RNA Interference-mediated Silencing of X11α and X11β Attenuates Amyloid β-Protein Levels via Differential Effects on β-Amyloid Precursor Protein Processing*

Received for publication, December 21, 2004, and in revised form, February 4, 2005
Published, JBC Papers in Press, February 7, 2005, DOI 10.1074/jbc.M414353200

Zhongcong Xie‡§‡, Donna M. Romano‡‡, and Rudolph E. Tanzi‡‡§†

From the ‡Genetics and Aging Research Unit, Massachusetts General Institute for Neurodegenerative Disease, and the Departments of §Neurology and ¶Anesthesia and Critical Care, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129-4404

Processing of the β-amyloid precursor protein (APP) plays a key role in Alzheimer disease neuropathogenesis. APP is cleaved by β- and α-secretases to produce APP-C99 and APP-C83, which are further cleaved by γ-secretase to produce amyloid β-protein (Aβ) and p3, respectively. APP adaptor proteins with phosphotyrosine-binding domains, including X11α (MINT1, encoded by gene APBA1) and X11β (MINT2, encoded by gene APBA2), can bind to the conserved YENPTY motif in the APP C terminus. Overexpression of X11α and X11β alters APP processing and Aβ production. Here, for the first time, we have described the effects of RNA interference (RNAi) silencing of X11α and X11β expression on APP processing and Aβ production. RNAi silencing of APBA1 in H4 human neuroglioma cells stably transfected to express either full-length APP or APP-C99 increased APP C-terminal fragment levels and lowered Aβ levels in both cell lines by inhibiting γ-secretase cleavage of APP. RNAi silencing of APBA2 also lowered Aβ levels, but apparently not via attenuation of γ-secretase cleavage of APP. The notion of attenuating γ-secretase cleavage of APP via the APP adaptor protein X11α is particularly attractive with regard to therapeutic potential given that side effects of γ-secretase inhibition due to impaired proteolysis of other γ-secretase substrates, e.g. Notch, might be avoided.

The study of amyloidogenic β-amyloid precursor protein (APP)‡ processing at the gene, protein, and cellular levels has been a major focus of Alzheimer disease neuropathogenesis research since the isolation of the APP gene in 1987 (see reviews in Refs. 1–3). Genetic, neuropathological, and biochemi-

cal findings indicate that excessive production and/or accumulation of the amyloid β-peptide (Aβ) plays a fundamental role in the pathogenesis of Alzheimer disease (see reviews in Refs. 1–3). Aβ is produced from APP through proteolytic processing by two proteases, β- and γ-secretase. Specifically, APP is first hydrolyzed in the extracellular domain, either between Met671 and Asp772 or between residues 682 and 683, by the aspartyl protease β-site APP-cleaving enzyme or β-secretase, a type I transmembrane glycosylated aspartyl protease found in post-Golgi membranes and at the cell surface (4–7). This cleavage by β-secretase generates a 99-residue membrane-associated C-terminal fragment (APP-C99). APP-C99 is further cleaved to release 4-kDa Aβ and the β-APP intracellular domain. This cleavage is achieved by an unusual form of proteolysis in which the protein is cleaved within the transmembrane domain (at residue +40 or +42) by γ-secretase (8–10). APP, a single-pass integral transmembrane protein, is more routinely cleaved by α-secretase, at the site close to the transmembrane domain and in the middle of the Aβ region of APP, to release a large ectodomain (APPα), leaving a C-terminal fragment of 83 amino acids (APP-C83) in the membrane. Whereas proteolysis of APP-C99 by γ-secretase produces Aβ, proteolysis of APP-C83 by γ-secretase produces p3, a peptide resembling an N-terminally truncated form of Aβ (Refs. 11 and 12; see review in Ref. 13). Presenilin and γ-secretase co-fractionate as a detergent-sensitive high molecular mass complex (14) that includes at least three other proteins, nicastrin/APH-2, APH-1, and PEN-2, all of which are necessary for γ-secretase activity (Refs. 15–17; see review in Ref. 18).

γ-Secretase cleavage of the cytoplasmic tail of APP generates the APP intracellular domain, which contains an absolutely conserved YENPTY motif present in the cytodomains of several tyrosine kinase receptors and in non-receptor tyrosine kinase. In tyrosine kinase receptors, the tyrosine residue of this motif is phosphorylated upon tyrosine kinase activation, and the YENPTY motif functions as a docking site for the phosphotyrosine-binding domain present in several adaptor proteins, including the X11 family. X11α (MINT1) and X11β (MINT2), encoded by genes APBA1 and APBA2, respectively, bind to the YENPTY motif of APP (Refs. 19 and 20; see review in Ref. 21).

Overexpression of X11α and X11β has been shown previously to inhibit APP catabolism. Borg et al. (22) and Sastre et al. (23) revealed that overexpression of X11α can increase the APP half-life and reduce the levels of the secreted N-terminal ectodomain of APP (APPα) and Aβ in non-neuronal cells. Like X11α, X11β also stabilizes cellular APP and diminishes the levels of APPs and Aβ (24, 25). X11α and X11β can interact with presenilin-1 via their PDZ domains (26). Recent studies have shown that overexpression of X11α can impair APP traf-
ficking and may inhibit Aβ production (27). Furthermore, King et al. (28) suggested that X11α may specifically interfere with γ-secretase (but not β-secretase)-mediated cleavage of APP and Aβ production.

