Membrane Topology and Nicastrin-enhanced Endoproteolysis of APH-1, a Component of the γ-Secretase Complex*

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APH-1, presenilin, nicastrin, and Pen-2 are proteins with varying membrane topologies that compose the γ-secretase complex, which is responsible for the intramembrane proteolysis of several substrates including the amyloid precursor protein. APH-1 is known to be necessary for γ-secretase activity, but its precise function in the complex is not fully understood, and its membrane topology has not been described, although it is predicted to traverse the membrane seven times. To investigate this, we used selective permeabilization of the plasma membrane and immunofluorescence microscopy to show that the C terminus of the APH-1 resides in the cytosolic space. Insertion of N-linked glycosylation sites into each of the hydrophilic loop domains and the N terminus of APH-1 showed that the N-terminal domain as well as loops 2, 4, and 6 could be glycosylated, whereas loops 1, 3, and 5 were not. Thus, APH-1 topologically resembles a seven-transmembrane domain receptor with the N terminus and even-numbered loops facing the endoplasmic reticulum lumen, and the C terminus and odd-numbered loops reside in the cytosolic space. By using these glycosylation mutants, we provide evidence that the association between nicastrin and APH-1 may occur very soon after APH-1 synthesis and that the interaction between these two proteins may rely more heavily on the transmembrane domains of APH-1 than on the loop domains. Furthermore, we found that APH-1 can be processed by several endoproteolytic events. One of these cleavages is strongly up-regulated by co-expression of nicastrin and generates a stable C-terminal fragment that associates with nicastrin.

The γ-secretase complex is responsible for cleaving several type I membrane proteins within their transmembrane (TM)

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** The abbreviations used are: TM, transmembrane; APP, amyloid precursor protein; Nct, nicastrin; PS, presenilin; HA, hemagglutinin;
is regulated can provide information about its maturation, trafficking, activity, and substrate selectivity. An important step in understanding the role of APH-1 in the complex is to learn its topology within the membrane and to determine whether it undergoes post-translational processing events. Analysis of the APH-1 sequence shows that it contains seven potential TM domains of unknown orientation. By using glycosylation and cleavage mutation strategies combined with selective membrane permeabilization and immunofluorescence microscopy, we found that APH-1 does in fact traverse the membrane seven times, with the N-terminal domain facing the extracytosolic space and the C-terminal domain facing the cytosol. We also found that APH-1 can be processed by several endoproteolytic events, with one of these cleavages being strongly up-regulated by co-expression of Nct. The resulting C-terminal fragment is stable and binds Nct, raising the possibility that APH-1 function, like that of PS, may be modulated by endoproteolytic processing.

EXPERIMENTAL PROCEDURES

Generation of Expression Vectors and APH-1 Mutants—The cloning of APH-1aS-V5 (22), APP-KK (8), Pen-2-HA, and S93N Pen-2-HA (22) has been described previously. Hemagglutinin (HA)-tagged Pen-2 was constructed similarly to the Nct-V5 described previously (26) but with a reverse primer encoding the HA epitope sequence and a stop codon to prevent translation of the V5 tag contained in the vector. The APH-1 point mutations described in Fig. 1B were introduced by QuikChange site-directed mutagenesis with overlapping mismatched PCR primers using the APH-1aS-V5 plasmid as the template. Loop extensions containing glycosylation sites were generated using standard overlapping PCR methodology, with the introduced sequences indicated in Fig. 4A. The N-terminal extension containing a glycosylation site was generated using standard PCR with the forward primer encoding the extension. The amino acid sequence of this extension (MTAGNGTGASAGHASP-GAT) was the same as a portion of the loop 6 extension. The loop extension and N-terminal extension PCR products were cloned into the pcDNA3.1/D/V5-His vector (Invitrogen) without a stop codon to allow expression of the V5/His tag. All primer sequences are available upon request.

Antibodies—V5-tagged APH-1 was detected with anti-V5 monoclonal antibody (mAb) (Invitrogen) on Western blots. Rabbit anti-V5 polyclonal antibody (pAb) (Sigma) was used for immunofluorescence detection. Immunoprecipitations were performed using a rabbit anti-V5 antisera produced at Cocalico (Reamstown, PA) by immunizing rabbits with a thioredoxin/V5 fusion protein. APP-KK was detected with the goat pAb Karen. HA-tagged proteins were detected with HA.11 mAb (Invitrogen) on Western blotting detection system (Amer- sham Biosciences) or SuperSignal West Fermo maximum sensitivity substrate (Pierce) on a FujiFilm (Stamford, CT) LAS-1000 camera.

