Front. Biol. China 2008, 3(1): 13–18
DOI 10.1007/s11515-008-0016-z

RESEARCH ARTICLE

Subcellular localization of PS1 based on PS1/GFP fusing protein

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Abstract Mutations in presenilin 1 (PS1) gene are closely associated with the early onset of familial Alzheimer's disease (EOFAD). The fusion genes, GFP-PS1 (recombinant plasmid pEGFP-C1-PS1) and PS1-GFP (recombinant plasmid pEGFP-N2-PS1) were constructed to study the subcellular localization of PS1 holoprotein. Recombinant plasmids were transiently transfected into two cell lines, HEK293 and CHO, respectively, using the green fluorescence from GFP (green fluorescence protein) as the PS1 localization signal. Then, we observed green fluorescence with a SPOT II (Olympus, BH2) and CONFOCAL microscope (Olympus, FV300) under 488 nm. The results show that PS1 located on the nuclear envelope. A few can be found on the cellular membrane and in the cytosol in a non-homogeneous distribution.

Keywords presenilin1, GFP, fusing protein, cellular distribution

1 Introduction

Alzheimer's disease (AD) is one of the most common human central nervous system degenerative diseases. Genetic analysis has identified three genes that are closely related to the incidence of EOFAD. These are β-amyloid precursor protein (APP) gene, presenilin 1 (PS1) gene and presenilin 2 (PS2) gene, which are located on chromosome 21, 14 and 1 respectively (Goate et al., 1991; Levy-Lahad et al., 1995).

The mutations of these genes lead to EOFAD in an autosomal dominant inheritance pattern. APP mutation is related to the 2%–3% of EOFAD. PS2 mutation is related to the 20% of EOFAD. PS1 mutation is related to 70%–80% of EOFAD. PS1 plays the most important role in EOFAD. Some studies found that the PS1 protein had 6–8 hydrophobic transmembrane (8-TM) helices (Lehmann et al., 1997; Li et al., 1998; Dewji et al., 2004). Mature PS1 is cut into an N-terminal fragment (NTF) and a C-terminal fragment (CTF). Then the two fragments form functional heterodimers (Gopal et al., 1996; Moliaka et al., 2004). Pathological studies of AD found that APP protein generates $A\beta_{1\text{-}40}$ multi-peptide (β-amyloid, Aβ) by γ-secretase. $A\beta_{1\text{-}40}$ is the main component of the "senile plaques" which is an important pathological characteristic of AD. PS1 is the active center of γ-secretase. Recent studies identified that there were four membrane proteins as components of γ-secretase: PS1, nicastrin, Pen-2 and Aph-1, all of which forms the functional activity of γ-secretase (Edbauer et al., 2002; Fraering et al., 2004; Niimura et al., 2005). PS1 mutation can make APP proteins, generating the peptide $A\beta_{42}$ which has a toxic effect on nerve cells and more easily assembles into non-degradable precipitates that are "senile plaques". And this will eventually lead to the occurrence of AD.

In-depth studies found that PS1, as the γ-secretase active center, acts as an important role not only in the cleavage of the APP protein, but also in cleavage of many transmembrane proteins, including Notch and E-cadherin. It then plays a role in nervous system development and in the interaction between cells (Marambaud et al., 2002; Selkoe et al., 2003). For the PS1 subcellular localization, researchers believe that PS1 located in the endoplasmic reticulum, plasma membrane, the cell surface and the membrane of some subcellular structures. But, there is dispute regarding whether PS1 exists in the Golgi membrane (Walter et al., 1996; Culvenor et al., 1997; Annaert et al., 1999; Ray et al., 1999; Kim et al., 2000; Pasternk et al., 2003). Some studies show that PS1 exists in the surface of some particles which are closely related to the Golgi. But we haven't seen the exact report yet. So we studied the subcellular localization of PS1 to

Translated from *China Biotechnology*, 2006, 26(6): 17–22 [译自: 中国生物工程杂志]

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help researchers find the proteins interacting with PS1 and deeply understand the function of PS1. Current studies of the PS1 subcellular localization mostly use the cell separation device and indirect immunofluorescence method. But the cell separation device may not reflect PS1 in the dynamic cell positioning and in indirect immunofluorescence. So, we constructed two PS1/GFP fusion proteins and observed the fusion proteins localization in HEK293 and CHO cell lines. We used GFP fluorescence as the signal to study the PS1 subcellular localization through SPOT II and CONFOCAL. We found that the PS1 can be found on the nuclear envelope with a few on the cell-cell contact cellular membrane and in the cytosol in a non-homogeneous distribution.

