APH-1 Interacts with Mature and Immature Forms of Presenilins and Nicastrin and May Play a Role in Maturation of Presenilin-Nicastrin Complexes*

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APH-1 and PEN-2 genes modulate the function of nicastrin and the presenilins in Caenorhabditis elegans. Preliminary studies in transfected mammalian cells overexpressing tagged APH-1 proteins suggest that this genetic interaction is mediated by a direct physical interaction. Using the APH-1 protein encoded on human chromosome 1 (APH-1; also known as APH-1a) as an archetype, we report here that endogenous forms of APH-1 are predominantly expressed in intracellular membrane compartments, including the endoplasmic reticulum and cis-Golgi. APH-1 proteins directly interact with immature and mature forms of the presenilins and nicastrin within high molecular weight complexes that display γ- and ε-secretase activity. Indeed APH-1 proteins can bind to the nicastrin Δ312–369 loss of function mutant, which does not undergo glycosylation maturation and is not trafficking beyond the endoplasmic reticulum. The levels of expression of endogenous APH-1, L can be suppressed by overexpression of any other members of the APH-1 family, suggesting that their abundance is coordinately regulated. Finally, although the absence of APH-1 destabilizes the presenilins, in contrast to nicastrin and PEN-2, APH-1 itself is only modestly destabilized in cells lacking functional expression of presenilin 1 or presenilin 2. Taken together, our data suggest that APH-1 proteins, and APH-1, in particular, may have a role in the initial assembly and maturation of presenilin-nicastrin complexes.

Presenilin 1 (PS1)1 (1), presenilin 2 (PS2) (2), and nicastrin (3) are components of high molecular weight protein complexes that are required for the intramembranous proteolysis of some type 1 transmembrane proteins, including the β-amyloid precursor protein (APP) (4), Notch (5–11), and ErbB-4 (12). Genetic screens in Caenorhabditis elegans have identified two additional proteins, APH-1 (13) and PEN-2 (14), in which loss of function mutations phenotypically modulate Notch signaling in a manner similar to that of null mutants in nicastrin and the presenilins. Preliminary studies in transfected cells overexpressing tagged APH-1 proteins suggest that the genetic interaction between APH-1 and the presenilin-dependent cleavage of Notch and APP is mediated by a direct physical interaction between APH-1 and nicastrin or the presenilins (14). We report here that both the APH-1 homologue on human chromosome 1 (termed APH-1,1 in Ref. 14, but here referred to as APH-1,1 for clarity to avoid confusion with labeling of alternate splice forms) and the APH-1 homologue on chromosome 15 (previously referred to as APH-1,2) are widely expressed in multiple tissues and that the APH-1,1 transcript is present as several different alternatively spliced forms (data not shown). We also report that endogenous APH-1,1 directly interacts with both immature and mature forms of the presenilins and nicastrin and that high molecular weight complexes containing these proteins display γ- and ε-secretase activity. Overexpression of any one member of the APH-1 family results in suppression of the levels of expression of all the endogenous APH-1 proteins, suggesting that their abundance is coordinately regulated by binding to the same limiting factors, presumably the presenilins, nicastrin, and/or PEN-2. In contrast to nicastrin and PEN-2, APH-1 is only modestly destabilized in cells lacking functional expression of PS1 or PS2. Our data suggest that the presenilins, nicastrin, and APH-1 proteins physically interact with each other within high molecular weight protein complexes and that APH-1,1 may have a role in the maturation of these PS1-nicastrin complexes.

EXPERIMENTAL PROCEDURES

APH-1 Expression Vectors—cDNA clones encoding human APH-1, S, APH-1, L, and APH-1,1 proteins were all generated by PCR from reverse-transcribed human brain or leukocyte DNA. After complete sequencing of both strands, the cDNAs were ligated into the expression vector, pCDNA4/myc-His(C) (Invitrogen).

Generation of Stable HEK293 Cell Lines Expressing Human APH-1 Proteins—HEK293 cells stably expressing APH-CTFP99 were transfected with the above plasmids or with empty vector (as a negative control) using LipofectAMINE, and stable transfectant cells were selected in 200 μg/ml zeocin. Stable cell lines were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum, 120 μg/ml zeocin, and 200 μg/ml G418.