Coomassie stainings—HEK293T cells in 6-well plates were transfected with APH-1-V5 plasmids plus or minus Nct-HA plasmids (1.5 μg of each plasmid per well) using GenePorter. For all co-transfection experiments, total DNA transfected per well was equalized by adding 1.5 μg of an irrelevant expression vector (DC-SIGN in pcDNA3.1) to the samples in which Nct was omitted. Cells were lysed ~24 h post-transfection with CHAPSO buffer (1% CHAPSO (Sigma), 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA with Complete protease inhibitors). Lysates were incubated for 30 min at 4 °C with rotation to allow complete lysis and then centrifuged (10,000 × g, 5 min, 4 °C) to remove insoluble material. Lysates were pre-cleared with protein A/G-agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) plus normal rabbit serum (NRS). Cleared lysates were divided into aliquots for immunoprecipitation at 4 °C for 3 h with HA.11 pAb, anti-V5 pAb, or NRS pre-bound to protein A/G-agarose beads and then washed three times in CHAPSO buffer after immunoprecipitation. For samples being processed for APH-1 detection, the proteins were eluted from the beads with immunoprecipitation elution buffer (50 mM Tris, pH 6.8, 2% SDS, 4 M urea, 10% glycerol) at 55 °C for 15 min. For samples being processed for Nct detection, the proteins were eluted with sample preparation buffer plus 2% β-mercaptoethanol at 100 °C for 10 min. For immunoprecipitations followed by Western blotting, the proteins were first eluted with 1% SDS, 50 mM Tris, pH 6.8, for 15 min at 55 °C, then treated with PNGase F after adding the appropriate buffers for 3 h at 37 °C, and then denatured in immunoprecipitation elution buffer at 55 °C for 15 min. Samples were electrophoresed and detected as described above for the glycosylation assays.

Cycloheximide Treatment—HEK293T cells were transfected with APH-1-V5 plus or minus Nct-HA, with the total DNA added per well equalized as described above. Cells were treated with 150 μg/ml cycloheximide (CHX, Sigma) in cell culture medium, then washed and lysed at various time points in RIPA buffer and prepared for Western blot analysis as described above.

RESULTS

C-terminal Domain of APH-1 Faces the Cytosol—APH-1 contains seven hydrophobic domains that are of sufficient length to span the lipid bilayer (Fig. 1A), giving rise to a model in which APH-1 traverses the membrane seven times. However, it is unclear whether all of these regions are in fact utilized as TM domains, nor is it known on which side of the membrane the N and C termini of the protein reside. To determine whether the C terminus of APH-1 lies on the cytosolic or luminal side of the ER membrane, we added a C-terminal V5 epitope tag to APH-1aS (the short splice variant of APH-1a), expressed the construct in HeLa cells, and visualized the epitope tag by immunofluorescence microscopy under various permeabilization conditions (Fig. 2). As a control, the cells were co-transfected with an APP construct containing a di-lysine ER retention motif (APP-KK). APP-KK was detected with an antisera that binds to epitopes within the APP ectodomain. In non-permeabilized cells, neither the APP ectodomain nor the APH-1 C-terminal tag was detectable (Fig. 2, upper panels). When the cells were treated with SLO to permeabilize selectively the plasma membrane, the APP ectodomain was not detected, indicating that the ER membrane remained intact, but the C-terminal tag of APH-1 was clearly visible (Fig. 2, middle panels). When all cellular membranes were permeabilized with

Complete protease inhibitors (Roche Applied Science) after washing in PBS. Lysates were sonicated and centrifuged (10,000 × g, 10 min, 4 °C) to remove insoluble material and then denatured in sample preparation buffer (2% SDS, 50 mM Tris, pH 6.8, 10% glycerol) at 55 °C for 15 min. For some experiments, the lysates were divided into 3 aliquots and treated with peptide N-glycosidase F (PNGase F) or endoglycosidase H (Endo H) (New England Biolabs, Beverly, MA), as described in the manufacturer’s instructions, or with PBS as a control. Digestions were carried out at 37 °C for 3 h, followed by denaturation in sample preparation buffer. Lysates were electrophoresed on 10–20% polyacrylamide Tris-HCl gels (Bio-Rad), transferrred to polyvinilidene difluoride membranes, and then probed with the appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Blots were stained with ECL Plus. Western blotting detection system (Amer- sham Biosciences) or SuperSignal West Fermo maximum sensitivity substrate (Pierce) on a FujiFilm (Stamford, CT) LAS-1000 camera.
Triton X-100, both APP and APH-1 were detected in a pattern characteristic of ER localization (Fig. 2, lower panels). These results indicate that the C terminus of APH-1 resides within the cytosol. Unfortunately, a variety of epitope tags placed on the N terminus of APH-1 were not detectable by immunofluorescence under any conditions, so we were unable to determine the location of the N terminus with this technique.

