Presenilin and nicastrin are essential components of the γ-secretase complex that is required for the intramembrane proteolysis of an increasing number of membrane proteins including the amyloid-β precursor protein (APP) and Notch. By using co-immunoprecipitation and nickel affinity pull-down approaches, we now show that mammalian APH-1 (mAPH-1), a conserved multipass membrane protein, physically associates with nicastrin and the heterodimers of the presenilin amino- and carboxyl-terminal fragments in human cell lines and in rat brain. Similar to the loss of presenilin or nicastrin, the inactivation of endogenous mAPH-1 using small interfering RNAs results in the decrease of presenilin levels, accumulation of γ-secretase substrates (APP carboxyl-terminal fragments), and reduction of γ-secretase products (amyloid-β peptides and the intracellular domains of APP and Notch). These data indicate that mAPH-1 is probably a functional component of the γ-secretase complex required for the intramembrane proteolysis of APP and Notch.

Regulated intramembrane proteolysis of the amyloid-β precursor protein (APP), Notch, and other selected membrane proteins is a conserved control mechanism essential for cell signaling (1). The cleavage of Notch within its transmembrane domain is preceded by a ligand-dependent “site-2” cleavage step in the extracytosolic domain. The Notch intracellular domain (NICD) liberated by the subsequent “site-3” intramembrane cleavage translocates into the nucleus to regulate transcription of specific target genes important in cell-fate decision. Intramembrane proteolysis of APP is also preceded by a cleavage step at the α- or β-site in the extracellular domain. Like NICD, the APP intracellular domain released by intramembrane cleavage at the γ-site undergoes nuclear translocation to regulate gene expression (2). The amyloid-β (Aβ) peptides released by the β- and γ-site cleavages are believed to be essential for the pathogenesis of Alzheimer’s disease.

Proteolytic cleavage within the hydrophobic transmembrane domains of Notch and APP are intimately associated with a multimeric γ-secretase complex that contains the presenilin-1 or presenilin-2 and the membrane glycoprotein nicastrin. First, presenilin and nicastrin interact stoichiometrically in a high molecular weight complex where the heavily glycosylated nicastrin and the amino- and carboxyl-terminal fragments (NTF and CTF) of the presenilin heterodimers are tightly regulated in a mutually dependent way (3–9). Second, γ-secretase inhibitors can capture both presenilin and nicastrin, and semipurified presenilin-nicastrin complex has γ-secretase activity (10–12). Third, when mutated in cells or animals, either presenilin or nicastrin affects APP and Notch processing (5, 13–20).

Genetic studies in Caenorhabditis elegans have demonstrated that the worm presenilin homologs, sel-12 and hop-1, along with two “anterior-pharynx-defective” genes, aph-1 and aph-2 (the nicastrin homolog), are essential for normal development (5, 21–23). Recent reports on the C. elegans aph-1 gene suggest that at the genetic level, aph-1 interacts with sel-12 and hop-1 and is an enhancer of the presenilin function (24–25). This function appears to be partially conserved during evolution in that human APH-1 can partially rescue some of the C. elegans mutant phenotypes. In addition, aph-1 is required for cell surface localization of APH-2 in C. elegans (24). In cultured Drosophila cells, aph-1 affects the accumulation of presenilin levels and cleavage of APP and Notch (25). Here we report that the mammalian APH-1 (mAPH-1) protein physiologically associates with nicastrin and the presenilin heterodimers in vivo, regulates the steady-state levels of the mature nicastrin and the presenilin endoproteolytic fragments, and is required for the intramembrane processing of APP and Notch in mammalian cells.

