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Biochemical and Biophysical Research Communications 327 (2005) 18–22
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Expression profiles of two human APH-1 genes and their roles in formation of presenilin complexes

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Received 19 November 2004
Available online 8 December 2004

Abstract

APH-1 is a polytopic membrane protein that functions as a component of presenilin–γ-secretase complexes. Two homologous genes of APH-1 exist in humans, APH-1a and APH-1b, and alternative splicing of the former generates two isoforms, APH-1aS and APH-1aL. We performed semi-quantitative reverse transcription-PCR analysis to investigate mRNA expression of these three APH-1 forms in human cell lines and tissues. We found that both APH-1a and APH-1b were expressed in almost all tissues, and that APH-1aS was 1.5–3 times more abundantly expressed than APH-1aL. We examined the effect of small interfering RNA-mediated knock down of APH-1a or APH-1b on APH-1 mRNA expression and presenilin complex protein expression. We found that knock down of APH-1a, but not APH-1b, resulted in impaired maturation of nicastrin and reduced expression of presenilin 1, presenilin 2, and PEN-2 proteins. These findings indicate that APH-1a plays an essential role in the formation of presenilin–γ-secretase complexes.

Keywords: Alzheimer's disease; APH-1; Nicastrin; PEN-2; Presenilin; RNAi; γ-Secretase

The accumulation of amyloid β-protein (Aβ) in the brain is considered to play a central role in the pathogenesis of Alzheimer's disease [1]. Two proteases, β-secretase and γ-secretase, are responsible for Aβ generation. β-Secretase has been identified as BACE1, and γ-secretase has been shown to be a multi-protein complex in which presenilin 1 (PS1) or presenilin 2 (PS2) constitutes the catalytic subunit [2], with PS1-containing complexes having more γ-secretase activity than PS2-containing complexes [3]. γ-Secretase also cleaves a number of type 1 integral membrane proteins in their intramembrane domain, resulting in release of intracellular signaling fragments [4]. Recent studies have identified three other γ-secretase complex components—nicastrin, PEN-2, and APH-1 [4,5].

The APH-1 gene was first isolated by screening *Caenorhabditis elegans* genes whose mutants induce an abnormal phenotype called Aph (anterior pharynx defective) [6,7]. Two human APH-1 genes exist, APH-1a and APH-1b, with two APH-1a isoforms, APH-1aS and APH-1aL, generated through alternative splicing of a single transcript (Fig. 1A) [8,9]. Both APH-1a and APH-1b are multi-pass membrane proteins with seven transmembrane domains [8–10], and APH-1aS and APH-1aL have different C-terminal sequences [8,9]. Recent evidence indicates that APH-1 associates with nicastrin early in γ-secretase complex assembly and maturation [9,11–13]. It is not clear whether APH-1a and APH-1b have different functions in γ-secretase complexes. In addition, regulation of APH-1aS, APH-1aL, and APH-1b mRNA expression is not well understood.

In the present study, we used reverse transcription (RT)-PCR analysis and RNA interference to show that APH-1aS is more abundantly expressed than APH-1aL in human tissues, and that APH-1a plays an essential role in formation of PS complexes.

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doi:10.1016/j.bbrc.2004.11.130

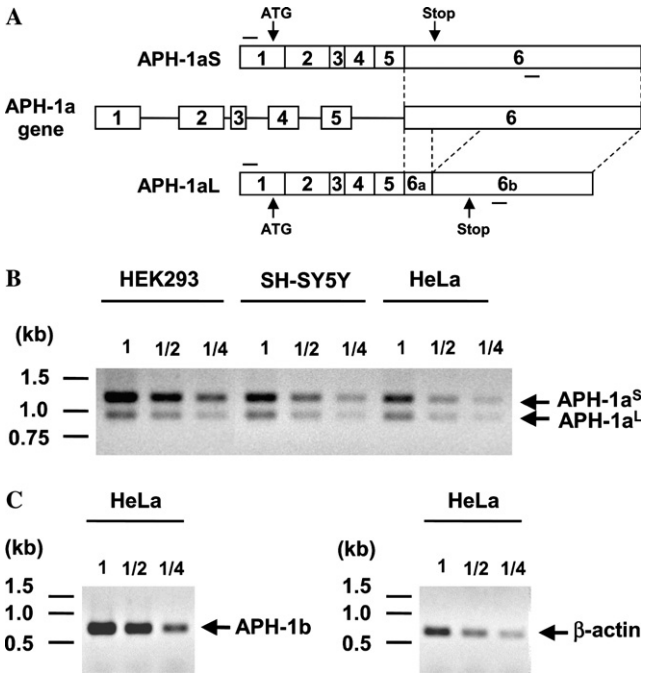

Fig. 1. Semi-quantitative RT-PCR analysis of APH-1a and APH-1b mRNA levels. (A) The APH-1a gene consists of 6 exons, with alternative splicing in exon 6 generating two isoforms, APH-1aS and APH-1aL. The positions of forward and reverse primers are indicated by the bars. (B) Total RNA was extracted from HEK293, SH-SY5Y, and HeLa cells, and subjected to RT-PCR analysis. Upper and lower bands correspond to APH-1aS and APH-1aL, respectively. Band intensities of these isoforms correlated with the amount of cDNA used in the reaction. (C) RT-PCR analysis of APH-1b (left) and β-actin (right) mRNA expression was performed using total RNA from HeLa cells. Band intensities were proportional to the amount of cDNA used.

