THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 24, pp. 16298–16307, June 12, 2009

APH1 Polar Transmembrane Residues Regulate the Assembly and Activity of Presenilin Complexes*[S]

Received for publication, March 2, 2009, and in revised form, April 10, 2009 Published, JBC Papers in Press, April 15, 2009, DOI 10.1074/jbc.M109.000067

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Complexes involved in the γ/ε-secretase-regulated intramembranous proteolysis of substrates such as the amyloid-β precursor protein are composed primarily of presenilin (PS1 or PS2), nicastrin, anterior pharynx defective-1 (APH1), and PEN2. The presenilin aspartyl residues form the catalytic site, and similar potentially functional polar transmembrane residues in APH1 have been identified. Substitution of charged (E84A, R87A) or polar (Q83A) residues in TM3 had no effect on complex assembly or activity. In contrast, changes to either of two highly conserved histidines (H171A, H197A) located in TM5 and TM6 negatively affected PS1 cleavage and altered binding to other secretase components, resulting in decreased amyloid generating activity. Charge replacement with His-to-Lys substitutions rescued nicastrin maturation and PS1 endoproteolysis leading to assembly of the formation of structurally normal but proteolytically inactive γ-secretase complexes. Substitution with a negatively charged side chain (His-to-Asp) or altering the structural location of the histidines also disrupted γ-secretase binding and abolished functionality of APH1. These results suggest that the conserved transmembrane histidine residues contribute to APH1 function and can affect presenilin catalytic activity.

The anterior pharynx defective-1 (APH1)[5] protein is an essential component of presenilin-dependent complexes required for the γ/ε-secretase activity (1). The multicomponent γ-secretase is responsible for the intramembrane proteolysis of a variety of substrates including the amyloid-β precursor protein (APP) and Notch receptor. Notch signaling is involved in a variety of important cell fate decisions during embryogenesis and adulthood (2). The γ/ε-secretase cleavage of APP protein is related to the pathogenesis of Alzheimer disease by releasing the 4-kDa amyloid β-peptide (Aβ) which accumulates as senile plaques in patients with Alzheimer disease (3, 4).

The γ-complexes are composed of multispanning transmembrane proteins that include APH1 (5, 6), presenilin (PS1 or PS2) (7–10), PEN2 (5), and the type 1 transmembrane nicastrin (NCT) (11). All four components are essential for proteolytic activity, and loss of any single component destabilizes the complex, resulting in the loss of substrate cleavage. Conversely, coexpression of all four components increases γ-secretase activity (12–14). During the maturation of the complexes, presenilins undergo an endoproteolytic cleavage to generate amino- and carboxyl-terminal fragments which remain associated as heterodimers in the active high molecular weight complexes (15–18). Although the exact function of presenilins has been debated (19, 20), it has been proposed that the presenilins are aspartyl proteases with two transmembrane residues constituting the catalytic subunit (21). Analogous aspartyl catalytic dyads are found in the signal peptide peptidases (21, 22). Contributions from the other components are under investigation, and it has been shown, for example, that the large ectodomain of NCT plays a key role in substrate recognition (23, 24). It has also been shown that other proteins can regulate activity such as TMP21, a member of p24 cargo protein, which binds to the presenilin complexes and selectively modulates γ but not ε cleavage (25, 26).

* This work was supported by grants from the Canadian Institutes of Health Research, Howard Hughes Medical Institute, Alzheimer Society of Ontario, The Ontario Research and Development Challenge Fund and the Canada Foundation for Innovation, the Weston and Garfield Foundation, and Fondation pour la Recherche Médicale and the Conseil Général des Alpes Maritimes.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5.

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[5] The abbreviations used are: APH1, anterior pharynx defective-1; APP, amyloid-β precursor protein; Aβ, amyloid β-peptide; AICD, amyloid intracellular domain; CTF, C-terminal fragment; NCT, nicastrin; MEF, mouse embryonic fibroblasts; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; TM, transmembrane; PS, presenilin; WT, wild type; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

APH1 is a seven-transmembrane protein with a topology such that the amino terminus is oriented with the endoplasmic reticulum and the carboxyl terminus resides in the cytoplasm (6, 27). It is also expressed as different isoforms encoded by two genes in humans (APH1a on chromosome 1; APH1b on chromosome 15) or three genes in rodents (APH1a on chromosome 3; APH1b and APH1c on chromosome 9). APH1a has 55% sequence similarity with APH1b/APH1c, whereas APH1b and APH1c share 95% similarity. In addition to these different genes, APH1a is alternatively spliced to generate a short (APH1aS) and a long isoform (APH1aL). These two isoforms differ by the addition of 18 residues on the carboxyl-terminal part of APH1aL (28, 29). Deletion of APH1a in mice is embryonically lethal and is associated with developmental and patterning defects similar to those found in Notch, NCT, or PS1 null embryos (30, 31). In contrast to the essential nature of APH1a, the combined APH1b/c-deficient mice survive into adulthood (31). This suggests that APH1a is the major homologue involved in presenilin-dependent function during embryonic development. In addition, these different APH1 variants are constituents of distinct, proteolytically active presenilin-containing complexes and may, therefore, make unique contributions to γ-secretase activity (30–32).

Despite their importance to complex formation and function, the exact role of the APH1 isoforms in presenilin-dependent γ/ε-secretase activity remains under investigation. In the current study, several highly conserved polar and charged residues located within the transmembrane domains of APH1 were identified. Mutagenesis of two conserved histidine residues embedded in TM5 and TM6 (His-171 and His-197) lead to alterations in γ-secretase complex maturation and activity. The histidine residues contribute to APH1 function and are involved in stabilizing interactions with other γ-secretase components. These key histidines may also be physically localized near the presenilin active site and involved in the γ-secretase activity as shown by the decreased activity of γ-secretase complexes that are assembled with the His-mutants.

EXPERIMENTAL PROCEDURES

Mutagenesis and cDNA Constructs—The human APH1aL cDNA was mutated by PCR site-directed mutagenesis using the QuikChange site-directed mutagenesis kit from Stratagene, then all constructs were confirmed by DNA sequencing. Expression constructs for human wild-type (WT) or mutant APH1aL were cloned without any tag in pcDNA4 vector. The PS1 construct in which aspartate 257 and/or 385 were mutated to alanine were cloned in pcDNA3 and obtained as previously described (63).

