JBC Papers in Press. Published on June 30, 2006 as Manuscript M601085200
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M601085200

INTERACTION BETWEEN PRESENILIN 1 AND UBIQUILIN 1 AS DETECTED BY FLUORESCENCE LIFETIME IMAGING MICROSCOPY AND A HIGH THROUGHPUT FLUORESCENT PLATE READER

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Running title: Interaction of PS1 and Ubiquilin 1 in intact cells

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Presenilin 1 (PS1) in its active heterodimeric form is the catalytic center of the γ-secretase complex, an enzymatic activity that cleaves amyloid precursor protein (APP) to produce amyloid beta (Aβ). Ubiquilin 1 is a recently described PS1 interacting protein, the overexpression of which increases PS1 holoprotein levels and leads to reduced levels of functionally active PS1 heterodimer. In addition, it has been suggested that splice variants of the Ubiquilin 1 gene are associated with an increased risk of developing Alzheimer's Disease (AD). However, it is still unclear whether PS1 and Ubiquilin 1 interact when expressed at endogenous levels under normal physiologic conditions. Here, we employ three novel fluorescence resonance energy transfer (FRET) based techniques to further investigate the interaction between PS1 and Ubiquilin 1 in intact cells. We consistently find that the Ubiquilin 1 N-terminus is in close proximity to several epitopes on PS1. We show that Ubiquilin 1 interacts both with PS1 holoprotein and heterodimer and that the interaction between PS1 and Ubiquilin 1 takes place near the cell surface. Furthermore, we show that the PS1 - Ubiquilin 1 interaction can be detected between endogenous proteins in primary neurons as well as in brain tissue of healthy controls and AD patients, providing evidence of its physiological relevance.

Alzheimer's Disease (AD) is a neurodegenerative disorder that is characterized by the deposition of amyloid plaques and neurofibrillary tangles in various brain regions. Amyloid plaques are mainly composed of Aβ species of different lengths, which are produced by cleavage of the amyloid precursor protein (APP) at different positions within its transmembrane region (for review, see (1)). After shedding of the APP ectodomain through β-secretase, intramembranous cleavage of APP occurs by the so-called γ-secretase complex in the amyloidogenic pathway. The γ-secretase complex is composed of aph-1, nicastrin, pen2 and presenilin1 (PS1), with PS1 being its catalytic center (for review, see (2)). PS1 is a 467 amino acid protein with predicted eight to nine transmembrane domains (3-7). Its N- and C-terminus as well as a large hydrophilic loop region between transmembrane domain 6 and 7 are predicted to protrude into the cytoplasm, although recent studies suggest that the C-terminus might be localized luminal/extracellularly (7,8). After being synthesized as a ~ 50 kDa holoprotein, PS1 undergoes endoproteolytic cleavage to generate a ~ 30 kDa N-terminal fragment (NTF) and a ~ 20 kDa C-terminal fragment (CTF) (9). Under physiological conditions, endogenous PS1 is predominantly present as a stable NTF-CTF heterodimer in a 1:1 stoichiometry, whereas the holoprotein is barely detectable. Recent data suggest that the

NTF-CTF heterodimer dimerizes or oligomerizes within the γ-secretase complex (10-12) . Only the NTF-CTF heterodimer is catalytically active, whereas the holoprotein prior to endoproteolysis does not possess any catalytic activity.

Apart from cleaving a wide range of substrates such as APP, APLP1 and APLP2, Notch, LRP, N-and E-cadherin and ErbB4, PS1 also interacts with a variety of molecules (for review, see (13)). Ubiquilin 1 is a recently described presenilin interactor that has been found in GST-pulldown and yeast-two-hybrid experiments to bind to both the loop region and the C-terminus of PS1 as well as PS2 (14). In humans, three Ubiquilin genes have been identified: *UBQLN1*, which is expressed ubiquituously, *UBQLN2* with a more restricted expression, and *UBQLN3*, which is expressed only in testis. Ubiquilin 1 is a 595 amino acid protein containing an N-terminal Ubiquitin-like (UBL) and a C-terminal Ubiquitin-associated (UBA) domain, the latter of which has been shown to be necessary and sufficient for its interaction with the presenilins. Although the exact role of Ubiquilin 1 is unknown, it is believed to promote the accumulation of PS1 full length protein and regulate its endoproteolysis while also modulating the levels of other members of the γ-secretase complex such as pen2 and nicastrin (14,15). Although there are contradictory data from case-control studies present (16,17), it has recently been shown that genetic variants in *UBQLN1* substantially increase the risk of developing AD (18-20). In their family-based cohort study, Bertram et al. have shown that several single nucleotide polymorphisms in *UBQLN1* are associated with AD, one of them (UBQ-8i) leading to alternative splicing of the gene in the brain (18).

The aim of this study was to investigate the interaction of Ubiquilin 1 with PS1 on endogenous level and to determine where in the cell their interaction occurs. Using three different fluorescence resonance energy transfer (FRET) based techniques that allow for the detection of intermolecular interactions in intact cells, we show that several epitopes on both the PS1 holoprotein and heterodimer are in close proximity to Ubiquilin 1. Furthermore, we demonstrate that the interaction between Ubiquilin 1 and PS1 takes place near the cell surface. Finally, we are able to show the interaction between Ubiquilin 1 and PS1 in brain sections from AD patients and controls, which further supports the physiological relevance of this interaction.

MATERIALS AND METHODS

Cell culture Conditions

Chinese Hamster Ovary (CHO) cells, PS70 cells (CHO cells stably overexpressing PS1 and APP (21)), D257A cells (CHO cells stably overexpressing D257A mutant PS1 and APP (21)) and primary neurons were used in this study. All cells were grown in an incubator at 37°C containing 5% CO2. CHO cells were grown in OPTI-MEM media containing 10% FBS. PS70 cells were grown in OPTI-MEM media containing 10% FBS, 200 µg/ml G418 and 2.5 µg/ml Puromycin. D257A cells were grown in OPTI-MEM media containing 10% FBS, 200 µg/ml G418 and 250 µg/ml Zeocin.

PS70 and D257A cells were plated on four-well glass slides (Nalge Nunc International, Naperville, IL) or 96 well poly-L-lysine-coated glass-bottom plates (Nalge Nunc International, Naperville, IL) 24 hours prior to immunostaining for the FLIM analysis and high throughput FRET assay, respectively.

Primary neuronal cultures were prepared as described elsewhere (22). In brief, mixed cortical-hippocampal neurons were generated from CD1 mice at embryonic day 15-16. The cells were plated on four-well poly-L-lysine-coated glass slides in chemically defined Neurobasal media (Gibco, Gaithersburg, MD) containing 10% FBS for one hour. The neurons were maintained in Neurobasal media containing 2% B27 supplement (Gibco, Gaithersburg, MD) for 7-12 days in vitro (DIV) prior to immunostaining. At 5-7 DIV, 5 µg/ml Cytosine Arabinoside (Sigma, St. Louis, MO) was added to the culture media to suppress the growth of non-neuronal cells.