Materials and methods

Cell culture. Human embryonic kidney 293 (HEK293) cells and HeLa cells were cultured in a humidified atmosphere of 5% CO_2/95% air at 37 °C in DMEM containing 10% fetal bovine serum. Human neuroblastoma SH-SY5Y cells were cultured as described previously [14].

RNA isolation. Total RNA was extracted from cells using a Gene Elute Mammalian Total RNA Miniprep Kit (Sigma). Tissue RNA samples were obtained from BD Biosciences Clontech (Human Total RNA Master Panel II) and Stratagene.

Reverse transcription-PCR. Reverse transcription (RT) was carried out in a reaction volume of 20 μl containing 1 μg total RNA and 25 μg/ml oligo(dT)15, using the ImProm II Reverse Transcription System (Promega) according to the manufacturer's instructions. PCR was carried out using 1 μl RT reaction mixture in the presence of 200 μM dNTPs, 0.5 μM primers, and 1 μl Advantage 2 Polymerase mix (Stratagene) in a final volume of 50 μl. The following primer pairs were used: APH-1a, 5′-CATTTGCCTGTCCTGGTCAGG-3′ and 5′-CATCTCCAGTTCTGGACAGTG-3′; APH-1b, 5′-TTTCCGCGGTGGCCATGACT-3′ and 5′-GAAGTGCTGGTTCCCTGAGG-3′; and β-actin, 5′-GCCAGCTCACCATGGATGATG-3′ and 5′-TTCTCCAGGGAGGAGCTGGAA-3′. PCR protocols were as follows: for APH-1a, 30 cycles at 95 °C for 1 min, 65 °C for 1 min, and 72 °C for 2 min; for APH-1b, 29 cycles at 95 °C for 1 min, 65 °C for 1 min, and 72 °C for 2 min; and for β-actin, 21 cycles at 95 °C for 1 min, 65 °C for 1 min, and 72 °C for 2 min. Amplification products were separated on 0.9% agarose gels, bands were visualized using ethidium bromide staining, and masses were quantified using an LAS-1000 image analyzer (Fuji Film).

RNA interference (RNAi). Single-stranded, gene-specific sense and antisense RNA oligomers were obtained from Qiagen. Small interfering RNAs (siRNAs) for APH-1a and APH-1b were directed towards the following target sequences: 5′-AAGAAGGCAGATGAGGGGTTA-3′ for APH-1a and 5′-AACAAAGATGGACCAACACAG-3′ for APH-1b [7]. The APH-1a siRNA oligonucleotides were predicted to target both APH-1aS and APH-1aL. Control, non-silencing siRNA was also purchased from Qiagen. SiRNA duplexes were transfected into HeLa cells using oligofectamine reagents (Invitrogen) according to the manufacturer's instructions. Transfections with siRNA involved two consecutive rounds in which cells underwent an initial transfection, and then 2 days later cells were replated, transfected again, and harvested 2 days later.

Antibodies. Rabbit polyclonal antibodies against nicastrin and APH-1aL were obtained from Sigma and Covance, respectively. Antibodies against PS1 (αPS1-Ext1) and PS2 (Ab555C) were described previously [15,16]. PEN-2 antiserum (PNT2) was generously provided by Dr. Thinakaran [17].

Western blot analysis. Western blotting was performed essentially as described previously [14]. For Western blot analysis of APH-1, PS1, and PS2, samples were incubated at 37 °C for 10 min prior to SDS–PAGE separation. For analysis of PEN-2, samples containing 100–150 μg protein were immunoprecipitated using PEN-2 antiserum, immunoprecipitated proteins were washed and subjected to immunoblotting using the PEN-2 antibody.

Results

We validated the RT-PCR analysis system using total RNA derived from human cell lines, including HeLa and HEK293 cells. PCRs using the primer pair flanking the APH-1aL coding region gave rise to two bands corresponding to APH-1aS and APH-1aL, and their intensities were proportional to the amount of cDNA used (Fig. 1B). Interestingly, in all cell lines, APH-1aS mRNA was expressed more highly than APH-1aL. We also detected one major band of APH-1b (Fig. 1C). HEK293 cells showed higher expression of APH-1a and lower expression of APH-1b than HeLa cells (Fig. 2A).

