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Analysis of Presenilin 1 and 2 interacting proteins in mouse cerebral cortex during development

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ABSTRACT

In our previous report, we showed that Presenilin (PS)1 and 2 have differential expression profile from early embryonic stages till adulthood in mouse cerebral cortex, suggesting that both of these proteins are crucial for brain development. Genetic manipulation studies have also shown the involvement of PS1 in brain development, but PS2 remains largely unexplored. In order to understand how PS1 and 2 mediate developmental functions, we have investigated the interaction of PS1 and 2 with proteins of mouse cerebral cortex during development. Co-immunoprecipitation (Co-IP) combined with MALDI-MS/MS analysis revealed 12 interacting partners of PS1 and 11 partners of PS2. The interacting proteins were different for PS1 and 2, and involved in cell division, glycolysis, cell adhesion and protein trafficking. Densitometric analysis of protein bands visualized after SDS-PAGE separation of Co-IP proteins revealed variation in their amount and degree of interaction during different developmental stages of mice. Further, immunoblot based validation of PS1 interacting protein Notch-1 showed maximum interaction at embryonic day (E) 12.5, decline at E18.5, upregulation from postnatal day 0 (P0) to P20 and thereafter reduction at P45 and 20 weeks. *In-silico* analysis of PS and its interacting proteins indicated conformation based interaction through common type of secondary structures having alpha helical, extended beta strand and random coil, and CK2, PKC phosphorylation and myristoylation motifs. Taken together, our study showed that PS1 and PS2 interact to varying extent with different proteins of mouse cerebral cortex and suggests their interaction based on specific conformation and involvement in diverse functions essential for the brain development.

1. Introduction

Presenilin (PS)1 and 2 are multi-pass transmembrane (TM) proteins of aspartyl protease family. Their primary sequence is evolutionarily conserved in eukaryotes, having homologues in organisms as distant as *C. elegans* (Levitan and Greenwald, 1995), Drosophila (Boulianne et al., 1997), and lower chordates (Martínez-Mir et al., 2001), suggesting functional conservation. Mammalian PS1 and 2 share extensive amino acid sequence identity and are synthesized as 50 kDa polypeptides (Hutton and Hardy, 1997). They span the membrane in a serpentine way and consist of nine TM domains. Their main domains are three stretches of hydrophilic domains comprising of N-terminal tail, C-terminal tail and a long loop between TM VI and TM VII (Tomita and Iwatsubo, 2013). Amino terminal and long loop domains of PS1 and PS2 are phosphorylated at serine residues (De Strooper et al., 1997).

PS1 and PS2 have been implicated in several brain functions (Zhang et al., 2013) through their interaction with amyloid precursor protein, Notch-1, β-catenine, calsenilin, cadherines and others (Chen and Schubert, 2002). With respect to brain development, PS1 knockout mice showed developmental deformities including cortical dysplasia and died before birth (Wen et al., 2005). Conditional knockouts of PS1 have shown its implication in several processes such as neuronal migration, cortical lamination, neurogenesis, neuronal differentiation (Wines-Samuelson et al., 2005), synaptogenesis (Uchihara et al., 2006), somitogenesis, gliogenesis (Saura, 2010), and growth (Nakajima et al., 2009). On the other hand, knockout of PS2 survived well without any defect in the brain morphology (Herreman et al., 1999), indicating that the protease might not contribute to brain developmental processes directly and may serve to compensate PS1 functions postnatally (Wang et al., 2003).

In our previous report, we showed that PS1 and PS2 expression varied significantly in the cerebral cortex during critical time of development. These findings indicated that PS1 and 2 might have independent involvement during different developmental

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stages. In order to understand such involvement, we have analyzed interacting partners of PS1 and 2 during brain development. We have co-immunoprecipitated (Co-IP) PS1 and PS2 specific complexes during different developmental stages, and the resulting proteins were identified by MALDI-MS/MS. Further, the interaction of Notch-1 in Co-IP proteins was validated with PS1 at different developmental stages. *In silico* analysis for secondary structure prediction of putative interacting proteins was done by bioinformatics GOR-4 and specific motifs were identified by Motif-scan.

