Nuclear transfer of perchloric acid–soluble protein by endoplasmic reticulum stressors

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Abstract

Perchloric acid–soluble protein (PSP) is highly conserved during evolution from bacteria to mammals. Although PSP has been recognized as an inhibitor of translation and proliferation in vitro, its precise biological role has not yet been elucidated. Since we previously found similar distributions for PSP and the endoplasmic reticulum (ER) and Golgi complex, the intracellular distribution of PSP was analyzed in more detail. Immunofluorescence studies indicated that PSP co-localized with the ER and Golgi complex, since the distribution pattern of PSP was well matched to both of these organelles. An immunoelectron microscopic study revealed PSP was located not only in the cytosol but also on the surface of the outer ER membrane. Since PSP was present on the ER, we speculated that it may be associated with ER function. Therefore, we analyzed whether or not the ER stress response, which is one of the ER functions, affected PSP expression. The results showed that various ER stressors (thapsigargin, A23187, tunicamycin, brefeldin A, and cisplatin) provoked a dramatic change in the localization of PSP from outside of the nucleus to inside the nucleus within 3 h. Moreover, the ER stressors induced PSP expression. These results suggest that PSP is involved in the cellular response to ER stressors, and that the change in localization of PSP from the ER to the nucleus may be associated with ER stress responses.

Keywords: perchloric acid; soluble protein; μ-calpain; endoplasmic reticulum; ER stress; thapsigargin

Perchloric acid–soluble protein (PSP) was initially isolated from the rat liver as a translational inhibitor (Oka et al. 1995). Subsequently, it was also found in various other species, including humans (Schmiedeknecht et al. 1996), mice (Samuel et al. 1997), goats (Ceciliani et al. 1996), *Escherichia coli* (Colombo et al. 1998), chicks (Nordin et al. 2001), *Saccharomyces cerevisiae* (Kim et al. 2001), pigs (Kaneki et al. 2003b), and so on. Since its gene (a member of the *YER057c/YJGF* family) is highly conserved during evolution, PSP may play important roles in the cell. In mammals, PSP has been reported to be associated with various functions, including ribonuclease activity (Morishita et al. 1999; Sawasaki et al. 2001), fatty acid-binding activity (Sasagawa et al. 1999), differentiation-dependent expression (Oka et al. 1995; Asagi et al. 1998; Nordin et al. 2001; Suzuki et al. 2001; Kaneki et al. 2003a), and repression of proliferation (Kanouchi et al. 2001). However, the precise biological function of PSP has not yet been elucidated.

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Abbreviations: PSP, perchloric acid–soluble protein; ER, endoplasmic reticulum; GRP78, 78 kDa glucose regulated protein/BiP; D-PSP, *Dorosophia* ortholog PSP.

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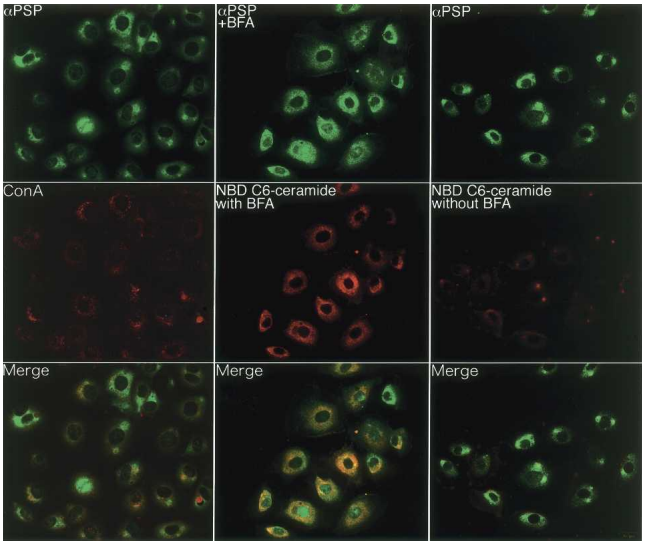