Immunocytochemistry of cells

Prior to the FLIM assay, cells were washed twice in PBS, fixed in ice cold methanol for

ten minutes and then blocked and permeabilized in 1.5% normal donkey serum (NDS) containing 0.1% Triton-X 100 for 45 minutes. Both primary and secondary antibodies were applied in 1.5% NDS for one hour at room temperature, and washed three times for 5 minutes each in PBS. After removal of the wells, slides were coverslipped using GVA mounting solution (Zymed, South San Francisco, CA). The same immunostaining protocol was performed prior to the high-throughput FRET assay with minor changes: The primary antibody was applied overnight at 4°C and the washing steps were extended to ten minutes each. The following antibodies were used: goat anti PS1 directed against amino acids 14-33 (Sigma, St. Louis, MO), mouse anti PS1 directed against an epitope in the loop domain between TM6 and TM7 (Chemicon, Temecula, CA) and biotinylated goat anti PS1 directed against amino acids 298-407 (R&D Systems, Minneapolis, MN) to label the PS1 N-terminus (NT), loop region and C-terminus (CT), respectively. The Ubiquilin 1 NT was labeled with rabbit anti Ubiquilin 1 antibody directed against amino acids 2-18 (Abcam, Cambridge, MA). Pairs of primary antibodies were labeled with secondary antibodies conjugated to Alexa 488 (Invitrogen, Gaithersburg, MD) and Cy3 (Jackson Immunoresearch, West Grove, PA) or Alexa 430 and Cy3 prior to the FLIM and high-throughput FRET assay, respectively.

Immunohistochemistry of brain sections

Fifty µm thick sections from temporal neocortex and hippocampus of three AD patients (1 male, 2 females, mean age 81 ± 3 years) and two cognitively healthy controls (2 females, mean age 67 ± 11.3 years) were fixed and stored in 15% glycerol at -20°C prior to immunostaining. After a short washing step in TBS, sections were permeabilized in 0.5% Triton-X 100 in TBS for 20 minutes and washed in TBS again prior to blocking in 1.5% NDS for one hour. Primary antibodies against the PS1 NT and the Ubiquilin 1 NT were applied in 0.1% Triton-X 100 in 1.5% NDS at 4°C overnight. Samples were washed three times for five minutes each before and after application of secondary antibodies, Alexa 488 and Alexa 546, in TBS. After immunostaining, slides were coverslipped using GVA Mounting Solution.

Detection of FRET using Fluorescence Lifetime Imaging Microscopy (FLIM)

Fluorescence Resonance energy transfer (FRET) occurs between two fluorophores if they are within close proximity of each other (~10nm). Upon activation of the donor fluorophore, some of its emission energy is non-radiatively transferred to the acceptor fluorophore. To detect FRET between PS1 and Ubiquilin 1, a validated FLIM technique based on multiphoton microscopy was employed (22-25). In this assay, the cells are immunostained for the two epitopes of interest, labeled with donor and acceptor fluorophore, respectively, and the donor fluorophore lifetime is monitored as an indicator for the presence or absence of FRET. If there is an acceptor fluorophore present within 10 nm of the donor fluorophore, the fluorescence lifetime of the donor fluorophore decreases in inverse relation to the distance between the donor and acceptor. As a negative control, the donor fluorophore lifetime is measured in the absence of FRET (i.e. no acceptor present or distance between donor and acceptor fluorophores greater than 10 nm). Positive controls consist of donor fluorophore stained cells that are further labeled with a secondary antibody against the species in which the donor fluorophore is raised (e.g. goat anti mouse Alexa 488 labeled with donkey anti goat Cy3; (22)). A multiphoton microscope (Radiance 2000, Bio-Rad, Hercules, CA) with a femtosecond pulsing mode-locked Ti:Sapphire Laser (Mai Tai; Spectra-Physics, Mountain View, CA) at 800 nm was used with a high-speed photomultiplier tube (MCP R3809; Hamamatsu, Hamamatsu City, Japan) and a time correlated single-photon counting (TCSPC) acquisition board (SPC 830; Becker&Hickl, Berlin, Germany) for lifetime imaging Data analysis was carried out using SPC Image (Becker&Hickl, Berlin, Germany). Donor fluorophore lifetimes were determined by fitting the data to one (negative control) or

two (positive control or experimental conditions) component exponential decay curves to allow for the calculation of the fraction of donor fluorophores within each pixel that does or does not interact with an acceptor fluorophore. A 128 x 128 and/or 256 x 256 pixel matrix was created for both single- and multi-exponential curve fit data for each image in order to display lifetimes in each pixel on a pseudocolor scale.

Detection of FRET using a high-throughput, time domain fluorescent plate reader

To detect interactions between Ubiquilin 1 and PS1 on a high-throughput screening level, we employed a TECAN FLT Ultraevolution system (Tecan Trading AG, Switzerland) that allows for the assessment of FRET using 96-well plates. The cells were grown on 96-well plates and immunostained as described above using Alexa 430 as donor and Cy3 as acceptor fluorophore. Excitation of the donor fluorophore was carried out by a 440 nm laser head with a high repetition rate (20 million pulses per second). TCSPC was employed to reconstruct the donor fluorophore decay curve with high temporal resolution (35ps). Data acquisition was performed using XFluor Software (Tecan Trading AG, Switzerland). Data analysis was carried out using a recently developed method for fitting fluorescence lifetime data (Jones et al., manuscript submitted for publication), which will be briefly reviewed here. The decay curve is assumed to follow the equation $A=A_I \exp\{(-t/\tau_I)^{\beta}\} + A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$, with A_I, A_1 and A_2 being the intensity of the instrument background autofluorescence, the non-FRETing, and FRETing donor fluorophores, respectively; and τ_I, β, τ_1 and τ_2 being the characteristic constants of each of the fluorophore decay components. To avoid cross-talk between decay components, the background and donor lifetimes are individually fit using a series of control experiments, first without using fluorophores and then with the donor fluorophore alone. FRET strength was calculated according to the formula A2/(A1+A2) as a measure of the number of molecules that are interacting in the respective sample.

Detection of FRET using a Photobleach Dequenching Assay

The presence of FRET in a given sample leads to the "quenching" of the donor fluorophore, i.e. upon excitation, the intensity of the emitted light is lower when compared to an "unquenched" fluorophore. The "dequenching" of the donor fluorophore by photobleaching of the acceptor fluorophore, leads to an increase in donor fluorophore intensity, which can be quantified and indicates the presence of FRET in the tested sample. Photobleach dequenching FRET measurements were performed using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany). Krypton-Argon and Helium-Neon lasers were used alternately to excite samples at 488 and 543 nm, respectively. After photobleaching of the acceptor fluorophore, Alexa 546, in an outlined area of the cell using the 543 nm laser line at 100% laser power, a second set of images was acquired. FRET was measured as previously described (22,26). The percent increase in donor fluorophore intensity after photobleaching of the acceptor fluorophore was normalized to the percent change in intensity in an unbleached region of the same cell.

