Altered binding of mutated presenilin with cytoskeleton-interacting proteins[1]

Amit A. Johnsingh[a], Jane M. Johnston[b], George Merz[b], Jiliu Xu[b], Leszek Kotula[b], J. Steven Jacobsen[c], Nikolaos Tezapsidis[a,b,*]

[a] *Department of Psychiatry, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, Box 1229, New York, NY 10029, USA*
[b] *NYS Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314, USA*
[c] *Department of CNS Disorders, CN-8000, Wyeth-Ayerst Research, Princeton, NJ 08543, USA*

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Abstract The majority of familial Alzheimer's disease (AD) cases are linked to mutations on presenilin 1 and 2 genes (*PS1* and *PS2*). The normal function of the proteins and the mechanisms underlying early-onset AD are currently unknown. To address this, we screened an expression library for proteins that bind differentially to the wild-type PS1 and mutant in the large cytoplasmic loop (PS1L). Thus we isolated the C-terminal tail of the 170 kDa cytoplasmic linker protein (CLIP-170) and Reed–Sternberg cells of Hodgkin's disease-expressed intermediate filament-associated protein (Restin), cytoplasmic proteins linking vesicles to the cytoskeleton. PS1L binding to CLIP-170/restin requires Ca^{2+}. Treating cells with thapsigargin or ionomycin increased the mutated PS1 in CLIP-170 immunoprecipitates. Further, PS1 and CLIP-170 co-localize in transfected cells and neuronal cultures.

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Key words: Alzheimer's disease; Presenilin; Mutation; 170 kDa cytoplasmic linker protein; Restin; Protein interaction

1. Introduction

All early-onset forms of familial Alzheimer's disease (FAD), inherited in an autosomal dominant fashion, are linked to three gene products: amyloid precursor protein (APP) [1], presenilin 1 (PS1) [2] and presenilin 2 (PS2) [3]. PS1, which is linked to approximately 50% of all known early-onset FAD cases, is expressed in somatodendritic processes of neurons [4,5]. The genetic and biochemical data from carriers of these mutations, of which more than 50 have been identified so far, suggest that the accelerated pathology and onset of the disease is caused by a dysfunctional PS1 which ultimately alters APP proteolytic processing. These conclusions are consistent with the amyloid cascade hypothesis [6]. However, the normal function(s) of PS1 and PS2 in terminally differentiated neurons, where both are most likely to be expressed, is currently unknown, as is the effect of the mutations.

A brain expression library was screened for peptides able to bind to the large cytoplasmic loop of PS1 (PS1L). One of the clones sustained, in the presence of Ca^{2+}, a higher binding affinity for two FAD-linked mutants (substitutions E280A and L286V) compared to the wild-type (wt) PS1. This clone corresponded to the C-terminal tail of 170 kDa cytoplasmic linker protein (CLIP-170)/Reed–Sternberg cells of Hodgkin's disease-expressed intermediate filament-associated protein (Restin).

2. Materials and methods

2.1. Screening

A single nucleotide (nt) substitution (E280A) was introduced into *PS1* by unique site elimination mutagenesis (Pharmacia). Dr. Bruce Sopher and Dr. George Martin provided a *PS1* cDNA with the L286V mutation. Dr. Wilma Wasco (Harvard Medical School) provided a *PS2* clone.

Human presenilins (or related fragments) were produced as fusion proteins to glutathione-S-transferase (GST) [4]. These peptides were produced in bacteria, transformed with a modified pGEX-KT vector [7] containing foreign cDNA in-frame to GST [8]. The GST fusion peptides, expressed by isopropyl β-D-thiogalactopyranoside (IPTG) induction, following purification, were labeled with ^{32}P using casein kinase II.

Plaques (10^6) from Y1090 cells infected with a human fetal brain λgt11 library (American Tissue Culture Collection #77436) were IPTG-induced and screened for the expression of β-galactosidase (β-Gal) fusion proteins that bind specifically to [^{32}P]GST-PS1L (260–407 amino acids (aa) of PS1). Nitrocellulose filters containing an imprint of the plaques were blocked in Tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.4), 3% dry milk powder and 100 μg GST/ml. Binding was performed using the probe at a specific activity of 4×10^6 counts per minute (cpm)/μg/ml. Clones that provided 100% positive plaques after tertiary screening were processed.