Figure 1. Immunofluorescence analysis of PSP in NRK-52E cells. NRK-52E cells were grown on coverslips, and PSP was labeled by immunofluorescence as described in Materials and Methods. The ER was labeled with 50 μg/mL of Alexa 594-Con A for 10 min after fixation (*left* panels). NRK-52E cells were incubated in medium containing 5 μg/mL of NBD C_6–ceramide–BSA at 4°C for 30 min. After rinsing twice with PBS, the cells were incubated in fresh medium at 37°C for 30 min, and then fixed (*right* panels). To accumulate NBD C_6–ceramide–BSA in the ER, medium containing NBD C_6–ceramide–BSA, PBS, and fresh medium containing 15 μM BFA were used (*middle* panels).

On the other hand, we previously reported that PSP is localized in the endoplasmic reticulum (ER) (Kanouchi et al. 2000), suggesting that an interaction with the ER may be involved in its function. Bovine PSP was reported to be a μ-calpain activator (Melloni et al. 1998). Calpains are intracellular nonlysosomal cysteine proteases that are regulated by calcium (Sorimachi et al. 1997). Although μ-calpain has been studied extensively (Huang and Wang 2001; Glading et al. 2002), its precise function is poorly understood. Recently, it was suggested that μ-calpain is related to the apoptosis induced by ER stresses, such as glucose deprivation, alterations in calcium homeostasis, and accumulation of misfolded proteins in the ER (Lee 2001; Lu et al. 2002). Since PSP is considered to be a μ-calpain activator, it may be involved in the functions of μ-calpain in response to ER stress. The cellular responses to ER stress have been reported to be attenuation of protein synthesis, up-regulation of genes encoding molecular chaperones, degradation of misfolded proteins, and induction of apoptosis (Kaufman 1999; Lu et al. 2002). The induction of these cellular responses is considered to be one of the important functions of the ER.

In this study, we examined the localization of PSP in the ER and investigated its transfer to the nucleus after treatment with ER stressors.

Results

PSP is localized on the ER membrane

We examined whether PSP was localized in the ER by immunocytochemistry of normal rat kidney-52E (NRK-52E) cells. The ER was stained by two chemicals, namely concanavalin A (Con A) or 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)sphingosine–bovine serum albumin complex (NBD C_6–ceramide–BSA). Con A labels mannose-terminated oligosaccharides that exist on the ER and nuclear envelope, while NBD C_6–ceramide–BSA is transferred to the Golgi complex via the ER in living cells. When cells were treated with brefeldin A (BFA), which inhibits ER-to-Golgi traffic, NBD C_6–ceramide–BSA accumulated in the ER. PSP and Con A showed similar localization patterns, although their co-localization was not complete (Fig. 1, left panels). NBD–C_6–ceramide–BSA (without BFA; Golgi complex) was recognized in some of the PSP expression sites (Fig. 1, middle panels). When cells were treated with BFA, NBD–C_6–ceramide–BSA became almost completely co-localized with PSP (Fig. 1, right panels). The cellular distribution of PSP in the rat liver was examined by electron microscopy. The results indicated that PSP was present not only in the cytosol but also on the outer membrane of the ER and the nuclear envelope (Fig. 2). To confirm the localization of PSP in the ER, a rat liver homogenate was separated into three fractions (containing nuclei/mitochondria, microsomes, and cytosol, respectively) by centrifugation and immunoblotted with an anti-PSP antibody. As shown in Figure 3, PSP was present in the microsomal and cytosolic fractions, although much more was present in the cytosolic fraction.

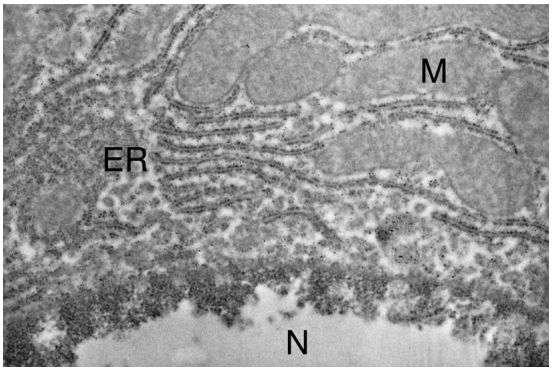

Figure 2. Immunoelectron microscopy for PSP in a normal rat liver section. PSP labeled by electron-dense gold particles is localized on the ER membrane. Nuclei (N), mitochondria (M), and the endoplasmic reticulum (ER) are marked.