Co-Immunoprecipitation

CHO cells were co-transfected with PS1 and v5-tagged Ubiquilin 1 transcript variant 1 (TV1, (18)) constructs. 48 hours post-transfection, cells were incubated with 2mM DSP crosslinker in PBS (Pierce Biotechnology, Rockford, IL) for 30 minutes. After incubation in 1M Tris pH 7.5 for 15 minutes to stop the crosslinking reaction, cells were lysed in 1% CHAPSO lysis buffer (27) and incubated with protein A sepharose beads (Sigma Aldrich, St. Louis, MO) and PS1 antibodies rb x81 and rb 4627 (a gift from Dr. Dennis Selkoe, Boston, MA) overnight at 4°C. After collection of supernatants, beads were washed with 1% CHAPSO lysis buffer and boiled in 2x Tris-Glycine SDS sample buffer (Invitrogen, Carlsbad, CA) to release the proteins. Negative controls consisted of lysates that were incubated with beads only to

detect unspecific pulldown by the sepharose beads and lysis buffer that was incubated with rb IgG to detect unspecific immunoreactive bands. Supernatants and immunoprecipitates were loaded on 4-20% Tris-Glycine polyacrylamide gels (Novex, San Diego, CA) and transferred to PVDF membranes (Millipore, Bedford, CA), which were immunoblotted using ms PS1 loop (Chemicon, Temecula, CA), and ms v5 (Invitrogen, Carlsbad, CA) and ms Ubiquilin 1 (Zymed, San Francisco, CA) antibodies to detect PS1 and Ubiquilin 1, respectively. Proteins were visualized after incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies using chemiluminescence (ECL Western Blotting Detection Reagent, Amersham Biosciences, Piscataway, NJ).

Western Blotting

Primary neurons, PS70 and D257A cells were lysed in 1% CHAPSO lysis buffer (27) and loaded onto a 10-20% Tris-Glycine polyacrylamide gel (Novex, San Diego, CA) for protein separation under reducing and denaturing conditions. Proteins were transferred onto PVDF membranes (Millipore, Bedford, CA) that were blocked in 5% milk in TBS-T prior to application of the primary antibody. An HRP-conjugated secondary antibody was applied and proteins were visualized using chemiluminescence (ECL Western Blotting Detection Reagent, Amersham Biosciences, Piscataway, NJ).

Statistical Analysis

Statistical analysis was performed using StatView for Windows, Version 5.0.1 (SAS Institute, Inc.). Differences between samples were determined using two-sample t-test or Fisher's PLSD ANOVA post-hoc test. Results were considered significant if $p<0.05$.

RESULTS

Ubiquilin 1 and Presenilin 1 colocalize in intact cells

Endogenous and transiently overexpressed Ubiquilin 1 has been shown to localize both to the nucleus and the cytoplasm of HeLa cells (14). In order to confirm these findings in the cell culture systems used in this study, PS70 cells and primary neurons were immunostained using an N-terminal anti Ubiquilin 1 antibody. As expected, endogenous Ubiquilin 1 showed a punctate staining pattern throughout the cells that was more pronounced in the nucleus (Figures 1B and E). A similar staining pattern of Ubiquilin 1 was observed in D257A cells. As PS1 is known to be expressed predominantly in the ER/Golgi area (Figures 1A and D), both proteins colocalize mainly in perinuclear regions of the cell (Figures 1C and F).

In primary neurons, the expression pattern of Ubiquilin 1 was very similar to that observed in PS70 cells (Figure 1E). However, levels of endogenous Ubiquilin 1 were much higher; a finding which is in agreement with the previously described high Ubiquilin 1 expression levels in brain tissue (Mah et al, 2000).

Taken together, these data show cellular colocalization of the proteins, but due to the relatively low resolution on light microscopic level do not allow for the assessment of an interaction between PS1 and Ubiquilin 1.

PS1 and Ubiquilin 1 coimmunoprecipitate in CHO cells

Employing yeast-two-hybrid and GST-pulldown experiments, it has recently been suggested that Ubiquilin 1 is a novel PS1 interactor (14). In order to confirm previous findings, CHO cells were co-transfected with PS1 and Ubiquilin 1 constructs and a coimmunoprecipitation experiment was performed. As is shown in Figure 2, immunoreactive bands of ~ 20 kDa for PS1 CT and ~ 64 kDa for Ubiquilin 1 were present in the immunoprecipitated samples, which points towards an interaction of the two proteins at some point during their trafficking/processing.

Ubiquilin 1 and Presenilin 1 interact in intact PS70 cells

Using several biochemical approaches, it has been suggested that Ubiquilin 1 and PS1 are interacting proteins (Figure 2, (14)). To verify that this interaction takes place in intact mammalian cells, we established a new FRET-

based assay using a high-throughput fluorescent plate reader, which allows for the rapid assessment of intermolecular interactions in intact cells on 96 well plates. In this assay, the proximity of the NT of Ubiquilin 1 to several epitopes on the PS1 molecule was determined. The NT of Ubiquilin 1 was labeled with the donor fluorophore Alexa 430, the lifetime of which was measured. A bi-exponential fit was then applied in the absence (negative control) or presence of Cy3, labeling the NT, loop region or CT of the PS1 molecule. Since FRET only takes place if the distance between the donor and the acceptor fluorophore is of the order of 10 nm, the presence of a second shorter lifetime indicates close proximity between a proportion of the two labeled epitopes. As is shown in Table 1, a significant shortening in donor fluorophore lifetime was observed in the experimental conditions when compared to the negative control, indicating close proximity between the Ubiquilin 1 NT and the PS1 NT, loop and CT.

To confirm the data generated in the plate reader FRET system, we developed a microscope-based FLIM assay in which the proximity of several epitopes on the PS1 molecule to the NT of Ubiquilin 1 were assessed within individual cells. As shown in Table 2, the lifetimes of the donor fluorophore labeling each of three PS1 epitopes in the absence of an acceptor fluorophore were similarly around 2100 psec. However, when the NT of Ubiquilin 1 was labeled with Cy3, the donor fluorophore lifetime dramatically shortened to about 1700 to 1400 psec, indicating close proximity between the Ubiquilin 1 NT and either PS1 N-terminal, loop or C-terminal epitopes.

Taken together, the data obtained both in the high-throughput FRET system and the FLIM assay provide strong evidence that the interaction between PS1 and Ubiquilin 1 takes place in intact PS70 cells.

Ubiquilin 1 and Presenilin 1 interact near the cell surface in intact PS70 cells

Since the donor fluorophore lifetimes that are determined by the FLIM assay can be displayed on a pixel-by-pixel basis in a pseudocolor coded image of the cell, this assay provides information about the subcellular localization of the detected interactions. The pseudocolor scale is aligned from red to blue, with red to green pixels indicating "FRETing" molecules with a shortened lifetime and blue pixels indicating "non-FRETing" molecules with an unchanged lifetime that is similar to the negative control.