2 Materials and methods

2.1 Materials

HEK293 cells, CHO cells, pEGFP-C1 plasmid and pEGFP-N2 plasmids were preserved by our laboratory.

2.2 Reagents

Restriction endonuclease *Eco*RI, *Bam*HI, *Sal*I, and DNA polymerase *Pyrobest*™ were purchased from TaKaRa. The Lipofectamine transfer agent was from Invitrogen and rabbit anti-PS1 antibody was obtained from the Beijing Xuanwu Hospital.

2.3 PS1-GFP fusion gene construction

The coding sequence of PS1 was amplified from pMD18-T-PS1 by PCR. To generate recombinant vector pEGFP-C1-PS1, PCR was used with the following primers: upstream primer: 5′– GCC GAA TTC TAT GAC AGA GAT ACC TGC ACC –3′ (30 bp); downstream primer: 5′– GAA GGA TCC GAT ATA AAA CTG ATG GAA TGC –3′ (30 bp), and cloned into pEGFP-C1 vector with *Eco*RI/*Bam*HI. The target sequence of PS1 was amplified by PCR with pEGFP-C1-PS1 as template and inserted into pEGFP-N2 with *Eco*RI/*Sal*I to generate the recombinant vector pEGFP-N2-PS1. All recombinant vectors were verified by sequencing.

2.4 PS1-GFP fusion gene expression and fluorescence observation

HEK293 and CHO cells were transfected with pEGFP-C1-PS1 or pEGFP-N2-PS1 vector with Lipofectamine according to the manufacturer's instructions. Cells were seeded on glass slips and placed in a 24-well plate at 1×10^5 cell/mL. Forty-eight hours after transfection, cells were washed twice with cold PBS and fixed with 3.7% formaldehyde for 10 min and were then stained with propidium iodide (PI) and, lastly, observed using SPOT II (Olympus, BH2) and CONFOCAL microscope (Olympus, FV300).

2.5 Western blot analysis

After transfection and culture for 48 hours, the cells were trypsinized and collected by centrifugation and washed in PBS. The cell pellet was re-suspended in a lysis buffer (50 mmol/L Tris-HCl, pH7.5, 1% Triton X-100, 150 mmol/L NaCl, 0.5 mmol/L EDTA, 0.5 mmol/L PMSF). After 5 min incubation at 4°C, the lysate was centrifuged at 13000 r/min, 4°C for 30 min and supernatant was preserved. Protein concentration was determined by Bradford protein assay. The protein which had been separated by 10% SDS-PAGE was transferred to PVDF membrane and probed with antibodies by using the mouse anti-GFP antibody as the first, and AP-anti-mouse antibody as the second. Then, the protein was stained by NBT/BCIP.

3 Results

3.1 PS1-GFP fusion gene construction

PS1 is a transmembrane protein with 467 amino acid residues with a length is 1401 bp. Studies found that the mature PS1 cut at 260–320 aa (the hydrophilic central between TM6-TM7) generated NTF and CTF, then two fragments formed the heterodimers with functional activity. We cloned the full-length of PS1 cDNA into two plasmids, pEGFP-C1 and pEGFP-N2, which were reinforced-GFP protein expression vector, constructed GFP-PS1 fusion gene (pEGFP-C1-PS1 recombinant plasmid, PS1 gene linked to the GFP gene C-terminal), and PS1-GFP fusion gene (pEGFP-N2-PS1 recombinant plasmid, PS1 gene linked to the GFP gene N-terminal). The expressed GFP-PS1 and PS1-GFP fusion protein structure is shown in Figure 1.

