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Perchloric acid-soluble protein is accumulated in nuclei by ER-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 a μ-calpain activator, its precise biological role has not yet been elucidated. Because the μ-calpain has been reported to act as an inducer of apoptosis in endoplasmic reticulum (ER)-stressed cells, we speculated that PSP might be associated with ER-stress. In this study, we used immunoelectron microscopy to demonstrate that PSP locates on the ER membrane. Moreover, various ER-stressors (thapsigargin, A23187, tunicamycin, brefeldin A and cisplatin) provoked dramatic change in localization of PSP from ER to nuclei. These results suggest that PSP is a novel ER-stress associated protein and may induce attenuation of translation and/or apoptosis in ER-stressed cells.

Key words: perchloric acid-soluble protein, p14.5, μ-calpain, endoplasmic reticulum, ER-stress, thapsigargin

Introduction

Perchloric acid-soluble protein (PSP) was initially isolated from rat liver as a 14,400 dalton inhibitor of translation [1]. Later it was found in various other species [1-9]. Its gene (*YER057c/YJGF* family) has been highly conserved during evolution. As highly conserved proteins usually have key cellular functions, PSP might also play important roles in the cell. In mammals, it has been reported that PSP is associated with various functions such as ribonuclease activity (1, 3, 10 and 11), fatty acid-binding activity [12], μ-calpain activation [5], differentiation-dependent expression [3, 6, 7, 13 and 14] and proliferation repression [15]. However, the crucial function of PSP is not yet known.

Because it was suggested that PSP might exist in ER from our previous study [22], the function of PSP seemed to connect with ER function. On the other hand, Melloni et al., reported that bovine PSP is a μ-calpain activator. Calpains are intracellular non-lysosomal cysteine proteases whose activity is regulated by calcium [16]. Both μ-calpain and m-calpain show ubiquitous and constitutive expression [16]. Although these calpains have been studied extensively [17 and 18], their precise functions are poorly understood. Recently, Lu et al [19] have suggested that calpains are related to apoptosis induced by ER-stress such as glucose deprivation, alterations in calcium

homeostasis and accumulation of misfolded proteins in ER [20]. Because PSP is a μ-calpain activator, it might be involved in the function of μ-calpain including response to ER-stress. The cellular responses to ER-stresses have been reported as the attenuation of protein synthesis, the up-regulation of genes encoding molecular chaperones, the degradation of misfolded protein and the induction of apoptosis [19 and 21]. These cellular responses are considered to be one of the important functions of ER [21].

In this investigation, we confirm further that PSP exists in ER and the change of expression pattern of PSP by ER-stressors.

Experimental Procedures

Materials

General chemicals were purchased from Nacarai Tesque (Kyoto, Japan). Cisplatin and tapsigargin were obtained from Wako Pure Chemical (Osaka, Japan). Anti-PSP rabbit serum and purified anti-PSP IgG was prepared as described previously [13]. Anti-glucose-regulated protein 78 rabbit polyclonal antibody and anti-β-actin antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Sigma (Saint Louis, MO), respectively. An ECL western blotting analysis system was purchased from Amersham (Little Chalfont, UK).

Cell culture

Normal rat kidney-52E (NRK-52E) cells were obtained from American Type Culture Collection (Manassas, VA). They were cultured in Dulbecco's modified Eagle's medium (Dainippon Pharmaceutical, Osaka, Japan) supplemented with 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.

Immunoelectron microscopy

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

Cell fractionation and determination of glucose-6-phosphatase activity

Rat liver (0.5g, 6 weeks old) was homogenized in 5 ml of a 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 x g and 4℃ for 30 min. The pellet was used as a nuclear and mitochondrial fraction. The supernatant was further centrifuged at 105,000 x g for 1 h. The precipitate was used as a microsomal fraction and the supernatant was used as a cytosolic fraction. Glucose-6-phosphatase activity was measured as described previously [23]. The protein concentration was determined with the BCA protein assay kit (Pierce, Rock Ford, IL). Bovine serum albumin was used as a standard.

Immunocytochemistry

Immunofluorescent cytochemistry was carried out as described previously [2]. Briefly, cells grown on glass coverslips were washed with PBS and fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. The fixed cells were treated with PBS containing 1% bovine serum albumin (BSA) for 30 min, and incubated with a 1: 2000 rabbit anti-PSP antiserum (1: 2000) at 4 ℃ overnight. After washing with PBS, the cells were incubated with Cy-2 conjugated goat anti-rabbit IgG 1: 3000 (Amersham) diluted in PBS containing 0.1% BSA at room temperature for 2 hr. The nuclear DNA was stained with 50 μg/ml propidium iodide at room temperature for 5 min. Finally, the coverslips were washed extensively, mounted onto glass slides with Vectashield (Vector, Burlingame, CA), and the cells were photographed using a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Immunoblotting

The nuclear and other organelle, post-nuclear fractions, were separated from NRK-52E cells by the Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA). Immunoblotting was performed according to the method described previously [22].