2.2. Binding assays

Lysogenized Y1089 cells, infected with candidate clones, were blotted onto nitrocellulose filters as described [9]. Blots were incubated in TBS containing [^{32}P]peptides (Figs. 1C and 2D). Filters were analyzed by autoradiography and densitometry.

*Corresponding author. Fax: (1)-718-698 1553.
E-mail: n_tezapsidis@prodigy.net

[1] This work is dedicated to Dr. Thomas Kreis.

Abbreviations: aa, amino acid; AD, Alzheimer's disease; APP, amyloid precursor protein; AT, PS1 N-terminus-directed antibodies; β-Gal, β-galactosidase; BSA, bovine serum albumin; CLIP-170, 170 kDa cytoplasmic linker protein; CT, PS1 C-terminus-directed antibodies; DMEM, Dulbecco's modified essential medium; EDTA, ethanyldiamino tetraacidic acid; ER, endoplasmic reticulum; FAD, familial Alzheimer's disease; FCS, fetal calf serum; GST, glutathione-S-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; K_d, dissociation constant; MIP, microtubule-interacting protein; MNR2, peptide corresponding to 311–330 amino acids of PS1; nt, nucleotide; PBS, phosphate-buffered saline; PS1, presenilin 1; PS2, presenilin 2; Restin, Reed–Sternberg cells of Hodgkin's disease-expressed intermediate filament-associated protein; SREBP, sterol regulatory binding protein; TBS, Tris-buffered saline; wt, wild-type; *Zeo*, zeocin resistance gene

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Saturation binding curves and Scatchard plots were obtained for GST-PS1L, GST-PS1L(L280V) and GST-PS1L(E286A) binding to #22197 fusion protein in the presence of 1 mM Ca^{2+} (total binding) or 2 mM ethanyldiamino tetraacidic acid (EDTA) (non-specific binding) [9]. Ca^{2+}-dependent binding was the difference.

2.3. Cell cultures

For the transfection of SY5Y cells, PerFect™ Pfx-8 (Invitrogen) was used. Stably transfected cell-lines were selected in 200 μg/ml zeocin for pcDNA3.1-*Zeo*-derived plasmids (Invitrogen) or 1000 μg/ml G418 for the pCI-neo-derived plasmids (Promega). Cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential aa, 100 IU/ml penicillin, and 100 μg/ml streptomycin and 20% fetal calf serum (FCS) in a 5% CO_2 incubator.

Neuro-2a cells were similarly grown in DMEM containing 10% FCS and transfected cell-lines were selected in 600 μg/ml G418.

Primary neuronal cultures were prepared from rat embryonic cortex (E16) as described previously [10] and cultured for 3 days in DMEM/Ham's F12 medium plus 10% FBS.

2.4. Immunofluorescent confocal microscopy

Cells were fixed (methanol or 2% paraformaldehyde, room temperature, 5 min) then blocked (20 min) in phosphate-buffered saline (PBS) containing 2% serum or 1% bovine serum albumin (BSA). For antibody incubations and staining, see Fig. 2 legend. Images were viewed with the Nikon PCM-2000 confocal microscope.

