Nicatrin, Presenilin, APH-1, and PEN-2 Form Active γ-Secretase Complexes in Mitochondria*

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Camilla A. Hansson‡, Susanne Frykman‡, Mark R. Farmery‡, Lars O. Tjernberg‡, Camilla Nilsbért‡, Sharon E. Pursglove§, Akira Ito¶, Bengt Winblad§, Richard F. Cowburn‡, Johan Thyberg, and Maria Ankarcróna***

From the ‡Karolinska Institutet and Sumitomo Pharmaceuticals Alzheimer Center (KAS PAC), Neurotec, Novum, SE-141 57 Huddinge, Sweden, the §School of Molecular and Microbial Biosciences, University of Sydney, Sydney, New South Wales 2006, Australia, the ¶Sumitomo Pharmaceuticals Co. Ltd., Osaka 541-8510, Japan, and the ||Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institutet, SE-17177 Stockholm, Sweden

Mitochondria are central in the regulation of cell death. Apart from providing the cell with ATP, mitochondria also harbor several death factors that are released upon apoptotic stimuli. Alterations in mitochondrial functions, increased oxidative stress, and neurons dying by apoptosis have been detected in Alzheimer’s disease patients. These findings suggest that mitochondria may trigger the abnormal onset of neuronal cell death in Alzheimer’s disease. We previously reported that presenilin 1 (PS1), which is often mutated in familial forms of Alzheimer’s disease, is located in mitochondria and hypothesized that presenilin mutations may sensitize cells to apoptotic stimuli at the mitochondrial level. Presenilin forms an active γ-secretase complex together with Nicatrin (NCT), APH-1, and PEN-2, which among other substrates cleaves the β-amyloid precursor protein (β-APP) generating the amyloid β-peptide and the β-APP intracellular domain. Here we have identified dual targeting sequences (for endoplasmic reticulum and mitochondria) in NCT and showed expression of NCT in mitochondria by immunoelectron microscopy. We also showed that NCT together with APH-1, PEN-2, and PS1 form a high molecular weight complex located in mitochondria. γ-Secretase activity in isolated mitochondria was demonstrated using C83 (α-secretase-cleaved C-terminal 83-residue β-APP fragment from BD8 cells lacking presenilin and thus γ-secretase activity) or recombiant C100-Flag (C-terminal 100-residue β-APP fragment) as substrates. Both systems generated an APP intracellular domain, and the activity was inhibited by the γ-secretase inhibitors L-685,458 or Compound E. This novel localization of NCT, PS1, APH-1, and PEN-2 expands the role and importance of γ-secretase activity to mitochondria.

Degeneration and death of neurons as well as accumulation of amyloid plaques and neurofibrillary tangles are typical features of Alzheimer’s disease pathology. Amyloid β-peptide (Aβ),1 the main constituent of amyloid plaques, is toxic to cells in culture when applied extracellularly or intracellularly (1). Aβ is released from the β-amyloid precursor protein (β-APP) by the consecutive proteolytic activities of BACE and γ-secretase (2). Several lines of evidence point to γ-secretase being a protein complex consisting of (at least) presenilin 1 (PS1)/presenilin 2 (PS2), Nicatrin (NCT), APH-1, and PEN-2 (3–6). The γ-secretase complex has been reconstituted in yeast (which lacks endogenous γ-secretase) by the co-expression of human PS, NCT, APH-1 and PEN-2 (7). The importance of PS for γ-secretase activity has been demonstrated in several ways (i) in PS-deficient cells (8, 9), (ii) by the use of γ-secretase inhibitors that bind to PS (10, 11), and (iii) by the substitution of either of two aspartyl residues in transmembrane domains 6 and 7 of PS1 (12). All these studies showed inhibited γ-secretase activity and lower production of Aβ.