Glycosylation Site Insertion Mutagenesis—To identify regions of APH-1 that lie within the ER lumen, we used site-directed mutagenesis to introduce the N-linked glycosylation sequon NX(S/T) into regions of APH-1 that are predicted to reside outside of the lipid bilayer (Fig. 1B). Because N-linked glycosylation occurs only within the lumen of the ER (30), proteins containing this motif will be glycosylated only if the sequon is contained within a luminally exposed region of the protein. This method of glycosylation-scanning mutagenesis has been used to probe the topology of other membrane proteins, including Pen-2 (22, 31–33). As a control for the assay, we expressed a previously characterized Pen-2 S93N mutant containing a glycosylation site within its luminal C-terminal domain (22). This protein became glycosylated and showed the expected ~4-kDa mobility shift above wild-type (wt) Pen-2 (Fig. 3). However, none of the seven APH-1 mutants exhibited mobility shifts characteristic of glycosylation (Fig. 3).

Because N-linked glycosylation may be inefficient when the NX(S/T) motif is in close proximity to adjacent membrane domains and because most of the predicted hydrophilic loops of APH-1 are short, we next constructed “glycosylation extended-loop” (GX-L) mutants of APH-1, in which each of the six hydrophilic loop regions was extended to a length of at least 30 amino acids with an NGT sequon in the middle (Fig. 4A). Because loops 2 and 4 are each ~15 residues long, GX-L2 and GX-L4 were created by doubling the loop sequence and placing the NGT sequon between the two repeats. The extensions on the other loops (GX-L1, GX-L3, GX-L5, and GX-L6) were created using random hydrophilic amino acids as spacers with NGT in the middle. When these GX-L mutants were expressed in cells and analyzed by Western blotting, GX-L2, GX-L4, and GX-L6 showed ~4–5-kDa shifts upward, whereas GX-L3 and GX-L5 did not (Fig. 4B). As expected, the mobilities of all of the mutants were somewhat shifted compared with wt APH-1 because of the added mass of the loop extensions themselves, each of which are ~1–2 kDa. GX-L1 was apparently an unstable mutant as it was detected poorly.

To determine whether the observed mobility shifts were due to glycosylation, we treated cell lysates with PNGase F, which removes all N-linked carbohydrate structures. We found that PNGase F treatment caused a downward mobility shift in GX-L2, GX-L4, and GX-L6 but did not alter the mobilities of wt APH-1, GX-L3, or GX-L5 (Fig. 5A). These results confirm that loops 2, 4, and 6 were glycosylated and therefore within the lumen of the ER, whereas loops 3 and 5 were not.

The addition of hydrophilic loop extensions could alter the topology of APH-1. For example, a significant length of additional hydrophilic residues could prevent a portion of the protein that would normally be luminal from transferring across the ER membrane. If this were the case, the remainder of the protein C-terminal to the extension might adopt a topology opposite of its normal orientation. To assess whether the topology of any of our GX-L mutants had been reversed, we used the V5 epitope tag at the C terminus of each of the GX-L constructs and performed immunofluorescence microscopy with selective membrane permeabilization as in Fig. 2. In each case, the C-terminal tag was detectable within the cytosol after selective plasma membrane permeabilization with SLO, similar to the wild-type protein, whereas the ectodomain of the control pro-
tein (APP-KK) was not (data not shown). We also assessed whether each of the mutants was able to associate with Nct in co-immunoprecipitation experiments. Each of the GX-L mutants (except for the poorly expressed GX-L1) was expressed in HEK293T cells along with Nct-HA, followed by immunoprecipitation of APH-1 (wt or GX-L mutants) and detection of co-immunoprecipitated Nct on Western blots with an anti-HA epitope tag antibody. Each of the APH-1 constructs interacted with Nct as judged by this co-immunoprecipitation assay (Fig. 5B). In addition, immunoprecipitation of Nct resulted in co-immunoprecipitation of each of the APH-1 constructs (data not shown). Because it is unlikely that Nct and APH-1 could associate if APH-1 were incorrectly folded or oriented in the membrane, these results provide strong evidence that the GX-L mutations did not significantly affect the folding or orientation of APH-1.

To assess whether these mutants might be useful for following post-ER trafficking of APH-1 and the γ-secretase complex, we also treated each of the mutants with Endo H, which removes only those N-linked carbohydrate structures that have not been modified in the medial Golgi apparatus. However, GX-L2, GX-L4, and GX-L6 were all sensitive to Endo H treatment (Fig. 5A), even when co-expressed with PS1, Nct, and Pen-2 (data not shown), suggesting either that the proteins were not trafficked beyond the ER or that they had moved to the Golgi but had not been modified by Golgi enzymes.