3. Results

3.1. Screening

Twenty-five clones provided 100% positive plaques upon tertiary screening using the [^{32}P]GST-PS1L probe. Among those, 21 were also able to bind to [^{32}P]GST-PS2L, as determined by dot-blots, and were classified as non-specific. The remaining four clones did not bind to [^{32}P]GST-PS2L and were all sequenced. Here, we describe the characterization of one of these candidates. Bacteriophage DNA from clone #22197 contained a 2.4 kb cDNA insert at the unique *Eco*RI site (Fig. 1A). Western blotting of lysogenized #22197-infected Y1089 cells, using anti-β-Gal antibodies, showed that a 170–175 kDa β-Gal fusion protein was expressed (Fig. 1B). The *Eco*RI site in λgt11 is located 53 nt upstream of the β-Gal termination codon. Since β-Gal is a 116 kDa protein, only part of the 2.4 kb insert was a reading frame. DNA sequence analysis [11] determined that 381 nt at the 5′ end of the insert were 100% identical to the 2689–3069 nt fragment of *CLIP-170* (GenBank: M97501) [12] and to the 2838–3218 nt fragment of *Restin* (EMBL: X64838) [13]. Restriction map analysis confirmed that the insert corresponded to the C-terminal portion of either *CLIP-170* or *Restin* with an additional 3′ untranslated fragment (results not shown).