To date, the effects of reduced expression of APBA1 and APBA2 or X11α and X11β on APP processing and Aβ production have not been assessed. For this purpose, we established RNA interference (RNAi) for APBA1 and APBA2 in H4 cells overexpressing either full-length APP (APP-FL) or APP-C99 and evaluated the effects of RNAi-mediated silencing of APBA1 and APBA2 on APP processing and Aβ production.

EXPERIMENTAL PROCEDURES

Cell Lines—We used H4 naïve human neuroglioma cells and H4 cells stably transfected to express either APP-FL or APP-C99. APP-C99 is the product of β-secretase and therefore contains α- and γ-cleavage (but not β-cleavage) sites. This cell line provides a valid system to assess whether any effects on APP processing are dependent on γ-secretase-mediated APP processing and independent of β-secretase-mediated APP processing. All cell lines were cultured in high glucose Dulbecco's modified Eagle's medium containing 9% heat-inactivated fetal calf serum, 2 mM glutamine. Stably transfected H4 cells were additionally supplemented with 200 μg/ml G418.

RNAi and DAPT Treatment—Small interfering RNA (siRNA) duplexes designed against human APBA1, the gene encoding X11α (5'-GGATCTCGAGTCATCTGC-3'), and APBA2, the gene encoding X11β (5'-GGTGAAGTCCTCAACTTGTTC-3'), were obtained from Dr. Drummton. Scrambled siRNA (5'-AAATCTGCTACGTCCTC-3') (29) was used as the control siRNA. siRNAs were transfected into cells by electroporation (Amaxa Inc., Gaithersburg, MD). We mixed 1 million cells, 100 μl of amaxa electroporation transfection solution, and 10 μl of 20 μM siRNA together, and we used the C-9 program in the amaxa electroporation device for cell transfection. The transfected cells were placed in a 6-well plate containing 1.5 ml of cell culture medium. The cells were harvested 48 h after siRNA treatment. The γ-secretase inhibitor DAPT (250 μM, 18-h treatment) was employed in the experiments as a positive control.

Cell Lysis and Protein Quantification—Cell pellets were detergent-extracted on ice using 10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 2 mM EDTA, and 0.5% Nonidet P-40 plus protease inhibitors (1 μg/ml apro- tinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). The lysates were collected, centrifuged at 12,000 rpm for 10 min, and quantified for total proteins using the BCA protein assay kit (Pierce).

Western Blot Analysis of APP Processing—Western blot analysis was performed as described by Xie et al. (30). Briefly, 40 μg of total protein from each sample was subjected to SDS-PAGE using 4–20% gradient Tris/glycine gels (Invtogen) under reducing conditions. Next, the proteins were transferred to a polyvinylidene difluoride membrane (Bio Rad) using a semidy electrode transfer system (Amersham Biosciences). Nonspecific proteins were blocked using 5% nonfat dry milk in Tris-buffered saline/Tween for 1.5 h. Blots were then incubated with a primary antibody, followed by a secondary antibody (horseradish peroxidase-conjugated anti-rabbit antibody, 1:10,000 dilution; Pierce). Blots were washed with 1× Tris-buffered saline/Tween for 30 min between steps. Antibody H-265 (1:200 dilution; Santa Cruz Biotechnol- ogy, Inc., Santa Cruz, CA) was used to recognize X11α (105 kDa), and antibody N-20 (1:100 dilution; Santa Cruz Biotechnology, Inc.) was used to detect X11β (135 kDa). Antibody A8717 (1:1000 dilution; Sigma) was used to visualize APP-FL (110 kDa), APP-C83 (12 kDa), and APP-C99 (10 kDa) in Western blot analysis. The intensity of signals was analyzed using the NIH Image Program (Version 1.62). We quantified the Western blot images as follows. We used the levels of β-actin to normalize the levels of X11α, X11β, APP-FL, and the APP C-terminal fragment (APP-CTF) (i.e. determining the ratio of the X11α amount to the β-actin amount) to control for loading differences in total protein amounts. We present the changes in the protein levels of X11α, X11β, APP-FL, APP-C99, and APP-C83 in the cells treated with APBA1 or APBA2 siRNA as a percentage of those in the cells treated with control siRNA.

Quantification of Aβ Using Sandwich Enzyme-Linked Immunosorbent Assay—Following treatment with saline, electroporation, and treatment with siRNA (control, APBA1, or APBA2), the conditioned medium was collected, and secreted Aβ was measured by a sandwich enzyme-linked immunosorbent assay as described by Xie et al. (30). Briefly, 96-well plates were coated with mouse monoclonal antibody specific to Aβ40 (antibody 266) or Aβ42 (antibody 21F12). Following blocking with bovine serum albumin, the wells were incubated overnight at 4 °C with test samples of the conditioned cell culture medium, and horseradish peroxidase-conjugated anti-Aβ antibody HR1 was added. The plates were then developed with tetramethylbenzidine reagent, and absorbance was measured at 450 nm. Aβ levels in test samples were determined by comparison with the signal from unconditioned medium supplemented with known quantities of Aβ40 and Aβ42.

Statistics—Analysis of variance with repeated measurements was employed to compare the differences from the control group. p < 0.05 was considered statistically significant.

