Immunohistochemical analysis of presenilin-1 expression in the mouse brain

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Abstract At least 22 different mutations associated with early-onset familial Alzheimer's disease (AD) in various kindreds have been reported to occur in a recently identified gene on chromosome 14, presenilin 1 (PS-1) (Sherrington et al. (1995) Nature 375, 754–760 [1] and reviewed by Van Broeckhoven (1995) Nat. Genet. 11, 230–231 [2]). In order to study the localization of PS-1 in the brain, we raised a polyclonal antiserum specific to a fragment of the predicted protein sequence of PS-1. PS-1 immunostaining was found intracellularly, in the perikaria of discrete cells, mostly neurons, appearing as thick granules, resembling large-size vesicles. These granules were located in the periphery of cell bodies and extended into dendrites and neurites. PS-1 expression was found to be broadly distributed throughout the mouse brain, not only in structures involved in AD pathology, but also in structures unaltered by this disease.

Key words: Neurodegenerative disease; Alzheimer's disease; Presenilin; Chromosome 14; Immunohistochemistry; Mouse brain; Amyloid

1. Introduction

The study of the inheritable forms of Alzheimer's disease (AD) has led to the identification of four genes linked to the development of the disease: the β-amyloid precursor protein (βAPP) on chromosome 21 [3], apolipoprotein E on chromosome 19 [4], and presenilin 1 and 2 (PS-1 and PS-2) on chromosomes 14 [1] and 1 [5,6] respectively. The proteins encoded by the presenilin genes are 467 (PS-1) and 448 (PS-2) amino acids long. They display a similar hydrophobicity profile, with at least seven highly hydrophobic stretches of approx. 20 residues which constitute putative transmembrane segments. PS-1 and PS-2 display a high homology in the putative membrane-spanning domains (61–100% identity). The primary structure of the presenilins suggests that these are integral membrane proteins, but their precise molecular function remains to be determined. The existence of a moderate but significant amino acid similarity between the presenilins and *spe4*, a *Caenorhabditis elegans* gene involved in the transport and partitioning of cellular constituents during spermatogenesis, has led to the proposal that the presenilins might participate in intracellular protein transport [1]. Homology with another *C. elegans* gene *sel12* (48% amino acid similarity), which facilitates signalling mediated by lin12/Notch, suggested that the presenilins might be involved in the transduction pathway of the lin12/Notch family [7].

The analysis of the expression pattern of the presenilins in the brain is an important step in the understanding of both the physiological function of these proteins and their role in the pathophysiology of AD. Furthermore, the subcellular localisation of the presenilins will provide insights into their biological function. We developed a specific polyclonal antiserum raised against a fragment of the predicted protein sequence of PS-1. We describe here the distribution and subcellular localisation of PS-1 protein in the mouse brain.

2. Materials and methods

2.1. Antiserum production

Peptide (340–356: EAQRDSHLGPHRSTPE) from the sequence of PS-1 protein (PS-1$_{(340-356)}$) was synthesized on a model 431 peptide synthesizer (Applied Biosystems) and then purified by reverse-phase chromatography. Before immunization, the peptide was coupled to RSA. Male New Zealand rabbits (2–3 kg) were immunized by subcutaneous injections of 1 mg of the immunogen emulsified in Freund's complete adjuvant; injections were repeated 28, 56, 84, and 112 days after the initial one. The animals were bled after each boost. Solid-phase ELISA was used to titrate the antibodies of all the bleedings (1–4). Plates were coated with the peptide PS-1$_{(340-356)}$ used at 10^{-3} mg well^{-1}. After incubation with the anti-PS-1$_{(340-356)}$ antiserum used at dilutions ranging from 1:100 to 1:100000 and washing, secondary antiserum (goat anti-rabbit IgG) coupled to alkaline phosphatase (1:1000 dilution) was added and, after the addition of 4-nitrophenyl phosphate in diethanolamine, the optical density was measured at 405 nm. For immunoprecipitation and immunohistochemistry experiments, we used the antiserum of rabbit 1331.