Orientation of the APH-1 N Terminus—The results thus far suggest that APH-1 lies in the membrane with the C terminus and odd-numbered loops facing the cytosol, whereas the even-numbered loops face the ER lumen. Assuming that the first hydrophobic region is utilized as a TM domain, this would suggest that the N terminus of the protein resides in the lumen. However, we were unable to detect tags placed on the N terminus by either immunofluorescence or Western blotting. We hypothesized that the N terminus of the protein may be cleaved, in which case N-terminal epitope tags would not be present on the mature protein. To test this, we added a 20-amino acid extension to the N terminus of APH-1, which would add a mass of ~1.7 kDa if it were to remain on the protein. In addition, we engineered the extension to contain an NGT glycosylation motif so that if the extension were not cleaved from the protein, we could assess the orientation of the N terminus based on its glycosylation status. As shown in Fig. 6A, when this “glycosylation extended-N terminus” (GX-N) mutant was expressed in cells and analyzed by Western blotting with detection of the C-terminal V5 tag, two major bands were present, both of which migrated more slowly than wt APH-1. Treating the cell lysates with either PNGase F or Endo H removed the upper band, indicating that this was a glycosylated species. In addition, the GX-N protein was able to co-immunoprecipitate Nct (Fig. 6B) and thus was most likely folded correctly. Therefore, we conclude that the N terminus of APH-1 remains on the protein and resides on the luminal side of the membrane.

Close inspection of our gels shows that APH-1 migrates as a doublet, with a major band that migrates at the expected ~31 kDa using V5-tagged APH-1αS and a fainter band that migrates slightly faster (Figs. 3 and 4). Others have shown that when APH-1αL (the longer splice variant of APH-1α) is expressed in cells, the upper band of this doublet corresponds to the in vitro translation product (23), suggesting that the lower band results from a cleavage or other post-translational modification in cells. To examine this more closely, we immunoprecipitated both wt APH-1 and the GX-N mutants (Fig. 6A, lower panel) and found that the lower band of the doublet was unaffected by the addition of the N-terminal extension in the GX-N mutant. Thus, the lower band of the doublet likely represents a minor APH-1 species that has been cleaved near the N terminus.

Modulation of APH-1 Glycosylation by Nct—We noticed in our co-immunoprecipitation experiments that co-expression of Nct resulted in less efficient glycosylation of GX-L6 (data not shown) which, when expressed alone, was almost completely glycosylated (Figs. 4B and 5A). Co-expression of Nct did not affect the glycosylation of GX-L2 or GX-L4 (data not shown). To study this more closely, we expressed GX-L6 in cells either alone or with co-transfected Nct and then analyzed both the association with Nct and the levels of glycosylated versus non-glycosylated GX-L6 (Fig. 7). When cells were transfected with only GX-L6, Western blotting of the lysates showed only the glycosylated form. In contrast, when cells were co-transfected with both GX-L6 and Nct, both glycosylated and non-glyco-
lated forms of GX-L6 were present in the lysates (Fig. 7, right panel). In cells co-expressing GX-L6 and Nct, immunoprecipitation of Nct resulted in the co-immunoprecipitation of only the non-glycosylated form of GX-L6, whereas direct immunoprecipitation of GX-L6 itself produced both glycosylated and non-glycosylated forms (Fig. 7, left panels). Thus, not only did Nct associate specifically with the non-glycosylated form of GX-L6, but co-expression of high levels of Nct with GX-L6 prevented, at least in part, the glycosylation of this protein. This suggests either that glycosylation of loop 6 prevents association with Nct because of a critical interaction between loop 6 and Nct, or that association with Nct prevents glycosylation of loop 6. We favor the latter explanation because our mutagenesis strategy resulted in the addition of 28 amino acids to loop 6 (which is predicted to contain only 6 amino acids in its native state), and this significant alteration did not impact association with Nct, suggesting that loop 6 does not contain residues that are critically important for Nct interactions. Because we have shown previously (26) that APH-1 interacts directly with Nct in the absence of PS1, we propose that Nct binds to APH-1 in a manner that covers loop 6 but is not directly dependent on interactions with loop 6, thus preventing glycosylation in the GX-L6 mutant. Because glycosylation typically occurs cotranslationally (30), this suggests that Nct and APH-1 associate very rapidly in the ER.