Results

PSP is localized on the ER membrane

Normal rat liver was used for detection of PSP molecules in an electron microscopic analysis. As a result, PSP was detected in not only cytosol but also ER membrane (Fig. 1). To confirm that PSP is localized in ER, the rat liver homogenate was separated into fractions containing nuclei and mitochondria, microsomes or cytosol by centrifugation. Figure 2 shows that PSP exist in the microsome and cytosol fraction, though more PSP was present in the cytosolic fraction. Enzymatic activity of glucose-6-phosphatase (a marker of rough ER) was checked to assert whether microsome

Figure 1. Immunoelectron microscopy for PSP in a rat liver section. PSP was detected by immunoelectron microscopy in the section obtained from the normal rat liver. Electron-dense gold particles are localized in ER membrane. Nuclei (N), mitochondria (M) and ER (endoplasmic reticulum) are marked.

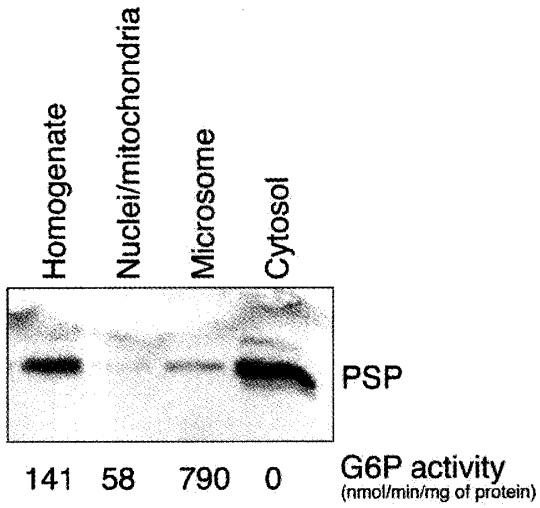

Figure 2. Subcellular distribution of PSP in rat liver cells. Twenty μg each of protein from homogenate and fractions containing nuclei and mitochondria, microsome, and cytosol were subjected to immunoblotting with an anti-PSP antibody. Glucose-6-phosphatase activity in each fraction was measured as a marker of rough ER.

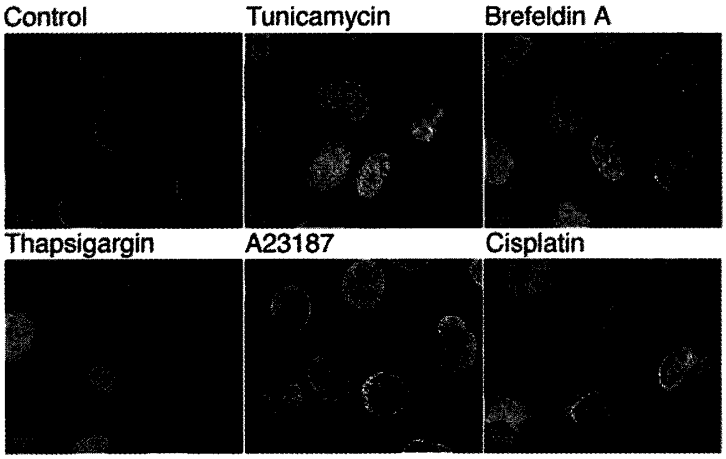

Figure 3. Intracellular distribution of PSP after the addition of various ER-stressors. NRK-52E cells were grown on coverslips in the medium containing various ER-stressors for 6 h. PSP was detected by immunofluorescent cytochemistry (green spots). Nuclear DNA was stained with propidium iodide (red spots).

was separated well from the other fraction.

PSP accumulates in nuclei after the treatment with ER-stressors

To determine whether the ER-stressors affect intracellular distribution of PSP, four types of ER-stressors were used, chemicals that disturb Ca^{++} homeostasis (thapsigargin and A23187), a chemical that inhibits glycosylation (tunicamycin), a chemical that inhibit protein traffic from ER to Golgi apparatus (brefeldin A), and one that acts as anti-cancer agent (cisplatin) [20 and 24]. PSP was visualized by immunocytochemical staining with an anti-PSP antibody and a Cy-2 conjugated secondary antibody (green spots, Fig. 3). Nuclear DNA was stained with propidium iodide (red spots) and yellow spots indicate overlaps of green spots and red spots. In untreated cells, PSP was