3.2 GFP fusion protein detected by Western blot

The molecular weight of PS1 protein is 51 kD, and that of GFP is 26 kD. The molecular weight of fusion protein

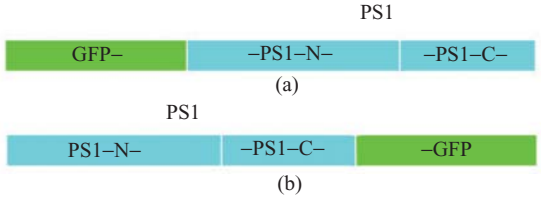

Fig. 1 Structure of fusing protein. (a): PS1-GFP fusing protein; (b): GFP-PS1 fusing protein

GFP-PS1 and PS1-GFP both are about 77 kD. Forty-eight hours after transfecting the fusion protein expression vector pEGFP-C1-PS1 and pEGFP-N2-PS1 into HEK293 cell lines, the Western blot results shows that two fusion proteins appeared as one band with a molecular weight of 77 kD, similar to the PS1-GFP or GFP-PS1 fusion protein (Fig. 2). It shows that the PS1 in the both fusion proteins was full-length, rather than the PS1 NTF or CTF. The band of 50 kD has α-tubulin as an internal reference.

3.3 Analysis of GFP fusion gene expression

As a control group, we transfected pEGFP-C1 plasmid into HEK293 cells, then observed the uniform green fluorescence in the cells with SPOT II (Figure 3). We then transfected the recombinant plasmid, pEGFP-C1-PS1, into HEK293 cells. Twenty-four hours later, we observed GFP spontaneously show green fluorescence with CONFOCAL microscopy at 488 nm excitation and discovered that the green fluorescent focused on: 1) the nuclear envelope; 2) the cytosol in a non-homogeneous distribution; and 3) on the cell membrane of cell-cell contacts to a lesser degree (Fig. 4). Transfecting recombinant pEGFP-N2-PS1 plasmid into HEK293 cells received the same results under the same conditions (Fig. 5). When we transfected the expression fusion gene vector into CHO cells and observed it under SPOTII, the results were similar (Fig. 6). Comparing the results of HEK293 cell lines with CHO cell lines, we found that the PS1 located on the nuclear envelope was clearer than that in the HEK293 cell line.

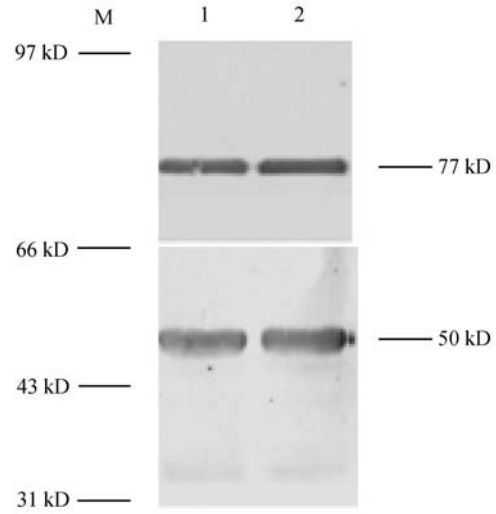

Fig. 2 Western blot analysis of fusing protein. M: Protein Marker; 1: Total protein of cells transfected with pEGFP-C1-PS1; 2: Total protein of cells transfected with pEGFP-N2-PS1