Cell Culture and Transfection—APH1-deficient mouse embryonic fibroblasts (MEF APH1$^{-/-}$) lacking both APH1a and APH1c expression were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. PS-deficient fibroblasts were obtained and cultured as previously described (64). Transient transfections were performed in cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Membrane Fraction Preparation and Co-immunoprecipitation—Fibroblasts were harvested and homogenized in buffer A (5 mM HEPES, pH 7.4, 1 mM EDTA, 0.25 M sucrose, and a protease inhibitor mixture from Roche Applied Science). Membranes were isolated and solubilized in 1% CHAPSO as previously described (25). Extracts (300 μg of total protein) were used for immunoprecipitation with the appropriate primary antibodies with overnight incubation at 4 °C and recovery using protein G-Sepharose.

SDS/PAGE and Western Blot Analyses—Whole cell lysates (50 μg of total protein), membrane lysates (25 μg of protein), and immunoprecipitated proteins were dissolved in SDS sample buffer, separated on Tris Tricine gels 16% or Tris-glycine gels 4–20% (Invitrogen) and transferred to nitrocellulose membranes. Target proteins were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences) with the following antibodies: mouse monoclonal anti-NCT (BD Biosciences), anti-PS1-loop (Chemicon), anti-Aβ 2H3 (provided by Dr. D. Schenk, Elan Pharmaceuticals, South San Francisco, CA), anti-FLAG (Sigma), and anti-tubulin (Zymed Laboratories Inc.) antibodies, rabbit polyclonal anti-PS1-Nt, anti-APH1aL (O2C2), anti-PEN2, and anti-APP-CTF antibodies.

γ-Secretase Cell-free Assay—After APH1 transfection in fibroblasts, membrane fractions were prepared and subjected to an *in vitro* γ-secretase assay as described previously (25) using exogenous recombinant APP-C100 FLAG peptide as substrate. The γ-secretase inhibitor difluoroketone-167 (50 μM) was also used as negative control. The products Aβ and AICD-FLAG were detected by Western blotting with anti-Aβ 2H3, anti-APP CTF, and anti-FLAG antibodies.

Preparation of the Recombinant C100 Fragment—cDNA encoding the carboxyl-terminal 99 amino acids of human βAPP was subcloned into a pet29c vector fused to FLAG tag at the carboxyl terminus and harboring an additional methionine at the amino terminus (provided by Dr. L. Mercken, Sanofi-Aventis). Recombinant protein was expressed in *Escherichia coli*, then the cell pellet was solubilized with Tris lysis buffer (20 mM, pH 7.5) containing EDTA (0.1 mM), lysozyme (1 mg/ml), and *N*-lauroylsarcozyne (1% v/v). After 3 h under agitation at 4 °C, lysate was spun at 5000 × *g* for 2 h at 4 °C. The supernatant was next spun for 75 min at 20,000 × *g* at 4 °C, then the resulting supernatant was subjected to filtration on a 30-kDa cut-off membrane.

RESULTS

Identification of Conserved Polar Residues in APH1 Transmembrane Domains—Inspection of the primary sequence and membrane topology of APH1 revealed several highly conserved polar or potentially charged residues within the selected transmembrane domains. Because of their hydrophilic properties, these particular amino acids are not as commonly found in membrane environments and may, therefore, be of functional significance. More specifically, residues identified are the negatively charged Asp-84, positively charged Arg-87, and polar Gln-83 located in TM3 and His-171 in TM5 and His-197 in TM6, both of which have imidazole side chains that can be positively charged under physiological conditions (Fig. 1*A*). All of these transmembrane residues are conserved across a broad

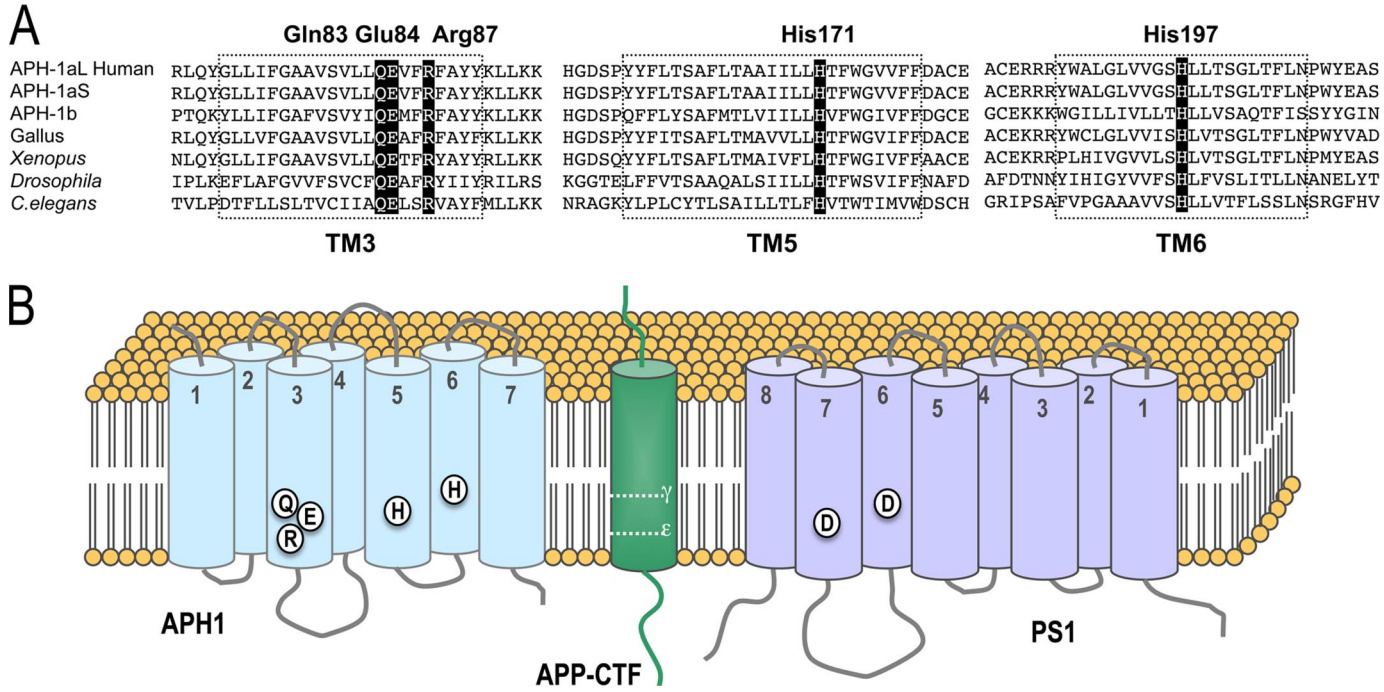

FIGURE 1. **Identification of conserved polar residues in APH1 transmembrane domains.** *A*, positively charged residues (His-171, His-197, and Arg-87), a negatively charged residue (Glu-84), and an uncharged polar residue (Gln-83) are highly conserved within species from human, chicken, *Xenopus*, *Drosophila* to *C. elegans*. *B*, TM topology of APH1, APP-CTF, and PS1 showing the location of the conserved polar/charged residues within the membrane. The polar/charged residues on APH1 are located at about the same distance from the cytoplasm as the proposed catalytic aspartate residues of PS1 (Asp-257 and Asp-385) relative to the γ- and ϵ-cleavage sites within the APP-CTF.

range of species from human to *Drosophila* and *Caenorhabditis elegans* and may be involved, for example, in protein-protein binding through electrostatic interactions or hydrogen bonding. Such interactions could be important for the intramolecular association of APH1 transmembrane (TM) domains to promote structural stability or could mediate interactions with other γ-secretase components. Alternatively, these membrane-embedded residues may contribute to functional aspects of APH1 as they relate to presenilin-mediated proteolysis.