**Nct-enhanced Endoproteolysis of APH-1**—Throughout our experiments, we noticed that in addition to the closely spaced APH-1 doublet at the ~31-kDa position, a fragment was consistently detected at ~22 kDa (Fig. 3). Because we detected APH-1 using the C-terminal V5 tag, this species must represent a C-terminal fragment (CTF) of APH-1. We hypothesized that this fragment was an intermediate in a degradative pathway and was perhaps an artifact of overexpressing APH-1 without a corresponding increase in the expression of other γ-secretase components. To test this, we co-transfected each of the other γ-secretase components, either singly or as a group, with APH-1. The levels of the ~22-kDa CTF were not altered by co-expression of Nct, Pen-2, or PS1 (data not shown). How-
ever, when APH-1 was co-transfected with Nct, a second and more prominent CTF of APH-1 appeared at ~19 kDa (Fig. 8A). This pattern was consistent when a different Nct construct with a V5 tag was used or when the other γ-secretase components were co-transfected in addition to Nct and APH-1 (data not shown). When APH-1 was expressed in HEK293T cells, this “lower” CTF was essentially undetectable in the absence of Nct co-transfection (as in Fig. 8A) or was detectable at very low levels (data not shown). When APH-1 was expressed in some other cell types (e.g. mouse embryonic stem cells), the lower CTF was more easily detectable without co-transfecting Nct, but it was always strongly induced upon overexpression of Nct (data not shown). These differences likely reflect different levels of endogenous Nct in various cell types.

To determine the approximate locations of the cleavages that produce these two CTFs, we co-transfected HEK293T cells with Nct and the GX-L mutants of APH-1 to see which loop extensions would increase the sizes of the two CTFs (Fig. 8B). When GX-L3 was used, the migration of the CTFs was unaffected (Fig. 8B, left lane). Thus, APH-1 cleavage must occur after loop 3. When GX-L4 was used, the “upper” CTF was shifted upward and was partially modified by glycosylation (Fig. 8B, middle lane), which we confirmed by PNGase F treatment (data not shown). In contrast, the lower CTF was not affected by the GX-L4 modification. Thus, the upper CTF is generated by endoproteolysis of APH-1 N-terminal to the loop 4 modification, whereas the lower CTF must be generated by endoproteolysis C-terminal to this domain. When GX-L5 was used, both the upper and lower CTFs were shifted upward (Fig. 8B, right lane). Thus, the cleavage that produces the upper CTF occurs between residue 103 in loop 3 and residue 154 in loop 4 (the positions into which the extensions were placed; see Fig. 4), and the cleavage producing the lower CTF occurs between residue 154 in loop 4 and residue 183 in loop 5 (Fig. 8E).

If either of the CTFs are physiologically relevant species, they should be relatively stable. To examine this, we tested the stability of the fragments by treating cells transfected with APH-1 alone or APH-1 plus Nct with cycloheximide (CHX) for up to 6 h, after which the cells were lysed, and the amounts of the various APH-1 species present were monitored by Western blot (Fig. 8C). This approach was taken because we were unable to metabolically label APH-1 sufficiently well to employ a pulse-chase protocol. The lower CTF was detected only when Nct was overexpressed and was stable throughout the time course of the experiment. Levels of full-length APH-1 also appeared to be unchanged throughout the time course, and its overall levels were increased when Nct was co-transfected, possibly due to stabilization of APH-1 by Nct or an increase in the production of APH-1 (prior to CHX treatment) in the presence of Nct. The upper CTF was less stable than full-length APH-1 or the lower CTF and decreased to nearly undetectable levels by the 6-h time point. We also tested whether either of the fragments was able to bind Nct. The lower CTF was present in the Nct immunoprecipitate, whereas the upper CTF was absent (Fig. 8D), suggesting that only the lower CTF is able to associate with Nct. These results suggest that the lower CTF is a stable product of endoproteolysis of APH-1 and is unlikely to be an intermediate of degradation.

**DISCUSSION**

PS constitutes the enzymatically active component of the γ-secretase complex (15, 16), but its maturation and activity are absolutely dependent upon the presence of Nct, APH-1, and Pen-2 (11–14, 19, 23, 27, 28, 34–37). Although the roles of these proteins in γ-secretase function are not well understood, one or more of these three integral membrane proteins could influence the structure of PS, modulate its enzymatic function, limit or regulate substrate accessibility, or control transport and targeting of the complex to sites distal from the ER. Our general approach to characterize the role of APH-1 in the γ-secretase complex is to introduce mutations that can ultimately be used to address functional questions. Prior to this, however, it is important to define the topology of APH-1 in the membrane in order to more accurately design mutagenesis strategies and to determine whether the protein undergoes any post-translational processing events. For example, attempts to identify...
potential interactions between the ectodomains of Nct and APH-1 will require knowledge of which regions of APH-1 face the lumen of the ER. The sequence of APH-1 contains seven domains that are of sufficient length and hydrophobicity to span the membrane. However, the sequence of APH-1 differs from that of most 7-TM domain receptors in that it lacks an identifiable hydrophilic N-terminal domain. This raises the possibility that APH-1 could exhibit a topology other than that seen for typical 7-TM domain receptors, which have an N-terminal domain that faces the lumen of the ER and a C-terminal domain that faces the cytosol.