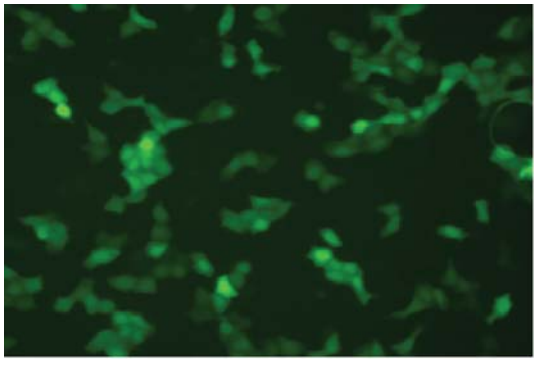

Fig. 3 SPOTII microscopy of HEK293 cells transiently transfected with pEGFP-C1

4 Discussion

PS1 is closely related to AD as an active center of γ-secretase which hydrolyzes proteins. PS1 as a multi-pass transmembrane protein contains 467 amino acid residues. It may be involved in interacting with a series of transmembrane proteins, particularly type I transmembrane protein, which is involved with numerous ways to control the protein and transmit the cell signal (Levy-Lahad et al., 1995; Rogaev et al., 1995). The structure of PS1 is still not completely clear, especially the number of PS1 transmembrane and its subcellular localization. Knowing the precise structure of PS1 can help us understand the function of PS1 better. The cellular localization of PS1 will help us find more proteins interacting with PS1 and learn more regarding its function.

Researchers used a variety of biochemical methods to detect the distribution of PS1 in the cell. They found that PS1 exits from the endoplasmic reticulum. Intermediates are transmitted from the endoplasmic reticulum to the Golgi apparatus, or more precisely, the smooth surface of Golgi apparatus, and then, the cell surface (Walter et al., 1996; Culvenor et al., 1997; Annaert et al., 1999; Ray et al., 1999; Kim et al., 2000). Akihiko and his colleagues, (Takashima et al., 1996) through the expression of the PS1 in abundance in COS-7 cells, clarified that the PS1 can be found in the cell membrane, the endoplasmic reticulum and the nuclear periphery using immuno-electron microscopy and biochemical methods. Recent studies also found that PS1 can be found in lysosomal membranes (Pasternk et al., 2003). Many studies have shown that the PS1 existed in the surface of some particles which are related to the Golgi apparatus. Seong and his colleagues (Kim et al., 2000) broke through the cells to the different cell debris, acquiring the localization information of PS1 by density-gradient centrifugation. Their findings show that PS1 and the Golgi apparatus were not found in the same debris. Therefore, the question on whether PS1 existed in the Golgi membrane still had not been determined. However, the biochemical