RESULTS

APBA1 RNAi Increases APP-CTF Levels and Decreases Aβ Levels in APP-FL-overexpressing H4 Cells—We first established conditions under which APBA1 siRNA treatment would successfully reduce X11α protein levels in H4 cells overexpressing APP-FL (H4-APP-FL cells). The cells were harvested 48 h after transfection with either control or APBA1 siRNA and then subjected to Western blot analyses in which antibody H-265 was used to visualize the protein levels of X11α. As shown in Fig. 1A, X11α immunoblotting revealed a visible reduction in the protein levels of X11α following APBA1 siRNA treatment (lanes 4–6) compared with control siRNA treatment (lanes 1–3). There was no significant difference in the amount of β-actin in control siRNA- or APBA1 siRNA-treated cells. We then quantified all Western blots using the NIH Image program. As shown in Fig. 1B, APBA1 siRNA treatment significantly reduced X11α protein levels by 61% (normalized to β-actin) compared with control siRNA treatment. These results indicated that RNAi for APBA1 significantly knocked down the protein levels of X11α.

We next assessed the effects of RNAi-mediated silencing of APBA1 on APP processing in H4-APP-FL cells by measuring the protein levels of APP-FL, APP-C99, and APP-C83 following APBA1 siRNA treatment. 48 h after transfection of APBA1 or control siRNA, the cells were harvested and subjected to Western blot analyses in which antibody A8717 was used to detect APP-FL, APP-C99, and APP-C83. The protein levels of APP-C99 and APP-C83 were increased in the cells treated with APBA1 siRNA (Fig. 1A, lanes 4–6) compared with those treated with control siRNA (lanes 1–3). As a positive control, the γ-secretase inhibitor DAPT was employed to induce the accumulation of APP-C99 and APP-C83 (lanes 7–9) compared with the control treatment (lanes 1–3). To confirm bands corresponding to APP-C99 and APP-C83, synthetic forms of APP-C99 and APP-C83 were employed as markers. No significant difference in the protein levels of APP-FL was observed in APBA1 siRNA-, control siRNA-, or DAPT-treated cells. We also assessed the protein levels of APP-FL, APP-C99, and APP-C83 in H4 naïve cells and found that they were less than those in H4-APP-FL cells. There was no significant difference in the amount of β-actin, in control siRNA-, APBA1 siRNA-, or DAPT-treated H4-APP-FL cells and H4 naïve cells.

Quantification of APP-FL, APP-C99, and APP-C83 (normalized to β-actin) revealed that APBA1 siRNA treatment led to a 270% increase in the ratio of APP-C99 to APP-FL (p < 0.05) (Fig. 1C) and a somewhat smaller increase (205%) in the ratio of APP-C83 to APP-FL (p < 0.05) (Fig. 1D) compared with control siRNA treatment. Used as a control, the γ-secretase inhibitor DAPT led to 310% (p < 0.05) (Fig. 1C) and 250% (p < 0.05) (Fig. 1D) increases in the ratios of APP-C99 and APP-C83 to APP-FL, respectively.

We next measured Aβ levels in the conditioned medium 48 h after treatment with control siRNA, APBA1 siRNA, or DAPT. Because Aβ42 was too low to be detected in many samples, we present only the changes in Aβ40 production from these experiments. As shown in Fig. 1E, both APBA1 siRNA (black bar) and DAPT (gray bar) treatment decreased Aβ levels to a sim-
FIG. 1. Effects of APBA1 RNAi on APP processing and Aβ levels in H4-APP-FL cells. In H4-APP-FL cells, APBA1 siRNA treatment decreased the protein levels of X11α, increased the protein levels of APP-C83 and APP-C99, and decreased Aβ production. A, APP processing in Western blot analyses. X11α immunoblotting showed reductions in the protein levels of X11α in the cells treated with APBA1 siRNA (lanes 4–6) compared with those treated with control siRNA (lanes 1–3) or saline (lanes 7–9). APP-FL immunoblotting revealed that there was no significant difference in the protein levels of APP-FL in the cells treated with control siRNA, APBA1 siRNA, or DAPT. Synthetic forms of APP-C99 and APP-C83 were used as markers to confirm the bands of APP-C99 and APP-C83 in the blot. APP-CTF immunoblotting showed increases in the protein levels of APP-C99 and APP-C83 in the cells treated with APBA1 siRNA (lanes 4–6) or DAPT (lanes 7–9) compared with those treated with control siRNA (lanes 1–3). The blot showing the band of APP-C83 only was the same blot with less exposure time in developing the film. The protein levels of APP-FL, APP-C83, and APP-C99 in H4-APP-FL cells (lanes 1–9) were higher than those in H4 naïve cells (lanes 10 and 11). There was no significant difference in the amount of β-actin in control siRNA, APBA1 siRNA, or DAPT-treated H4-APP-FL cells and H4 naïve cells. B, X11α protein levels assessed by quantifying X11α in the Western blot. APBA1 siRNA treatment (black bar) significantly decreased the protein levels of X11α compared with control siRNA treatment (white bar), *, p < 0.05 (normalized to β-actin). C, APP processing assessed by quantifying the ratio of APP-C99 to APP-FL in the Western blot. APBA1 siRNA (black bar) and DAPT (gray bar) treatment significantly increased the ratio of APP-C99 to APP-FL compared with control siRNA treatment (white bar), *, p < 0.05 (normalized to β-actin). D, APP processing assessed by quantifying the ratio of APP-C83 to APP-FL in the Western blot. APBA1 siRNA (black bar) and DAPT (gray bar) treatment significantly increased the ratio of APP-C83 to APP-FL compared with control siRNA treatment (white bar), *, p < 0.05 (normalized to β-actin). E, effects of APBA1 RNAi on Aβ production in H4-APP-FL cells. APBA1 siRNA (black bar) and DAPT (gray bar) treatment decreased Aβ40 production compared with control siRNA treatment (white bar), *, p < 0.05.
APBA2 RNAi Decreases Aβ Levels but Does Not Alter APP Processing in H4-APP-FL Cells—We next investigated whether the other X11 family protein, X11β (encoded by gene APBA2), can similarly affect APP processing and Aβ production in a manner similar to that of DAPT.