We used two independent approaches to probe the membrane topology of APH-1, with our results showing that APH-1 does, in fact, resemble a 7-TM domain receptor topologically (Fig. 8E). By introducing glycosylation sites or epitope tags, we were able to identify the locations of five of the eight extramembranous domains of APH-1. The N-terminal domain as well as all three putative extracellular loops could, following mutagenesis, accept the addition of an N-linked carbohydrate structure, indicating that these domains face the lumen of the ER. Co-immunoprecipitation experiments showed that our APH-1 mutants could specifically interact with Nct, providing strong evidence that our modifications did not significantly influence APH-1 structure. In addition, we were able to show that the C-terminal domain of APH-1 resides in the cytosol. This finding, coupled with the presence of the N-terminal domain in the ER lumen, indicates that APH-1 must span the membrane an odd number of times.

Despite using three different experimental approaches, we were unable to provide definitive evidence that loops 1, 3, and 5 of APH-1 actually reside in the cytosol. N-Linked glycosylation sites introduced either by point mutation or in the context of loop extensions failed to become glycosylated in loops 1, 3, or 5. Although consistent with a cytosolic location, the lack of glycosylation cannot be taken as definitive evidence for a cytosolic orientation because not all N-linked consensus sites in proteins are necessarily utilized (30). Unfortunately, epitope tags introduced into loops 1, 3, and 5 could not be detected by immunofluorescence microscopy, even though two different types of tags (Au1 and V5) were used (data not shown). We also inserted factor Xa protease cleavage sites into each of the loops to determine whether the odd-numbered loops were uniquely accessible to this protease after selective plasma membrane permeabilization. However, no cleavage by factor Xa was detected for any of these mutant proteins under any conditions, possibly because the sites were contained in inaccessible portions of the protein or were hidden by other members of the complex. Although we cannot say with absolute certainty that loops 1, 3, and 5 of APH-1 reside in the cytosol, we feel that alternative models are exceedingly unlikely, as they would entail topologies in which APH-1 contains one, three, or five TM domains and would require extensive regions of hydrophobic sequence to reside within the ER lumen. Thus, we conclude that APH-1 is a 7-TM domain protein with a topology essentially identical to that of many other well studied 7-TM domain receptors.

With the topology of APH-1 now having been defined, it is apparent that the known components of the γ-secretase complex have an impressive total of 18 distinct TM domain segments, using the most widely accepted model for PS topology (with eight TM domains of PS (38–40), one TM domain of Nct (19, 20), and two TM domains of Pen-2 (22)). It will not be surprising if interactions between the components of the γ-secretase complex are largely dependent upon interactions between these TM domain regions. We have shown recently that the C-terminal region of Nct, containing the last 50 amino acids of the ectodomain, the TM domain, and the cytosolic domain, plays a key role in mediating interactions with other γ-secretase components, including direct interactions with APH-1 (26). APH-1 possesses several charged and polar residues within its TM segments. The presence of charged residues within the TM domains of proteins that span the membrane multiple times is not unusual, and for other 7-TM domain proteins such residues have been shown or postulated to play roles in ligand binding, signal transduction, and maintenance of receptor structure (41–43). These residues in APH-1 may therefore be structurally important or may be critical for inter-
exception of the loop 1 mutation (which apparently destabilized the protein), had no apparent effect on the ability of the protein to interact with Nct. Interestingly, we found that Nct associated with only the non-glycosylated form of the APH-1 loop 6 mutant (GX-L6) and that co-transfection of Nct with GX-L6 significantly decreased glycosylation of this mutant. We interpret this finding as evidence that Nct and APH-1 associate very rapidly in the ER and that this association prevents glycosylation of the GX-L6 mutant. This supports the idea that APH-1 and Nct form an early subcomplex prior to linking the other γ-secretase components (25–27).

We found that APH-1 can undergo at least three distinct endoproteolytic cleavage events, one of which is more likely than the others to be relevant for APH-1 function or regulation. We and others (23) have noted that APH-1 migrates as a closely spaced doublet, with a less abundant species migrating slightly more quickly than full-length APH-1. We found that the lower molecular weight species lacks a small portion of the APH-1 N terminus and therefore likely represents a proteolytic fragment of the protein. In vitro translation in the absence of microsomes does not produce this N-terminally truncated version of APH-1 (23). This truncated form was not as stable as full-length APH-1; it did not appear to associate with Nct (data not shown), and its production did not correlate with co-expression of the other γ-secretase components. Thus, we feel that this form of APH-1 represents a degradative intermediate.