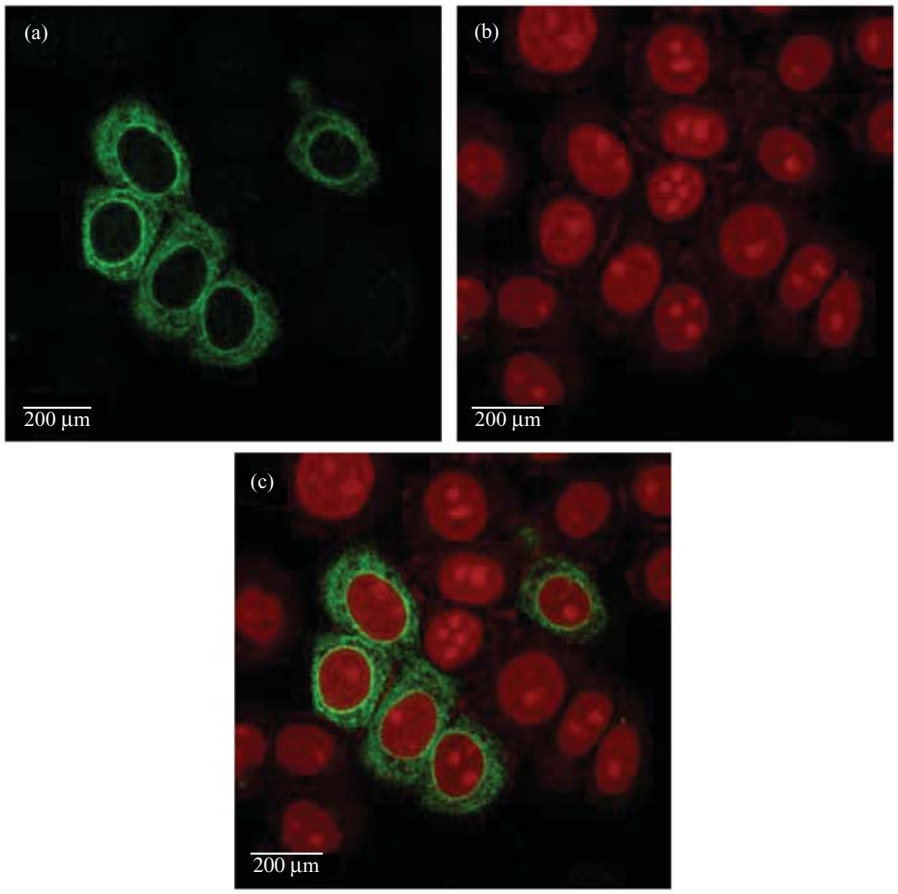

Fig. 4 HEK293 cells transfected with recombinant plasmid pEGFP-C1-PS1. (a): 24 h after transfection, cells were observed by CONFOCAL microscopy (Green:GFP); (b): PI staining after transfection shows nucleus (red); (c): merged view of (a) and (b).

methods to extract broken cell elements and the classification of the different elements, used in many studies, cannot directly reflect the accuracy of the information regarding the PS1 protein *in vivo* and the cellular localization of the dynamic processes.

To simply study the subcellular localization of PS1, we have constructed a PS1 fusion protein (pEGFP-C1-PS1 and pEGFP-N2-PS1). Western blot results show that the fusion protein of PS1 had the full-length PS1 protein instead of the NTF or CTF of PS1. We observed the fluorescence from transfected cells and found the patterns of green fluorescence from the transfected cells with fusion protein were significantly different from the control group (pEGFP-C1). The result of the subcellular localization of PS1 shows that PS1 is distributed in the region near the nuclei and located in the nuclear envelope. The nuclear envelope, as the separator of nuclear and cytoplasmic organs, is a relatively independent organ that is similar to the cell membrane. However, there is a special structure called the nuclear pore complex in the nuclear membrane which can selectively allow proteins to pass. This is different in the cell membrane. The preliminary findings in this study show that PS1 can be found on the nuclear envelope and indicated that PS1 might interact with certain proteins in the nuclear membrane. PS1 protein could be divided into different segments which can be a constructed fusion protein with GFP. Then, the exact segments of PS1 which are found on the nuclear envelope could be confirmed. Furthermore, the PS1 gene used in this study is from mouse genes. The expression of PS1 gene in the mouse-CHO cells was higher than in human-HEK293 cells. So PS1 localization in the nuclear membrane shown in Figure 6 is not as clear as in Figures 4 and 5.

At the same time, we found that PS1 was distributed unevenly in the cytosol which suggests that PS1 can be found on the surface of various organelles with membrane structure. Some studies indicated that PS1 exists in the endoplasmic reticulum and in some secretory vesicles, but not in the Golgi apparatus. These results are similar to the subcellular localization of PS1 we found. A large number of PS1 exists in the cytosol not to exercise its function, but to prepare the formation of γ-secretase complex. Moreover, the latest research found that PS1 had to combine with at least three other proteins, nicastrin, Pen-2 and Aph-1, to be active as γ-secretase

