacgagaaata-3′).

Statistical analysis

Data were analyzed by Student's t test to evaluate the significance of differences.

Results

Expression of PSP in transfected cells

PSP expression was analyzed using immunoblotting, 40 h after inoculation (fig. 1A). In all cell lines transfected with pcDNA-S-PSP, PSP protein was over-expressed. On the other hand, the expression of PSP in pcDNA-AS-PSP-transfected cells did not differ from that of pcDNA-E-transfected cells. The expression of PSP mRNA was also examined in NRK-52E cells 40 h after the inoculation using RT-PCR methods followed by Southern hybridization (fig. 1B). The expression level of PSP remained high even at 68 h, because the mammalian expression vector pcDNA3.1 provides strong expression levels from the CMV promoter. Abundant sense or antisense PSP mRNA was detected in the cells transfected with pcDNA-S-PSP and pcDNA-AS-PSP but not in pcDNA-E-transfected cells. Expression of GAPDH mRNA as a control was similar in all the groups.

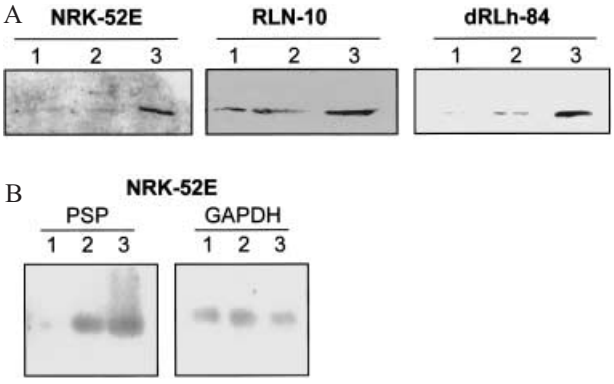

Figure 1. PSP expression in PSP expression vector-transfected cells. PSP expression levels in NRK-52E, RLN-10, and dRLH-84 cells were detected 40 h after electroporation. Total protein (20 µg) from transfected cells was electrophoresed on 15% SDS-PAGE gels. Electrophoresed protein was immunoblotted using anti-PSP antiserum (*A*). PSP and GAPDH mRNA levels were analyzed using quantitative RT-PCR followed by Southern hybridization in NRK-52E cells (*B*). Lane 1, pcDNA-E-transfected cells; lane 2, pcDNA-AS-PSP-transfected cells; lane 3, pcDNA-S-PSP-transfected cells.

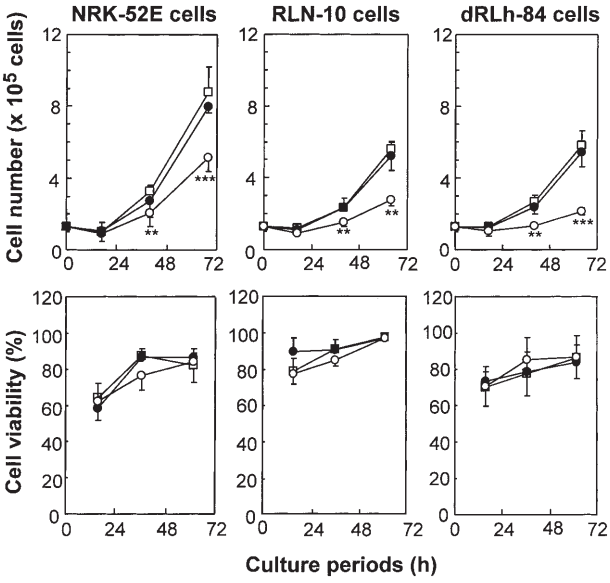

Figure 2. Over-expression of PSP has a dominant-negative effect on cell proliferation. Rat kidney NRK-52E, rat hepatocyte RLN-10, and rat hepatoma dRLh-84 cells were transfected with pcDNA-E, pcDNA-AS-PSP, or pcDNA-S-PSP by electroporation and the cells were seeded at an initial density of 1.3×10^5 viable cells/6-cm dish. After inoculation, viable cells were counted using a hemocytometer in the presence of trypan blue at 17, 40, and 68 h. pcDNA-E-, pcDNA-AS-PSP, and pcDNA-S-PSP-transfected cells are shown as open squares, filled circles and open circles, respectively. Data are shown as the mean SD for three to six samples. ** and *** denote significant differences from a respective mock value at $p < 0.01$ and $p < 0.001$, respectively.