PS70 cells were immunostained for PS1 (labeled with Alexa 488), and Ubiquilin 1 (labeled with Cy3), and the donor fluorophore lifetimes were measured in the absence or presence of an acceptor fluorophore. As expected, the pseudocolor image of cells in the negative control reveals only one "non-FRETing" population of Alexa 488-labeled PS1 molecules, which is color coded in blue (Figure 3B). The pseudocolor image of the cells dramatically changed once Cy3-labeled Ubiquilin 1 was introduced in the experimental condition (Figure 3D). Although the average lifetime throughout the whole cell significantly decreased (Table 2), the most pronounced shortening in lifetime was observed at or near the cellular surface. We consistently observed two different "populations" of the "FRETing" molecules, one of which showed a very pronounced decrease in donor fluorophore lifetime to < 1000 psec (red pixels, Figure 3B), whereas the second one showed a moderate decrease in donor fluorophore lifetime to about 1000 to 1800 psec (green pixels, Figure 3D). Since the decrease in donor fluorophore lifetime is proportional to the distance between donor and acceptor fluorophores, we suggest that PS1 - Ubiquilin 1 complexes may exist in two different conformational states, with red pixels representing tight PS1 - Ubiquilin 1 interactions.

Taken together, these data indicate that PS1 and Ubiquilin 1 interact in intact PS70 cells and that this interaction predominantly takes place near the cell surface.

PS70 cells stably overexpress PS1, therefore both holoprotein and heterodimer are present in considerable amounts (Figure 4, lane 2). Since the antibodies that were used in the FRET-based assays stain both PS1 species, we cannot differentiate to what extent Ubiquilin 1

interacts with the PS1 holoprotein or heterodimer in PS70 cells. To address this question, a series of experiments were conducted in D257A cells, which stably overexpress a mutant PS1 molecule that does not undergo endoproteolysis (21) (Figure 4, lane 3) and in primary neurons, in which PS1 predominantly exists as a heterodimer due to rapid processing and degradation of the PS1 holoprotein (Figure 4, lane 1).

Ubiquilin 1 and Presenilin 1 interact near the cell surface in D257A cells

To test if Ubiquilin 1 interacts with the PS1 holoprotein, a series of experiments in D257A cells were conducted. The cells were stained in the same manner as PS70 cells and both the high-throughput FRET-based screen and the FLIM assay were performed as described above. As shown in Tables 3 and 4, both assays revealed close proximity between the NT of Ubiquilin 1 and the PS1 NT, loop region and CT, as indicated by the pronounced shortening in donor fluorophore lifetime.

The FLIM analysis, which determines the subcellular localization of FRET between PS1 holoprotein and Ubiquilin 1 NT, revealed the closest proximity between PS1 and Ubiquilin 1 near the cellular surface in D257A cells (data not shown).

Since in D257A cells almost no PS1 heterodimer is present, these data provide strong evidence that several epitopes on the PS1 holoprotein are in close proximity to the Ubiquilin 1 NT.

Ubiquilin 1 and Presenilin 1 interact near the cell surface in primary neurons

To determine if PS1 interacts with Ubiquilin 1 on an endogenous level and to assess where in the cell their interaction occurs, we performed the FLIM assay in primary neurons. The introduction of Cy-3 labeled Ubiquilin 1 led to a pronounced shortening of donor fluorophore lifetime from 2146 ± 46 psec in the negative control to 1559 ± 372 psec in the experimental condition (data pooled for all conditions, total n=82 cells for negative control and n=129 cells for experimental conditions). This shortening in lifetime was independent of the epitope on PS1 that was labeled with the donor fluorophore, indicating that the NT, loop region and CT of endogenous PS1 are in close proximity to the NT of endogenous Ubiquilin 1. The most pronounced decrease in the donor fluorophore lifetime occurred near the surface of the neuronal cell body and was present in neurites as well (Figure 5).

These data indicate that endogenous Ubiquilin 1 is in close proximity to the endogenous PS1 molecule.

Interaction of PS1 and Ubiquilin 1 in brain tissue

To further test the physiological relevance of the finding that Ubiquilin 1 interacts with PS1, we analyzed their proximity in human brain tissue. Control and AD brain sections were stained for PS1 and Ubiquilin 1 and their proximity was assessed using a photobleach dequenching FRET assay. After photobleaching, a mean increase in donor fluorophore intensity of 10.1 ± 5.9% was observed (n=29 cells, $p<0.0001$ vs. a no FRET control). No significant difference between AD and control tissue was observed ($p=0.9$). These data indicate that the interaction of PS1 and Ubiquilin 1 takes place in human brain tissue in both healthy and diseased individuals. While these three different FRET based assays are consistent with results from yeast-two-hybrid assays (using purified proteins) suggesting a PS1 - Ubiquilin 1 interaction, multiple attempts to co-immunoprecipitate PS1 and Ubiquilin 1 from overexpressing cells or from brain homogenates were unsuccessful, suggesting that their interaction may be detergent-sensitive or otherwise unstable under co-IP conditions.

DISCUSSION

Alzheimer's Disease is a neurodegenerative disorder that is characterized by severe memory impairment due to synaptic dysfunction and neuronal loss. Several genes have been identified that are either associated with an increased risk of developing late onset AD (e.g. APOE, α2Macroglobulin) or lead to early onset of the disease (i.e. APP, PS1 and PS2)(for review, see (28)). In a search for presenilin interactors, Ubiquilin 1 was identified using yeast-two-hybrid,

colocalization and GST-pulldown assays (14). Moreover, it has recently been suggested that genetic variants in *UBQLN1* may be associated with an increased risk of AD (18), although other studies could not confirm the association (16,17). Very little is known so far about the function of Ubiquilin 1 in the brain. To better understand the possible involvement of Ubiquilin 1 in the pathogenesis of AD, it is important to determine whether Ubiquilin 1 and PS1 interact on endogenous level in neuronal cells *in situ*.

In the current study, we use three FRET-based techniques to analyze the interaction between PS1 and Ubiquilin 1 in intact mammalian cells without disrupting the normal physiological environment of the proteins: (1) a fluorescent plate reader to detect the presence of the PS1 - Ubiquilin 1 interaction in intact cells; (2) a FLIM assay to analyze the subcellular distribution of the interacting molecules and (3) a photobleach dequenching FRET assay to study the interaction in human brain tissue. We found that: (i) endogenous Ubiquilin 1 interacts with endogenous and stably overexpressed PS1 in intact cells, (ii) the NT of endogenous Ubiquilin 1 comes into close proximity to the NT, CT and loop domains of PS1, (iii) endogenous Ubiquilin 1 interacts with both functionally inactive PS1 holoprotein and mature PS1 heterodimer, (iv) despite greatest colocalization of endogenous Ubiquilin 1 and PS1 in perinuclear compartments, the closest proximity between the proteins was detected near the cellular surface and (v) FRET was present between PS1 and Ubiquilin 1 in primary neurons and brain tissue of healthy controls and AD patients, confirming the possible physiological relevance of the interaction.