Gradient Centrifugation—Glycerol velocity gradient centrifugation was performed as described previously (15). For subcellular fractionation, membrane fractions were isolated from HEK293 cells or mouse brain homogenates as described previously (16) and were applied to the
top of an 11.5-ml 5–25% (v/v) linear iodixanol gradient (Accurate). Following centrifugation, 0.8-ml fractions were collected and analyzed by Western blotting. Specific marker proteins of the ER (calnexin) and Golgi (GM130) were detected using monoclonal antibodies (anti-calnexin, StressGen; anti-GM130, Sigma).

Immunoprecipitation—HEK293 cells were lysed with 1% digitonin in buffer containing 10% glycerol, followed by centrifugation at 100,000 g for 30 min at 4 °C (15). All immunoprecipitation steps were performed at 4 °C. After preclearing with protein G-Sepharose CL-4B (Amersham Biosciences) for 1 h, cells were lysated with antibody for 1 h. The immunoprecipitates were recovered by overnight incubation with protein G-Sepharose CL-4B. The beads were washed four times with 1% digitonin in the same buffer.

Immunocytochemistry—For immunofluorescence microscopy, the cells were plated onto 18-mm round coverslips coated with mouse collagen, type 4 (BD Biosciences) in a 35-mm dish. After a brief rinse in PBS, the cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min and permeabilized with 0.3% Triton X-100 in PBS, the cells were incubated with 5% acetic acid in ethanol for 30 min at room temperature for 20 min and then incubated overnight with each primary antibody. The primary antibodies used in this study were as follows: mouse monoclonal antibodies anti-my′c (Invitrogen); mouse monoclonal antibodies directed to organelar-marker proteins BiP (an endoplasmic reticulum marker) (StressGen, Victoria, BC, Canada), to GM130 (Transduction Laboratories), and to PS1 (NT1 from Paul M. Mathews); and rabbit polyclonal antibody to PS1-NTF (Ab14) and to human APH-1 (O2C2). The cy2-conjugated goat anti-mouse IgG or cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibodies. Nuclei were visualized by staining with 2 μM ToPro3 (Molecular Probes). Specimens were examined with a Bio-Rad laser scanning confocal imaging system (Microradiance R2000/AG-2) equipped with the Lasersharp2000 software (Bio-Rad). Images were processed using the Lasersharp2000 post-processing software and Adobe Photoshop (Adobe Systems).

Presenilin-γ-Secretase Complex Purification—Microsomal membranes were lysed in 1% CHAPSO-containing IP buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, and 2 mM EDTA plus a mixture of protease inhibitors (Sigma)). The lysates were centrifuged at 45,000 rpm for 1 h, the supernatants were immunoprecipitated with anti-PS1, anti-myc, or anti-APH-1-L antibodies, and the generation of e-stubs and amyloid β-peptide was then performed as a cell-free assay system by incubation at 37 °C for 2 h (e-stubs) or 4 h (amyloid β-peptide) (17–19). Control samples were kept on ice.

RESULTS AND DISCUSSION

In addition to the two APH-1 members reported on chromosome 1 (APH-1L) and chromosome 15 (APH-1L15) (14), a data base search and molecular cloning revealed a longer isoform of APH-1 arising from alternative splicing of the transcript from chromosome 1. Here we designate them as APH-1-S (short isoform) and APH-1-L (long isoform), respectively (Fig. 1).

Overexpression of exogenous presenilin or nicastrin proteins causes the artifactual accumulation of immature forms of these proteins because of the fact that the stoichiometry and abundance of components within the mature presenilin complexes are highly regulated (15, 20, 21). Consequently, we initially elected to investigate the biology of APH-1 in native cells expressing endogenous APH-1. Polyclonal sera were raised against epitopes at the C terminus of APH-1-L, and of APH-1-S. Analysis of Western blots expressing exogenous APH-1 with or without the myc-His tag indicated that the antibody was specific for APH-1-L, and that APH-1-S co-fractionates with nicastrin and PS1 on velocity gradient centrifugation. Membrane preparation from HEK293 cells were solubilized in 1% digitonin and fractionated by centrifugation through 10–30% linear glycerol density gradients; the fractions were analyzed by immunoblotting for APH-1-L (O2C2), PS1 (Ab14), and nicastrin (PA1–758). APH-1-L co-fractionates best with immature and mature nicastrin but also with PS1.