CLIP-170 is a 1392 aa microtubule-interacting protein

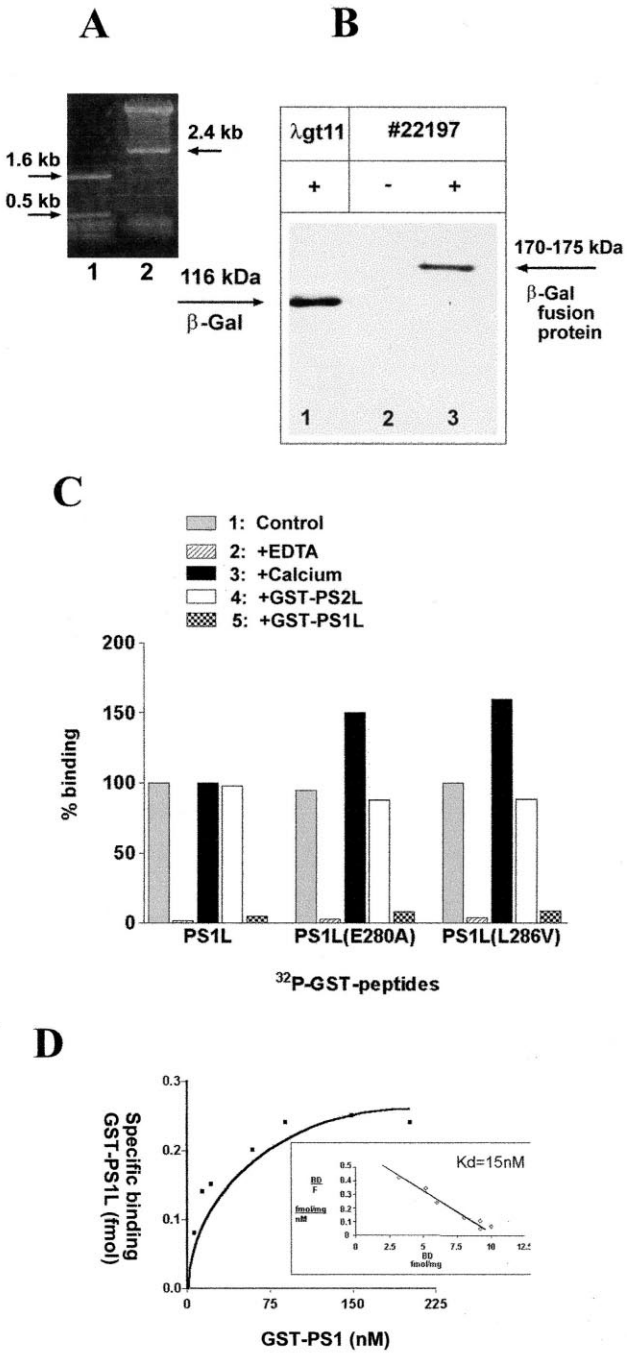

Fig. 1. Characterization of clone #22197. A: #22197 clone contains 2.4 kb insert. Lane 1: pBR322 digested with *Hin*FI. Lane 2: Purified phage DNA from #22197-infected bacteria, digested with *Eco*RI and applied to a 1.2% agarose gel. B: #22197 encodes a 170–175 kDa β-Gal fusion protein. Y1089 bacterial cells were infected with λgt11 (lane 1) and clone #22197 (lanes 2 and 3). Lysogens were either induced by IPTG (lanes 1 and 3) or not (lane 2). Western blotting was performed using antibodies to β-Gal (Promega). C: Specificity of binding to GST-PS1L; effect of Ca^{2+} and FAD-linked mutations. Extracts (25 μg) from lysogens of Y1089 cells infected with the #22197 clone, induced with IPTG, were blotted onto a nitrocellulose filter. Blots were cut, placed in wells of a microtiter plate and incubated with ^{32}P-labeled GST peptides (*x*-axis) in the absence or presence of 2 mM EDTA or 1 mM $CaCl_2$ or excess unlabeled peptides. Each blot was incubated with 4×10^6 cpm/ml of [^{32}P]peptides (1 μg/ml) for 1 h. Filters were exposed to XAR-5 film (Kodak) overnight. Radioactive signals were quantified by densitometry and expressed as a percentage of controls (first group), set at 100%. D: Saturation curves and Scatchard plots. Extracts from #22197 lysogens were applied onto nitrocellulose as in (C), blocked and then incubated with different amounts of [^{32}P]GST-PS1L(L286V) in the presence of 1 mM $CaCl_2$ or 2 mM EDTA. Ca^{2+}-dependent binding was determined as the difference. The amount of bound probe was determined from specific activity. K_d values were determined from Scatchard plots of the saturation data (inset). Bound/free (B/F) and bound probe are expressed as fmol/mg protein per nM and fmol/mg of protein, respectively. Similar curves were obtained for [^{32}P]GST-PS1L and [^{32}P]GST-PS1L(E280A) (not shown).