**EXPERIMENTAL PROCEDURES**

mAPH-1 cDNAs—Full-length mAPH-1 cDNAs were amplified by PCR from either the human glioblastoma U118-MG cDNA library (26) or the I.M.A.G.E. clones (numbers 3457595, 4177580, and 264668). The U118-MG cDNA library was obtained from D. Young. Expression constructs were generated by inserting the mAPH-1 cDNAs in-frame with the Myc and His epitope of pcDNA4 (Invitrogen) at the carboxyl terminus of mAPH-1. Chromosomal localizations and genetic map positions were obtained from public genetic and transcriptional maps (www.ncbi.nlm.nih.gov). Sequence alignment was performed by using the Multalin program (prodes.toulouse.inra.fr/multalin).

Cell Lines and Transfection—HEK293 cells stably or HeLa cells transiently transfected with the Myc-His-tagged mAPH-1 were used to...
FIG. 1. Protein sequences and gene structures of mAPH-1. A, predicted amino acid sequences of Homo sapiens (Hs) and Mus musculus (Mm) mAPH-1, and Drosophila melanogaster (Dm), Anopheles gambiae (Ag), Caenorhabditis elegans (Ce), and Arabidopsis thaliana (At) APH-1 orthologs. GenBank™ accession numbers are: AAH01230 (Hs_mAPH-1α), AAH08732 (Hs_mAPH-1β), AAH20905 (Hs_mAPH-1β), AAH24111 (Mm_mAPH-1α), AAH12406 (Mm_mAPH-1β), AAF51212 (Dm_APH-1), EAA14158 (Ag_APH-1), CAA16282 (Ce_APH-1), and AAL36063 (At_APH-1). The Ce_APH-1 is predicted to contain an additional 17-amino acids at the carboxyl terminus, which were trimmed in this alignment. mAPH-1α and mAPH-1β are identical except at the carboxyl terminus. mAPH-1a is ~50% identical to mAPH-1b. Dots depict identical amino acid residues to Hs_mAPH-1α. Putative transmembrane domains are underlined. Amino acid sequences of the peptides used as antigens for generating the mAPH-1 antisera are highlighted. B, the gene structures and alternative splicing mechanism of human mAPH-1. Exons are boxed. Shading indicates coding sequences. Empty boxes indicate non-coding regions. The lines between exons indicate introns. mAPH-1α maps to a region of chromosome 1 that has been previously linked to an Alzheimer’s disease susceptibility locus in a full genome scan (32). mAPH-1β maps to chromosome 15.
evaluate the interactions of mAPH-1 with presenilin and nicastrin. HeLa cells were stably transfected with a pcDNA3.1/APP<sup>swe</sup>-<sup>resw</sup> construct to generate the HeLa/APP<sup>swe</sup>-<sup>resw</sup> stable cell line used for detecting APP processing in the small interfering RNA (siRNA) studies. Native HeLa cells were also used in the siRNA experiments.

**Antibodies**—H2B (H2B-1 and H2B-2), H2D (H2D-1 and H2D-2), and H2E (H2E-1 and H2E-2) were raised in rabbits against hydrophilic mAPH-1a peptides (Fig. 1A, the antigen sequences are highlighted). H2D specifically recognizes mAPH-1b<sup>-a</sup> in co-immunoprecipitation and Western blotting experiments, but H2B and H2E have so far not been useful for detecting mAPH-1a. H2D was affinity-purified and used to demonstrate the interaction of endogenous mAPH-1a<sup>-a</sup> with endogenous presenilin and nicastrin in brain tissues. Myc-His-tagged mAPH-1a protein were visualized using the monoclonal anti-Myc antibody 9E10 (ATCC) and were immunoprecipitated with rabbit polyclonal anti-Myc antibody A-14 (Santa Cruz Biotechnology). Other antibodies include rabbit polyclonal and mouse monoclonal antibodies against presenilin-1 and presenilin-2 amino- and carboxyl-terminal fragments (Chemicon and Oncogene, respectively), rabbit polyclonal anti-nicastrin antibodies (ABR and Calbiochem, respectively), antibodies against full-length APP and COOH-terminal α- and β-secretase derivatives (Sigma and Chemicon, respectively), and anti-α-tubulin (Sigma). Polyclonal antibody and monoclonal antibody against Aβ were kindly provided by E. Koo.