These particular residues are also of interest because of their predicted location within the bilayer relative to the catalytically active presenilin aspartyl groups. The pair of aspartate residues, Asp-257 and Asp-385, involved in γ and ϵ cleavage, are located on TM6 and TM7 of PS1. Based on the predicted topology of PS1, the polar/charged residues in APH1 are situated at similar positions on their respective transmembrane helices (Fig. 1*B*). The APH1 charged and polar TM residues may, therefore, face the catalytic residues and could potentially affect the activity of the γ-secretase complex.

Loss of Polarity Substitutions and Effects on γ-Secretase Maturation—Initial attempts to express mutant APH1 constructs in normal, wild-type cells did not result in suppression of the endogenous APH1 or incorporation of the mutants into mature γ-secretase complexes (data not shown). Therefore, exploring the effects of the various substitutions required expression in a knock-out background. This was accomplished using mouse embryonic fibroblasts ablated for APH1a and APH1b expression as previously described (30). These cells still express low levels of the murine APH1c isoform but are unable to form APH1aL-containing complexes which are explored in the current investigation. Loss of the major APH1 species results in impaired NCT maturation as well as reduced PEN2 expression and PS1 endoproteolysis (29–32).

The primary objective of this investigation is to examine the effect of loss of polarity mutants for these particular residues. Alanine substitutions were, therefore, used as this change is not predicted to alter the helix necessary for the transmembrane domain. It is probable that more dramatic substitutions for helix breaking residues, such as proline, may have dramatic effects on APH1 function. However, these types of structural disruptions may result in activity changes that are unrelated to the function of the polar and charged residues identified in this study.

Mutant APH1 proteins containing alanine substitutions of the two potentially charged (E84A and R87A) or polar (Q83A) residues located in TM3 were expressed at levels similar to wild-type APH1 (Fig. 2*A*). Similar replacements of His-171 (H171A) or His-197 (H197A) also did not affect expression with monomeric and dimeric forms being observed for all APH1 variants. Dimerization has been previously reported for cells expressing high levels of APH1 (28). Although expressed at comparable levels, the APH1 mutants displayed different abilities to rescue the phenotypic characteristics of the knock-out fibroblast. Expression of the TM3 mutants (E84A, R87A, Q83A) led to a partial rescue of NCT maturation as well as PEN2 expression and restored PS1 endoproteolysis to the same level as that seen for wild-type APH1 (Fig. 2*A*). In contrast, the two histidine substitutions, H171A and H197A, were less efficient in rescuing the γ-secretase deficiencies associated with the APH1 knock-out fibroblasts. Maturation of NCT was slightly restored, but a significant decrease in PS1 endoproteolysis was observed (Fig. 2*A*). Quantification of PS1-NTF levels

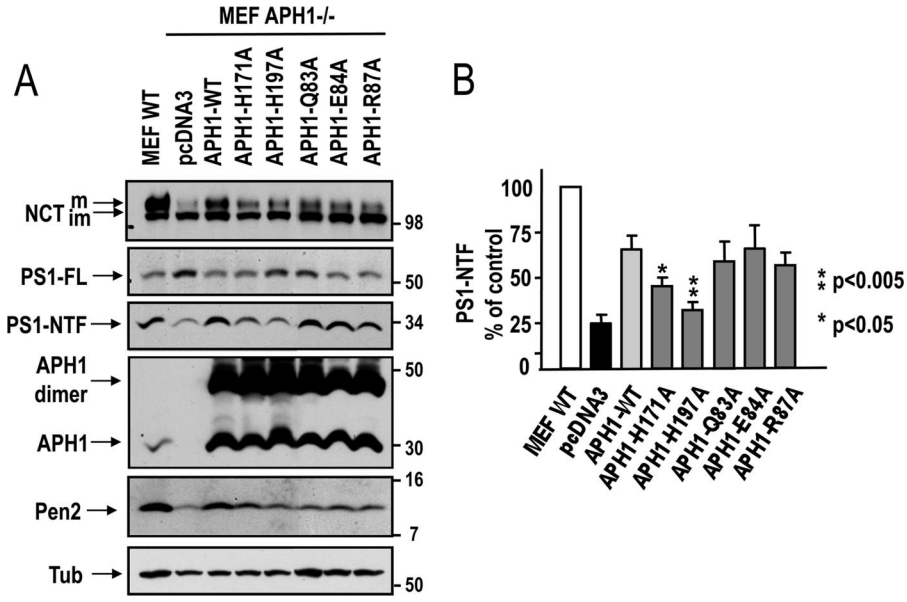

FIGURE 2. **APH1 mutants and rescue of γ-secretase phenotype in knock-out fibroblasts.** *A*, APH1 knock-out fibroblasts (MEF APH1$^{-/-}$) expressing wild-type (WT) or mutated APH1 H171A, H197A, Q83A, E84A, and R87A were subjected to immunoblotting analysis of NCT, PS1, PEN2, and APH1. Mutant APH1 proteins showed modest rescue of NCT maturation and PEN2 expression relative to wild-type protein but varied in the amount of PS1 amino-terminal fragment (PS1-NTF) generated. *B*, quantification of the level of PS1 endoproteolysis as measured by levels of the PS1-NTF, indicating the most significant changes were observed for the H171A and H197A substitutions. Mutation of the polar residues in TM3 did not affect PS1 processing. *m*, mature; *im*, immature; *Tub*, tubulin used as loading control.