The initial report on the interaction between the Ubiquilin 1 CT and PS1 loop region or CT had been based on yeast-two-hybrid and GST-pulldown experiments in a cell-free system (14). To confirm this finding in intact mammalian cells, we used novel morphologic FRET-based approaches that allow for the detection of protein-protein interactions without disrupting the proteins' physiological cellular environment (22,24). In accordance with the previously reported data, the FRET assays demonstrate close proximity between Ubiquilin 1 and the PS1 CT, loop region and NT. These data suggest that there might be more than one step in the association of PS1 and Ubiquilin 1. It is plausible that Ubiquilin 1 initially comes into close proximity to one epitope on the PS1 molecule and then moves along to a different epitope (similarly to PS1-APP interaction, where APP first binds to a docking site on PS1/gamma-secretase, presumably on the NT-CT interface (22,29) and then moves to the active site near the loop domain (21)). Alternatively, the stoichiometry could be that the Ubiquilin 1 epitope is configured at the interface of PS1 dimers (10-12), thus bringing it into close proximity with several PS1 epitopes.

To further characterize the interaction between PS1 and Ubiquilin 1, we took advantage of the capability of the FLIM assay to provide information about the subcellular localization of proteins in close proximity. Although the majority of Ubiquilin 1 and PS1 molecules colocalize in perinuclear and cytoplasmatic regions, the strongest FRET was observed at the cell periphery, as indicated by significant shortening of the donor fluorophore lifetime, suggesting that the two molecules come into closest proximity near the cellular surface. Interestingly, two populations of the "FRETing" molecules were detected, suggesting that the PS1 - Ubiquilin 1 complex may exist in different conformations.

By using cell lines that predominantly express D257A PS1 holoprotein, we found that Ubiquilin 1 also associates with the catalytically inactive form of PS1. These data suggest that PS1 endoproteolysis is not required for the interaction between PS1 and Ubiquilin 1, thus indicating that an association of the proteins might occur early in the secretory pathway and possibly prior to assembly of the γ-secretase complex. Overexpression of Ubiquilin 1 has been shown to increase PS1 holoprotein levels and to decrease levels of PS1 NTF and CTF as well as pen2 and Nicastrin (14,15), suggesting that Ubiquilin 1 might play a role in PS1 endoproteolysis and the regulation of the γ-secretase complex.

The exact role of the PS1 - Ubiquilin 1

interaction is unknown. The association of Ubiquilin 1 with PS1 through its entire maturation process is in accordance with previous data suggesting that Ubiquilin 1 might act as a molecular chaperone and may affect protein degradation. It has been demonstrated that both the Ubiquilin 1 UBL and UBA domains can bind to the S5a subunit of the proteasomal cap (30,31). Moreover, the UBA domain, which has been shown to be necessary and sufficient to bind PS1, is present in a variety of proteins that are involved in the proteasome pathway, further endorsing a possible role of Ubiquilin 1 in proteosomal degradation of proteins.

In the present study, we show for the first time that the interaction between Ubiquilin 1 and PS1 takes place between endogenous proteins in intact cells, therefore providing evidence of its presence in a physiological, non-overexpressing setting. In addition, we demonstrate that the interaction between Ubiquilin 1 and PS1 occurs in brain tissue of both healthy individuals and AD patients, thus showing its possible significance in neuronal cells. Since Ubiquilin 1 might be a regulator of the γ-secretase activity and, thus, may present a possible therapeutic target, better understanding of the interaction between Ubiquilin 1 and PS1 may potentially have therapeutic implications. Thus, the FRET-based screen that has been introduced in this study may allow for a rapid monitoring of the PS1 – Ubiquilin 1 interaction and provides an interesting tool to perform screens of large chemical libraries to modify this interaction.

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FOOT NOTES

We wish to thank Dr. Katrin Lindenberg for helpful discussions. This work was supported by NIH Grant 5 P01 AG015379-08 (BTH), Mass Alzheimer's disease Research Center pilot grant 218683 (OB), NIMH, NIA and Extendicare Foundation (MH, RET) and a research fellowship from the Deutsche Forschungsgemeinschaft to AVT (TH 1129/1-1).

The abbreviations used are: PS1, Presenilin 1; APP, amyloid precursor protein; FLIM, Fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; TCSPC, time correlated single photon counting

FIGURE LEGENDS

Table 1 – FRET between Ubiquilin 1 and three epitopes on PS1 as detected by a high-throughput fluorescent plate reader in PS70 cells

After immunostaining of PS70 cells for Ubiquilin 1 NT, which was labeled by Alexa 430 and PS1 NT, loop or CT that were labeled by Cy3, FRET was determined on a high-throughput fluorescent plate reader. A significant shortening in donor fluorophore lifetime was observed in all experimental conditions, indicating close proximity between a fraction of the epitopes of interests. Note, that in the positive control, in which Alexa 430-labeled Ubiquilin 1 NT was directly labeled with Cy3, the most pronounced decrease in donor fluorophore lifetime as well as a mean FRET strength of more than 90% was observed, proving the sensitivity and validity of the method.

Each experiment was performed at least in triplicate with 8 wells being read per condition. n = total number of wells. Statistical analysis was performed using Fisher's PSLD ANOVA.

Table 2 – FRET between Ubiquilin 1 and three epitopes on PS1 as detected by FLIM in PS70 cells

PS70 cells were immunostained for PS1 NT, loop or CT (labeled by Alexa 488) and Ubiquilin 1 NT (labeled by Cy3). Close proximity was detected between all epitopes on PS1 and the Ubiquilin NT, as indicated by the significant shortening in lifetime when compared to the negative control in which the acceptor fluorophore was not present.

Results from one representative experiment are shown; experiments were replicated at least four times and a total of 57-62 cells (negative controls) and 76-104 cells (experimental conditions) were analyzed. Statistical analysis was performed using Fisher's PSLD ANOVA.

Table 3 – FRET between Ubiquilin 1 and three epitopes on PS1 as detected by a high-throughput fluorescent plate reader in D257A cells

Ubiquilin 1 and PS1 NT, loop or CT were labeled with Alexa 430 and Cy3, respectively, and FRET was determined using a high-throughput fluorescent plate reader. Close proximity was detected between all epitopes on the PS1 holoprotein and the Ubiquilin 1 NT, as indicated by the pronounced shortening in lifetime in all experimental conditions.

Each experiment was performed at least in triplicate with 8 wells being read per condition. n = total number of wells. Statistical analysis was performed using Fisher's PSLD ANOVA.

Table 4 – FRET between Ubiquilin 1 and three epitopes on PS1 as detected by FLIM in D257A cells

D257A cells were immunostained for several epitopes on PS1 (labeled by Alexa 488) and Ubiquilin 1 (labeled with Cy3). The pronounced shortening in lifetime that is observed in every experimental condition shows close proximity between the NT, loop region and CT of the PS1 holoprotein and the N-terminus of Ubiquilin 1.