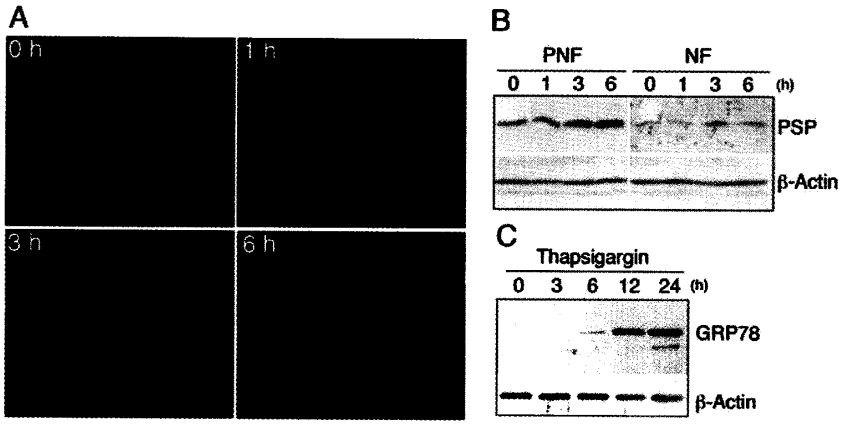

Figure 4. Time course of intracellular localization of PSP in NRK-52E cells treated with thapsigargin. (A) NRK-52E cells were grown on coverslips in the medium containing 1 μM of thapsigargin. After the addition of tapsigargin, NRK-52E cells were fixed at 0, 1, 3, and 6 h. PSP was detected by immunofluorescent cytochemistry. (B) NRK-52E cells were cultured under the same conditions as in Fig. 3. Nucleic fraction (NF) and post-nuclear fraction (PNF) were separated from the cells and subjected to immunoblotting using anti-PSP antibody. (C) PNF was subjected to immunoblotting using anti-GRP78 antibody.

localized outside of nuclei, probably in ER membrane. After treatment with tunicamycin (1 μg/ml), brefeldin A (1 μM), thapsigargin (1 μM), A23187 (1 μM) and cisplatin (10 μM) for 6 h, PSP was clearly detected in the nuclei as well as outside of nuclei. There were no differences in type of ER stressors. The expression of Glucose-regulated protein 78 (GRP78) as a marker of ER-stress was measured by immunoblotting. All ER-stressors excepting cisplatin induced the expression of GRP78.

We next examined when PSP moved into nuclei by treatment with thapsigargin. PSP was detected using the immunocytochemical method (Fig. 4A). PSP was obviously detected outside of nuclei, and the contours of nuclei were clear in untreated cells and at 1 h after the addition of thapsigargin (1 μM). At 3 h after the addition of thapsigargin, PSP was observed in both cytosol and nuclei, and the contours of nuclei became indistinct. At 6 h after the addition of thapsigargin, PSP was mainly detected in the nuclei. To confirm the existence of PSP in nuclei, we tried to detect PSP by the immunoblotting method after separating nuclear and post nuclear fractions (Fig. 4B). PSP was induced in both cytosol and nuclei from 3 h after addition of thapsigargin. The amount of PSP in cytosol was higher than that in nuclei. GRP78 was induced from 6 h after addition of thapsigargin (Fig. 4C).

Discussion

In this study, we elucidated that PSP exists on the ER membrane, although PSP was originally discovered as a cytosolic and nucleic protein [1-7, 13 and 14]. Until now, PSP and its homologues were purified from the cytosolic fraction. In the present immunoblotting study, we also found a high level of PSP in the cytosolic fraction of the rat liver. However, immunoelectron microscopy indicated that a lot of PSP seemed to exist on the ER membrane. Thus, there was a discrepancy between the results of microscopic examinations and immunoblotting analysis. We speculate that PSP weakly

binds to the ER membrane. PSP has a tetra peptide sequence (RIEI), which is similar to an ER-extensional signal (KDEL) [25] from, a. a. residue 115. Now we are investigating whether this sequence is important for localization in ER.

As PSP is localized in ER, it might be associated with ER functions. Bovine PSP activates μ-calpain that is related to cellular responses to ER-stress [5 and 19]. Therefore, to determine whether PSP is related to ER-stress, we investigated the effects of various ER-stressors on the expression pattern of PSP. As a result, PSP dramatically moved from outside of nuclei to the inside of nuclei in the presence of ER-stressors. This change was earlier than induction of GRP78 as one of the indicators of cellular response to ER-stress. We could not detect an obvious accumulation of PSP in nucleic fraction under the ER-stress by the immunoblotting method. PSP might leak from nuclei during the preparation of nucleic fraction. Up-regulation of PSP was also recognized by tapsigargin. It was reported that paired box gene 6 (PAX6) that is one of the transcription factors was induced by ER-stress, and PAX6 induces Hrp12, mouse PSP homologue [26 and 27]. Because the investigation was with cDNA microarray analysis, more study may be needed. Indeed, there is a PAX6 binding site upstream of the human PSP gene.

Under the ER-stress condition, cells seems to attenuate of translation and/or apoptosis. PSP is an inhibitor of translation and inducer of μ-calpain [1, 5, 10 and 11]. These activities might act as a response to ER-stress. It is not clear from our observations whether the translocation of PSP is associated with the attenuation of translation and/or the activation of calpain. Further investigation is necessary to reveal the physiological significance of translocation of PSP from ER to nuclei.

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Footnotes

Abbreviations: PSP, perchloric acid-soluble protein; ER, endoplasmic reticulum; G6P, Glucose-6-phosphatase; PAX6, paired box gene 6; GRP78, glucose-regulated protein 78.