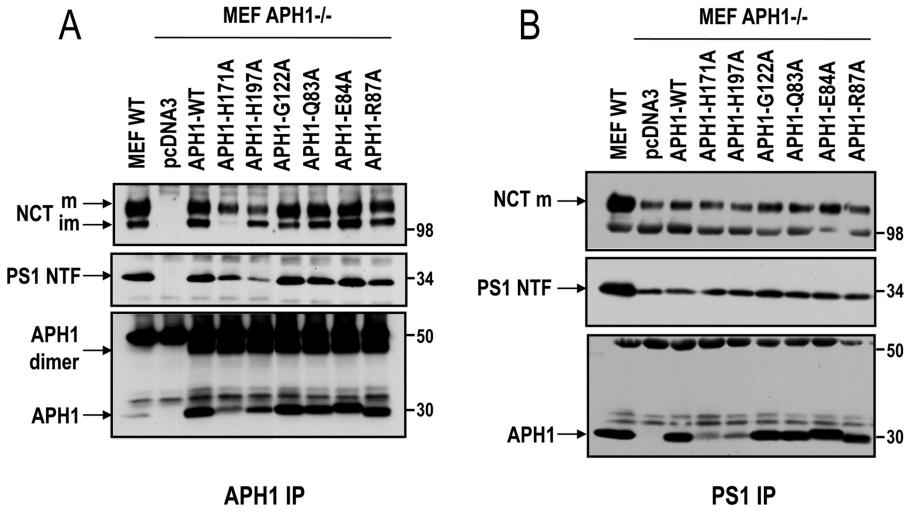

FIGURE 3. **Effects of APH1 substitutions on γ-secretase complex assembly.** CHAPSO membrane lysates from APH1 knock-out fibroblasts (MEF APH1$^{-/-}$) expressing WT or mutant proteins (H171A, H197A, Q83A, E84A, R87A) were subjected to immunoprecipitation (*IP*) using APH1 (*A*) or PS1-loop (*B*) antibodies. Western blotting indicated that substitution of either His-171 or His-197 significantly affected their binding to PS1 and the maturation of NCT.

indicated that loss of His-171 resulted in an ~30% reduction in PS1 cleavage, and the H197A substitution had a more pronounced effect, leading to an ~50% decrease in PS1-NTF compared with wild-type APH1 (Fig. 2*B*).

APH1 TM Mutants and γ-Secretase Complex Assembly—To examine the interaction of the APH1 mutants with other γ-secretase complex components, CHAPSO-solubilized cell membranes were used for immunoprecipitation studies with both anti-APH1 and anti-PS1 antibodies. Immunoprecipitation of APH1 from cells expressing wild-type protein resulted in the expected pulldown of APH1 monomers and dimers as well as PS1-NTF along with both mature and immature NCT (Fig. 3*A*). Wild-type APH1 was incorporated into the γ-secretase complex to a similar degree as seen in normal mouse fibroblasts (MEF WT). Immunoprecipitation of the TM3 mutants (Q83A, E84A, or R87A) resulted in a comparable co-isolation of PS1-NTF and mature/immature NCT, as was found for wild-type APH1 (Fig. 3*A*). These mutants were compared with a control alanine substitution (G122A) located in the *GXXXG* transmembrane dimerization motif. As described previously, this substitution also had no effect on γ-secretase assembly (33). A similar situation was observed after immunoprecipitation of endogenous PS1, where wild-type APH1 co-precipitated with the PS1-NTF and mature NCT (Fig. 3*B*). No significant differences were observed for the TM3 mutants (Q83A, E84A, or R87A), which associated with PS1-NTF and mature NCT at levels comparable with wild-type APH1 or the control G122A mutant. Quantification of the co-precipitated proteins as determined by image analysis supports the normal interaction of these mutants with PS1 and NCT (supplemental Fig. 1). The results from these reciprocal immunoprecipitation studies suggest that the polar/charged residues in TM3 are not essential for assembly of the γ-secretase complex.

Substitution of His-171 or His-197 resulted in the most significant effects on the assembly of the γ-secretase complex, which are consistent with the observed changes in PS1 endoproteolysis and NCT maturation. Immunoprecipitation of the H171A mutant resulted in the isolation of a slightly decreased amount of the monomeric species as compared with wild-type or the TM3 mutants, which could be because of partial occlusion of the epitope employed for immunocapture or subtle changes in structure (Fig. 3*A*). The dimeric APH1 H171A mutant was readily immunoprecipitated, indicating that the APH1 antibodies are effective for immunoprecipitation. However, the H171A mutant was unique in that it co-precipitated only with mature NCT. This is unusual as APH1 and immature NCT form a precomplex that is independent of other γ-secretase components (13, 15, 28, 33).

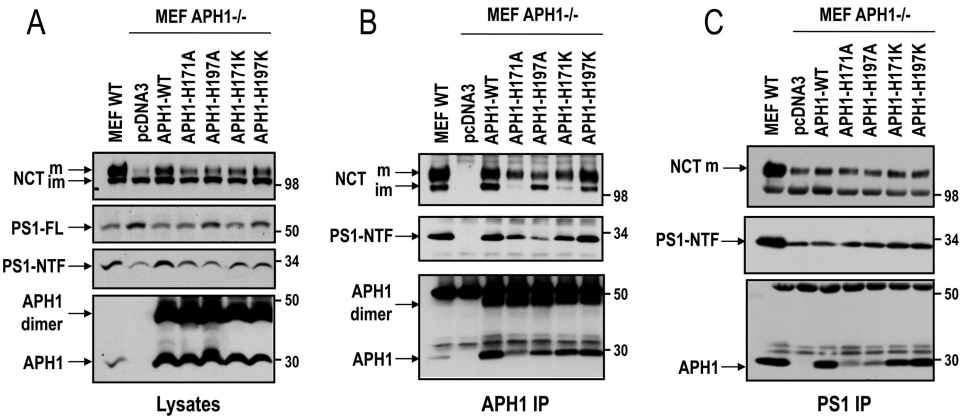

FIGURE 4. **APH1 histidine-to-lysine substitutions and effects on γ-secretase.** *A*, expression of H171K or H197K resulted in modest rescue of NCT maturation, and H197K resulted in a significant increase in PS1 endoproteolysis as compared with His → Ala mutants. *B*, immunoprecipitation of APH1 confirmed binding of both H171K and H197K to PS1 NTF. H197K was co-precipitated with both mature (*m*) and immature (*im*) NCT, whereas H171K was bound almost exclusively to mature NCT. *C*, immunoprecipitation (*IP*) of PS1 revealed a strong binding to both H171K and H197K as compared with the nonpolar alanine substitutions.