We analyzed mRNA expression of the three APH-1 forms in various human tissues. We found that both APH-1a and APH-1b were expressed ubiquitously, and that APH-1aS expression was higher than that of APH-1aL (Fig. 2A). Furthermore, APH-1a expression appeared to be higher in adult tissues compared to fetal tissues (brain and liver). The highest expression of APH-1b was observed in the testis. We calculated the ratios of APH-1aS to APH-1aL expression in cell lines and tissues. Levels of APH-1aS were higher than those of APH-1aL, with the ratio being higher in the kidney (∼3), brain and liver (∼2.5) than in the uterus and testis (∼1.5) (Fig. 2B). The ratios in HEK293, SH-SY5Y, and HeLa cells were similar to those of their respective tissue origins.

We investigated the effect of RNAi-mediated down-regulation of APH-1a or APH-1b on mRNA expression of the counterpart form. Using RT-PCR, we measured APH-1a and APH-1b mRNA expression in HeLa cells treated with siRNAs specific for APH-1a, APH-1b or

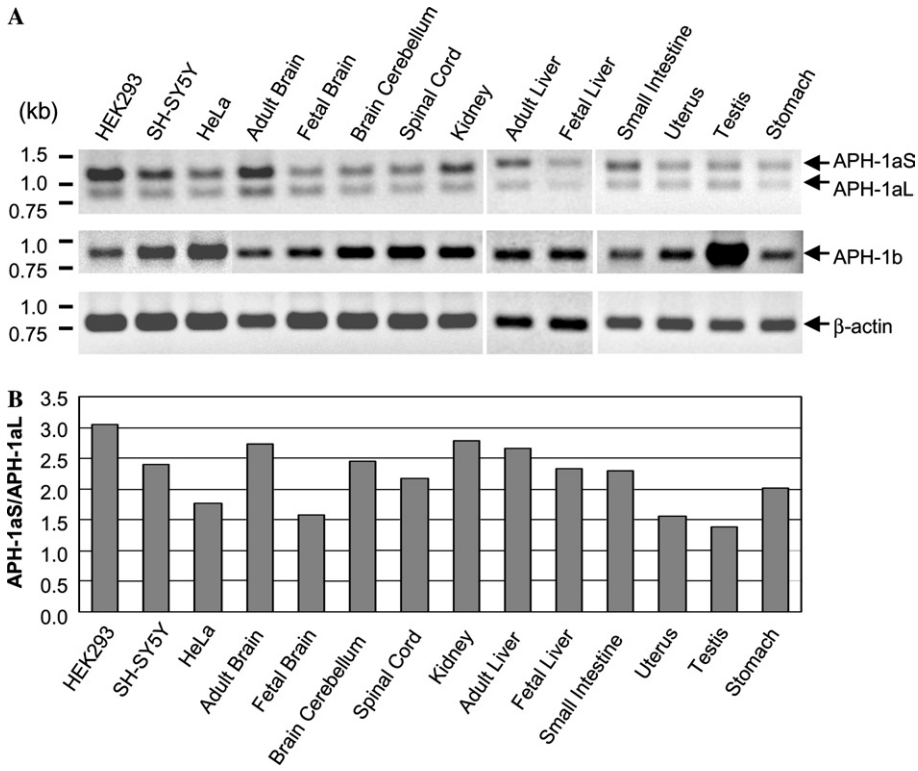

Fig. 2. RT-PCR analysis of APH-1a and APH-1b mRNA expression in human cells and tissues. (A) RNA extracted from the indicated human cells and tissues was subjected to RT-PCRs, as in Fig. 1. Reproducible results were obtained in at least two independent experiments. (B) The band intensities in (A) were quantified, and the relative ratios of APH-1aS to APH-1aL were calculated and plotted.

control siRNAs. We found that down-regulation of APH-1b did not significantly affect APH-1aS or APH-1aL expression, and that similarly, down-regulation of APH-1a had almost no effect on APH-1b expression (Fig. 3A). Thus, down-regulation of one APH-1 form did not appear to cause a compensatory increase in mRNA expression of another form.