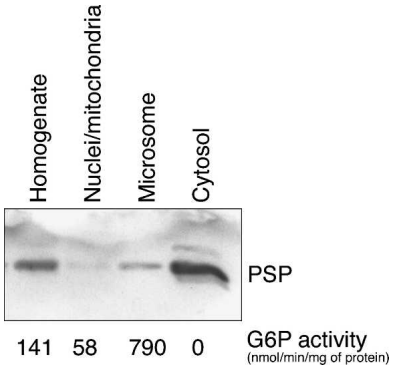

Figure 3. Subcellular distribution of PSP in rat liver cells. Aliquots (containing 20 μg protein) of the homogenate and the three fractions (containing nuclei/mitochondria, microsomes, and cytosol, respectively) were subjected to immunoblotting with an anti-PSP antibody. The G6P activity in each fraction was measured as a marker for the rough ER.

Transfer of PSP to the nucleus after treatment with ER stressors

To determine the effects of ER stressors on the intracellular distribution of PSP, four types of ER stressors were used: namely, two chemicals that disturb Ca^{++} homeostasis (thapsigargin and A23187), one that inhibits glycosylation (tunicamycin), one that inhibits protein traffic from the ER to the Golgi apparatus (brefeldin A), and one that acts as an anti-cancer agent (cisplatin) (Lee 2001; Mandic et al. 2003). PSP was visualized by immunocytochemical staining with an anti-PSP antibody and a Cy-2-conjugated secondary antibody (Fig. 4, green spots), while nuclear DNA was stained with propidium iodide (Fig. 4, red spots). Yellow spots indicate overlapping of the green and the red spots. In untreated cells, PSP was localized outside of the nucleus, probably on the ER membrane. After treatment with tunicamycin (1 μg/mL), brefeldin A (1 μM), thapsigargin (1 μM), A23187 (1 μM), or cisplatin (10 μM) for 6 h, PSP was clearly detected inside the nucleus as well as on the outside. There were no differences among the different types of ER stressors used. The expression of GRP78 (78-kDa glucose-regulated protein/BiP) as a marker for ER stress was examined by immunoblotting. All the ER stressors except for cisplatin induced the expression of GRP78 (data not shown).

Next, we performed an immunocytochemical study to clarify the nuclear transfer of PSP induced by thapsigargin. PSP was obviously detected on the outside of the nucleus, and the contours of the nucleus were clear, in both untreated cells and cells at 1 h after the addition of thapsigargin (1 μM) (Fig. 5A). At 3 h after the addition of thapsigargin, PSP was observed in both the cytosol and the nucleus, and the contours of the nucleus became indistinct. At 6 h after the addition of thapsigargin, PSP was mainly detected in the nucleus. To confirm the nuclear localization of PSP, we performed an immunoblotting study after separating the nuclear and post-nuclear fractions (Fig. 5B). PSP was present in both the cytosolic and nuclear fractions from 3 h after the addition of thapsigargin. The amount of PSP was higher in the cytosol than in the nucleus. The ER stress marker GRP78 was induced from 6 h after the addition of thapsigargin (Fig. 5C). Since the level of PSP appeared to greatly increase during the exposure to thapsigargin, we analyzed whether the other ER stressors also affected the level of PSP expression (Fig. 6). The results revealed that tunicamycin, brefeldin A, thapsigargin, and A23187 all induced the expression of PSP, although the induction levels of PSP were much lower than those of GRP78. Cisplatin did not affect the expression of either PSP or GRP78.