Co-precipitation of the PS1-NTF with H171A mutants was also observed, suggesting that loss of the TM5 His-171 polar side chain may promote incorporation of APH1 into mature γ-secretase complexes. This was confirmed after immunoprecipitation of endogenous PS1, which resulted in the expected co-precipitation of mature NCT but also a small amount of H171A APH1 (Fig. 3*B*). None of the dimeric APH1 (mutant or wild type) bound to PS1-NTF or mature NCT. Therefore, loss of His-171 permits the partial rescue of PS1 endoproteolysis and the formation of some complexes containing PS1-NTF and mature NCT. However, based upon the level of H171A detected after PS1 immunoprecipitation, these complexes are of relatively low abundance, and they represent only a small proportion of the total PS1-NTF and APH1 (Fig. 3*B* and supplemental Fig. 1). Therefore, it would be predicted that the amount of fully mature, active γ-secretase containing all four components would be reduced by the H171A mutation.

Immunoprecipitation of the TM6 H197A mutant efficiently pulled down a significant proportion of the APH1 species (monomer and dimer), which displayed the normal association with both mature and immature NCT (Fig. 3*A*). However, progression to the more mature γ-secretase complex was impaired, as only a small amount of PS1-NTF was bound to the H197A mutant as demonstrated by APH1 immunoprecipitation studies (Fig. 3*A*). Cells expressing H197A mutants did have less PS1-NTF and, therefore, decreased amounts of the fragment available for binding. However, the failure of H197A to incorporate into a potentially active γ-secretase was supported by PS1 co-immunoprecipitation. This revealed that only a small amount of H197A APH1 was incorporated into complexes containing both PS1-NTF and mature NCT (Fig. 3*B*). The results indicate that loss of the His-197 residue does not inhibit the formation of the APH1-NCT pre-complex but does reduce PS1 endoproteolysis and limits APH1 binding with PS1. Quantification of the levels of co-immunoprecipitated proteins supported the significant effects of the His mutants on interactions of APH1 mutants with both PS1 and NCT (supplemental Fig. 1). The H171A- and H197A-induced changes to the γ-secretase components and the assembly of the multisubunits are considered to be because of alterations in APH1 structure and/or the loss of hydrogen bonding and/or electrostatic interactions that stabilize the physical association of APH1 within the γ-secretase complex.

Histidine-to-lysine Substitutions and γ-Secretase Assembly—To determine whether the observed effects are dependent on positively charged side chains, His-to-Lys substitutions (H171K, H197K) were also examined. Both H171K and H197K were highly expressed, which resulted in an increase of mature NCT (Fig. 4*A*). However, PS1-NTF immunoblotting revealed that lysine substitution of His-171 and His-197 did not have the same effect on PS1 endoproteolysis. Although H171K displayed roughly the same level of endoproteolysis as H171A, the H197K mutant displayed a more substantial restoration of presenilin endoproteolysis where levels approached those seen for wild-type APH1 (Fig. 4*A*). This was supported by quantification of PS1-NTF of the mutants as compared with APH1-WT and normal mouse fibroblasts (supplemental Fig. 2*A*). These findings indicate that the positive charge contributed by His-197 is able to rescue the characteristics of the APH1 knock-out phenotype, most notably PS1 endoproteolysis.

Maintaining a charged side chain on the histidine residues also affected binding of APH1 to other γ-secretase components. Immunoprecipitation with APH1-directed antibodies resulted in a high level of recovery for the H171K and H197K monomers (Fig. 4*B*). The H171K also exhibited the same tendency as H171A to associate primarily with the mature form of NCT. Therefore, any substitution, nonpolar or charged, resulting in the loss of His-171 effectively eliminates the early pre-complex formed by APH1 and immature NCT. Similar interactions with the PS1-NTF were also observed for both H171A and H171K mutants after APH1 immunoprecipitation (Fig. 4*B*). In contrast, significant levels of PS1-NTF interact with H197K as compared with H197A (Fig. 4*B*), which is consistent with the observed restoration of PS1 endoproteolysis. Quantification of the co-immunoprecipitated PS1-NTF levels indicated a similar level of interaction as compared with wild-type APH1 (see (supplemental Fig. 3). The reciprocal immunoprecipitation with PS1-directed antibodies indicated additional differences between the APH1 lysine and alanine mutants. Both H171K and H197K mutants displayed a much stronger binding affinity to PS1 as compared with the corresponding His-to-Ala loss of charge mutants (Fig. 4*C* and supplemental Fig. 3). The increase in complexes containing both PS1-NTF and APH1-H197K mutant is probably due in part to the increased amount of the presenilin fragment that is produced by these mutants. However, in comparing PS1-NTF in total lysates for the H171A and H171K proteins, the levels are not dramatically increased (Fig. 4*A* and supplemental Fig. 2*A*). The observed increase in co-immunoprecipitation of APH1-H171K mutants with PS1 must,

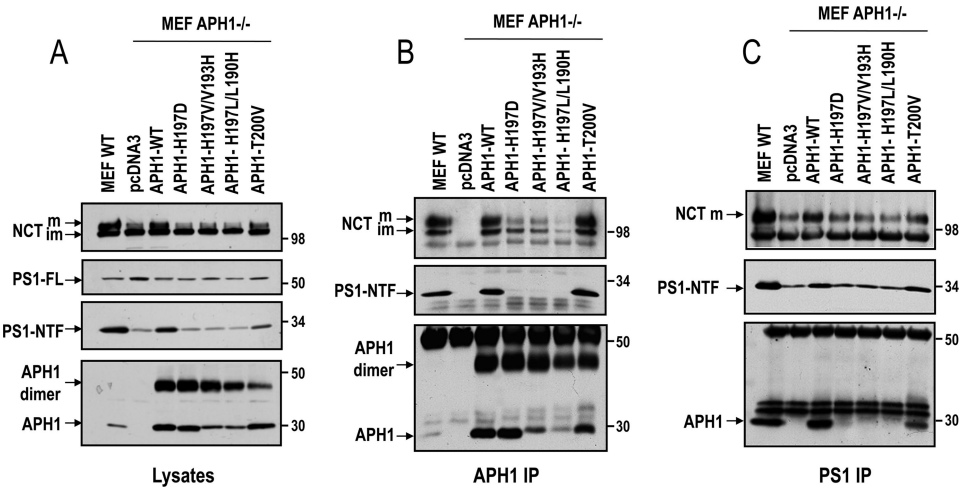

FIGURE 5. **Histidine charge reversal and translocations mutants.** *A*, expression of the negatively charged H197D mutant or shifting the His-197 residue down the transmembrane helix failed to rescue NCT maturation and PS1 endoproteolysis to generate the amino-terminal fragment (PS1-NTF). *B*, immunoprecipitation of APH1 indicated a reduced binding of H197D and the translocation mutants (H197V/V193H, H197L/L190H) to NCT and the absence of PS1 interactions as compared with the wild-type or the control T200V mutant. *C*, PS1 immunoprecipitation revealed that the His-197 mutants did not bind PS1. *m*, mature; *im*, immature.

therefore, be the result of more stable protein-protein interactions. This could be mediated by hydrogen-bonding networks or possibly via electrostatic salt bridges that stabilize APH1 binding to the γ-secretase complex components. Taken together, these findings indicate that alterations in His-171 can promote incorporation of APH1 into mature γ-secretase complexes and that the presence of positively charged residues at the 171 and 197 sites promote stable binding to PS1.