Results from one representative experiment are shown. Each experiment was performed at least in triplicate with a total number of 22-35 cells analyzed in the negative controls and a total number of 36-42 cells analyzed in the experimental conditions. Statistical analysis was performed using Fisher's PSLD ANOVA.

Figure 1 – Colocalization of PS 1 and Ubiquilin 1
PS70 cells (top row) and primary neurons (bottom row) were double immunostained with Alexa 488-labeled mouse anti PS1 loop antibody (A, D) and Cy3-labeled rabbit anti Ubiquilin 1 antibody (B, E). Images C and F show colocalization of PS1 and Ubiquilin 1.
Images were acquired on a Zeiss LSM 510 confocal microscope using the multitrack mode to minimize crosstalk between the fluorophores.

Figure 2 – Coimmunoprecipitation of PS1 and Ubiquilin 1.
CHO cells were cotransfected with PS1 and v5-tagged Ubiquilin 1 constructs and immunoprecipitation was performed after crosslinking. As is shown in lane B, pull down of PS1 resulted in specific coimmunoprecipitation of Ubiquilin 1, which could be detected using both v5 and Ubiquilin 1 antibodies. No specific pulldown was shown in the absence of antibodies (lane C) or of cell lysate (lane D).

Figure 3 – Ubiquilin 1 and PS1 interact near the cell surface in PS70 cells
PS70 cells were stained for PS1 NT that was labeled by Alexa 488 and for Ubiquilin 1 NT that was labeled by Cy3. Lifetime of Alexa 488 was assessed in absence (A,C) and presence (B,D) of the acceptor fluorophore Cy3. FRET between PS1 NT and Ubiquilin 1 NT occurs near the cell surface, as indicated by the red and green pixels (D).

Figure 4 – Proportion of PS1 holoprotein and PS1 heterodimer in primary neurons, PS70 and D257A cells
As shown in the left column, PS1 predominantly exists as a cleaved heterodimer in primary neurons, whereas the holoprotein is not detectable due to rapid degradation. In PS70 cells (middle column), where PS1 is stably overexpressed, both holoprotein and heterodimer are present, whereas in D257A cells (right column), which stably overexpress a mutant form of PS1 that does not undergo endoproteolysis, only holoprotein is detectable. PS1 was detected with mouse anti PS1 loop antibody.

Figure 5 – Ubiquilin 1 and PS1 interact near the cell surface in primary neurons
Primary neurons were grown for 12 DIV and immunostained for PS1 NT and Ubiquilin 1 NT. Proximity between the epitopes was assessed by FLIM and displayed in a pseudocolor image, in which red and green pixels indicate FRET, whereas blue pixels represent "non FRETing" molecules. Accordingly, in the negative control (A,C) the cell appears in blue due to the absence of the acceptor fluorophore (C). In the experimental condition (B,D), Ubiquilin 1 NT and PS1 NT are in close proximity predominantly near the cell surface, as indicated by the presence of red and green pixels near the cell surface and in the neuronal processes (D).

Table 1

FRET DONOR (Alexa 430)	FRET ACCEPTOR (Cy3)	ALEXA 430 LIFETIME (mean ± SD, psec)	FRET STRENGTH (mean ± SD, %)
Ubiquilin NT (n=56)	none	3910 ± 481	-
Ubiquilin NT (n=24)	Cy3 anti Alexa 430	1059 ± 51	92 ± 3
Ubiquilin NT (n=31)	PS1 NT	1276 ± 124	65 ± 13
Ubiquilin NT (n=45)	PS1 loop	1254 ± 296	28 ± 8
Ubiquilin NT (n=31)	PS1 CT	1231 ± 181	53 ± 23

Table 2

FRET DONOR (Alexa 488)	FRET ACCEPTOR (Cy3)	ALEXA 488 LIFETIME (mean ± SD, psec)	p-value (compared to Alexa488 alone)
PS1 NT (n=10)	none	2034 ± 64	-
PS1 NT (n=20)	Ubiquilin 1 NT	1698 ± 174	p<0.0001
PS1 loop (n=11)	none	2140 ± 14	-
PS1 loop (n=21)	Ubiquilin 1 NT	1470 ± 207	p<0.0001
PS1 CT (n=11)	none	2127 ± 20	-
PS1 CT (n=17)	Ubiquilin 1 NT	1627 ± 330	p<0.0001

Table 3

FRET DONOR (Alexa 430)	FRET ACCEPTOR (Cy3)	ALEXA 430 LIFETIME (mean ± SD, psec)	FRET STRENGTH (mean ± SD, %)
Ubiquilin NT (n=40)	none	3953 ± 494	-
Ubiquilin NT (n=24)	Cy3 anti Alexa 430	1069 ± 51	92 ± 5
Ubiquilin NT (n=36)	PS1 NT	1307 ± 184	52 ± 25
Ubiquilin NT (n=22)	PS1 loop	1197 ± 213	34 ± 16
Ubiquilin NT (n=23)	PS1 CT	1194 ± 181	47 ± 32

Table 4

FRET DONOR	FRET ACCEPTOR	ALEXA 488 LIFETIME	p-value
(Alexa 488)	(Cy3)	(mean ± SD, psec)	(compared to Alexa488 alone)
PS1 NT (n=8)	none	2056 ± 31	-
PS1 NT (n=16)	Ubiquilin 1 NT	1408 ± 129	p<0.0001
PS1 loop (n=7)	none	2156 ± 20	-
PS1 loop (n=17)	Ubiquilin 1 NT	1213 ± 146	p<0.0001
PS1 CT (n=8)	none	2062 ± 28	-
PS1 CT (n=14)	Ubiquilin 1 NT	1472 ± 145	p<0.0001