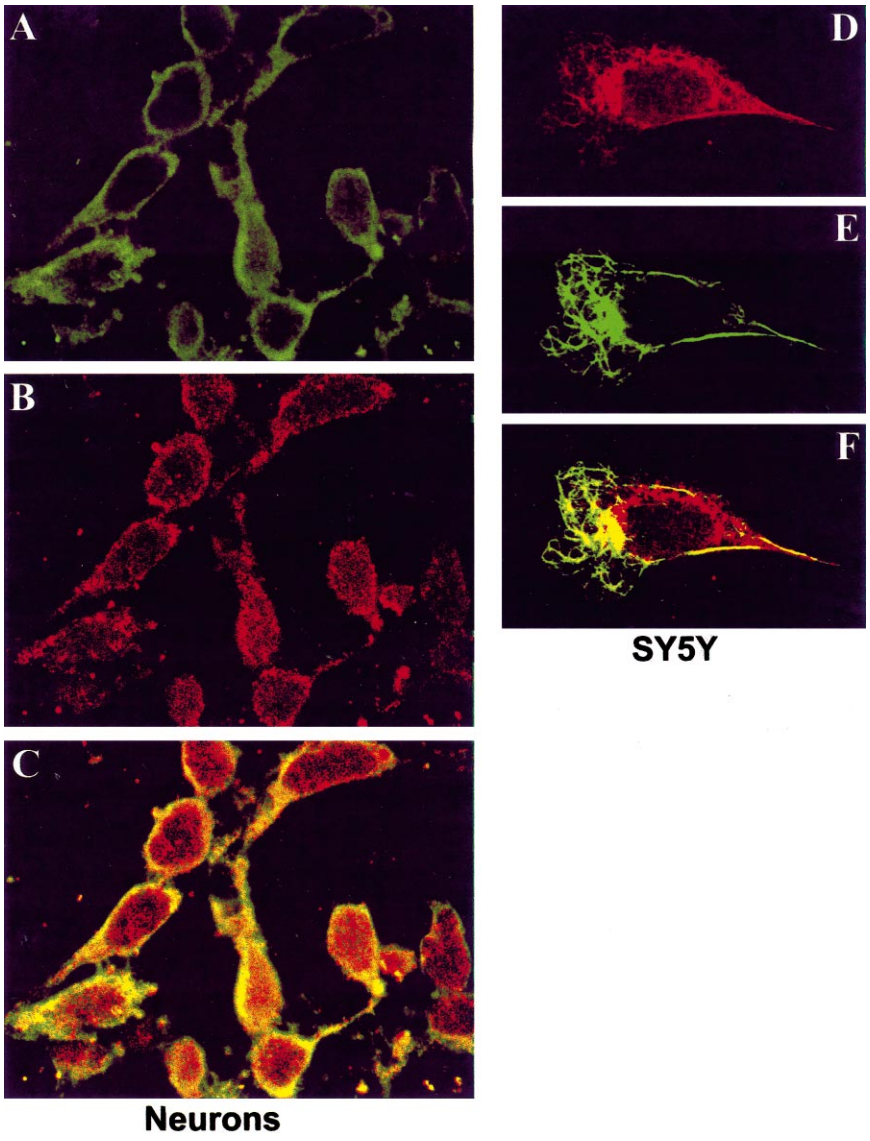

Fig. 2. PS1 co-localizes with CLIP-170/Restin. Primary neuronal cultures from rat embryo cortex (E16) were fixed in paraformaldehyde and blocked in PBS containing 1% BSA. For PS1 detection, the primary antibody used was as described blow. For CLIP-170, a mouse monoclonal antibody was used (1:100 dilution) [14]. Staining was performed with Alexa 488-conjugated donkey anti-goat and Alexa 594-conjugated donkey anti-mouse antibodies. A: PS1. B: CLIP-170. C: PS1/CLIP-170 co-localization in yellow. SY5Y cells were transfected with pSG5-*Myc-CLIP-170*. At 24 h post-transfection, the cells were methanol-fixed, blocked with 2% donkey serum and incubated (1 h) with affinity-purified goat anti-PS1 antibodies (AT antibodies, 1:100) and mouse monoclonal anti-Myc antibodies (Invitrogen). Staining was performed with Texas red-conjugated donkey anti-goat IgG and FITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories). D: PS1. E: Myc-CLIP-170. F: PS1/Myc-CLIP-170 co-localization in yellow.

(MIP) [12] known to link membrane organelles to microtubules [14]. Restin is identical to CLIP-170 except that it contains additional 35 aa (residues 457–491). Restin is overexpressed in Reed–Sternberg cells of Hodgkin's disease and is believed to be an intermediate filament-associated protein with a similar function to CLIP-170.

The binding of the probe was abolished only in the presence of excess unlabeled GST-PS1L (Fig. 1C, fifth bar in each group) and not GST-PS2L (Fig. 1C, fourth bar in each group). The latter corresponds to a 50 aa segment of PS2, within the large hydrophilic loop, a non-homologous region between PS1 and PS2.

The C-terminal domain of CLIP-170/Restin contains a consensus sequence for a metal binding motif, CX2CX3GHX4C (1373–1386 aa in CLIP-170), conserved among other MIPs; CLIP-170/Restin, BIK1 and HIV1-GAG [12]. Therefore, the binding of [^{32}P]GST-PS1L to CLIP-C/Restin-C (carboxyl-terminal fragment of CLIP-170/Restin, respectively) was examined either in the presence of a metal chelator or excess Ca^{2+}. Inclusion of 1 mM EDTA abolished binding, consistent with the requirement for a divalent cation (Fig. 1C, second bars in each group). Both mutant probes (E280A or L286V substitution) exhibited a stronger autoradiographic signal in the presence of 1 mM Ca^{2+} (Fig. 1C, third bars of each group). Thus, it appears that the interaction of PS1L with CLIP-C/Restin-C requires a metal which can be chelated by EDTA and substituted by Ca^{2+}.