Histidine Charge Reversal and Translocation on the Transmembrane Helix—Reversing the histidine charge by substitution with aspartic acid or relocating the side chain vertically along the TM domain was also investigated to further understand the contributions of these residues to APH1 function. Reversing the charge in an H197D mutant did not have an appreciable effect on expression (Fig. 5*A*). However, no rescue of NCT maturation or increase in PS1 endoproteolysis was observed. Quantification of the PS1-NTF indicated that cells expressing the H197D mutant were not statistically different from the background APH1 knock-out fibroblasts (supplemental Fig. 2*B*). Assembly of the γ-secretase complex was also compromised by the charge reversal as shown by co-immunoprecipitation studies. Precipitation of the H197D mutant recovered the APH1 monomers and dimers, but the amount of associated mature as well as immature NCT was reduced and no binding to PS1 was observed (Fig. 5*B*). Lack of binding to PS1 was confirmed by PS1 immunoprecipitation where virtually none of the H197D mutant was detected (Fig. 5*C*). This may be due in part to the low level of the PS1-NTF (70% reduction), which is significantly different from H197A. However, the complete failure of the H197D mutant to associate with PS1-NTF suggests that the charged His side chain plays a role not only in PS1 endoproteolysis but also in the assembly of a normal γ-secretase complex.

The importance of His-197 spatial location on TM6 was also investigated to determine whether the simple presence of a charged side chain is crucial, or is its function dependent on a particular structural position on the transmembrane helix? Helical wheel models of TM6 demonstrated that His-197, Val-193, and Leu-190 are located on the same face of the transmembrane helix (supplemental Fig. 4). The position of His-197 on TM6 was switched with these residues to generate two additional mutants, H197V/V193H and H197L/L190H. The resulting APH1 proteins are compositionally identical to wild type, but the location of His-197 is shifted down the helix closer to the cytoplasmic interface. Both H197V/V193H and H197L/L190H were expressed but at reduced levels, possibly indicating an inherent instability, and did not rescue NCT maturation or PS1 endoproteolysis (Fig. 5*A*). Co-immunoprecipitation of APH1 or PS1 demonstrated a greatly reduced interaction of the mutants with NCT and the absence of any binding to PS1 (Fig. 5, *B* and *C*). Another TM6 mutant where Thr-200 located on the same face of the helix upstream of His-197 was substituted by valine (T200V) was examined as an unrelated mutation. This substitution displayed the same rescue as wild type of APH1 binding to the other γ-secretase components (NCT, PS1) and assembly of a mature γ-secretase (Fig. 5). Therefore, the effect of translocating the His residues appears to be because of specific changes in the structural location of His-197, consistent with the critical nature of this residue.

Effects of PS1 Aspartate Mutants on APH1 Interaction—The conserved PS1 aspartyl residues (Asp-257 and Asp-385) are required for both PS endoproteolysis as well as γ/ε cleavage activity by serving as the active site for the presenilin complexes (21) Given their position within the bilayer relative to APH1 His-171 and His-197, one potential explanation for the observed effects is the formation of electrostatic interactions between these side chains (see Fig. 1*B*). To test this hypothesis, the binding between PS1 and APH1 was examined using PS1/PS2 double-knock-out fibroblasts complemented with wild-type or Asp mutant PS1. Individual (D257A, D385A) or double (PS1DD-AA) PS1 aspartyl-substituted proteins exist as the predominantly uncleaved holoprotein, and the level of endogenous APH1 is unaffected by these PS1 mutations (Fig. 6*A*). Immunoprecipitation of wild-type as well as aspartyl-mutated PS1 results in the co-precipitation of endogenous APH1 (Fig. 6*B*). These findings indicate that direct interactions between PS1 and APH1 are not dependent upon salt-bridge formation by the complementary aspartate and histidine residues. However, this does not rule out the possibility that APH1 His-197 or His-171 participate in electrostatic interactions or form hydrogen-bonding networks with the other polar transmembrane residues in PS1 that stabilize multisubunit γ-secretase.

Effects of APH1 TM Mutants on γ-Secretase Activity—Given their effects on γ-secretase proteins and complex assembly, the activities associated with His-171 and His-197 mutants were

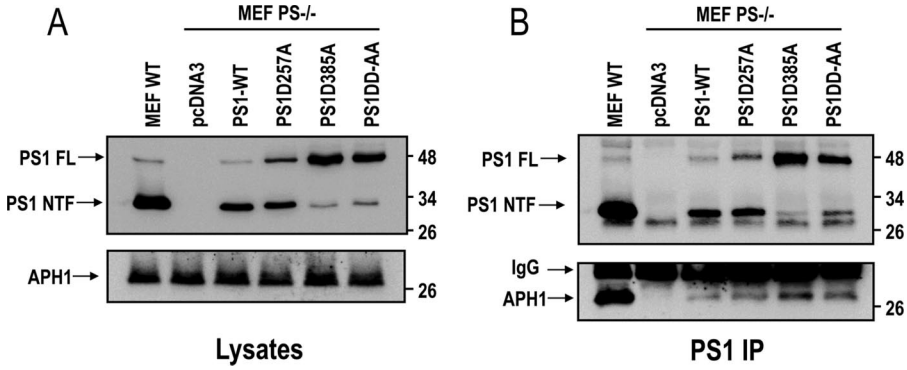

FIGURE 6. **PS1 aspartate mutants and APH1 interaction.** *A*, expression of the PS1 mutants (D287A, D385A, DD-AA) in double-knock-out fibroblasts (MEF $PS^{-/-}$) showed the typical lack of endoproteolysis, and these mutants did not alter the level of endogenous APH1 as compared with wild-type and mock-transfected cells. *B*, immunoprecipitation (*IP*) of PS1 demonstrated that the Asp mutants had no effect on binding to endogenous APH1. FL, full-length; NTF, N-terminal fragment.