The γ-secretase complex cleaves β-APP, Notch, and other type I transmembrane proteins such as the receptor tyrosine kinase ErbB4 (2). The components of the γ-secretase complex are transported from the ER, through the Golgi compartment, to the cell surface where many of the substrates are located and processed. It appears that NCT matures on its way through the ER/Golgi and is fully glycosylated in the γ-secretase complex (13). The glycosylation and trafficking of NCT through the Golgi apparatus is PS-dependent (14, 15), and PS interacts preferentially with mature NCT (16). The absence of PS1 leads to dramatic reductions in the level of mature glycosylated NCT and the redistribution of NCT away from the cell surface. In comparison, the absence of PS2 gives only modest reductions in the levels of immature NCT (17). However, γ-secretase activity does not require mature NCT, because the inhibition of oligosaccharide processing with mannosidase I inhibitors does not inhibit the γ-secretase cleavage of β-APP and Notch (14).

Although the substrates for γ-secretase are mainly located in the plasma membrane it is possible that γ-secretase activity and cleavage of transmembrane proteins also occurs in other cellular compartments. Indeed, Siman and Velji (18) demonstrated the localization of PS:NCT complexes and γ-secretase

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** To whom correspondence should be addressed: Karolinska Institutet, Neurotec, KAS PAC, Novum, 5th floor, SE-141 57 Huddinge, Sweden. Tel.: 46-8-585-83622; Fax: 46-8-585-83610; E-mail: maria.ankarcrona@neurotec.ki.se.

1The abbreviations used are: Aβ, amyloid β-peptide; β-APP, β-amyloid precursor protein; PS, presenilin; ER, endoplasmic reticulum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; BN-PAGE, blue native PAGE; CHAPSO, 3-[3-cholamidopropyl]dimethylammonio]-2-hydroxy-1-propanesulfonate; BisTris, 2-[bis(2-hydroxyethyl]-amino]-2-hydroxymethylpropane-1,3-diol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; AICD, β-APP intracellular domain; L-685,458, [1s-benzyl-4R-[1s-1-carbonyl-2-phenylethylcarbamoyl]-1s-3-methylbutylcarbamoyl]-2R-hydroxy-5-phenylpentyl)carnbamic acid tert-butyl ester.
activity to the trans-Golgi network. In addition, PS1 and /H9252-APP are located in lysosomes, and /H9253-secretase activity has been detected in this organelle (19, 20).

We have shown previously that PS1 is located in rat brain and liver mitochondria (21). That study was undertaken on the basis that PS mutations are known to sensitize cells to apoptotic stimuli in vitro (2) and that mitochondria are central in the regulation of apoptosis (22, 23). We hypothesize that PS located in mitochondrial membranes could have a role in, for example, the opening of megapores during permeability transition or cytochrome c release and that PS1 mutations could facilitate such processes making cells more vulnerable at the mitochondrial level. It is also possible that PS1 is part of a /H9253-secretase-like complex in mitochondria and that such activity could cleave and activate proteins involved in the initiation of apoptosis. How PS1 is imported into mitochondria is presently unclear, because it lacks a mitochondrial targeting sequence at the N terminus (as predicted by iPSORT). It is therefore possible that PS1 is co-transported into mitochondria together with other proteins. Because it is known that PS1 is part of the /H9253-secretase complex (3), we did similar iPSORT searches for mitochondrial targeting sequences in NCT, APH-1, and PEN-2. Interestingly, NCT appears to have dual signaling peptides: one for ER and one for mitochondria. In the present study we therefore investigated the expression and activity of the /H9253-secretase complex in isolated rat mitochondria.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The following antibodies were used for detection of components of the /H9253-secretase complex: NCT raised against C-terminal residues 693–709 of human NCT (N1660, Sigma), PS1 raised against residues 2–12 of human PS1 (Prof. John Hardy, Mayo Clinic, Jacksonville, FL), Ab14 directed against the N-terminal fragment of PS1 (Dr. Sam Gandy, Thomas Jefferson University, Philadelphia, PA), PS1 C-terminal raised against residues 263–378 of human PS1 (MAB5232, Chemicon, Temecula, CA), UD1 raised against the N-terminal residues 15-256 of PEN-2 (Dr. Jan Na¨slund, Karolinska Institutet, Sweden), and H2D-2 raised against human APH-1aL (Dr. Gang Yu, University of Texas Southwestern Medical Center, Houston, TX). The antibody for detection of /H9252-APP C1/6.1, directed against the C-terminal of /H9252-APP was from Dr. Paul M. Mathews, Nathan Kline Institute. The following antibodies were used as markers for different subcellular compartments: mitochondria, Hsp60 (SPA-806, StressGen Biotechnologies Corp., Victoria, Canada); ER, KDEL (SPA-827, Nordic BioSite, Ta¨by, Sweden); cis-Golgi, GM130 (610822, BD Biosciences); early endosome, Syntaxin-13 (VAM-SV026, StressGen Biotechnologies Corp., Victoria, Canada); plasma membrane, N-cadherin (610920, BD Biosciences).
the 40 and 26% Percoll layers were collected using a syringe and later identified as pure mitochondria by immunoblot analysis (see below). Mitochondria were washed in buffer A to remove the Percoll and then used for the different experiments described below.