To test this observation, Ca^{2+}-dependent saturation curves of GST-PS1L binding to lysogenized bacterial extracts, infected by #22197, were obtained [9]. The mutant peptides

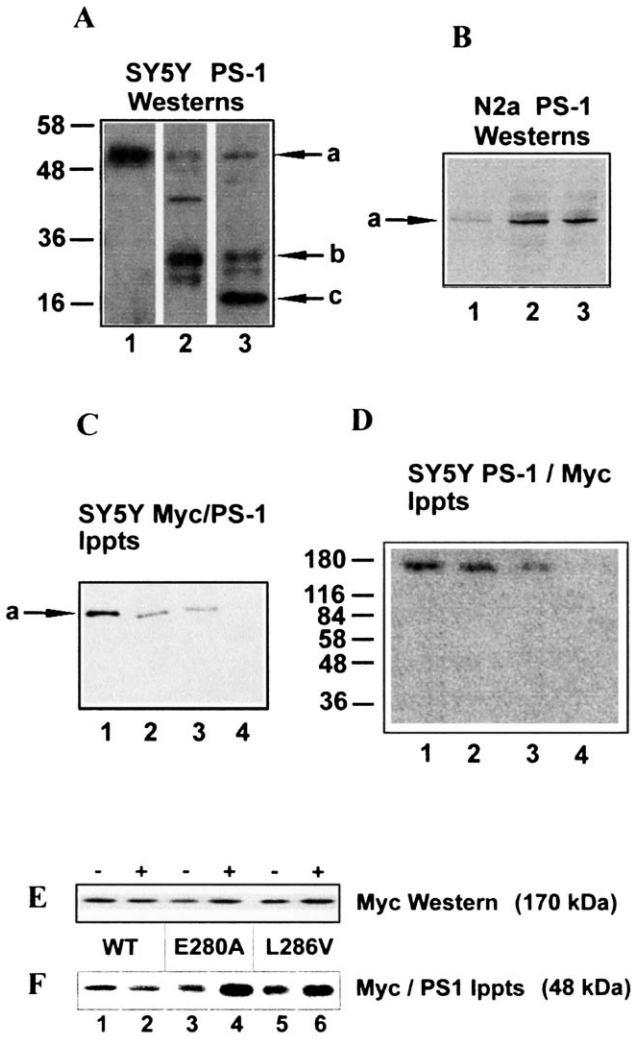

Fig. 3. Co-immunoprecipitation of PS1 with CLIP-170. $[Ca^{2+}]_i$ and the E280A/L286V PS1 mutations. A: Western blot analysis of PS1. Extracts from SY5Y cells were subjected to Western blotting as described [4] using the following antibodies: Lane 1, anti-MNR2 antibodies; Lane 2, AT antibodies; Lane 3, CT antibodies (see Section 2). B: Detection of PS1 in Neuro-2a cells transfected with full-length *PS1* cDNA using pCI-neo: Lane 1, transfected cells with unmodified vector; lanes 2 and 3, extracts from two individual clones transfected with vector containing *PS1* cDNA. C: Extracts from SY5Y cells transiently transfected with *Myc-CLIP-170* cDNA were immunoprecipitated with anti-Myc antibodies (Invitrogen) as described before [36]. Pellets were further analyzed by Western blotting using the anti-MNR2 antibodies (lanes 1 and 4) or the AT antibodies (lane 2) or the CT antibodies (lane 3). In lane 4, the MNR2 antibody was pre-absorbed with GST-PS1L. The anti-PS1 antibodies were radiolabeled with ^{125}I by the chloramine T method. Blots were developed by autoradiography. D: Extracts as in (C) were immunoprecipitated with anti-MNR2 (lanes 1 and 4) or AT antibodies (lane 2) or CT antibodies (lane 3) and analyzed by Western blotting/autoradiography using [^{125}I]anti-Myc antibodies. In controls, GST-PS1 was included in the reaction (lane 4). E: Neuro-2a cells stably transfected with pCI-neo containing the *PS1* cDNA with the wt sequence (lanes 1 and 2) or with the E280A (lanes 3 and 4) or L286V mutation (lanes 5 and 6) were transiently transfected with pSG5 containing *Myc-CLIP-170* cDNA and were subjected to Western blotting using anti-Myc antibodies. Cultures were treated for 2 h with 0.1% DMSO (lanes 1, 3 and 5) or 1 μM ionomycin, 48 h post-transfection with the pSG5-*Myc-CLIP-170* cDNA. F: Extracts from (E) were immunoprecipitated with anti-Myc antibodies, then, PS1 was detected in the pellets by Western blotting using anti-MNR2 antibodies, as described in (C). PS1 was not detected in Myc immunocomplexes derived from cells that had not been transfected with pSG5-*Myc-CLIP-170* or transfected with unmodified pSG5 vector (not shown).
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had a higher affinity for CLIP-C/Restin-C, as indicated by lower dissociation constant (K_d) values: K_d(E280A) = 14 nM and K_d(L286V) = 15 nM (Fig. 1D) compared to K_d(wt) = 25 nM. K_d values were determined by Scatchard plots (insert in Fig. 1D). The ability of CLIP-C/Restin-C to bind GST-PS1L in the absence of additional calcium must have been acquired during the incubation with the blocking solution.