Fig. 1. Amino acid sequences of human APH-1 proteins. Peptide residues used for raising antisera are underlined. The putative transmembrane domains are shaded. The novel C-terminal residues in the long isoform of APH-1-L (APH-1L) are in italics. The abundance of APH-1-L and APH-1-S is similar.

Fig. 2. Western blot analysis of endogenous and transfected APH-1 proteins. A, lysates (~3 μg of total protein) from HEK293 cells stably transfected with both APP-C99 and one of the myctagged human APH-1s or empty vector (Vector) were fractionated by SDS-PAGE and analyzed by immunoblotting with anti-myc antibody. B, lysates from mouse brains and kidney (~6 μg of total protein/lane) and 1% digitonin extracts of crude membrane preparations from mouse brain and kidney (~2 μg of membrane protein) were Western-blotted with the antiserum to the C terminus of APH-1-L (O2C2). The myctagged exogenous species migrate with higher apparent molecular masses because of the myc-His tag.
FIG. 4.  

**a**, endogenous APH-1,L, PS1, and nicastrin co-immunoprecipitate. Membrane preparations from HEK293 cells were solubilized in 1% digitonin, immunoprecipitated with pre-immune serum (lane 3), anti-PS1 N-terminal antiserum (Ab14, lane 4), or anti-APH-1,L antiserum (O2C2, lane 5). The immunoprecipitation products were then immunoblotted with anti-APH-1,L antiserum (top panel), monoclonal anti-PS1 N-terminal (NT1) or anti-PS2 N-terminal (DT2) (middle two panels), and polyclonal anti-nicastrin antibody (PA1-758 from Affinity Bioreagents; bottom panel). Lane 1, lysates of HEK293 cells transiently transfected with human APH-1,L were included as a positive control (diluted 50 times). Lane 2, lysate of untransfected HEK293 cell. Loading represents about 10% of the starting digitonin lysate utilized for immunoprecipitation in lanes 3–5.  

**b**, endogenous APH-1,L also co-immunoprecipitates presenilin holoproteins. Lysates from cells expressing endogenous APH-1,L and stably overexpressing wild type PS1 (lane 1), PS1 D385A loss of function mutant (lane 2), wild type nicastrin (lane 6), or nicastrin Δ312–369 loss of function mutant (lane 7) were immunoprecipitated with pre-immune serum (lane 3), anti-PS1 antibodies (lanes 4 and 5), or anti-V5 antibody (to precipitate V5-tagged nicastrin; lanes 8 and 9). The IP products were Western-blotted with anti-APH-1,L antibody (O2C2, top row), anti-PS1 antibody (NT1, middle two rows), and anti-nicastrin antibody (PA1-758, bottom row). Endogenous APH-1,L co-precipitates with both PS1-NTF/CTF (predominant PS1 species in PS1 wild type cells, lane 4) and PS1 holoprotein (predominant PS1 species in PS1 D385A cells, lane 5). The apparent overabundance of APH-1,L in the anti-PS1 immunoprecipitation products from PS1 D385A cells reflects the ability of APH-1,L to bind both the PS-NTF/CTF and the holoprotein and the relative overabundance of the holoprotein in PS1 D385A cells. Although
without myc tags in HEK293 cells revealed that one antiserum (O2C2) specifically detected the longer isoform of APH-1, but did not detect the shorter isoform of APH-1, or APH-1\textsubscript{5} (Fig. 2, A and B). This antibody also detected significant levels of expression of APH-1, in brain and kidney. This endogenous APH-1, immunoreactive band had an apparent molecular weight that is in good agreement with that predicted from the primary amino acid sequence of APH-1, and that is identical to that of the untagged APH-1\textsubscript{1L} transiently overexpressed in HEK293 cells as a positive control. These results indicate that, in contrast to nicastrin (22, 23) and the presenilins (24), it is unlikely that APH-1\textsubscript{1L} undergoes significant post-translational modifications.