Cell proliferation of PSP expression vector-transfected cells

Figure 2 shows the effect of the transfected PSP expression vector on cell proliferation in rat kidney NRK-52E cells, rat hepatocyte RLN-10 cells, and rat hepatoma dRLh-84 cells. The proliferation ability of all pcDNA-E-transfected cell lines was similar to that of nontransfected cells (data not shown). At 17 h after inoculation, cell numbers of pcDNA-AS-PSP- and pcDNA-S-PSP-transfected cells decreased slightly, but these numbers were not significantly different from pcDNA-E-transfected cells, for all cell lines. Effects began to be seen at 40 h. For NRK-52E cells, the cell numbers of pcDNA-S-PSP-transfected cells were 63 and 59% of pcDNA-E-transfected cells at 40 and 68 h, respectively. In RLN-10 cells, cell numbers of pcDNA-S-PSP-transfected cells were reduced to 60 and 50% of pcDNA-E-transfected cells at 40 and 68 h, respectively, after the inoculation. In dRLh-84 cells, the corresponding values were 50 and 40%. The greatest suppression of cell proliferation by the transfection with pcDNA-PSP was observed in dRLh-84 cells. Cell numbers of pcDNA-AS-PSP- and pcDNA-E-transfected cells did not differ.

For all the lines, cell viability was not significantly different among the cells transfected with pcDNA-E, pcDNA-AS-PSP, and pcDNA-S-PSP. Cell viability of transfected cells was about 60% at 17 h after electroporation, and thereafter was maintained up to 84% at 68 h.

Under an optical microscope, transfection with pcDNA-E, pcDNA-AS-PSP, and pcDNA-S-PSP showed no morphological effects on the cells (data not shown).

Discussion

PSP has been highly conserved through evolution, but its biological role has not been clearly elucidated. Previous studies showed that PSP and H-p14.5 are more highly expressed in the differentiated than in the undifferentiated state [7, 9]. Recently, we reported that PSP expression was lower in the proliferating state and gradually increased into the stationary phase, and the pattern of this expression was negatively correlated with the activity of cellular protein synthesis [5]. These results might indicate that PSP expression is related to cell proliferation.

To assess whether or not over-expression of PSP affects cell proliferation, we introduced a PSP expression vector into various rat cell lines. Cell proliferation of pcDNA-S-PSP-transfected cells was remarkably suppressed compared with that of pcDNA-E-transfected cells in all the cell lines tested. These results suggested that PSP has an activity that suppresses cell proliferation. The cell viability of transfected cells was slightly reduced at 17 h after inoculation because of the electroporation shock, but there were no differences between pcDNA-E, pcDNA-AS-PSP, and pcDNA-S-PSP. Thus PSP appears to suppress cell proliferation without influencing cell viability. We also examined the effect of transfection with pcDNA-

AS-PSP, but it showed no effect in cell proliferation. Despite ample expression of antisense PSP mRNA, the PSP protein level did not change compared with that of pcDNA-E transfected cells.

Here, we revealed a novel role for the PSP protein as a suppressor the cell proliferation, which may be related to an inhibitory activity on protein synthesis [6, 10]. We are currently studying to elucidate how PSP suppress the cell proliferation. Expression of PSP has been reported to be completely downregulated in tumor cells [7, 9]. According to our findings, the low expression of PSP in tumor cells may explain their proliferation.This study suggested that the inhibitory activity of PSP on cell proliferation was higher in hepatoma cells than in hepatocytes. High suppression of cell proliferation in hepatoma cells seems to be due to the lower intrinsic expression of PSP in hepatoma cells than in hepatocytes. The relationship between PSP expression and cell proliferation in the tumor cells has yet to be studied, but overexpression of PSP in tumor cells may contribute to new strategies for cancer therapy.

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