Figure 1

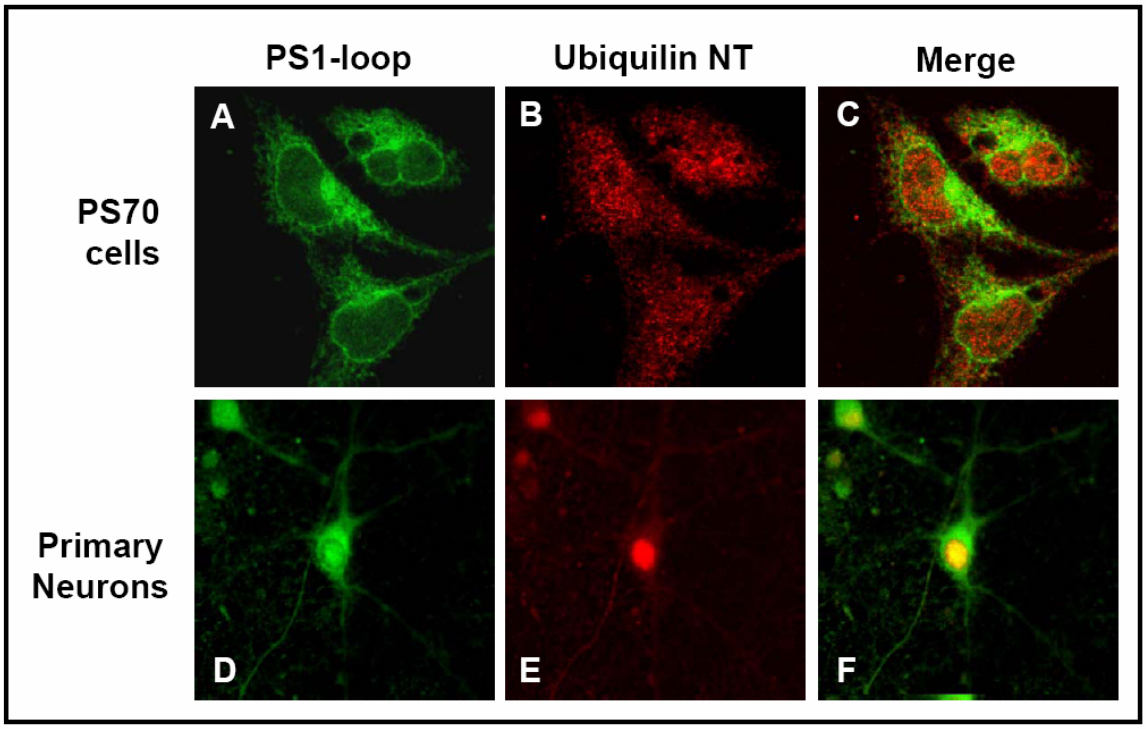


Figure 2

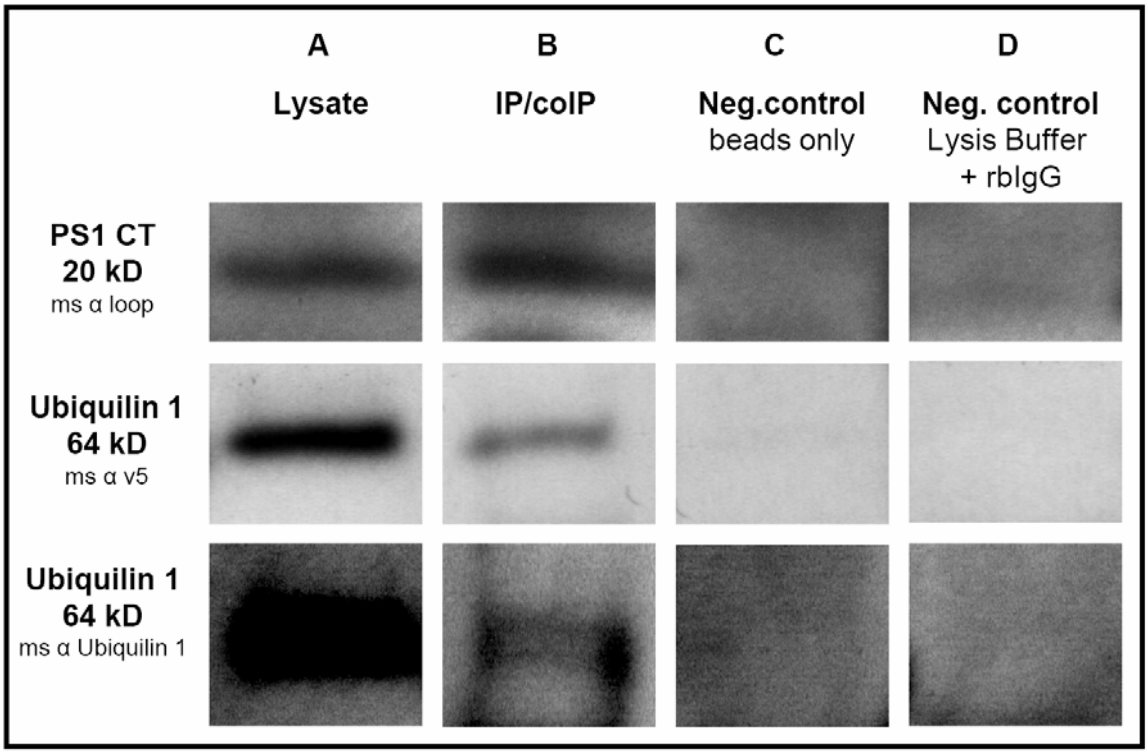


Figure 3

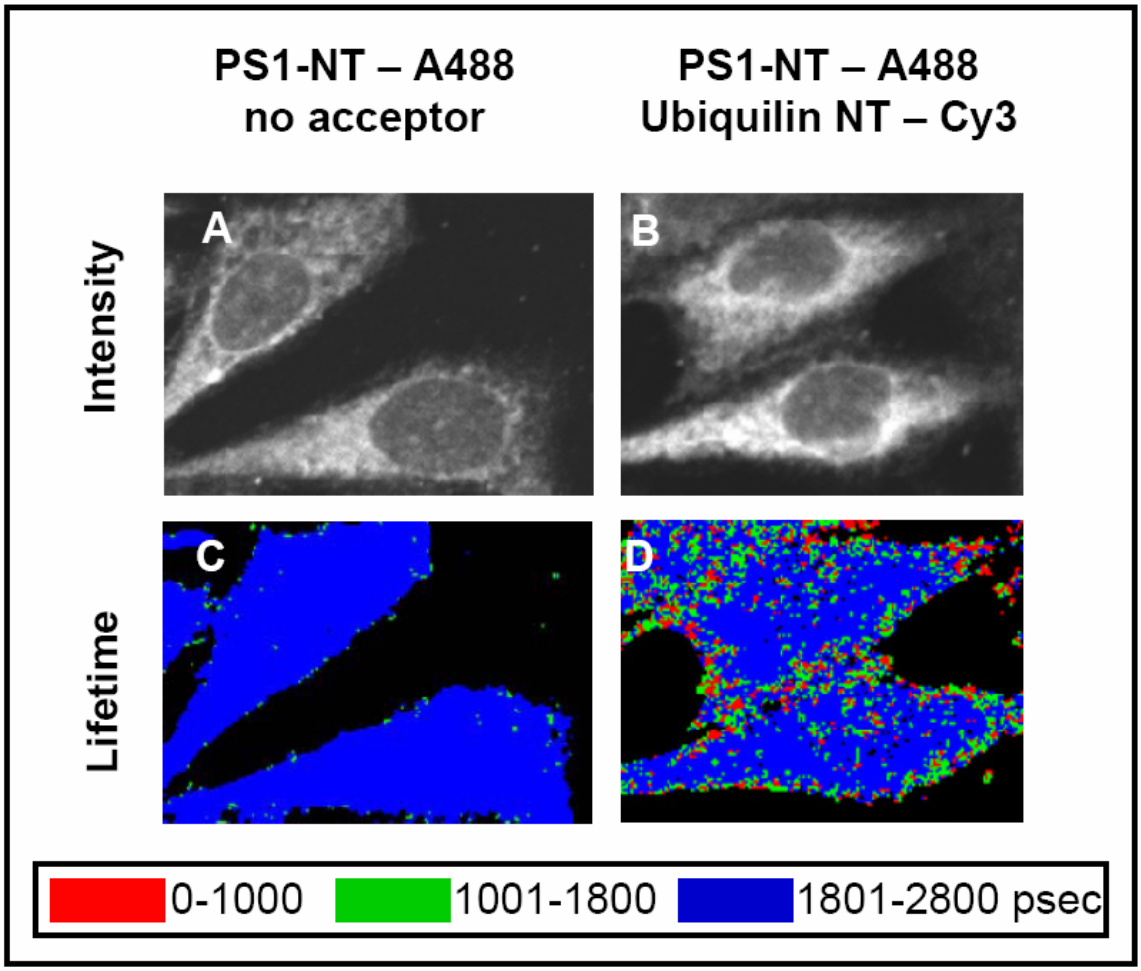


Figure 4

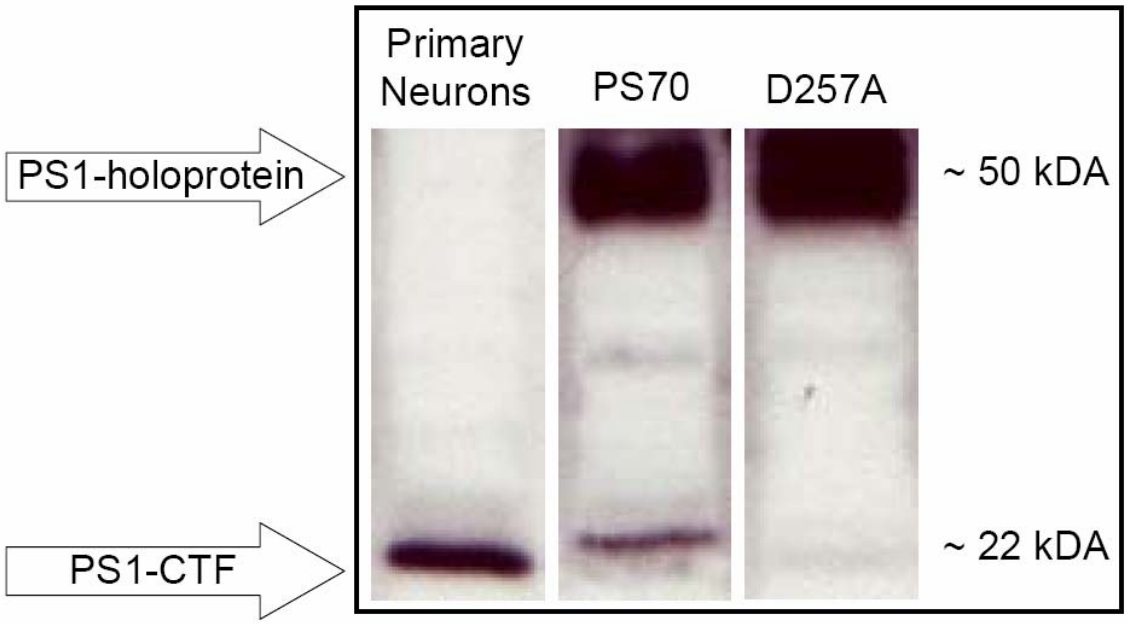