2.2. Immunoprecipitation of PS-1 protein from in vitro translated material

The coding sequence of PS-1 was amplified from human cell line cDNA (KCN-SMS) using standard PCR techniques. After subcloning in pCRII (Invitrogen), the amplified cDNA was sequenced and the 5' end of the coding sequence modified by introducing a Kozak translation initiation sequence. The coding sequence was further subcloned in the pBTG vector in between the 5' and 3' untranslated regions of the ß-globin gene. For in vitro transcription/translation, the DNA template was transcribed using T3 RNA polymerase (Promega) as described in the suppliers manual. Translation of the mRNA transcript in a cell-free rabbit reticulocyte lysate (Promega) was performed according to supplier's instructions. The reaction was carried out for 60 min at 30°C in the presence of 40 µCi of [^{35}S]methionine and 0.5–1.0 µg transcript. For immunoprecipitation, in vitro translatated PS-1 was diluted with 300 µl of medium buffer (100 mM Tris pH 8.0, 10 mM EDTA, 0.5% NP-40, 0.5% Triton X-100) and incubated for 2 h at room temperature with 2 mg of protein A-Sepharose together with 5 µl of undiluted rabbit polyclonal anti-PS-1 antiserum. Unbound proteins were removed from Sepharose beads by washing three times with 10 mM Tris (pH 7.5), 150 mM NaCl, 0.2% NP-40, 2 mM EDTA and subsequently twice with 10 mM Tris (pH 7.5), 500 mM NaCl, 0.2% NP-40, 2 mM EDTA. The precipitate was incubated at room temperature for 20 min with Laemmli buffer and fractionated by 8–16% gradient SDS-PAGE (Novex). The gels were analyzed by fluorography (Amplify, Amersham).

2.3. Immunohistochemistry

Male Balb C mice (20–25 g) were anaesthetized with sodium pentobarbital (50 mg/kg ip) and ketamine (40 mg/kg ip). They were perfused through the ascending aorta, first with saline and then with

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fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.5). Brains were removed, immersed in the same fixative solution for 3 h, then stored for 18 h in 0.1 M phosphate buffer containing 20% saccharose, and finally frozen at $-30°C$. Transverse sections of 25 μm thickness were cut with a cryomicrotome (Leica) and placed in glass vials for immunohistochemistry. Sections were preincubated with 0.03% hydrogen peroxide for 30 min, washed and then incubated with normal goat serum (10% in PBS) at 19°C for 30 min. They were further incubated at 4°C for 24 h with the PS-$1_{(340-356)}$ antiserum (1331) diluted to 1:100, 1:500, 1:2500, 1:5000 or 1:10000 in PBS containing 2% normal goat serum and 0.3% Triton X-100. After washing with PBS, sections were incubated for 1 h with goat anti-rabbit IgG coupled to biotin diluted to 1:400, washed and then incubated for 1 h with the complex avidin-horseradish peroxidase (Vector Laboratories). Sections were finally incubated for 5–30 min in peroxidase substrate solution containing 0.01% hydrogen peroxide, 0.05% diaminobenzidine tetrahydrochloride (DAB) in 0.1 M Tris buffer, pH 7.2. They were mounted on slides and dehydrated.

Control experiments were performed (1) by omitting the primary antiserum, (2) by using the preimmune serum, (3) by immunoabsorpion of the primary antiserum with the synthetic peptide PS-$1_{(340-356)}$ (preincubation for 18 h of the primary antiserum diluted to 1:2500 in presence of 1–1000 μg of the peptide PS-$1_{(340-356)}$) before performing immunohistochemistry as described above.

3. Results

3.1. Immunoprecipitation of PS-1 in vitro

The polyclonal antiserum 1331 was raised against a synthetic peptide corresponding to the amino acid stretch 340–356 of the predicted protein sequence of human PS-1. To test whether this antiserum had a specific affinity for the full length PS-1 protein, we immunoprecipitated in vitro translated human PS-1 protein (Fig. 1). PS-1 protein was detected after precipitation with the antiserum 1331, but not with the preimmune serum of the same rabbit. The Antiserum 1331 did not precipitate in vitro translated human PS-2 (not shown).

The predicted molecular weight of PS-1 is 53 kDa. In addition to the expected band at around 50 kDa, we found an additional band at 85 kDa and a diffuse high molecular weight band. The addition of urea into the loading reduced the intensity of the diffuse 85 kDa band and that of diffuse high molecular weight and increased the intensity of the 50 kDa band (data not shown). Therefore, it is likely that the additional band at 85 kDa and the diffuse high molecular weight band resemble aggregated forms of PS-1.