Discussion

We (Oka et al. 1995) and Schmiedeknecht et al. (1996) previously observed that a small fraction of PSP was occasionally localized in the nucleus of liver tissue cells in immunohistochemical studies. However, the physiological significance of this nuclear PSP localization was not elucidated. In the current study, we clarified that PSP exists in not only the cytosol but also the ER, and that it seemed to be attached to the outer membrane of the ER and periphery of the nucleus in cells. However, the majority of PSP was detected in the cytosolic fraction by immunoblotting, although some was also detected in the ER by immunocytochemistry. This inconsistency may arise via weak attachment of PSP to the ER, such that it becomes detached from the ER during the organelle fractionation. However, it was reported that PSP in the liver and its

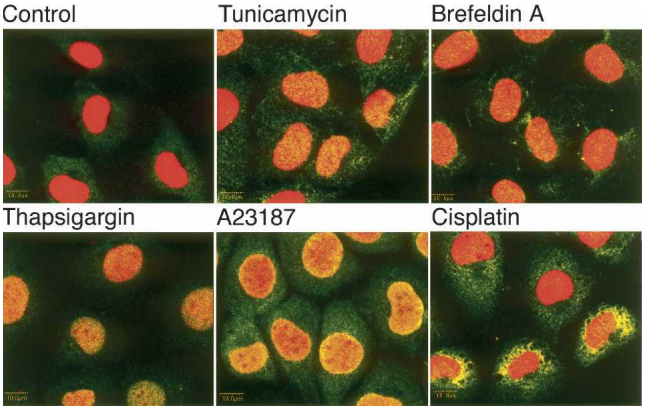

Figure 4. Intracellular distribution of PSP after treatment with various ER stressors. NRK-52E cells were grown on coverslips in medium containing various ER stressors for 6 h. PSP was detected by immunofluorescence cytochemistry (green spots). Nuclear DNA was stained with propidium iodide (red spots).

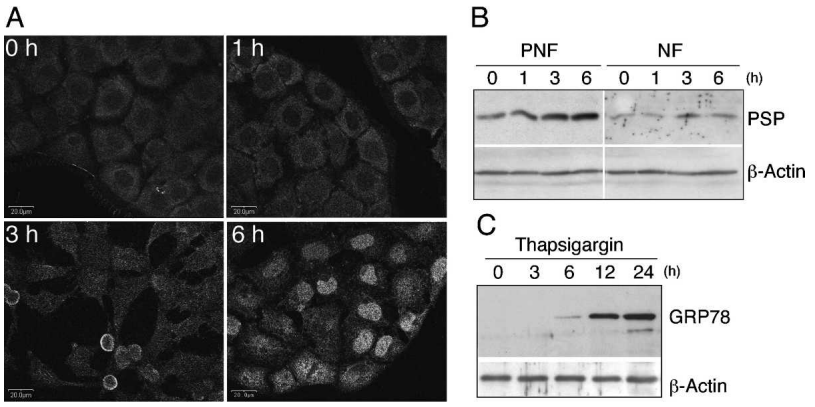

Figure 5. Time course of the intracellular localization of PSP in NRK-52E cells treated with thapsigargin. (*A*) NRK-52E cells were grown on coverslips in medium containing 1 μM thapsigargin. After the addition of thapsigargin, the cells were fixed at 0, 1, 3, and 6 h. PSP was detected by immunofluorescence cytochemistry. (*B*) NRK-52E cells were cultured under the same conditions as described for *A*. The nuclear fraction (NF) and post-nuclear fraction (PNF) were separated from the cells and subjected to immunoblotting using an anti-PSP antibody. (*C*) The PNF was subjected to immunoblotting using an anti-GRP78 antibody.

homolog in goats are very sticky (Mistiniene et al. 2003), and the hydrophobicity of PSP may affect its attachment to membranes. On the other hand, PSP has a tetrapeptide sequence (RIEI) at amino acid residue 115 that appears to be similar to the ER extension signal KDEL (Peterson et al. 2000). Therefore, PSP may be retained on the outer membrane of the ER through this RIEI tetrapeptide sequence. Substitution experiments investigating the function of RIEI as an ER retention signal are currently in progress.