3.2. Co-localization

Overlapping of endogenously expressed PS1 and either the endogenous CLIP-170/Restin-like and recombinant Myc-CLIP-170 immunofluorescent signals was observed in primary rat neuronal cultures and SY5Y cells, respectively.

In primary neuronal cultures, co-localization of endogenous PS1 (Fig. 2A) and CLIP-170/Restin (Fig. 2B) and immunofluorescent staining was observed in perinuclear regions and neuritic extensions (Fig. 2C). The signal for endogenous CLIP-170/Restin was considerably weaker compared to that found in the transfected cells (Fig. 2D,E).

PS1-like immunoreactivity in SY5Y cells was predominant in a perinuclear region. Staining was observed throughout the cytoplasm and neuritic extensions (not shown) as reported previously [4]. When these cells were transfected with the *Myc-CLIP-170* cDNA, PS1-like immunoreactivity was organized throughout the cytoplasm in arrays, resembling a cytoskeletal network (Fig. 2D). Staining for Myc-CLIP-170 in these cells was strong throughout the cytoplasm as tubular structures (Fig. 2E). The staining for the two proteins overlapped at a perinuclear region (Fig. 2F). Additional overlap was seen near the cell surface (Fig. 2C). The redistribution of PS1 staining towards cytoskeletal structures upon overexpression of CLIP-170 is also reminiscent of a possible association of PS1 with a MIP.

3.3. Co-immunoprecipitations

Co-immunoprecipitations of PS1 with CLIP-170/Restin were performed using extracts from SY5Y and Neuro-2a cells. A pSG5 vector containing a *Myc-CLIP-170* cDNA insert [15] was used for transfecting SY5Y or stably transfected Neuro-2a cells expressing human wt or mutated PS1. The various anti-PS1 antibodies used were as follows: anti-MNR2 [4], directed against the PS1L (311–330 aa), recognized prominently a 48 kDa protein in SY5Y (Fig. 3A, lane 1) and transfected Neuro-2a cell extracts (Fig. 3B). PS1 N-terminus-directed antibodies (AT) (South Western Immunology) directed against the N-terminus of PS1 (14–33 aa) and PS1 C-terminus-directed antibodies (CT) (Santa Cruz) directed against the C-terminus of PS1 (448–467 aa) detected, in addition to the full-length protein, lower molecular weight fragments, representing the 30 kDa N-terminal and 16 kDa C-terminal derivatives of PS1, respectively (Fig. 3A, lanes 2 and 3).

The only PS1 species co-immunoprecipitated with Myc-CLIP-170 by anti-Myc antibodies was the full-length protein, as detected by all of the above three antibodies (Fig. 3C, lanes 1–3). Conversely, Myc-CLIP-170 was detected in the immunocomplex obtained using anti-MNR2, AT or CT antibodies

(Fig. 3D). Pre-absorption of the anti-MNR2 antibody with GST-PS1L prevented co-immunoprecipitation of Myc-CLIP-170 (Fig. 3D, lane 4) and abolished detection of PS1 by Western blotting (Fig. 3C, lane 4).