3.2. Distribution of PS-1 protein in the mouse brain (Fig. 2)

Widespread expression of PS-1 protein was observed in the mouse brain. Cells in which PS-1 immunostaining was extremely dense were found in high numbers in the piriform cortex, entorhinal cortex, layers V and II of all cortical areas, hippocampus, dentate gyrus, amygdaloid nuclei, olfactory bulb, thalamus (paraventricular and mediodorsal nuclei), septum, hypothalamus (preoptic, supraoptic and paraventricular nuclei), basis pontis, and in moderate number in the pallidum, thalamus (ventromedial and lateral nuclei) substantia nigra ars reticulata, reticulotegmental nucleus pontis, lateral vestibular nucleus, cerebellum (lateral and medial nuclei). Neurons with much lower density of PS-1 immunostaining were found in moderate numbers in the striatum, all layers of colliculis, medial geniculate nucleus and cerebellum (granule cells of dentate fascia), and in much lower numbers in all the other examined structures, including for example the layer VI of the cortex and substantia nigra pars compacta. No immunostaining was found in the white matter (internal and external capsules).
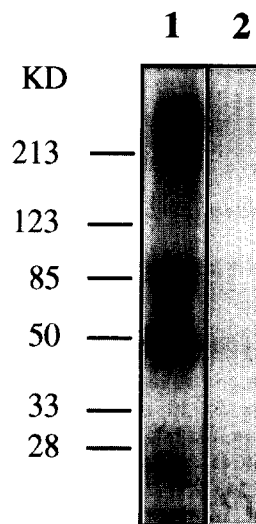

Fig. 1. Immunoprecipitation of in vitro translated PS-1. Human PS-1 mRNA was transcribed from the corresponding plasmid and translated in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. IVT PS-1 was immunoprecipitated and analysed by SDS-PAGE as described in section 2. Lane 1, IVT PS-1 immunoprecipitated with polyclonal antiserum 1331; lane 2, IVT PS-1 immunoprecipitated with the corresponding preimmune serum; KD, kilo Dalton.

There was no cellular immunostaining when the primary antiserum was omitted or when the preimmune serum was used. Likewise, there was no cellular immunostaining when the primary antiserum was first incubated with the synthetic peptide at the concentration of 1 mg/ml (Fig. 2).

3.3. Subcellular localization of PS-1 protein in mouse brain (Fig. 2)

PS 1 immunostaining was found intracellularly, in the perikaria of discrete cells, mostly neurons. It appeared as thick granules resembling vesicles, mainly at the periphery of cell bodies and in dendrites and neurites. In some, but not all neurons, the immunostaining was found around the nucleus. When the antiserum was preincubated with increasing amounts of PS-$1_{(340--356)}$ synthetic peptide, PS-1 immunostaining decreased in all subcellular compartments in a dose-specific manner.

Fig. 2. Immunohistochemical images showing the distribution and subcellular localization of PS-1 protein in the mouse brain. PS-1 immunostaining is found in neurons located in various structures. (A,C) Transverse sections showing neurons with high density of PS-1 immunostaining in the cortex (Ctx), the hippocampus (Hip) the globus pallidus (GP). Neurons with lower density are seen in the striatum (St). No immunostained cells are found in the white matter, internal and external capsule (ic, ec) ($\times 50$). (B) Section adjacent to A incubated with the antiserum following absorption with the synthetic peptide PS-$1_{(340-356)}$ ($\times 50$). (D) PS-1 immunostaining is found intracellular in neurons of the hippocampus ($\times 200$); (E) section adjacent to D showing no intracellular immunostaining after immunoabsorption. (F,G) PS-1 immunostaining appears at high magnification ($\times 1200$) as thick granules resembling large-size vesicles in the cell bodies (arrow in F) and in neuronal processes (arrows in G). For absorption, 1 mg of synthetic peptide PS-$1_{(340-356)}$ was incubated with 2 μl of antiserum in 5 ml of PBS for 18 h (4°C). This solution was then used (without centrifugation) to perform immunohistochemistry on brain sections.
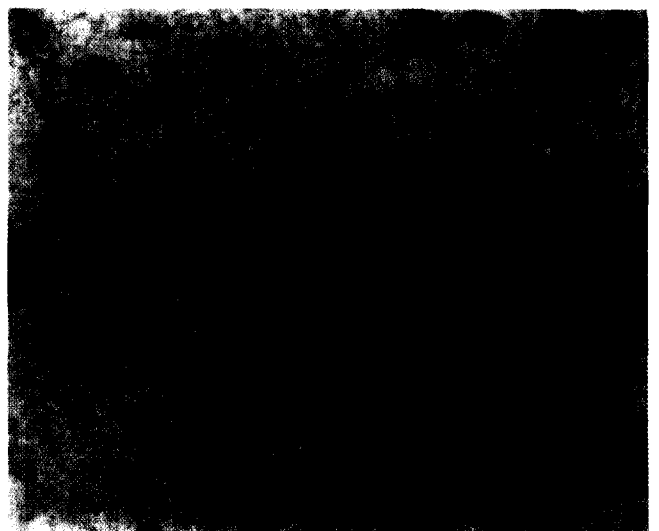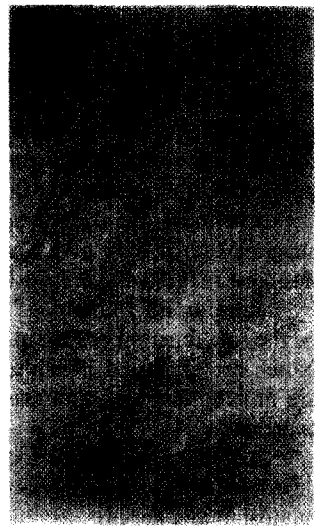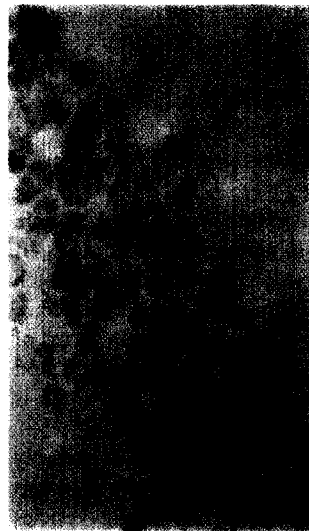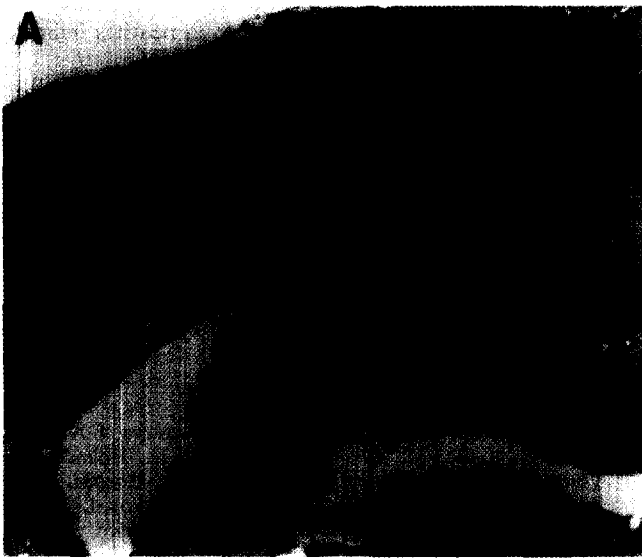
4. Discussion