APBA2 RNAi treatment did not alter APP processing. However, 109 pg/ml (APBA1 siRNA) and 70 pg/ml (DAPT) versus 116 pg/ml (control siRNA). Collectively, these data indicated that RNAi silencing of APBA1 affected APP processing and Aβ production in a manner similar to that of DAPT.

APBA2 RNAi Decreases Aβ Levels in H4-APP-C99 Cells—To eliminate increased β-secretase cleavage of APP-FL as a possible explanation for increased APP-C99, we employed H4-APP-C99 cells. APP-C99 is the product of β-secretase and harbors α- and γ-cleavage (but not β-cleavage) sites. 48 h after transfection of H4-APP-C99 cells with APBA1 or control siRNA, the cells were harvested and subjected to Western blot analyses in which antibody H-265 was used to detect X11a. As shown in Fig. 3A, X11a immunoblotting revealed a visible reduction in the protein levels of X11a by 50% (p < 0.05) (Fig. 3B).

We next assessed the effects of RNAi-mediated silencing of APBA1 on processing of APP-C99. 48 h after transfection with APBA1 or control siRNA, the cells were harvested and subjected to Western blot analyses with antibody A8717. APBA1 siRNA treatment (Fig. 3A, lanes 5–7) did not alter the protein levels of APP-FL compared with control siRNA treatment (lanes 2–4). APP-CTF immunoblotting revealed visible increases in the protein levels of both APP-C99 and APP-C83 in the H4-APP-C99 cells treated with APBA1 siRNA (lanes 5–7) compared with those treated with control siRNA (lanes 2–4). We also assessed the protein levels of APP-C99 and APP-C83 in H4 naïve cells and found that they were less than those in H4-APP-C99 cells. There was no significant difference in the amount of β-actin in control siRNA- or APBA1 siRNA-treated H4-APP-C99 cells and H4 naïve cells. Quantification of APP-FL, APP-C99, and APP-C83 revealed that APBA1 siRNA treatment led to a 224% increase in the ratio of APP-C99 to APP-FL (p < 0.05) (Fig. 3C) and a 273% increase in the ratio of APP-C83 to APP-FL (p < 0.05) (Fig. 3D) compared with control siRNA treatment.

We then measured Aβ levels in the conditioned medium from H4-APP-C99 cells 48 h after transfection with either control or APBA1 siRNA. As shown in Fig. 3E, APBA1 siRNA decreased Aβ production compared with control siRNA treatment: 110 pg/ml (APBA1 siRNA) versus 164 pg/ml (control siRNA) (p < 0.05). These findings suggest that APBA1 RNAi inhibits γ-secretase (but not β-secretase)-mediated cleavage of APP.

APBA2 RNAi Does Not Alter APP Processing or Aβ Levels in H4-APP-C99 Cells—Finally, we assessed the effects of APBA2 RNAi on APP processing and Aβ production in H4-APP-C99 cells. 48 h after transfection with APBA2 or control siRNA, the cells were harvested and subjected to Western blot analyses in which antibody H-265 was used to detect the protein levels of X11β. X11β immunoblotting revealed a visible reduction in the protein levels of X11β (encoded by gene APBA2) in the cells treated with APBA2 siRNA (Fig. 4A, lanes 6–8) compared with those treated with control siRNA (lanes 3–5). There was no significant difference in the amount of β-actin in control siRNA- or APBA2 siRNA-treated cells. Quantification of X11β (normalized to β-actin) revealed that APBA2 siRNA treatment decreased the protein levels of X11β by 86% (p < 0.05) (Fig. 4B) compared with control siRNA treatment.

Immunoblotting with anti-APP antibody A8717 revealed no detectable difference in the protein levels of APP-FL, APP-C99, and APP-C83 in the cells treated with APBA2 siRNA (Fig. 4A, lanes 6–8) compared with those treated with control siRNA (lanes 3–5). We also assessed the protein levels of APP-C99 and APP-C83 in H4 naïve cells and found that they were less than those in H4-APP-C99 cells. There was no significant difference in the amount of β-actin in control siRNA- or APBA2 siRNA-treated H4-APP-C99 cells and H4 naïve cells. Quantification of APP-FL, APP-C99, and APP-C83 revealed that APBA2 siRNA treatment led to a 224% increase in the ratio of APP-C99 to APP-FL (p < 0.05) (Fig. 3C) and a 273% increase in the ratio of APP-C83 to APP-FL (p < 0.05) (Fig. 3D) compared with control siRNA treatment.