2. Materials and methods

2.1. Animals

Swiss albino strain mice were inbred and maintained at 25 ± 2 °C with 12 h light and 12 h dark schedule with *ad libitum* access to standard mice feed and drinking water in the animal house of the Department of Zoology, Banaras Hindu University, Varanasi, India. They were used according to guidelines of the institutional animal ethical committee, Banaras Hindu University, Varanasi, India. Male mice ($n = 54$) were used for the present study and the detailed information is provided in the supplementary section.

To analyze the interacting proteins of PS during development, mice of embryonic day (E) 12.5, E18.5, postnatal day (P) 0, P20, P45 and 20 weeks (w) were used. The mice were decapitated and embryos were collected by flushing out from uterus for prenatal ages. The telencephalon from E12.5 and the cerebral cortex from other developmental stages were dissected out.

2.2. Preparation of protein extract

Cytosolic protein extract was prepared from the mouse cerebral cortex of different developmental ages. Briefly, 10% homogenate was prepared in RIPA buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40 and 0.5% sodium deoxycholate] with protease inhibitors (Sigma–Aldrich, USA) at 4 °C (Kim et al., 2012; Jang et al., 2011). The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C. The post-nuclear supernatant was collected in separate microfuge tube and stored at −70 °C. The amount of protein in the preparation was estimated by Bradford (1976) method.

2.3. Co-IP

For Co-IP, the protein (100 μg) was incubated with protein A Sepharose beads at 4 °C for 2 h to avoid nonspecific binding, and then centrifuged at 3000 × *g* for 10 min. The precleared supernatant was incubated overnight with 2 μg of anti-PS1 antibody (Alpha Diagnostics International, USA) or anti-PS2 antibody (Alpha Diagnostics International, USA) in 500 μl of IP buffer (50 mM Hepes, pH 7.4, 50 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM PMSF). IgG was used as a negative control. Next day, protein A Sepharose beads were added to the reaction mix at 4 °C for 4 h. Thereafter, the beads containing bound proteins were precipitated by centrifugation, washed three times in Co-IP buffer. Co-immunoprecipitate was denatured by boiling in SDS-sample buffer, resolved on 10% Tris–glycine SDS-PAGE and analyzed by silver staining. To analyze Co-IP bands, the signal intensity of each stained band was calculated as Integrated Density Value (IDV) by spot densitometry tool AlphaEaseFC software (Alpha Innotech Corp., USA).

2.4. Immunoblotting

Initially, the input level of both Notch-1 full length (Notch-1 FL) and PS1 was determined by their protein expression during development. Briefly, 20 μg cytosolic protein from each developmental stages was denatured, resolved by 10% Tris–glycine SDS-PAGE, transferred onto PVDF membrane. The membrane was blocked in 5% (w/v) nonfat milk prepared in 1× PBS for 2 h and incubated overnight with rabbit anti-Notch-1 antibody in 1:1000 dilutions (Developmental Studies Hybridoma Bank, USA). After washing twice in 0.1% PBST, the membrane was incubated with horse-radish peroxidase conjugated goat-anti-rabbit secondary antibody in 1:2000 dilutions (Bangalore Genei, India), washed twice in 0.1% PBST and detected by ECL. Immunoblotting of input PS1 was done as mentioned previously (Kumar and Thakur, 2012). Internal control β-actin was detected by reprobing the membrane with antimouse β-actin HRP conjugate, 1:10,000 dilutions (Sigma–Aldrich, USA). In order to analyze interaction level of Notch-1 with PS1 protein, equal amount of PS1 Co-IP protein from different developmental stages was subjected to immunblotting of Notch-1 Intracellular domain (Notch-1 NICD). The signal intensities for input Notch-1 FL, input PS1 and Co-IP Notch-1 NICD fragment were normalized against signal intensity of β-actin and represented as a histogram with mean of three values calculated as RDV (IDV of Notch-1 FL/β-actin, PS1/β-actin and Notch-1 NICD/β-actin).