**Brain Sections**—A male Sprague-Dawley rat (200 g) was anesthetized with an overdose of chloral hydrate (5%). A needle was inserted into the left ventricle, and a cut was made in the right atrium. The rat was first perfused with 50 ml of PBS and then with 500 ml of fixation solution (2% formaldehyde, 0.1% glutaraldehyde in PBS, pH 7.3). The brain was dissected out of the body and left in a fixation solution for 2 h. Pieces of the cerebral cortex were put in PBS and azide and stored at 4 °C. Samples were subsequently prepared for immunoelectron microscopy as described below.

**Immunoelectron Microscopy**—Isolated rat brain mitochondrial pellets and rat brain tissue were fixed for 1–2 h in 2% formaldehyde and 0.1% glutaraldehyde in PBS, pH 7.3. After repeated rinsing in PBS, the specimens were dehydrated in ethanol (70, 95, 100%), and embedded in LR White (London Resin Company). They were first incubated in a mixture of equal parts ethanol and LR White (v/v) for 30 min and then in pure resin for 12–15 h at 4 °C. After two additional incubations in LR White (30 min each), they were put into gelatin capsules filled with resin and placed in an UV light polymerization unit (Agar Scientific) for 12 h. Thin sections were cut with diamond knives on a Leica Ultracut and picked up on nickel grids coated with formvar film. For immunogold staining, the grids were placed on droplets of PBS, 2% BSA for 30 min to block unpecific binding sites, transferred to primary antibodies diluted in PBS, 2% BSA, and incubated for 2 h in a humid box. After rinsing with PBS, 2% BSA, they were placed on droplets of gold-labeled secondary antibodies diluted in PBS, 0.5% BSA (goat anti-rabbit IgG, Sigma) for 1 h. The sections were then rinsed with PBS, 0.5% BSA followed by PBS, postfixed with 2% glutaraldehyde in PBS for 5 min, rinsed with PBS followed by water, and air-dried. Controls without primary antibodies or with unrelated antibodies were negative. Preabsorption of the Nicastrin antibody with the peptide used for immunization (KADVLFIAFPRPGAVSY) as well as an unrelated peptide (TEL-PAPLSYFQN) in a 1:1 molar relationship was done as a control for binding specificity. Contrast staining was made with aqueous uranyl acetate for 5 min and lead citrate for 5 s. The specimens were examined in a Philips CM120 electron microscope at 80 kV and photographed using a Kodak MegasPlus CCD camera.

**Immunoblotting**—Mitochondrial pellets were lysed in lysis buffer (Tris-buffered saline, 0.2% Nonidet P-40, 2 mM EDTA, 2% protease inhibitor mixture complete EDTA-free (Roche Diagnostics), 2% Triton X-100 and 2% IGEPAL CA-630), and the protein concentration was determined using BCA reagents (Pierce). Proteins were resolved by electrophoresis through 12% self-cast SDS-polyacrylamide gels or 4–20% precast criterion SDS-PAGE (Bio-Rad) and then transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat milk before overnight incubations with antibody. The membranes were subsequently incubated with secondary antibody (horseradish peroxidase-linked antibody, Amersham Biosciences) and developed using the Pierce Super Signal West Pico chemiluminescence substrate kit.