We next measured Aβ levels in the conditioned medium from H4-APP-C99 cells 48 h after transfection with either control or APBA2 siRNA. As shown in Fig. 3E, APBA2 siRNA decreased Aβ production compared with control siRNA treatment: 110 pg/ml (APBA1 siRNA) versus 164 pg/ml (control siRNA) (p < 0.05). These findings suggest that APBA1 RNAi inhibits γ-secretase (but not β-secretase)-mediated cleavage of APP.
FIG. 2. Effects of APBA2 RNAi on APP processing and Aβ levels in H4-APP-FL cells. In H4-APP-FL cells, APBA2 siRNA treatment decreased the protein levels of X11β; did not alter the protein levels of APP-FL, APP-C83, and APP-C99; and decreased Aβ production. A, APP processing in Western blot analyses. X11β immunoblotting showed reductions in the protein levels of X11β in the cells treated with APBA2 siRNA (lanes 4–6) compared with those treated with control siRNA (lanes 1–3) or saline (lanes 7–9). APP-FL immunoblotting revealed that there was no significant difference in the protein levels of APP-FL in the cells treated with control siRNA, APBA2 siRNA, or DAPT. Synthetic forms of APP-C99 and APP-C83 were used as markers to confirm the bands of APP-C99 and APP-C83 in the blot. APP-CTF immunoblotting showed increases in the protein levels of APP-C99 and APP-C83 in the cells treated with APBA2 siRNA (lanes 4–6), compared with controls (lanes 1–3). The protein levels of APP-FL, APP-C83, and APP-C99 in H4-APP-FL cells (lanes 1–9) were higher than those in H4 naïve cells (lanes 10 and 11). β-Actin immunoblotting revealed that there was no significant difference in the amount of β-actin in the control siRNA-, APBA2 siRNA-, or DAPT-treated H4-APP-FL cells and H4 naïve cells. B, X11β protein levels assessed by quantifying X11β in the Western blot. APBA2 siRNA treatment (black bar) significantly decreased the protein levels of X11β compared with control siRNA treatment (white bar). *, p < 0.05 (normalized to β-actin). C, APP processing assessed by quantifying the ratio of APP-C99 to APP-FL in the Western blot. APBA2 siRNA treatment (black bar) did not alter the ratio of APP-C99 to APP-FL compared with control siRNA treatment (white bar) (normalized to β-actin). DAPT treatment (gray bar) increased the ratio of APP-C99 to APP-FL compared with control treatment. *, p < 0.05 (normalized to β-actin). D, APP processing assessed by quantifying the ratio of APP-C83 to APP-FL in the Western blot. APBA2 siRNA treatment (black bar) did not alter the ratio of APP-C83 to APP-FL compared with control siRNA treatment (white bar) (normalized to β-actin). DAPT treatment (gray bar) increased the ratio of APP-C83 to APP-FL compared with control treatment, *, p < 0.05 (normalized to β-actin). E, effects of APBA2 RNAi on Aβ production in H4-APP-FL cells. Both APBA2 siRNA (black bar) and DAPT (gray bar) treatment decreased Aβ40 production compared with control siRNA treatment (white bar). *, p < 0.05.
FIG. 3. Effects of APBA1 RNAi on APP processing and Aβ levels in H4-APP-C99 cells. In H4-APP-C99 cells, APBA1 siRNA treatment decreased the protein levels of X11α, increased the protein levels of APP-C83 and APP-C99, and decreased Aβ production. A, APP processing in Western blot analyses. X11α immunoblotting showed reductions in the protein levels of X11α in the cells treated with APBA1 siRNA (lanes 5–7) compared with those treated with control siRNA (lanes 2–4). APP-FL immunoblotting revealed that there was no significant difference in the protein levels of APP-FL in the cells treated with control or APBA1 siRNA. Synthetic forms of APP-C99 and APP-C83 were used as markers (lane 1) to confirm the bands of APP-C99 and APP-C83 in the cells treated with APBA1 siRNA (lanes 5–7) compared with those treated with control siRNA (lanes 2–4). The blot showing the band of APP-C83 only was the same blot with less exposure time in developing the film. The protein levels of APP-C83 and APP-C99 in H4-APP-C99 cells (lanes 2–7) were higher than those in H4 naïve cells (lanes 8 and 9). There was no significant difference in the amount of β-actin in the control siRNA- or APBA1 siRNA-treated H4-APP-C99 cells and H4 naïve cells. B, X11α protein levels assessed by quantifying X11α in the Western blot. APBA1 siRNA treatment (black bar) significantly decreased the protein levels of X11α compared with control siRNA treatment (white bar). *, p < 0.05 (normalized to β-actin). C, APP processing assessed by quantifying the ratio of APP-C99 to APP-FL in the Western blot. APBA1 siRNA treatment (black bar) significantly increased the ratio of APP-C99 to APP-FL compared with control siRNA treatment (white bar). *, p < 0.05 (normalized to β-actin). D, APP processing assessed by quantifying the ratio of APP-C83 to APP-FL in the Western blot. APBA1 siRNA treatment (black bar) significantly increased the ratio of APP-C83 to APP-FL compared with control siRNA treatment (white bar). *, p < 0.05 (normalized to β-actin). E, effects of APBA1 RNAi on Aβ production in H4-APP-C99 cells. APBA1 siRNA treatment (black bar) decreased Aβ40 production compared with control siRNA treatment (white bar). *, p < 0.05.
FIG. 4. Effects of APBA2 RNAi on APP processing and Aβ levels in H4-APP-C99 cells. In H4-APP-C99 cells, APBA2 siRNA treatment decreased the protein levels of X11β, but did not alter the protein levels of APP-FL, APP-C83, and APP-C99 or Aβ production. A, APP processing in Western blot analyses. X11β immunoblotting showed reductions in the protein levels of X11β in the cells treated with APBA2 siRNA (lanes 6–8) compared with those treated with control siRNA (lanes 3–5). APP-FL immunoblotting revealed that there was no significant difference in the protein levels of APP-FL in the cells treated with control or APBA2 siRNA. Synthetic forms of APP-C99 and APP-C83 were used as markers (lane 1) to confirm the bands of APP-C99 and APP-C83 in the blot. APP-CTF immunoblotting showed no significant difference in the protein levels of APP-C99 and APP-C83 in the cells treated with APBA2 siRNA (lanes 6–8) compared with those treated with control siRNA (lanes 3–5). The blot showing the bands of APP-C83 only was the same blot with less exposure time in developing the film. The protein levels of APP-C83 and APP-C99 in H4-APP-C99 cells (lanes 3–8) were higher than those in H4 naive cells (lanes 2 and 9). There was no significant difference in the amount of β-actin in the control siRNA- or APBA2 siRNA-treated H4-APP-C99 cells and H4 naive cells. B, X11β protein levels assessed by quantifying X11β in the Western blot. APBA2 siRNA treatment (black bar) significantly decreased the protein levels of X11β compared with control siRNA treatment (white bar). *, p < 0.05 (normalized to β-actin). C, APP processing assessed by quantifying the ratio of APP-C99 to APP-FL (normalized to β-actin). D, APP processing assessed by quantifying the ratio of APP-C83 to APP-FL (normalized to β-actin). E, effects of APBA2 RNAi on Aβ production in H4-APP-C99 cells. APBA2 siRNA treatment (black bar) did not change Aβ40 production compared with control siRNA treatment (white bar).
treated H4-APP-C99 cells and H4 naive cells. Quantification of APP-FL, APP-C99, and APP-C83 revealed that APBA2 siRNA treatment did not significantly alter the ratios of APP-C99 (Fig. 4C) and APP-C83 (Fig. 4D) to APP-FL. We next measured Aβ levels in the conditioned medium from H4-APP-C99 cells 48 h after treatment with control or APBA2 siRNA. As shown in Fig. 4E, APBA2 siRNA treatment did not significantly alter Aβ production compared with control siRNA treatment: 173 pg/ml (control siRNA) versus 167 pg/ml (APBA2 siRNA). Collectively, these results suggest that, in contrast to APBA1 RNAi, APBA2 RNAi does not alter APP processing or Aβ production in H4-APP-C99 cells.