**Biochemical Methods**—Co-immunoprecipitation and Western blotting were performed as described previously (3–5, 10–11) with the exception that the SuperSignal West Femto Reagents (Pierce) were used. Nickel-nitrilotriacetic acid (Ni-NTA)-agarose affinity pull-down experiments were performed according to the manufacturer’s instructions (Qiagen).

**Reverse Transcriptase-PCR**—RNA was purified from HeLa cells using RNeasy column (Qiagen), and first-strand cDNA synthesis was generated using the Thermoscript cDNA kit (Invitrogen). PCR was performed using primer pairs specific for either mAPH-1a (5'-GCTGG-GGTGTCTTTCGGG-3' and 5'-GTCCTCATTGACCTCACC-3'), mAPH-1b (5'-AATGACTGCGGCTGATCC-3' and 5'-CCATGACTGGCCACCGTG-3'), presenilin-1 (5'-CAAGCCACCGGAAACTGCCG-3'), presenilin-1 (5'-CGGTGCTGAGCAAGATGA-3') and 5'-TAAATGATATGCTGCTGAC-3'), or 18 S rRNA (Ambion). Please note that the primer pair for mAPH-1a is predicted to detect both mAPH-1a<sup>-a</sup> and mAPH-1a<sup>-b</sup>.

**RNA Interference**—Small interfering RNA oligonucleotides (University of Texas Southwestern RNA Synthesis Core) directed against mAPH-1a (5'-GAAGGCACGATACCCGTTTT-3' and 5'-GATACCCA-CTCTCCATCG-3'), mAPH-1b (5'-TACCCATCTGACTCCTTGTTT-3' and 5'-CAAGGAGTACCAACACAGTT-3'), nicastrin (5'-GGGCAAATGTTCCGCGCGATG-3'), or presenilin-1 (5'-GGTCCTACCTGATCGTGT-3') were transfected into native HeLa cells or HeLa/APP<sup>swe</sup>-<sup>resw</sup> stable cell lines according to the guidelines recommended by Elbashir et al. (27). Please note that the mAPH-1a siRNA oligonucleotides used in the study are predicted to target both mAPH-1a<sup>-a</sup> and mAPH-1a<sup>-b</sup>. APP and Notch Cleavage Assays—Native HeLa cells were transfected with mAPH-1, nicastrin, or presenilin-1 siRNAs or control RNA oligonucleotides for 56–96 h to inactive target genes. The resultant cells were subsequently transfected with proper reporter constructs and analyzed as previously described for APP and Notch cleavage in either the luciferase reporter gene assays (28, 29) or the β-estradiol immobilizing activity (15, 17, 29). Luciferase reporter systems were provided by T. Sudhof and J. Lundkvist. NAE and NICD constructs were from R. Kopan. For Aβ analysis, HeLa/APP<sup>swe</sup>-<sup>resw</sup> stable cell lines were treated with the appropriate siRNAs for 56–96 h and then changed to fresh media for 18 h. Aβ was immunoprecipitated from the conditioned media by polyclonal antibody, separated on Bis/Tra SDS gels (30), and immunoblotted with Aβ monoclonal antibody. All experiments in this paper were performed at least four times.