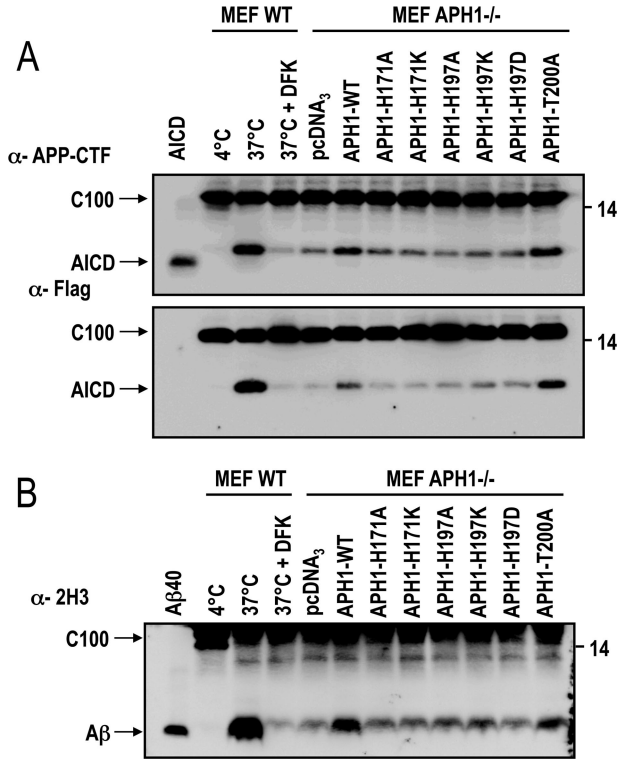

FIGURE 7. **γ-Secretase activity in $APH1^{-/-}$ cells complemented with APH1 mutants.** *A*, processing of recombinant FLAG-tagged C100 to generate the AICD fragment was used to monitor activity of the complexes from normal mouse fibroblasts (*MEF WT*) at 4 and 37 °C and in the presence of the inhibitor, difluoroketone-167 (*DFK*). Western blotting with antibodies directed to the APP-CTF (α-APP-CTF) and FLAG-tag (α-FLAG) indicated AICD generation by wild-type APH1 (APH1-WT) and T200V control proteins. A synthetic peptide corresponding to the AICD fragment (without FLAG tag) was also used as a positive control. No processing of the C100 to produce the AICD fragment was observed for any of the APH1 His-mutants (H171A/K, H197A/K/D). *B*, examination of Aβ peptides generated from the C100 substrate indicated significant amounts in normal mouse fibroblast (MEF WT) and APH1 knock-out cells expressing wild-type APH1 or T200V, but levels in cell expressing His-mutants (H171A/K, H197A/K/D) were not significantly elevated over background.

assessed using a cell-free assay. Membrane preparations from knock-out fibroblasts expressing the APH1 mutants were combined with recombinant FLAG-tagged C100, and processing to AICD and Aβ was determined. Knock-out fibroblasts expressing wild-type APH1, control T200V mutant, or normal mouse fibroblasts produced significant levels of the AICD fragment after incubation of membrane preparations at 37 °C (Fig. 7*A*). This processing was prevented by the addition of difluoroketone-167, which is a substrate-based difluro ketone inhibitor of the γ-secretase (34). The TM3 mutants (Q83A, E84A, and R87A) also display activity levels similar to wild-type APH1, which is consistent with their ability to restore PS1 endoproteolysis and assembly into a mature γ-secretase complex (supplemental Fig. 5).

Assaying similar membranes from knock-out cells expressing H171A or H197A revealed low levels of AICD generation as determined by immunoblotting with antibodies which recognize the APP-CTF or FLAG tag (Fig. 7*A*). No significant increase in Aβ processing was observed for either of the His-to-Ala mutants, which displayed signals similar to the background of untransfected fibroblasts (Fig. 7*B*). This low level of activity is consistent with the small pool of complexes containing the APH1 His mutants (H171A or H197A), PS1-NTF, and mature NCT as demonstrated by PS1 co-immunoprecipitation (see Fig. 4*C*). Lack of AICD and amyloid processing was also observed for the H197D mutants, which is consistent with the inability of this mutant to restore NCT maturation or PS1 endoproteolysis and assemble into a mature γ-secretase complex.

Assaying the activity of the His-to-Lys mutants using the cell-free assay indicated a similar lack of C100 processing. Combining membrane preparations from cell expressing H171K or H197K with the APP substrate indicated that AICD and Aβ levels were not significantly elevated above these seen after mock-transfection (Fig. 7). This was unexpected given that both H171K and H197K mutants restored PS1 endoproteolysis and assembled into complexes containing PS1-NTF and mature NCT. Taken together, these findings are consistent with the involvement of His-171 and His-197 in the physical assembly of the complex possibly via electrostatic or H-bonding interactions. In addition, the loss of activity in the apparently normal complexes for the H171K and H197K mutants suggests that the His residues also have the ability to modulate the PS1-mediated proteolytic processing.

DISCUSSION

Polar or ionizable residues occur with only modest frequency in TM domains because of the generally unfavorable thermodynamics of embedding them in the hydrophobic lipid bilayer. These residues are, therefore, often associated with critical functional and/or structural characteristics of membrane proteins. This is supported by the fact that missense mutations resulting in either the loss or gain of hydrophilic TM residues show a statistically higher propensity of being associated with a disease phenotype (35). In the current study several highly conserved polar residues were identified in APH1 that were inves-

tigated for their potential functional contributions to γ-secretase-mediated amyloid processing.

Similar TM residues are responsible for maintaining aqueous pores in channel-forming and transport proteins. They can also play key function roles in catalytic activities, for example, the aspartyl residues in the presenilins and signal peptide peptidases (21, 22). Alternatively, charged side chains within TM domains such as rhodopsin are essential for binding the functional retinal co-factor (36). Polar and ionizable amino acids are also involved in protein stabilization and protein-protein interactions, which are mediated by ionic salt bridges and hydrogen bonding. These structural features have been shown to be important for intramolecular association of TM domains to increase proteins stability at high temperatures (35). Salt bridges between two complementary residues mediate TM-TM associations within multisubunit protein complexes, for example, in T cell receptor assembly with CD3 (37). Similar electrostatic interactions have also been reported in stabilizing the cystic fibrosis transmembrane conductance regulator and other related ion channels and membrane transport proteins (38–42).