Control siRNA or Electroporation Does Not Affect APP Processing or Aβ Levels in H4-APP-FL and H4-APP-C99 Cells—In a control experiment, we also assessed the effects of control siRNA or electroporation on APP processing and Aβ levels in H4-APP-FL and H4-APP-C99 cells. We found that control siRNA or electroporation did not affect APP processing or alter Aβ levels compared with saline treatment in H4-APP-FL and H4-APP-C99 cells (data not shown). These results confirmed that the effects of APBA1 or APBA2 RNAi on APP processing and Aβ levels in our experiments were not due to electroporation or control siRNA (scrambled siRNA), but to the reduction in the protein levels of X11α and X11β.

**DISCUSSION**

Aβ, the key component in senile plaques, is derived from APP via cleavage by two proteases, β- and γ-secretase (4–7). Cleavage by β-secretase first generates APP-C99, which is further cleaved by γ-secretase to release Aβ and the APP intracellular domain (8–10). APP is also cleaved by α-secretase to release a large ectodomain (APPα) and APP-C83, and APP-C83 is sequentially cleaved by γ-secretase to produce β and the APP intracellular domain (Refs. 11 and 12; see review in Ref. 13). Several APP adaptor proteins (see review in Ref. 21), including X11α and X11β, have been shown previously to affect APP processing and Aβ production following overexpression (22–25, 27, 28, 31). Specifically, overexpression of X11α and X11β has been shown to increase APP levels, to prolong the APP half-life, to decrease APPs, and to decrease Aβ levels (22–25, 27, 28, 31). However, the effects of knockdown of X11α and X11β on APP processing and Aβ production have not been reported previously. We have shown here, for the first time, that RNAi silencing of APBA1 and APBA2 (encoding X11α and X11β, respectively) significantly affects APP processing and Aβ levels.

RNAi silencing of APBA1 led to increased levels of APP-C99 and APP-C83 without alterations in APP-FL (Fig. 1A, C, and D). Moreover, RNAi for APBA1 significantly decreased Aβ levels (Fig. 1E). Theoretically, the observed increases in APP-C99 and APP-C83 levels following APBA1 siRNA treatment could be due either to increases in the activities of β- and/or α-secretase or to decreases in γ-secretase cleavage of APP-C99 and APP-C83. To discern between these two possibilities, we have shown that, in H4 cells overexpressing APP-C99 (the β-secretase cleavage product of APP), APBA1 siRNA treatment still increased the protein levels of APP-C99 and APP-C83 and decreased the levels of Aβ in the conditioned medium. These data indicate that the observed changes in APP processing and Aβ levels following APBA1 RNAi are independent of β-secretase-mediated cleavage of APP and are most likely due to inhibition of γ-secretase-mediated cleavage of APP.

We next investigated whether the effects of APBA1 RNAi on γ-secretase-mediated cleavage of APP processing and Aβ production could be mimicked by RNAi silencing of X11β (encoded by APBA2). APBA2 RNAi treatment decreased Aβ levels (Fig. 2E), but did not alter the protein levels of APP-FL (Fig. 2A), APP-C99 (Fig. 2, A and C), or APP-C83 (Fig. 2, A and D) in H4-APP-FL cells. In contrast, APBA2 RNAi treatment of H4-APP-C99 cells did not alter the protein levels of APP-FL (Fig. 4A), APP-C99 (Fig. 4, A and C), or APP-C83 (Fig. 4, A and D) or Aβ levels (Fig. 4E). These findings suggest that, unlike the situation with APBA1, the alterations in Aβ levels following RNAi silencing of APBA2 most likely do not involve alterations in γ-secretase cleavage, but may involve changes in β-secretase cleavage of APP. It is also possible that APBA2 RNAi regulates Aβ levels through other mechanisms such as Aβ degradation. Further studies will be necessary to discern between these possibilities.