**RESULTS AND DISCUSSION**

From a human glioblastoma cDNA library, we isolated two mAPH-1 (mAPH-1<sup>a</sup> and mAPH-1<sup>b</sup>) genes that are homologous to worm aph-1. mAPH-1<sup>a</sup> has at least two splice variants: 1) mAPH-1a<sup>-a</sup> has seven exons and encodes a longer (L) open reading frame of 265 residues; and 2) mAPH-1a<sup>-b</sup> has six exons and encodes a shorter (S) open reading frame of 247 residues (Fig. 1A, B). mAPH-1b has six exons and encodes 257 amino acids (Fig. 1A, A and B). We made C49A DNA constructs that express Myc and His epitope-tagged mAPH-1a<sup>-a</sup>, mAPH-1a<sup>-b</sup>, and mAPH-1b. Western blots of lysates from HEK293 cells stably transfected with these cDNAs revealed Myc-immunoreactive bands with relative molecular masses of approximately 30 kDa, which are the predicted sizes of mAPH-1 amino acid sequences (Fig. 2A, lane 1) (data not shown). Additional Myc-immunoreactive bands at 13–15 and 50 kDa can sometimes be detected (data not shown).

To determine whether mAPH-1 physically associates with the γ-secretase complex, HEK293 cells stably expressing mAPH-1a<sup>-a</sup>-Myc-His were used in reciprocal co-immunoprecipitation experiments. We found that in cell lysates extracted with CHAPSO, a detergent compatible with γ-secretase activity, mAPH-1a<sup>-a</sup> co-precipitated with endogenous nicastrin and endogenous presenilin-1 NTF and CTF heterodimers (Fig. 2A, lanes 2–4). Conversely, endogenous presenilin-1 NTF and CTF and nicastrin (data not shown) co-precipitated with mAPH-1a<sup>-a</sup>-Myc-His (Fig. 2A, lane 5). Presenilin-2 NTF also co-precipitated with mAPH-1a<sup>-a</sup>-Myc-His and nicastrin, but not presenilin-1 NTF or CTF (Fig. 2A, lanes 2–5), suggesting that the two presenilin proteins are in separate complexes (3) that share other common components including nicastrin and mAPH-1<sup>a</sup> and that the interactions revealed in our co-immunoprecipita-
tion experiments are highly specific. The specificity of the mAPH-1aL, presenilin, and nicastrin interaction was corroborated by the absence of co-immunoprecipitation of these proteins with preimmune serum (Fig. 2A, lane 6) or with antibodies to irrelevant proteins such as calnexin and Rab5 (Fig. 2A, lanes 1b-Myc-His but not LacZ-Myc-His (Fig. 2A). The endogenous presenilin-2 NTF co-precipitated with mAPH-1b, whereas nicastrin-associated mAPH-1aL and the presenilin heterodimers were stable after mAPH-1 siRNA treatment (Fig. 3, lane 5). In contrast, H2B (H2B-1 and H2B-2) and H2E (H2E-1 and H2E-2) antisera or H2D-2 preimmune serum failed to precipitate presenilin-1 CTF (Fig. 3, lanes 3, 6–8). Taken together, we concluded that mAPH-1 specifically interacts with nicastrin and the presenilin heterodimers, two known components of the γ-secretase complex.

To study the role of endogenous mAPH-1 in presenilin- and nicastrin-associated γ-secretase activity, we used 21-nucleotide duplexes of siRNAs and performed RNA interference studies in HeLa cells (27) to inactivate endogenous mAPH-1. Reverse transcriptase-PCR experiments indicated that mAPH-1b mRNA is hardly detectable, whereas mAPH-1a mRNA can be easily detected in HeLa cells (Fig. 4A, lanes 2 and 3). mAPH-1a but not mAPH-1b siRNAs significantly reduced mAPH-1a mRNA levels in HeLa cells (Fig. 4A, lanes 4 and 5). The fact that siRNAs against a highly homologous gene did not affect mAPH-1a illustrates the specificity of the siRNA technique. We next examined whether the presenilin NTF:CTF heterodimers are stable after mAPH-1 is inactivated in mammalian cells. We found a dramatic reduction of presenilin-1 and presenilin-2 but not other proteins such as α-tubulin in mAPH-1a and mAPH-1a plus mAPH-1b siRNA-treated HeLa cells (Fig. 4, B and C). Consistent with the low expression level of mAPH-1b in HeLa cells, a minor reduction of presenilin proteins was observed in cells treated with mAPH-1b siRNAs (Fig. 4B). The down-regulation of the steady-state presenilins by mAPH-1
siRNAs is most probably at the protein levels in that mAPH-1 siRNAs did not cause major changes in presenilin-1 mRNA levels (Fig. 4A). As expected (7–8), we found that nicastrin siRNAs reduced the steady-state levels of presenilin-1 and presenilin-2 and that presenilin-1 siRNAs reduced mature nicastrin (Fig. 4, B and C). However, we did not observe significant changes in the overall nicastrin levels in cells treated with mAPH-1 siRNAs (Fig. 4C). This is probably because of the incomplete inhibition of mAPH-1 by the mAPH-1 siRNA oligonucleotides we used. Indeed, further investigation into the mAPH-1a plus mAPH-1b siRNA-treated cells did reveal a reduction in the level of mature nicastrin (Fig. 4D). Together with the observation that mAPH-1 co-immunoprecipitates with both presenilin and nicastrin, these data suggest that mAPH-1 functionally and physically associates with the γ-secretase complex under physiological conditions.