**Co-immunoprecipitation**—Percoll-purified rat brain mitochondria were prepared as above and solubilized in 20 mM Hepes, pH 7.0, 150 mM KCl, 2 mM EDTA, 2 mM EGTA, 1% CHAPSO with protease inhibitor mixture for 30 min on ice. Unsolubilized material was removed by centrifugation at 16,000 × g for 5 min, and the supernatant was preincubated with protein A-Sepharose (Amersham Biosciences) under rotation for 1 h at 4 °C followed by incubation with the PS1 antibody Ab14, anti-NCT antibody, or pre-immune rabbit serum, overnight at 4 °C. Protein A-Sepharose was added and incubated for an additional 2 h. The precipitates were washed twice with solubilization buffer with

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**Fig. 2.** A, all the known γ-secretase components are present in mitochondria. Immunoelectron microscopic analysis of brain tissue and isolated mitochondria is shown. Sections were incubated with antibodies directed against NCT (dilution 1:50), PS1 (dilution 1:20), APH-1 (dilution 1:250), and PEN-2 (dilution 1:50) and subsequently treated with gold-labeled secondary antibodies. Bars = 200 nm. Note the lack of NCT staining in the nucleus. B, preabsorption of NCT antibody. Immunoelectron microscopic analysis of brain tissue and isolated mitochondria is shown. Sections were incubated with NCT antibody alone or with NCT antibody preabsorbed with related or unrelated peptides. Bars = 500 nm.
0.2% CHAPSO and once without CHAPSO, resuspended in the sample buffer, heated at 60 °C for 5 min, and loaded on a SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose filter and analyzed with an antibody against the C-terminal fragment of PS1.

Blue Native (BN)-PAGE—BN-PAGE was performed as described (24). Mitochondrial pellets were resuspended in extraction buffer (0.75 M aminocaproic acid and 50 mM BisTris, pH 7.0, 20 μl/100 μg of protein), and n-dodecyl-β-D-maltoside was added to give a final concentration of 1% w/v. The mixture was incubated for 20 min on ice. The samples were centrifuged at 100,000 g for 20 min, and Coomassie Brilliant Blue G-250 (Serva, Heidelberg) in 0.75M aminocaproic acid was added to the supernatant to give a final concentration of 0.3% w/v. The samples were separated for 17 h at 75 V on a 4–12% BisTris gel (Invitrogen) using 50 mM BisTris as an anode buffer and 15 mM BisTris, 50 mM Tricine containing 0.02% Coomassie Brilliant Blue G-250 as a cathode buffer. Molecular weight standards (high molecular weight calibration kit, Amersham Biosciences) were resuspended in extraction buffer (200 μl/250-μg vial) plus 25 μl of 10% n-dodecyl-β-D-maltoside and 12 μl of 5% Coomassie Blue. Before blotting, the gel was soaked in transfer buffer for 30 min (0.02M Tris-HCl, 0.12M glycine, 20% methanol, 0.05% w/v SDS), where after the proteins were transferred to polyvinylidene difluoride membranes at 140 mA for 2 h and probed with specific antibodies.