Taken together, our findings demonstrate, for the first time, that RNAi knockdown of X11α attenuates γ-secretase-mediated cleavage of APP, leading to increased accumulation of APP-C99 and APP-C83 and decreased Aβ levels. Overexpression of X11α has also been shown to inhibit APP catabolism (22–25, 27, 28, 31), including increases in APP-C99 levels and decreases in Aβ levels (28). Thus, it is curious that, in this study, RNAi-mediated silencing of X11α led to similar and not opposite effects on APP processing and Aβ levels compared with the X11α overexpression studies. The phosphorytosine-binding domain of X11α (Ref. 19; see review in Ref. 21) has been shown to bind the YENPTY motif, located in the APP-CTFs, whereas the PDZ domain of X11α (26) has been reported to bind to presenilin-1 (Ref. 14; see review in Ref. 21). Based on these findings, one might expect that overexpression of X11α could, on one hand, impair clathrin-coated pit internalization of APP by binding the YENPTY motif and consequently lower Aβ levels by blocking entry of APP into the endocytic pathway and could, on the other hand, conceivably enhance γ-secretase cleavage of APP by enhancing its interaction with the γ-secretase component presenilin-1. Previous studies employing overexpression of X11α revealed the net result to be increased accumulation of APP-CTFs and lower levels of Aβ, consistent with decreased γ-secretase-mediated APP processing (22–25, 27, 28, 31). This suggests that the potential effects of X11α on APP endocytosis outweigh those on interactions with presenilin. Further studies will be necessary to investigate this possibility.

Along similar lines, knockdown of X11α expression might be expected to attenuate γ-secretase-mediated APP processing based on decreased interaction with presenilin while potentiating γ-secretase-mediated APP processing by allowing for greater access to the YENPTY motif utilized for clathrin-mediated endocytosis of APP. Our APBA1 RNAi silencing experiments revealed the net result to be similar to that of the previous X11α overexpression studies: increased accumulation of APP-CTF and reduced Aβ levels due to decreased γ-secretase-mediated APP processing. Collectively, the combined X11α overexpression and knockdown studies suggest that X11α levels must be carefully regulated (neither too high nor too low) to properly maintain normal γ-secretase-mediated APP processing and Aβ production. Interestingly, RNAi silencing of X11β led to a moderate decrease in Aβ levels in H4-APP-FL cells, but not in H4-APP-C99 cells. In addition, APBA2 RNAi treatment did not alter APP processing in either cell line, indicating that γ-secretase-mediated APP processing was most likely not affected. One possibility is that the reduction in Aβ levels following APBA2 siRNA treatment involves alterations in either β-secretase-mediated APP processing or Aβ degradation. Further investigation will be necessary to address these possible explanations.

X11α and X11β can also interact with proteins other than APP, including Munc18-1, a protein essential for synaptic vesicle exocytosis (32), and neurexins, which are neuron-specific cell-surface proteins that act as receptors for the excitatory neurotransin α-lactotoxin (32). Both X11α and X11β bind the
cytoplasmic tail of neurexin I, presumably via their PDZ domains (32). X11α (but not X11β) interacts with CASK-Veli to form a heterotrimERIC complex, mediating transmembrane receptor proteins in polarized cells (33, 34). The PDZ domain of X11α has also been reported to interact with several other proteins, including presenilin-1 (26), a presynaptic voltage-gated calcium channel (35), spinophilin/neurabin II (36), the copper chaperone of superoxide dismutase-1 (37), and the dendritic kinesin KIF17 (38). Because many of these proteins can mediate synaptic function, X11α may have a scaffolding/adapter function in the pre- and postsynaptic complex (see review in Ref. 21). It will be important in future studies to assess the effects of RNAi for X11 on the metabolism of these other X11-interacting proteins.

In conclusion, RNAi-mediated silencing of the APP adaptor protein X11α attenuates γ-secretase-mediated cleavage of APP, leading to decreased Aβ levels. In contrast, RNAi silencing of X11β also lowers Aβ levels, but without effects on γ-secretase-mediated APP processing. Our findings, together with those of previous studies, suggest that pharmacological modulation of X11α, X11β, and perhaps other APP adaptor proteins might potentially serve as a novel therapeutic approach to treating and preventing Alzheimer disease. The notion of attenuating γ-secretase cleavage of APP via APP adaptor proteins such as X11α is particularly attractive given that potential unwanted effects of γ-secretase inhibition due to impaired proteolysis of other γ-secretase substrates, e.g. Notch, might be avoided. Future studies will be necessary to further investigate these findings and to assess the feasibility of such a therapeutic strategy aimed at lowering Aβ levels.

Acknowledgment—We thank Dr. Weiming Xia (Director, Aβ ELISA Core Facility, Center for Neurological Diseases, Harvard Institute of Medicine, Harvard Medical School, Boston, MA) for the measurement of Aβ levels.