2.5. Sample preparation for matrix assisted laser desorption/ionization (MALDI)-time of flight (ToF) mass spectrometry

The protein bands were excised, minced into 1 mm^3 pieces and transferred into a sterile microcentrifuge tube. It was washed three times with 500 μl of wash solution (50% acetonitrile, 50 mM ammonium bicarbonate) and incubated at room temperature for 15 min with gentle agitation (vortex mixer on lowest setting). Further, the gel was dehydrated in 100% acetonitrile for 5 min. Acetonitrile was removed and the gel was dried at room temperature for 10–20 min. It was rehydrated in 150 μl reduction solution (10 mM DTT, 100 mM ammonium bicarbonate) for 30 min at 56 °C. The reduction solution was discarded and added 100 μl alkylation solution (50 mM iodoacetamide, 100 mM ammonium bicarbonate) and incubated for 30 min in the dark at room temperature. The alkylation solution was discarded with a pipette, added 500 μl of wash solution and incubated at room temperature for 15 min with gentle agitation. The gel was dehydrated in 100 μl 100% acetonitrile for 5 min. Further, acetonitrile was discarded and gel dried at room temperature.

The gel was rehydrated with a minimal volume of protease digestion solution (20 μg/ml in 50 mM ammonium bicarbonate solution) and digested overnight at 37 °C. Next day, it was centrifuged at 12,000 × *g* for 30 s. The supernatant was transferred to a sterile centrifuge tube. Further, 25–50 μl of extraction solution (60% acetonitrile, 0.1% TFA) was added to gel pieces, agitated gently by vortexing at lowest setting for 10 min and it was centrifuged at 12,000 × *g* for 30 s. The extracted peptides were pooled and dried to near dryness by centrifugal evaporation. The peptides were resuspended in 5 μl of resuspension solution (50% acetonitrile, 0.1% TFA) and agitated gently on a vortex at lowest setting.

2.6. MALDI-TOF-MS

The samples were spotted (1 μl) on MALDI target plate [MTP 384 ground steel (Bruker Daltonics, Germany)] followed by 0.5 μl of alpha-cyano-4-hydroxycinnamic acid matrix [10 mg/mL in 50% acetonitrile, 0.1% TFA (Sigma–Aldrich, USA)]. The peptide spectra was acquired in an AUTOFLEX speed MALDI TOF/TOF instrument (Bruker Daltonics, Germany) having Nd:YAG smart Laser beam of 335 nm wavelength. External calibration was done with peptide calibration standard supplied by Bruker, with masses ranging from 1046 to 3147 Da. The obtained spectra were acquired using Flex Control version 3.3 software in reflectron ion mode with an average of 2000 laser shots at mass detection range between 700 and 4000 m/z. The most three abundant peaks were subjected to further fragmentation using LIFT method. The data were analyzed using Flex Analysis software version 3.3 (Bruker Daltonics, Germany) and searched in MASCOT web server (Matrix Science; http://www.matrixscience.com) using Biotools version 3.2 software (Bruker Daltonics, Germany).

2.7. In silico analysis

Secondary structure of the predicted proteins obtained from MALDI-MS analysis were analyzed by GOR4 (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html, Combet et al., 2000). In order to identify the motifs in the predicted proteins, MOTIF-SCAN (http://myhits.isb-sib.ch/cgi-bin/motif_scan) was done.

2.8. Statistical analysis

Each experiment was repeated three times ($n = 3 \times 3 = 9$ mice/group). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by *post hoc* test of Student–Newman–Keuls method through Jindel Scientific SigmaPlot for Windows (standard version 2.0). Values were reported as mean ± SEM and p-values <0.05 were considered as significant.

3. Results

3.1. Co-IP of PS interacting proteins in the cerebral cortex of mice during development

With the optimized conditions for PS1 (Fig. 1A) and PS2 (Fig. 2A), Co-IP analysis showed that interacting proteins varied differentially both for PS1 (Fig. 1B) and PS2 (Fig. 2B) with respect to number, amount and degree of interaction in the cerebral cortex of mice during development. Out of these proteins, 12 partners of PS1 (Fig. 1C) and 11 of PS2 (Fig. 2C) showed significant alterations in their level of interaction in prenatal and postnatal stages as compared to P0. Predicted molecular weight of PS1 interacting partners was in the range of 30–342 kDa while the range was 24–193 kDa for PS2. One way ANOVA analysis showed significant F values for all the proteins (details of F values included in supplementary section).