To discover the native conformation of the APH-1 proteins and to examine the possibility that APH-1 proteins may form components of the presenilin-nicastrin complexes, we used glycerol velocity gradient centrifugation in cells expressing endogenous APH-1\textsubscript{1L}. These studies revealed that, in digitonin-solubilized lysates, endogenous APH-1\textsubscript{1L} co-fractionated with PS1 and nicastrin (Fig. 3). However, the distribution of APH-1\textsubscript{1L} most closely resembled that of immature forms of nicastrin, although there was substantial overlap with both the distribution of mature nicastrin and PS1-NTF. As expected, solubilization with Triton X-100 causes the nicastrin-PS1-APH-1\textsubscript{1L} complex to be completely disassembled (data not shown). These results are in agreement with the observation that APH-1\textsubscript{1L} can be co-immunoprecipitated with both immature and mature forms of nicastrin (see below). These results also suggest that APH-1\textsubscript{1L} is likely to be a component of both the immature and the higher molecular weight mature (functional) presenilin-nicastrin complexes (15).

To discern whether endogenous APH-1\textsubscript{1L} interacts directly with presenilin and nicastrin, reciprocal co-immunoprecipitation studies were performed in HEK293 cells expressing native presenilin, APH-1\textsubscript{1L}, and nicastrin proteins. Immunoprecipitation with pre-immune serum failed to bring down APH-1\textsubscript{1L}, PS1, PS2, or nicastrin. However, immunoprecipitation with antibodies to the N terminus of PS1 (Fig. 4A, lane 4) brought down endogenous APH-1\textsubscript{1L} and the mature form of nicastrin. Conversely, the reciprocal immunoprecipitation with anti-APH-1\textsubscript{1L} antibodies (Fig. 4A, lane 5) brought down stoichiometric quantities of APH-1\textsubscript{1L}, PS1 holoprotein and PS1-NTF, PS2 holoprotein and PS2-NTF, and both mature and immature forms of nicastrin. The interaction of APH-1\textsubscript{1L} with PS1 holoprotein was independently confirmed by analysis of anti-PS1 immunoprecipitation products from cells overexpressing the PS1 D385A loss of function mutant (Fig. 4B, lane 5). The PS1 D385A and PS1 D257A mutations and the equivalent mutations in PS2 were shown previously to generate only the intermediate, ~180-kDa, immature, non-functional complexes containing holoprotein (25), which do not mature into the higher molecular weight (>350 kDa) functional complex containing PS1-NTF/CTFs (25). The apparently larger amount of APH-1\textsubscript{1L} co-immunoprecipitated with PS1 D385A compared with wild type PS1 (Fig. 4B, compare lanes 4 and 5), likely reflects the much greater and unregulated abundance of the PS1 D385A

**Fig. 5.** APH-1\textsubscript{1L} is a component of the catalytically active PS1-nicastrin complex. A, complexes were immunopurified with anti-PS1 (lane 1), anti-APH-1\textsubscript{1L} (lanes 6 and 7), or anti-APH-1\textsubscript{5} antibodies (lanes 2–5) from CHAPS-solubilized microsomal membrane preparations from cells expressing endogenous APH-1 proteins (lanes 1, 6, and 7) or from cells overexpressing exogenous myc-tagged APH-1 proteins (lanes 2–5). B, Western blot of immunopurified PS1 complexes from CHAPS-solubilized membranes, showing that APH-1 is also a component of the biochemically active complexes.
holoprotein and does not imply a preferential interaction of APH-1, L with presenilin holoproteins.

Although it is not clear what the intermolecular binding interactions are within the presenilin complexes, analysis of APH-1, L co-immunoprecipitation products in cells expressing mutant nicastrin molecules suggests that APH-1, L may preferentially bind directly to immature nicastrin in the ER. Thus, in cells expressing the nicastrin Δ312–369 mutant, APH-1, L co-immunoprecipitates the mutant nicastrin molecule but not PS1 (Fig. 4B, lane 9). Significantly, the nicastrin Δ312–369 mutant blocks trafficking and maturation of nicastrin in the ER, inhibits interaction of nicastrin with PS1, and causes loss of function of the presenilin-nicastrin complex.