γ-Secretase Activity Assays—The purified mitochondria and membrane pellet from the rat brain were solubilized in a solubilization buffer (20 mM Hepes, pH 7.0, 150 mM KCl, 2 mM EGTA, 1% CHAPSO, and 2× protease inhibitor mixture) and incubated for 1 h at 4 °C with rotation. BD8 cells are blastocyst cells derived from a PS1/PS2 knock-out mouse (25). These cells lack γ-secretase activity and accumulate C83 (C-terminal 83 residues of β-APP), and to a minor extent C99 (C-terminal 99 residues of β-APP), and were here used as substrate in the γ-secretase activity assay. BD8 cells were cultured in ES medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2.4 mM L-glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.1 mM mercaptoethanol, and non-essential amino acids) and washed in PBS before they were harvested in PBS and centrifuged at 1500 × g for 5 min. The pellet was resuspended in buffer A, and the cells were homogenized by several strokes at 1800 rpm with a high torque mortar-driven pestle. All centrifugations were carried out at 4 °C. Unbroken cells and nuclei were collected by centrifugation at 100 × g for 15 min. BD8 membranes were collected from the supernatant by centrifugation at 140,000 × g for 1 h. The pellet was solubilized in solubilization buffer and incubated 1 h at 4 °C with rotation. Unsolubilized membranes were spun down at 140,000 × g for 15 min. The protein concentration was measured by spectrophotometer analysis. The solubilized mitochondria and membrane fractions were treated with γ-secretase inhibitors L-685,458 (0.25, 1, and 5 μM) (Bachem) or Compound E (0.25, 1, and 5 μM) (Merck) or Me2SO and left on ice for 10 min before the solubilized BD8 fractions were added in a ratio 5:3 and incubated at 37 °C for 16 h. Control samples without inhibitors were either incubated at 37 °C for 16 h or frozen down at −20 °C. The γ-secretase cleavage products were separated on 16.5% self-cast SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. The production of β-APP intracellular domain (AICD) was detected with monoclonal antibody C1/6.1 raised against the C-terminal of APP. Recombinant C100-Flag (tagged at the C-terminal of APP, Recombinant C100-Flag (tagged at the C-terminal of APP) was used as an alternative substrate. C100-Flag was purified essentially as described by Li et al. (26). Briefly, C100-Flag was PCR-amplified from a C99 expression plasmid using the primers 5′-ATTACCATGGATGCGAATTCCGA-3′ and 5′-ATATCCTGAGCCTA-3′. The amplification products were cloned into the pET28a vector and expressed in E. coli. The expressed protein was purified and used as a substrate in the γ-secretase activity assay.
RESULTS

Identification of a Mitochondrial Targeting Sequence in NCT—An iPSORT data base search for targeting sequences in NCT revealed dual targeting sequences. Amino acids 8–32 in the N-terminal region are an ER-targeting domain. In the sequence immediately following, five positively charged residues that resemble a mitochondrial targeting peptide are found at positions 38, 39, 46, 52, and 58 (Fig. 1). Similar dual targeting sequences have been found in other proteins like P4501A1 (28), P4502B1 (29), P4502E1 (30), and P4502F1 (31) (Fig. 1).

Immunoelectron Microscopy Studies of Brain Mitochondrial Fractions and Brain Tissue Slices—Immunoelectron microscopy studies of mitochondrial fractions and brain tissue slices showed an abundance of NCT in mitochondria (Fig. 2A). NCT was detected with a C-terminal antibody and found to be located in the inner parts of the mitochondria with gold particles tending to form clusters. The results from the tissue section clearly show that immunolabeling of NCT is predominantly located in mitochondria and that the fractionation procedure did not affect this staining pattern (Fig. 2A). Preabsorption of the NCT antibody with the peptide used for immunization (KADVLFIA-PREPAGVSY) abolished NCT labeling in mitochondria (Fig. 2B). Preabsorption of the NCT antibody with an unrelated peptide (TELAPLASYFQN) resulted in NCT immunolabeling similar to controls (Fig. 2B). Our previous demonstration of PS1 being located in mitochondria (21) was confirmed both in fractions and tissue (Fig. 2A). The levels of PS1 were lower as compared with NCT. Similar results were obtained when mitochondrial fractions or brain tissue were stained with antibodies directed to APH-1 and PEN-2 (Fig. 2A). Both APH-1 and PEN-2 were clearly located in mitochondria but at lower levels as compared with NCT.