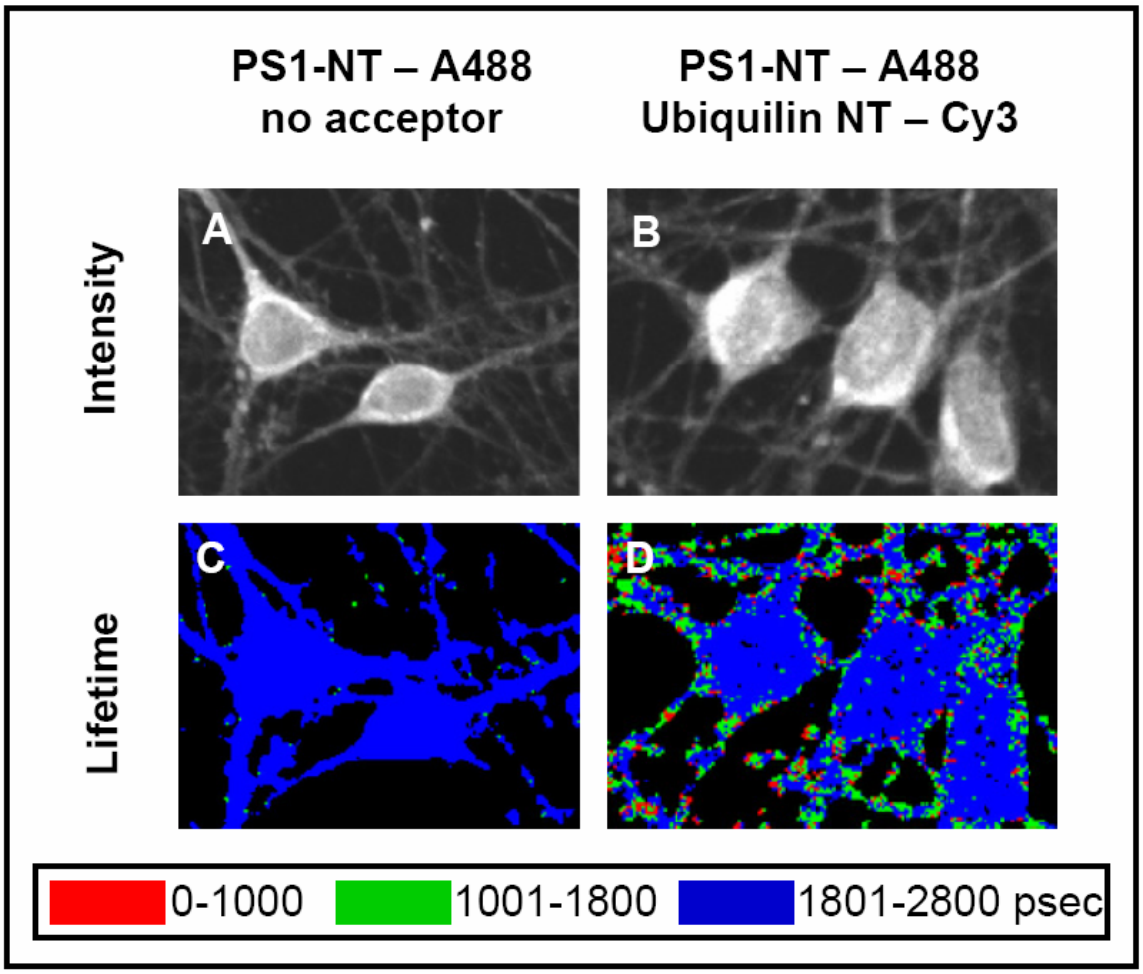


Figure 5


PS1-NT – A488
no acceptor
PS1-NT – A488
Ubiquilin NT – Cy3
Intensity
Lifetime
A
B
C
D
0-1000
1001-1800
1801-2800 psec