Because we were unable to generate antibodies of acceptable quality to APH-1, S and to APH-1, S, we examined the interaction of these proteins with PS1 and nicastrin using immunoprecipitation studies in cell lines stably expressing myc-tagged APH-1, S, APH-1, L, or APH-1, S. Anti-PS1 antibodies immunoprecipitated stoichiometric quantities of APH-1, S, APH-1, L, and APH-1, S (Fig. 4C, lanes 5–7). The reciprocal immunoprecipitations using the anti-myc monoclonal antibody revealed that APH-1, S, APH-1, L, and APH-1, S all co-immunoprecipitated PS1, PS2, mature nicastrin, and immature nicastrin (Fig. 4D, lanes 6–8). However, it was also apparent that small quantities of endogenous APH-1, L co-immunoprecipitate with the exogenous myc-tagged APH-1, S and APH-1, S (Fig. 4D, lane 7). This interaction was confirmed by the reciprocal immunoprecipitation. Thus, the myc-tagged APH-1, S could be detected in the immunoprecipitation products when endogenous APH-1, L was immunoprecipitated with antibody O2C2 (which does not detect APH-1, S) (Fig. 4E, lane 4).

Inspection of the levels of endogenous APH-1, L in cell lines overexpressing APH-1, S, APH-1, L, or APH-1, S also revealed that endogenous APH-1, L expression was suppressed by the overexpression of the exogenous APH-1 (Fig. 4D, second panel from the top), compare the signal intensities for the endogenous APH-1, L band in lanes 2–4 with that in vector-transfected cells in lane 1).

Several conclusions can be drawn from these results. First, APH-1 isoforms interact with both mature and immature forms of nicastrin and the presenilins, and the overall abundance of APH-1 proteins is tightly regulated (presumably because of competition for binding to the same, limited-abundance components within the presenilin-nicastrin complex). Second, our IP data, at least in cells overexpressing individual APH-1 proteins, suggest that they may directly interact physically with each other, in contrast to PS1 and PS2, which do not physically

Fig. 6. APH-1, L is distributed in intracellular membrane compartments including the endoplasmic reticulum and cis-Golgi on iodixanol gradient centrifugation. Iodixanol gradient fractions of HEK293 cells expressing endogenous APH-1, L were investigated with anti-APH-1, L (top panel), anti-PS1 (second panel), anti-GM130 antibodies as a marker of the cis-Golgi (third panel), or anti-calnexin antibodies as a marker of the ER (bottom panel).

Fig. 7. APH-1, L fractionates in the endoplasmic reticulum and cis-Golgi. KNS-42 glioma cells were investigated by confocal microscopy after being prepared, fixed, and co-stained with anti-APH-1, L antibody (O2C2) and anti-BiP (StressGen; a marker of the ER), anti-GM130 antibody (O2C2) and anti- GM130 antibodies as a marker of the cis-Golgi, anti-TGN38 antibody (Transduction Laboratories; a marker of the trans-Golgi network), or anti-PS1 (NT1) antibodies as described under “Experimental Procedures.” APH-1, L staining shows best co-localization with GM130 and BiP, but there is considerable APH-1, L immunoreactivity that is independent of any marker.

Fig. 8. Western blots of endogenous APH-1, L in PS1−/− and PS2−/− brain tissue. APH-1, L levels are minimally reduced in cell lysates from brains of 2-month-old PS1−/− mice compared with lysates from brains of 2-month-old wild type mice (two independent animals for each, 25 μg of protein/ lane with equal protein loading verified by blotting with anti-actin and anti-flotillin antibodies). Lysates from PS2−/− mice brain show no change in APH-1, L levels.
interact but which are capable of displacing each other from their native complexes, again by competing for the same shared limiting cofactors (20). The absence of a 1:1 ratio of APH-1-L to APH-1-S or APH-1-L in the immunoprecipitation products of cells overexpressing APH-1 proteins raises the possibility that the co-precipitation may be artifactual. However, the fact that overexpression of any one member of the APH-1 family reduces the level of expression of the other APH-1 protein family members (by a “displacement effect”) provides an equally plausible explanation for the absence of a 1:1 stoichiometry. This issue will be best resolved when antibodies specific to APH-1-S or APH-1-L become available, allowing analysis of endogenous APH-1-S or APH-1-L.