Immunoblot Studies of Percoll-purified Mitochondria—The mitochondrial fractions obtained from centrifugations in a mannitol buffer were further purified on a Percoll gradient. The purity of the fractions was examined by Western blot using markers for different organelles. These fractions were enriched in mitochondria as indicated by a high reactivity to anti-Hsp60 (Fig. 3). The lack of KDEL, GM130, Syntaxin-13, and N-cadherin immunoreactivity in the mitochondrial fractions indicated that these were free from ER, cis-Golgi, early endosomes, and plasma membranes, respectively (Fig. 3). Immunoblot studies (SDS-PAGE) revealed the expression of NCT, PS1, APH-1, and PEN-2 in the Percoll-purified mitochondria. It thus appears that all four components of the γ-secretase complex are located in mitochondria (Fig. 4A, top panel). To investigate whether the observed γ-secretase components are associated in a protein complex we performed BN-PAGE on Percoll-purified mitochondrial fractions. With this technique it is possible to analyze the intact γ-secretase complex, because the gel is run under non-denaturing conditions. This technique was used in our laboratory to detect γ-secretase complexes in homogenates from the human brain (32). BN-PAGE showed that NCT, PS1, APH-1, and PEN-2 could be detected in high molecular mass complexes running at ~500 kDa (Fig. 4B, bottom panel). In addition, we also detected PS2 in complexes running at the same molecular mass indicating that both PS proteins are present in mitochondria (not shown). Co-immunoprecipitation experiments, using NCT or PS1-NTF (Ab14) antibodies for precipitation and PS1-CTF antibody for detection, also revealed that these proteins interact in mitochondria (Fig. 4B).
37 °C with or without γ-secretase inhibitors for 16 h. Immunoblotting with antibodies directed to the C-terminal of β-APP revealed a band at 6 kDa corresponding to the molecular mass of AICD (Fig. 5A). Shorter incubation times, up to 6 h, resulted in low amounts of AICD hardly detectable on the film (not shown). AICD was not formed at −20 °C, and AICD formation was prevented in the presence of the γ-secretase inhibitors L-685,458 or Compound E. AICD (6 kDa) formation at 37 °C was detected on SDS-PAGE with a C-terminal APP antibody C1/6.1. No AICD was produced at −20 °C or in the presence of γ-secretase inhibitors at 37 °C.

**FIG. 5. CHAPSO-solubilized mitochondria show γ-secretase activity.** Solubilized membranes from BD8 cells (BD8) were mixed with either Percoll-purified and solubilized rat brain mitochondria (A) or with solubilized rat brain membranes (B) and incubated for 16 h in the absence or presence of γ-secretase inhibitors (L-685,458 or Compound E). AICD (6 kDa) formation at 37 °C was detected on SDS-PAGE with a C-terminal APP antibody C1/6.1. No AICD was produced at −20°C or in the presence of γ-secretase inhibitors at 37 °C.

**FIG. 6. SDS-PAGE for detection of AICD formation using C100-Flag as a substrate.** Recombinant C100-Flag (10 μg/μl, ~14 kDa) was treated with 4.5 M urea plus 50 mM Hepes. 1 μg C100-Flag was mixed with Percoll-purified and solubilized rat brain mitochondria and incubated for 16 h in the absence or presence of L-685,458 (1 μM). AICD formation was prevented in the presence of the γ-secretase inhibitors L-685,458 or Compound E at 37 °C. Membrane preparations from rat brain were used as a control for γ-secretase activity and showed similar results as mitochondria (Fig. 5B). Recombinant C100-Flag protein was used as an alternative substrate to C83. γ-Secretase activity in mitochondria derived from rat brain was detected as AICD formation also with C100-Flag as a substrate (Fig. 6). C100-Flag or mitochondria alone did not produce AICD, whereas a mixture of the two generated AICD when incubated at 37 °C for 16 h. The presence of L-685,458 (1 μM) prevented AICD formation.