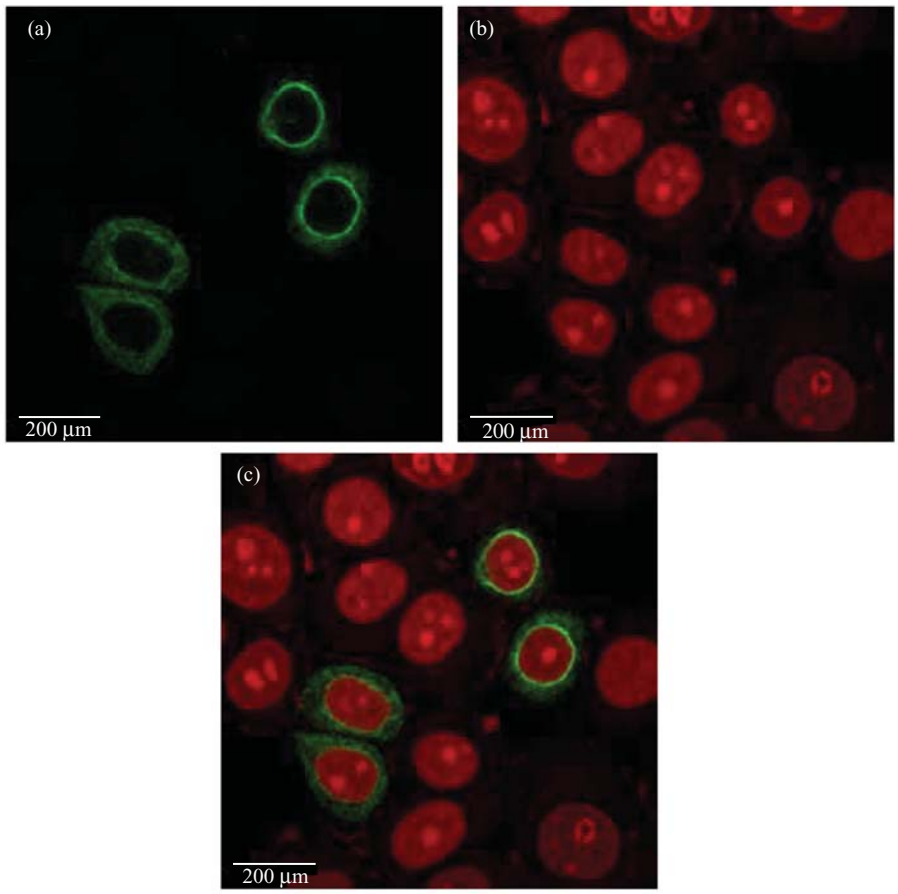

Fig. 5 HEK293 cells transfected with recombinant plasmid pEGFP-N2-PS1. (a): 24 h after transient transfection, cells were observed by CONFOCAL microscopy (Green:GFP); (b): PI staining after transfection shows the nucleus (Red); (c): merged view of (a) and (b).

(Goutte et al., 2002). PS1 itself is not modified but only spliced by PSase to form heterodimers. We speculate that the non-homogeneous PS1 distribution in the cytosol is likely to be the PS1 in a transport procession. But further exploration is needed to determine whether it is the functional positioning or not.

PS1 is one of the most important proteins closely related to AD. Research on the subcellular localization of PS1 provides useful evidence to explore its functions for us. Currently, some studies found that PS1 played the proteolytic role of γ-secretase to splice the proteins, such as APP, Notch, E-Cadherin and GHR. These transmembrane proteins all are hydrolyzed by γ-secretase at the transmembrane area, generates the intracellular protein fragments to cytosol which interacts with other proteins or are transferred to the nucleus. They play their roles as a transcription factor (Mumm et al., 2000; Haass et al., 2002; Marambaud et al., 2002; Cowan et al., 2005). In

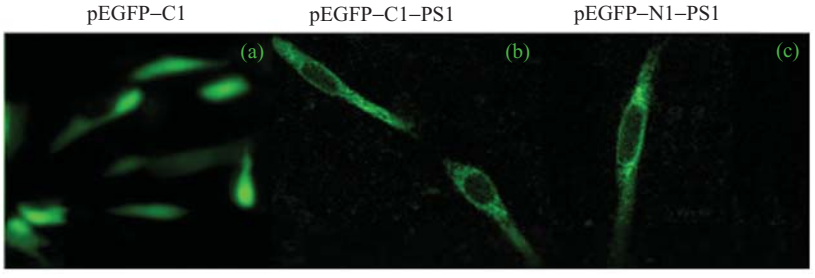

Fig. 6 Subcellular localization of the fusion protein in CHO cells. (a): SPOTII microscopy of control cells transfected with pEGFP-C1; (b): CONFOCAL microscopy of cells transfected with pEGFP-C1-PS1; (c): CONFOCAL microscopy of cells transfected with pEGFP-N2-PS1.

this study, we provided a new subcellular localization for PS1 to carry out its functions. We speculated that PS1 on the nuclear envelope might be involved with some of the proteins on the nuclear envelope and should be important in further understanding the roles of PS1 in Alzheimer's Disease.

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