In order to elucidate the specific function of PS-1 and its participation in AD physiopathology, we raised a polyclonal antiserum (1331) that recognized PS-1 protein both by immunoprecipitation and by immunohistochemical analysis on brain sections. This antiserum was raised against a region which is conserved between mouse and human PS-1 protein sequence. The epitope is not shared by PS-2 and, as expected, the antiserum recognized PS-1 and not PS-2.

Previously a widespread pattern of PS-1 expression had been suggested on the basis of Northern-Blot analysis of PS-1 mRNA transcript expression [1]. Here we show by immunohistochemical analysis a broad neuronal distribution of the PS-1 protein in the brain. The pattern of distribution of PS-1 in mice described here is similar to that previously described for βAPP in rats [8], but the exact colocalization of these 2 proteins will require double labelling studies. PS-1 is found not only in regions of the brain that display senile plaques and/or neurofibrillary tangles in AD, such as the cortex and the hippocampus, but also in regions little or not at all affected by the disease, such as the striatum and the cerebellum. However, preamyloid deposits are much more widely distributed than senile plaques throughout the central nervous system [9]. All these data together may indicate either that expression of βAPP and PS-1 proteins are not the only factors responsible for neurodegeneration in affected structures, or that there are endogenous mechanisms protecting neurons in unaffected stuctures in AD brains.

Furthermore, the wide neuronal expression of PS-1 protein suggests that it may play a physiological role in neurons. It has been suggested that PS-1 interacts with mammalian Notch by studies on PS-1 homologs in nematode [7], but this cannot be the sole function of PS-1 protein. The mammalian forms of Notch are expressed in limited neuronal structures [10], and are not found in most of the neurons where PS-1 protein is expressed.

PS-1 immunostaining is localized intracellularly as recently described in transfected cells by Kovacs et al. [11]. PS-1 immunostaining appears as thick granules resembling vesicles located mainly at the periphery of cell bodies, and occasionally around the nucleus. This granular staining extended into neuronal processes, an observation that further suggests its vesicular sublocalization. The intracellular localization of PS-1 protein in neurons and its presence in neurites indicate that it may participate in neuritic transport.

The determination of the distribution and subcellular localization of PS-1 protein in mouse brain should be of value in clarifying the mechanisms by which mutations in the PS-1 gene lead to AD and therefore the selective neuronal vulnerability observed in AD patients.

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