The presenilin proteins and nicastrin are components of high molecular weight, membrane-bound protein complexes that co-purify in cell-free systems with γ-secretase and ε-secretase activity (17, 26). To ascertain whether the APH-1 proteins are also components of these functionally active complexes, we generated functionally active γε-secretase complexes by immunopurification of the complexes from cell-free membrane preparations, as described previously (17, 19, 26), using anti-PS1 (antibody NT1.1), anti-APH-1, antibody O2C2 in cells expressing endogenous APH-1-L, or anti-γε antibodies (in cells expressing myc-tagged APH-1 proteins). In each instance, an aliquot of the complexes was examined for its molecular components by Western blotting, whereas the remainder was assayed for the presence of γ- and ε-secretase catalytic activities by observing the generation of both amyloid β-peptide (data not shown) and ε-stubs (Fig. 5) as described previously (17, 19, 26). These studies reveal that functionally active complexes were recovered and contained APH-1 proteins regardless of whether they were purified by anti-PS1 immunoprecipitation (Fig. 5, lane 1), immunoprecipitation of the endogenous APH-1-L (Fig. 5, lanes 6 and 7), or immunoprecipitation of exogenous APH-1-L, APH-1-S, or APH-1-L in transfected cell lines (Fig. 5, lanes 2–5).

The intracellular distribution of endogenous APH-1-L was investigated using both biochemical methods in HEK293 cells and confocal imaging in KNS-42 human glioma cell line (27). On iodixanol gradient fractionation of lysates from native HEK293 cells, endogenous APH-1-L was broadly co-distributed across the gradient but co-fractionated with markers of the endoplasmic reticulum (BiP) and cis-Golgi apparatus (GM130) (Fig. 6). The results of these biochemical studies were supported by confocal imaging studies of endogenous APH-1-L in KNS-42 glioma cell lines, which showed a diffuse, vesicular, perinuclear distribution of APH-1-L immunoreactivity that co-localized best with markers of the cis-Golgi (GM130) (Fig. 7). These data are in agreement with the observed interaction between APH-1-L and both immature and mature forms of nicastrin and the presenilins. However, a significant amount of the APH-1-L immunoreactivity decorates vesicular intracellular structures that do not contain either secretory pathway markers or presenin/nicastrin immunoreactivity.

The functional role of APH-1 in the nicastrin/presenilin complexes cannot be deduced from its primary amino acid sequence. Absence of APH-1, whether induced by null mutations or by RNA interference, results in destabilization of PS1, PS2, nicastrin, and PEN-2 and in the inhibition of Notch and APP processing (13, 14). Conversely, absence of the presenilins destabilizes nicastrin (28, 29). The absence of PS1 does reduce the endogenous levels of APH-1-L (Fig. 8). This reduction is independent of age, being equivalent in 2-month-old (Fig. 8), as well as 6-day-old and 3-month-old, PS1−/− mice. However, unexpectedly, in PS1−/− mouse brain, the degree of reduction in endogenous APH-1-L is small compared with the reported reduction in nicastrin levels (28, 29). The absence of PS2 has minimal if any effect on APH-1-L levels, presumably because of the presence of adequate amounts of PS1 in the brain of PS2−/− mice (Fig. 8).

Taken together, our results suggest that APH-1-L is likely to play an important role in the early maturation of the presenilin/nicastrin complexes, possibly as the initial scaffold molecule. This conclusion is supported by the localization of APH-1-L in the early secretory pathway (but also in some as yet uncharacterized perinuclear vesicular structures), its interaction with immature and mature forms of presenilins and nicastrin proteins, its comparative stability in the absence of the presenilins, and the dependence of the presenilins and nicastrin on the prior presence of APH-1-L.

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APH-1 Plays a Role in the Maturation of Presenilin Complexes

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