**DISCUSSION**

Mitochondria have a central role in the cell death process functioning as a switch between necrosis and apoptosis depending on the levels of ATP (33). Mitochondria also contain several “death molecules,” e.g., cytochrome c, Omi/HtrA2, SMAC/Diablo, and apoptosis-inducing factor, which are released upon apoptotic stimuli (22). Impairment of mitochondrial energy metabolism (34–36) and increased production of reactive oxygen species have been suggested as early alterations of cellular functions detected in Alzheimer’s disease (37). PS mutations sensitize cells to apoptotic stimuli, and the inhibition of mitochondrial functions causes an increased production of ROS in cells overexpressing PS1 mutant protein (38). Moreover, Aβ is a pro-oxidant and may participate in the increase of reactive oxygen species seen in Alzheimer’s disease (39). PS1 is located in mitochondria (21), and it is possible that PS1 has direct effects on mitochondrial functions. In the present study we demonstrated that the other components of the γ-secretase complex, i.e., NCT, APH-1, and PEN-2, are also located in mitochondria and that they together with PS1/PS2 form an active γ-secretase complex. Immunoelectron microscopy studies showed a clear localization of NCT to mitochondria. Studies of tissue sections confirmed the results obtained from fractionated mitochondria and showed that the fractionation procedure did not alter the localization. Immunogold labeling of PS1, APH-1, and PEN-2 were also observed in mitochondria, however, at lower levels as compared with NCT. These differences could depend on the antibodies used and how different epitopes are exposed, but it is also possible that these results reflect the actual situation. NCT has dual targeting sequences for ER and mitochondria. However, no mitochondrial targeting sequences were found in PS1/PS2, APH-1, and PEN-2. One possibility is that these constituents of the γ-secretase complex are transported into mitochondria together with NCT. Our data from BN-PAGE showed that PS1/PS2, NCT, APH-1, and PEN-2 form complexes with a molecular mass of ~500 kDa. This is in agreement with another study from our laboratory where the molecular mass of the γ-secretase complex isolated from the human brain was estimated to be ~500 kDa (32). The exact stoichiometry of the γ-secretase complex has yet to be determined. Schroeter et al. (40) have suggested that a PS heterotetramer forms the core of the complex with a suggested 2:1:1:1 (PS:NCT:APH-1:PEN-2, ~250 kDa) stoichiometry (40). Two such γ-secretase complexes could then in turn form a dimer corresponding to 500 kDa as detected in this and other studies (6, 32).

Interestingly, γ-secretase activity was detected in mitochon-
**γ-Secretase Activity in Mitochondria**

Using β-APP from BD8 cells or recombinant C100-Flag as substrates, the formation of AICD was abolished in the presence of well-characterized γ-secretase inhibitors indicating a specific γ-secretase activity. These results showed that γ-secretase complexes located in mitochondria are active and that they can cleave β-APP. β-APP has been located in the mitochondrial outer membrane (31) and appears to have targeting sequences for both ER and mitochondria. However, the insertion of β-APP into the mitochondrial membrane seems to be different compared with other membranes. Import assays with β-APP and isolated mitochondria show that β-APP is incompletely translocated through the mitochondrial outer membrane and the region containing amino acids 220–290 acts as a barrier for complete import. Therefore it is suggested that a 22-kDa region (N-terminal) of β-APP is located inside the mitochondria and that the remaining 73-kDa portion, containing the Aβ sequence and γ-secretase cleavage site, is exposed on the cytoplasmic side (C-terminal) (31). The Aβ sequence and γ-secretase cleavage site have to be in the transmembrane region for a correct processing by the γ-secretase. It is possible that this hydrophobic region of β-APP either inserts itself in neighboring ER membranes or loops back into the mitochondrial outer membrane. However, the C99 fragment of β-APP was not detected in the mitochondria after import studies (31). If this orientation of β-APP is correct, β-APP is not likely to be the substrate for the mitochondrial γ-secretase. Interestingly, Aβ interacts with Aβ-binding alcohol dehydrogenase in the mitochondria of Alzheimer's disease patients and transgenic mice (41). Whether Aβ is produced in mitochondria or reaches the mitochondria from other subcellular locations is presently unclear according to the discussion above.

In conclusion, we showed that NCT has dual targeting sequences and is abundantly expressed in brain mitochondria. We also demonstrated that NCT, PS1, APH-1, and PEN-2 form an active γ-secretase complex expressed in mitochondria. If β-APP is not the substrate for γ-secretase in mitochondria such protease activity may instead contribute to neurodegenerative pathways distinct from or upstream of Aβ generation and plaque formation. The γ-secretase cleaves several other type I transmembrane proteins, and future studies are required to determine which is the true substrate for the mitochondrial γ-secretase.

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