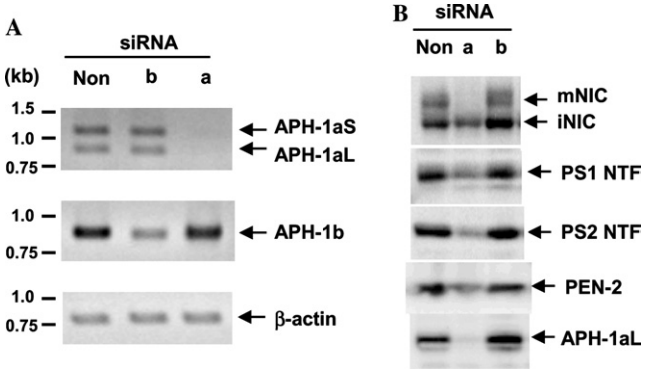

Fig. 3. Effect of RNAi-mediated down-regulation of APH-1a or APH-1b on APH-1 mRNA expression and PS complex protein expression. (A) HeLa cells were transfected with siRNAs specific for APH-1a or APH-1b or control non-silencing siRNAs. APH-1a and APH-1b mRNA expression was analyzed using RT-PCR, as in Fig. 2. (B) Cell lysates from siRNA-treated HeLa cells were subjected to immunoblot analysis using relevant antibodies. mNIC, mature nicastrin; iNIC, immature nicastrin; and NTF, N-terminal fragment.

Using immunoblot analysis we analyzed the effect of APH-1 down-regulation on expression of the PS–γ-secretase complex proteins nicastrin, PS1, PS2, and PEN-2. In HeLa cells treated with APH-1a-specific siRNAs, APH-1a down-regulation caused a marked reduction in the level of the mature form of nicastrin, while levels of the immature form of nicastrin were modestly reduced, compared to control cells. Endogenous expression of PS1, PS2, and PEN-2 proteins was also much lower in APH-1a siRNA-treated cells (Fig. 3B). In contrast, mature nicastrin, PS1, PS2, and PEN-2 were unaffected in cells treated with APH-1b-specific siRNAs (Fig. 3B). While levels of immature nicastrin and APH-1aL sometimes increased slightly in APH-1b siRNA-treated cells (Fig. 3B), these changes were not consistently observed. These results suggest that APH-1a and APH-1b play different roles in the formation of PS–γ-secretase complexes.

Discussion

In the present study, we demonstrate using RT-PCR analysis that both APH-1a isoforms, as well as APH-1b, are expressed ubiquitously in human tissues, and that APH-1aS is more abundantly expressed than APH-1aL. Similar ubiquitous expression of APH-1 was recently reported in mice [18]. Interestingly, the relative ratios of APH-1aS to APH-1aL varied considerably in different

tissues, suggesting that there is tissue-specific regulation of the alternative splicing of the APH-1a gene. Our data are consistent with the recent observation that APH-1aS protein levels are significantly higher than those of APH-1aL in HEK293 cells [19]. Our observations do not appear to agree with those of a previous report, suggesting that APH-1b mRNA expression was barely detectable in HeLa cells [8]. This contrast in observations may be due to technical differences regarding detection.

Analysis of mRNA expression in siRNA-treated cells in the present study suggests that down-regulation of APH-1a or APH-1b does not significantly influence the mRNA expression of its counterpart. Immunoblot analyses clearly showed that down-regulation of APH-1a, but not APH-1b, impaired nicastrin maturation and diminished endogenous expression of PS1, PS2, and PEN-2, suggesting an essential role of APH-1a in the formation of PS complexes. Down-regulation of PS1, PS2, and PEN-2 expression occurs most probably at the protein level [8]. Observations similar to those shown here have recently been reported [8,19], with one such study showing that nicastrin maturation was not clearly inhibited by APH-1a siRNA possibly due to incomplete RNAi [8].

PS1-deficient cells exhibit impaired nicastrin maturation similar to that observed in APH-1a knock-down cells [20–22]. Furthermore, APH-1aL protein is still expressed in PS1-deficient cells [9]. Thus, both APH-1a and PS1 appear to be essential for nicastrin maturation. Since nicastrin seems to form a subcomplex with APH-1 at an early step in PS complex assembly [12,13], nicastrin may start to mature at this step. It is likely that APH-1–nicastrin associates with PS1 to complete the nicastrin maturation process. This view is consistent with the observation that both APH-1 and PS are required for proper cell-surface localization of APH-2/nicastrin in *C. elegans* embryos [6].

APH-1a and APH-1b were very recently shown to be incorporated into distinct PS complexes [18,19]. However, the physiological role of APH-1b-containing PS complexes is less clearly understood than APH-1a-containing complexes. Further research is required to clarify the significance of APH-1b-containing PS complexes in γ-secretase function. It also remains to be determined whether APH-1aS-containing and APH-1aL-containing PS complexes have identical or subtly different functions. Further detailed analysis of the functional role of APH-1 should facilitate elucidation of the molecular mechanisms underlying intramembranous proteolysis by γ-secretase, as well as the pathomechanism of Alzheimer's disease.

Acknowledgments

We are grateful to Dr. Gopal Thinakaran for providing anti-PEN-2 antisera, Drs. Taisuke Tomita and Noriko Takahashi for helpful suggestions, Ms. Yumiko Ishii for technical assistance, and Dr. Takeshi Tabira for cooperation. This work was supported in part by grants from the Ministry of Health, Labour and Welfare in Japan, and the Organization for Pharmaceutical Safety and Research.

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