We also investigated the effects of various ER stressors on the expression of PSP. The results revealed that PSP was dramatically transferred from outside of the nucleus to inside the nucleus after treatment with ER stressors. This change occurred earlier than the induction of GRP78, which is often utilized as an indicator of cellular responses to ER stress. PSP may be imported into the nucleus by other molecules or via an as-yet unidentified nucleus import sequence in PSP itself. However, PSP was not observed in the nuclear fraction after treatment with ER stressors in the immunoblotting analysis. We speculate that PSP may leak from the nuclei during the preparation of the nuclear fraction. ER stressors also induced PSP expression. Two previously reported independent cDNA microarray analyses may explain how PSP is induced by ER stressors. First, a comparison of mRNA expressions in HeLa cells under various ER stresses revealed that HeLa cells under amino acid depletion and those expressing the active form of ATF4 showed ER stress-induced *PAX6* gene expression, probably via ATF4 (Okada et al. 2002). Second, a comparison of mRNA expressions between PAX6 overexpressing mouse lens and wild-type mouse lens revealed that PAX6 induced mouse PSP mRNA expression (Chauhan et al. 2002). Thus, the most probable pathway of PSP induction by ER stress is as follows: (1) ER stress provokes phosphorylation of eukaryotic translation initiation factor 2a (eIF2a) via phosphorylation of RNA-dependent protein kinase-like ER kinase (PERK) (Kaufman 1999); (2) the phosphorylated eIF2a causes attenuation of translation, thereby leading to induction of ATF4; (3) ATF4 induces PSP via PAX6.

There are further pieces of evidence suggesting that PSP is involved in ER stress responses. Melloni et al. (1998) reported that bovine PSP activates μ-calpain, which is related to the cellular responses to ER stress. However, Farkas et al. (2004) indicated that the *Drosophila* ortholog of PSP (D-PSP) is not a calpain activator. Hence, there is a conflict regarding whether or not PSP is a calpain

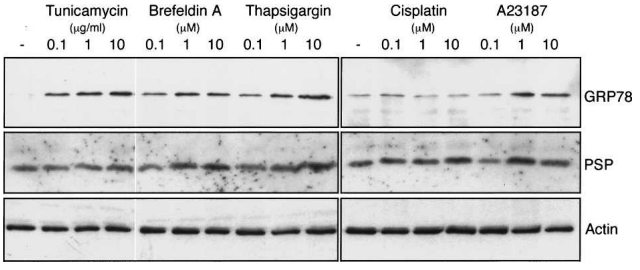

Figure 6. Effect of ER stressors on the expression of PSP. NRK-52E cells were cultured in medium containing various ER stressors at the indicated concentrations for 6 h. The cells were then collected and lysed in cell lysis buffer (140 mM NaCl, 0.5% Triton X-100, 0.2 mM PMSF, 0.2 units/mL aprotinin, 10 mM Tris-HCl [pH 8.0]). Each cell lysate was analyzed by immunoblotting with anti-PSP, anti-GRP78 and anti-β-actin antibodies.

activator. This conflict may arise due to species differences between vertebrates and invertebrates. Although the proliferation of the *Drosophila* Schneider (S2) cell line suffered during heat shock, another ER stress, it was rescued by overexpression of D-PSP. Therefore, even if D-PSP is not a calpain activator, it still appears to be related to ER stress. What is the role of the PSP transfer to the nucleus? It is possible that PSP may transmit information regarding ER stress in order to provoke cellular responses against the ER stress. PSP has been reported to act as an inhibitor of translation and proliferation in vitro and in vivo (Oka et al. 1995; Kanouchi et al. 2001; Himeno et al. 2005). Indeed, translation attenuation and growth arrest are recognized under ER stress conditions, and these cellular responses may be associated with PSP.