Examination of the APH1 membrane topology revealed three conserved hydrophilic resides on TM3 located in an analogous relative position within this transmembrane domain as the catalytic aspartic acid residues in the presenilins. These are potentially of functional significance and/or could mediate structurally important interactions. Polar asparagine and glutamine residues such as APH1 Gln-83 have been implicated in the association of TM domains through their ability to form strong hydrogen-bonding networks (43, 44). This type of interaction may be involved, for example, in the PS1-PEN2 binding, which is dependent upon an Asn-Phe (NF) motif (45). The neighboring charged residues in APH1, Glu-84 and Arg-87, have the potential to form stable salt bridges with TM domains either within APH1 or other components of the γ-secretase complex. However, loss of any of these residues after substitution with a nonpolar alanine had no readily detectable effect on assembly and activity of γ-secretase. These mutant proteins (Q83A, E84A, R87A) also rescued the impairments of NCT maturation and PS1 endoproteolysis in APH1 knock-out fibroblasts, indicating that they are functionally active. These findings support the conclusion that the TM3 polar residues are not essential for APH1 incorporation into the γ-secretase complex. However, two conserved histidine residues located on TM5, His-171, and TM6, His-197, were also investigated and had more pronounced effects.

Histidine imidazole side chains are unique in their ability to provide exchangeable protons and are, therefore, key functional sites in H^+ transporters and pH-sensing transmembrane proteins (46–49). They are also part of the His-Ser catalytic dyad of the Rhomboid family of proteins that mediates intramembrane proteolysis similar to that of the presenilins (for review, see Ref. 50). In addition to these functional roles, transmembrane His residues participate in metal chelation, for example, of Zn^{2+} in rhodopsin (51) and Fe^{2+} in bacterial redox electron transport proteins (52). Also relevant to the γ-secretase complex, His residues can be responsible for the binding of substrates within the active membrane core of multi-spanning TM proteins. This function is found for ubiquinol binding to cytochrome *c* (53) and sterol binding to acyl-coenzyme A:cholesterol acyltransferase 1 (54). Therefore, there is considerable precedence for histidine residues to be involved in similar functional and/or structural contexts as the APH1 His-171 and His-197 within γ-secretase.

Substitution of His-171 for either alanine or lysine had a dramatic effect on the binding of APH1 to NCT. Wild-type APH1 assembles into an initial scaffolding subcomplex with immature NCT, which subsequently binds with PS1 and PEN2 to form the active γ-secretase (13, 15, 28, 55). APH1-NCT binding is dependent upon the G*XXX*G transmembrane dimerization motif located in TM4 of APH1 (33). However, both H171A and H171K mutants coprecipitated exclusively with mature NCT and appeared to circumvent formation of the subcomplex with immature NCT. This phenomenon may be because of changes in APH1 trafficking resulting in the redirection of the mutants to different subcellular compartments. Alternatively, substitution of His-171 may accelerate APH1 shuttling out of the endoplasmic reticulum before interactions with immature NCT can occur.

Another possible explanation is that loss of His-171 prevents binding to other regulatory proteins which control APH1 movement within the cell. The existence of regulatory proteins has been proposed previously based on the G122D mutation of the G*XXX*G motif (33). This kind of regulatory process would be similar to a proposed activity of the endoplasmic reticulum retention factor, Rer1, which appears to bind directly to PEN2 and modulates its trafficking and incorporation into the presenilin complex (56). Other investigations have similarly revealed that Rer1p associates with immature NCT through its TM domain and can prevent formation of the APH1-NCT initial complex (57). Loss of the His-171 in APH1 may prevent binding to regulatory proteins which facilitate early association with immature nicastrin.

Changes in His-197 located on TM6 also had unique effects on γ-secretase assembly and activity. Loss of the imidazole side chain after H197A substitution did not alter APH1 binding to either mature or immature NCT but did result in reduced PS1 endoproteolysis. Co-immunoprecipitation demonstrated decreased binding of H197A to PS1 which is rescued by the His to Lys substitution. The contribution of the His-197 charge was confirmed by the H197D substitution which completely abolished PS1 endoproteolysis and APH1-PS1 binding. The relative position of His-197 within the transmembrane domain was also found to be critical, as translocation along the transmembrane helix disrupted both γ-secretase binding and protein stability.

The reduced pool of complexes containing the APH1 His-mutants (H171A and H197A) correlated with lower activity as monitored by a cell-free assay. Maintaining the charged side chain by Lys mutations (H171K and H197K) restored PS1 endoproteolysis and generated stable complexes containing mature NCT as well as PS1-NTF. However, even though complexes were formed, little or no activity was observed when assayed by monitoring processing of C100 to generate AICD and Aβ. The reasons for this could be that the Lys substitutions create subtle changes in the structure of APH1 that impair its function within the complex. Alternatively, Lys mutants may

still be able to participate in protein-protein interactions but replacement with the larger and more bulky side chain could create steric hindrance or structural disruptions within the PS1 catalytic site. This could, for example, reflect the involvement of the TM His residues in the movement of water into the PS1 active site.

It is expected that a hydrophilic oxyanionic channel is required for the entry of water into the protease and represents an essential feature for intramembrane hydrolysis proteases. It has been shown by cysteine-scanning approaches that PS1 has a water-accessible channel originating on its lumenal side within the mature multisubunit complex (58, 59), and APH1 may participate in maintaining this hydrophilic core. This is not to suggest that the APH1 His residues are actively involved in the catalytic site as is the case for the rhomboid Ser-His dyad but, instead, are secondary to the actual catalysis. This view is supported, for example, by the rhomboid crystal structures which implicate non-catalytic His residues in the movement of water into the protease active site (60–62). Co-crystals of the bacterial rhomboid GlpG with an inhibitor have identified changes in bound water that suggest His-150 and Asn-154 are involved in oxyanion binding (61). A similar role for the respective His residues in APH1 would be in agreement with the fact that the rhomboid residues are highly conserved and that mutagenesis results in a decrease in protease activity.

Taken together, these results reveal that the conserved histidine residues within the transmembrane domains of APH1 (His-171 and His-197) play key roles in stabilizing γ-secretase complex assembly and can affect proteolytic activity. Mutational analyses of TM domains from multisubunit protein assemblies such as the γ-secretase are informative and point to the importance of specific residues, as shown by the PS1 aspartyl residues. However, these are inherently complex, and determination of a high resolution structure of the γ-secretase complex will reveal whether the observed effects are because of the contribution of the imidazole side chain to the hydrophilic environment of the active site or whether these histidine residues participate in electrostatic and/or hydrogen-bonding interactions that stabilize APH1 and its function within the γ-secretase complex. The findings of the altered activity and assembly of the APH1 H171 and H197 mutants nevertheless point to an important role in the function of this component of the complex.

Acknowledgments—Development of the knockout mice was supported by National Institutes of Health Grants R01 NS45150 and P01 NS047308 (NINDS).

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