To test whether mAPH-1 like presenilin or nicastrin modulates γ-cleavage of APP within its transmembrane domain, we examined whether the α- or β-cleaved carboxyl-terminal fragments (C83 and C99, respectively), the immediate substrates of the γ-secretase complex, accumulated when endogenous mAPH-1 was inactivated in the mammalian cells. mAPH-1a siRNA treatment of HeLa cells stably expressing full-length APP_Swedish protein (HeLa/APP_Swedish) resulted in marked increase of both C83 and C99 (Fig. 5A). Inactivation of mAPH-1a in native HeLa cells also caused the accumulation of C83 and C99 (data not shown). Similarly, and as expected when γ-secretase activity is inhibited, C83 and C99 accumulated when the cells were treated with either nicastrin or presenilin-1 siRNAs. In contrast, we observed no significant change in the total level of full-length APP or α-tubulin (Figs. 4C and 5A). We next used Ab-specific antibodies to determine whether amyloid-β peptides, the products of γ-secretase complex, are inhibited in mAPH-1a siRNA-treated HeLa/APP_Swedish cells. Immunoprecipitation followed by immunoblotting experiments revealed a significant reduction of total Ab in the conditioned media from mAPH-1a siRNA-treated cells as compared with the control cells (Fig. 5A).

We also tested whether mAPH-1a is required for the production of APP intracellular domain, another product of the
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γ-secretase activity. To do so, we used a previously established cell-based assay (2, 18, 28). In this assay, a UAS-luciferase target gene was co-expressed with a chimeric protein (C99-GV), which was generated by inserting a transcription factor composed of the yeast Gal4 DNA-binding domain and the viral VP16 transcriptional-activating domain (GV) at the junction between the transmembrane and intracellular domains of the APP γ-secretase complex. However, when overexpressed, neither mAPH-1, nicastrin, nor mAPH-1 plus nicastrin was able to boost the levels of the presenilin NTF:CTF heterodimers in the mammalian cells (data not shown). This suggests that mAPH-1 and/or nicastrin are not sufficient to account for the "limiting cellular factors" (31) and that at least one additional protein is required for the restricted assembly of the active γ-secretase complex. One such protein is probably PEN-2 (25). Indeed, our preliminary data suggest that presenilin, nicastrin, and mAPH-1 can be co-isolated with Myc-His epitope-tagged mammalian PEN-2 in Ni-NTA-agarose pull-down experiments (data not shown). Future studies to understand how mAPH-1 interacts with the other components (presenilin, nicastrin, and possibly PEN-2) during the folding, assembly, and trafficking processes to form an active γ-secretase complex should shed light on the molecular mechanism of this unusual enzyme in the intramembrane proteolysis and in the pathogenesis of Alzheimer's disease.

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