The precise physiological function of PSP in mammals has not yet been elucidated, since it has various activities. Hence, the intracellular localization of PSP should also be borne in mind while considering the physiological role of PSP. Further investigations are required to reveal the physiological significance of the transfer of PSP to the nucleus.

Materials and methods

Animals

Male Sprague-Dawley rats (8 wk of age) were purchased from Seac Yoshitomi. All experiments were carried out under the guidelines for Animal Experiments of the Faculty of Agriculture's Graduate Course, Kyushu University, and the Law (no. 105) and Notification (no. 6) of the Japanese Government.

Materials

General chemicals were purchased from Nacalai Tesque. Cisplatin and thapsigargin were obtained from Wako Pure Chemicals. NBD C_6–ceramide–BSA and BFA were purchased from Molecular Probes. The rabbit anti-PSP anti-serum and purified anti-PSP IgG were prepared as described previously (Asagi et al. 1998). The anti-GRP78 rabbit polyclonal antibody and anti-β-actin antibody were purchased from Santa Cruz Biotechnology Inc. and Sigma, respectively. The ECL Western blotting analysis system was purchased from Amersham.

Cell culture

NRK-52E cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (Dainippon Pharmaceutical) supplemented with 50 μg/mL streptomycin, 50 units/mL penicillin, and 5% fetal bovine serum. Cells were replated before they reached confluency, and the medium was exchanged for fresh medium every 3 d.

Immunoelectron microscopy

Each rat liver was dissected out and a small piece was immediately fixed in 2% paraformaldehyde (PFA)/2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 2 h. This sample was then rinsed in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide in the same buffer at 4°C for 2 h, and routinely embedded in Epon 812. Semithin sections (1 μm thick) were cut using an ultramicrotome and incubated with rabbit anti-PSP IgG (1:500 dilution) in 0.1 M phosphate buffer at 4°C for 8 h. After rinsing in 0.1 M phosphate buffer, the sections were incubated with gold-conjugated anti-rabbit IgG (1:200 dilution) in 0.1 M phosphate buffer, and then stained with uranyl acetate and lead citrate. Finally, the sections were observed using an H-7000KU transmission electron microscope (Hitachi Co.) at 75 kV.

Cell fractionation and determination of glucose-6-phosphatase activity

Rat liver (0.5 g) from rats at 6 wk of age was homogenized in 5 mL of buffer (0.25 M sucrose, 25 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA and 50 mM Tris-HCl [pH 7.5]) using a glass-Teflon homogenizer, and centrifuged at 11,000*g* at 4°C for 30 min. The pellet was used as a nuclear and mitochondrial fraction. The supernatant was further centrifuged at 105,000*g* for 1 h. The pellet was used as a microsomal fraction and the supernatant was used as a cytosolic fraction. Glucose-6-phosphatase (G6P) activity was measured as described previously (Baginski et al. 1967). The protein concentration was determined using a BCA protein assay kit (Pierce), with BSA as the standard.

Immunocytochemistry

Immunofluorescence cytochemistry was performed as described previously (Kanouchi et al. 2000). Briefly, cells grown on glass coverslips were washed with phosphate-buffered saline (PBS) and then sequentially fixed at room temperature with 4% PFA in PBS for 20 min and 50% methanol for 10 min. The fixed cells were treated with PBS containing 1% BSA for 30 min, and then incubated with a rabbit anti-PSP anti-serum (1:2000 dilution) at 4°C overnight. After washing with PBS, the cells were incubated with Cy-2-conjugated goat anti-rabbit IgG (Amersham; 1:3000 dilution) in PBS containing 0.1% BSA at room temperature for 2 h. Nuclei were stained with 50 μg/mL propidium iodide at room temperature for 5 min. Finally, the coverslips were washed extensively and mounted on glass slides with Vectashield (Vector). The cells were photographed using a confocal laser scanning microscope (Olympus).

Immunoblotting

Nuclear, other organelle, and post-nuclear fractions were separated from NRK-52E cells using a Nuclear/Cytosol Fractionation Kit (BioVision). Immunoblotting was performed as previously described (Kanouchi et al. 2000).

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