3.4. Ca^{2+} homeostasis and FAD-linked mutations

We investigated whether addition of ionomycin and thapsigargin in cell cultures could influence the association of mutant PS1 with CLIP-170. Neuro-2a cells stably transfected with either wt or mutant *PS1* cDNA (expressing equivalent amounts of PS1, Fig. 3B, lanes 2 and 3) were transiently transfected with *Myc-CLIP-170* cDNA. At 48 h post-transfection, cells were treated (2 h) with 0.1% DMSO (vehicle) or 1 μM ionomycin [16]. Myc-CLIP-170 was immunoprecipitated from the cell extracts using anti-Myc antibodies and PS1 was detected in the immunocomplex as above. A higher amount of PS1 was detected in ionomycin-treated cells expressing mutant PS1 (Fig. 3F, lane 4, 225 ± 19%; lane 6, 194 ± 25%, $n = 4$), compared to wt PS1 (Fig. 3F, lane 2). The amount of PS1 recovered from vehicle-treated cells (Fig. 3F, lanes 1, 3 and 5) was averaged and taken as 100%. The Ca^{2+} ionophore did not alter the cellular levels of PS1 (not shown) and samples were normalized to contain the same amount of Myc-CLIP-170 (Fig. 3E, lanes 1–6). Comparable results were obtained with 1 μM thapsigargin (not shown) which blocks the endoplasmic reticulum (ER) Ca^{2+}-ATPase.

4. Discussion

CLIP-170 and Restin most likely derive from the same gene [17] mapped to chromosome 12 [18] by differential splicing. They are both cytoplasmic proteins, believed to conjoin membrane organelles to the microtubules [15] and the intermediate filaments [13], respectively. Our findings of the interaction of PS1 with CLIP-170/Restin imply that PS1 may be involved in cellular trafficking, in agreement with its homology to SPE-4 [2].

The requirement of Ca^{2+} for the binding of PS1 to CLIP-170/Restin and the augmented binding of the mutants may be of physiological significance, since mechanisms involving sustained increased intracellular Ca^{2+} have been associated with toxicity [19], increased production of Aβ [20], direct effect on cellular proteases [21] and aberrations in the structure and dynamics of the cytoskeleton [22].

Recent studies have suggested that the normal function of PS1 is either directly or indirectly linked to γ-secretase (or a co-factor) necessary for the proteolytic cleavage that generates the C-terminal end of Aβ [23–26]. Further, these studies showed that PS1 is pivotal not only for the proteolytic processing of APP, but for the activation of Notch [27] as well.

The data presented here can provide a possible scenario of PS1's involvement in APP metabolism (and possibly Notch) as an anchoring protein of membrane vesicles traveling along the cytoskeletal network. The interaction of PS1 with either CLIP-170 or Restin could allow the attachment of vesicles to the microtubules or intermediate filaments (or neurofilaments in neurons), respectively.

It has been demonstrated that PS1 co-localizes with the 53 kDa ER-Golgi intermediate compartment protein [28], an ER-resident protein, also found in the intermediate compartment between ER and Golgi. Others have shown that PS1 is found in the nuclear membranes and kinetochores [29]. Our findings of an association of PS1 with CLIP-170/Restin are consistent with both locations.

It is unknown how all disease-linked mutations, spanning the entire sequence of PS1, may impose the same phenotypic alteration via an abnormal PS1/CLIP-170/Restin interaction. The PS1 binding domain to CLIP-170/Restin may consist of a number of residues spread throughout the cytoplasmic portions of PS1 or mutations within the transmembrane or lumenal portions of PS1 may be linked to disruption of regulatory mechanisms of the binding or they may distort crucial structural elements of PS1. For example, a mutation on sterol regulatory binding protein (SREBP) cleavage activating protein affects its binding to SREBPs [30] at a site distant from the binding domain.

PS1 has been found to interact with a number of proteins, including APP [31], catenins [32], filamin [33], glycogen synthase kinase-3β, tau [34] and Notch [35]. Further studies are required to determine whether there is interplay among all these candidates and how this may ultimately affect PS1's function and role in AD.

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