The remaining two endoproteolytic fragments of APH-1 appear to result from two different cleavages between residues 104 and 183. Expression of APH-1 in several cell types resulted in the constitutive production of an ~22-kDa C-terminal fragment due to cleavage between loops 3 and 4. This fragment was not stable and did not associate with Nct and therefore likely represents a degradative intermediate of APH-1. A similar and perhaps identical CTF was documented by Kimberly et al. (23) while studying HA-tagged APH-1aL (the longer splice variant of APH-1), which they suggested is not part of the mature complex because it did not co-migrate with PS and Nct in glycerol velocity gradients and did not bind a γ-secretase inhibitor resin. However, we also observed a second and far more abundant APH-1 CTF that was strongly induced by Nct overexpression. This Nct-induced CTF was produced by cleavage between loops 4 and 5 of APH-1 and was observed when Nct was overexpressed with APH-1. Importantly, the Nct-induced CTF was as stable as full-length APH-1 and could be immunoprecipitated with Nct. Because we were unable to metabolically label APH-1 sufficiently well to perform pulse-chase experiments, we could not determine whether APH-1 endoproteolysis occurs prior to or after Nct association. However, because Nct co-expression strongly up-regulated the levels of this fragment, we think it is likely that the cleavage occurs after association with Nct. Because Nct can bind full-length APH-1 (Fig. 7 and data not shown), cleavage of APH-1 must not be a prerequisite for Nct association.

In conclusion, we have demonstrated that APH-1 exhibits a serpentine topology similar to 7-TM domain receptors. We have also provided evidence that Nct and APH-1 associate very rapidly in the ER and that association between the two proteins may not rely on the hydrophilic loop regions of APH-1. Finally, we have shown that APH-1 is processed by at least three endoproteolytic cleavages and that one of these is strongly induced by Nct expression and produces a stable C-terminal fragment that associates with Nct. Assessment of the relevance of this cleavage event for γ-secretase function will require additional experimental tools not available at this time, including antibodies capable of detecting N- and C-terminal regions of endogenous APH-1 and APH-1-deficient cells, which

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**Fig. 8. C-terminal fragments of APH-1.** A, HEK293T cells were transfected with APH-1 alone or APH-1 plus Nct-HA. Cells were lysed in RIPA buffer and analyzed by Western blotting for the V5 tag of APH-1. The APH-1 ~31-kDa doublet and the ~22-kDa upper CTF were present with or without Nct co-transfection. The ~19-kDa lower CTF was detected only when Nct was co-transfected. B, cells were transfected with Nct and GX-L3, GX-L4, or GX-L5. The sizes of the upper and lower CTFs were unaffected by the extension on loop 3. The upper CTF migrated more slowly with extensions on loops 4 or 5, whereas the lower CTF was shifted upward only by the extension on the loop 3. C, cells transfected with APH-1 alone or APH-1 plus Nct-HA were treated with CHX for up to 6 h, then lysed in RIPA buffer at the indicated time points, and analyzed by Western blotting. Tfn, transfection. D, cells transfected with APH-1 alone or APH-1 plus Nct were lysed in 1% CHAPS, and the lysates were immunoprecipitated (IP) with anti-HA pAb (for Nct) or anti-V5 pAb (for APH-1) or with NRS as a control. The upper CTF (arrow) was precipitated by the V5 antibody but did not co-precipitate with Nct. The lower CTF (arrowhead) was precipitated by the V5 antibody and also co-precipitated with Nct. E, diagram of APH-1 membrane topology and approximate cleavage locations. The approximate locations of the cleavages producing the upper and lower CTFs are represented by dashed lines and dotted lines, respectively.
will make it possible to determine whether cleavage-deficient mutants of the protein affect \(\gamma\)-secretase activity.

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REFERENCES

1. Hardy, J., and Selkoe, D. J. (2002) Science 297, 353–356
2. Sisodia, S. S., and St George-Hyslop, P. H. (2002) Nat. Rev. Neurosci. 3, 281–290
3. Budke, J. D., Liu, K. N., Luo, Y., Slack, J. L., Stocking, K. L., Peschon, J. J., Johnson, R. S., Castner, B. J., Cerretti, D. P., and Black, R. A. (1998) J. Biol. Chem. 273, 27665–27671
4. Slack, B. E., Ma, L. K., and Seah, C. C. (2001) Nat. Rev. Neurosci. 2, 778–788
5. Masters, C. L., Simms, G., Geun, N. A., Malchau, G., McDonald, B. L., and Beattie, E. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4245–4249
6. De Strooper, B. (2003) Annu. Rev. Biochem. 72, 785–824
7. Lee, S. F., Shah, S., Li, H., Yu, C., Han, W., and Yu, G. (2002) J. Biol. Chem. 277, 39062–39065
8. Li, X., and Greenwald, I. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 13039–13044
9. Li, X., and Greenwald, I. (1996) Neuron 17, 181–190
10. De Strooper, B., and Haass, C. (2000) Nature 407, 775–779
11. Steiner, H., Winkler, E., Edbauer, D., Prokop, S., Basset, G., Yamasaki, A., Selkoe, D. J., Kim, T. W., Yu, G., and Xu, H. (2003) J. Biol. Chem. 278, 27765–27767
12. Steiner, H., and Haass, C. (2000) Science 289, 741–745
13. Lee, V. M. Y., and Doms, R. W. (1997) J. Biol. Chem. 272, 28142–28145
14. Podlisny, M. B., Citron, M., Amaranthe, P., Sherrington, R., Xia, W., Zhang, J., Diehl, T., Levesque, G., Fraser, P., Haass, C., Koo, E. H., Seubert, P., St. George-Hyslop, P., Teplow, D. B., and Selkoe, D. J. (1997) Neuron 19, 356–359
15. Li, Y., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y. Q., Rogeaux, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D. S., Holmes, E., Milman, P., Liang, Y., Zhang, D. M., Xu, D. H., Sato, C., Rogeaux, E., Smith, M., Janus, C., Zhang, Y., Asherlaid, B., Farrow, L. S., Sorbi, S., Bruni, A., Fraser, P., and St. George-Hyslop, P. (2000) Nature 407, 48–54
16. Gupte, C., Tuszynski, M., Hale, V. A., and Priess, J. R. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 775–779
17. Fraser, P. E., and Selkoe, D. J. (2003) J. Biol. Chem. 278, 28155–28142
18. Crystal, A. S., Morais, V. A., Piers, C. A., Pijak, D. S., Carlin, D., Lee, V. M. Y., and Doms, R. W. (2002) J. Biol. Chem. 277, 20117–20123
19. Kimberly, W. T., LaVoie, M. J., Otsasewski, B. L., Ye, W., Wolfe, M. S., and Selkoe, D. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 6382–6387
20. Gu, Y., Chen, P., Sanjo, N., Kawarai, T., Hasegawa, H., Duthie, M., Li, W., Ruan, X., Luthra, A., Mount, H. T., Tandon, A., Fraser, P. E., and St. George-Hyslop, P. (2003) J. Biol. Chem. 278, 7374–7380
21. LaVoie, M. J., Fragering, F. C., Otsazewski, B. L., Ye, W., Kimberly, W. T., Wolfe, M. S., and Selkoe, D. J. (2003) J. Biol. Chem. 278, 37213–37222
22. Morais, V. A., Crystal, A. S., Pijak, D. S., Carlin, D., Costa, J., Lee, V. M. Y., and Doms, R. W. (2003) J. Biol. Chem. 278, 43284–43291
23. He, Y., and Fortini, M. E. (2003) J. Cell Biol. 161, 685–690
24. Liao, W. J., Wang, H., Li, H., Kim, B. S., Shah, S., Lee, H. J., Thirakaran, G., Kim, T. W., Yu, G., and Xu, H. (2003) J. Biol. Chem. 278, 7850–7854
25. Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Nimura, M., Takahashi, Y., Thirakaran, G., and Iwatsubo, T. (2000) Nature 422, 438–441
26. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664
27. Chang, X. B., Hou, Y. X., Jensen, T. J., and Ronson, J. R. (1994) J. Biol. Chem. 269, 18752–18757
28. Hamilton, S. R., Yao, S. Y., Ingram, J. C., Hadden, D. A., Ritzel, M. W., Gallagher, M. P., Henderson, P. J., Cass, C. E., Young, J. D., and Baldwin, S. A. (2001) J. Biol. Chem. 276, 27981–27988
29. Vannier, B., Zhu, X., Brown, D., and Birnbaumer, L. (1998) J. Biol. Chem. 273, 8675–8679
30. Hu, Y., Ye, Y., and Fortini, M. E. (2002) Dev. Cell 2, 69–78
31. Chang, H. M., and Struhl, G. (2001) Nat. Cell Biol. 3, 1129–1132
32. Li, T., Ma, G., Cai, H., Price, D. L., and Wong, P. C. (2003) J. Neurosci. 23, 3272–3277
33. Lopes-Schier, H., and St. Johnston, D. (2002) Dev. Cell 2, 79–89
34. Deo, A., Thirakaran, G., Borchert, D. R., Slunt, H. H., Ratovitskyy, T., Podlisny, M., Selkoe, D. J., Seeger, M., Gandy, S. E., Price, D. L., and Siddiqui, S. S. (1996) Neuron 17, 785–794
35. Li, X., and Greenwald, I. (1996) Neuron 17, 1015–1021
36. Li, X., and Greenwald, I. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7109–7114
37. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., LeTrong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
38. Audoly, L., and Breyer, R. M. (1997) Mol. Pharmacol. 51, 61–68
39. Murphy, P. M. (1994) Annu. Rev. Immunol. 12, 593–633
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