REFERENCES

1. Tanzi, R. E., and Bertram, L. (2001) Neuron 32, 181–184
2. Selkoe, D. J. (2001) Physiol. Rev. 81, 741–766
3. Sisodia, S. S., and St. George-Hyslop, P. H. (2002) Nat. Rev. Neurosci. 3, 281–290
4. Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loeffer, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lilo, J., Jarosinski, M. A., Biere, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Treanor, J., Rogers, G., and Citron, M. (1999) Science 286, 735–741
5. Hussain, I., Powell, D., Howlett, D. R., Tew, D. G., Meek, T. D., Chapman, C., Gisser, J. S., Murphy, K. E., Southan, C. D., Ryan, D. M., Smith, T. S., Simmons, D. L., Walsh, F. S., Dingwall, C., and Christie, G. (1999) Mol. Cell. Neurosci. 14, 419–427
6. Sinha, S., and Lieberburg, I. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11049–11053
7. Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brashear, J. R., Stratman, N. C., Mathews, W. R., Buhl, A. E., Carter, D. B., Tomasselli, A. G., Parodi, I. A., Heinrikson, R. L., and Gurney, M. E. (1999) Nature 402, 53–57
8. Gu, Y., Misonou, H., Sato, T., Dohmae, N., Takin, K., and Ihara, Y. (2001) J. Biol. Chem. 276, 35235–35238
9. Sastre, M., Steiner, H., Fuchs, K., Capell, A., Multhaup, G., Condron, M. M., Teplow, D. B., and Haass, C. (2001) EMBO Rep. 2, 835–841
10. Yu, C., Kim, S. H., Ikeuchi, T., Xu, H., Gasparini, L., Wang, R., and Sisodia, S. S. (2001) J. Biol. Chem. 276, 43756–43760
11. Lee, J. H., Lau, K. F., Perkinton, M. S., Standen, C. L., Shemilt, S. J., Mercken, M., Maxwell, M., Hai, B., Ellis, M. C., Parks, A. L., Xu, W., Li, J., Gurney, M., Myers, R. L., Himes, C. S., Hiebsch, R., Ruble, C., Nye, J. S., and Curtis, D. (2002) Dev. Cell 3, 85–97
12. Steiner, H., Winkler, E., Edsberg, D., Prokop, S., Basset, G., Yamashita, A., Kostka, M., and Haass, C. (2002) J. Biol. Chem. 277, 39062–39065
13. Yu, G., Nishimura, M., Arawaka, S., Levine, D., Zhang, L., Tandon, A., Song, Y. Q., Roganov, E., Chen, F., Kawarai, T., Supapa, A., Levesque, L., Yu, H., Yang, D. S., Holmes, E., Milman, P., Liang, Y., Zhang, D. M., Xu, D. H., Sato, C., Roganov, E., Smith, M., Janus, C., Zhang, Y., Aebersold, R., Farrer, L. S., Sorbi, S., Bruni, A., Fraser, P., and St. George-Hyslop, P. (2000) J. Biol. Chem. 275, 2629–2641
14. De Strooper, B. (2003) Neuron 38, 9–12
15. Borg, J.-P., Oon, J., Levy, E., and Margolis, B. (1996) Mol. Cell. Biol. 16, 6229–6241
16. McLoughlin, D. M., and Miller, C. C. (1996) FEBS Lett. 397, 197–200
17. King, G. D., and Scott Turner, R. (2004) Exp. Neurol. 185, 208–219
18. Borg, J.-P., Yang, Y., De Taddeo-Borg, M., Margolis, B., and Turner, R. S. (1998) J. Biol. Chem. 273, 14761–14766
19. Sastre, M., Steiner, H., and Turner, E. (1998) J. Biol. Chem. 273, 22351–22357
20. McLoughlin, D. M., Irving, N. G., Brownlee, J., Brion, J. P., Leroy, K., and Miller, C. C. (1999) Eur. J. Neurosci. 11, 1888–1904
21. Biedere, T., Cao, X., Sudhoff, T. C., and Liu, X. (2002) J. Neurosci. 22, 7340–7346
22. Lau, K. F., McLoughlin, D. M., Standen, C., and Miller, C. C. (2004) Mol. Cell. 17, 7347–7354
23. King, G. D., Perez, R. G., Steinhilb, M. L., Gaut, J. R., and Turner, R. S. (2003) Neuroscience 120, 143–154
24. King, G. D., Cherian, K., and Turner, R. S. (2004) J. Neurochem. 88, 971–982
25. Luo, W. J., Wang, H., Li, H., Kim, B. S., Shah, S., Lee, J. H., Shinakara, G., Kim, T. W., Yu, G., and Xu, H. (2003) J. Biol. Chem. 278, 7750–7754
26. Xie, Z., Moir, R., Romano, D. M., Tesco, G., Kovacs, D., and Tanzi, R. (2004) Neurodegenerative Dis. 1, 29–37
27. Lee, J. H., Lau, K. F., Perkins, M. S., Standen, C. L., Shemilt, S. J., Mercken, L., Cooper, J. D., McLoughlin, D. M., and Miller, C. C. (2003) J. Biol. Chem. 278, 47025–47029
28. Biedere, T., and Sudhoff, T. C. (2000) J. Biol. Chem. 275, 39803–39806
29. Borg, J.-P., Lopez-Figueroa, M. O., De Taddeo-Borg, M., Krom, D. E., Turner, R. S., Watson, S. J., and Margolis, B. (1999) J. Neurosci. 19, 1307–1316
30. Whitfield, C. W., Benard, C., Barnes, T., Hekimi, S., and Kim, S. K. (1999) J. Biol. Chem. 274, 43756–43760
31. Ide, N., Hata, Y., Irie, M., Deguchi, M., Yao, I., Satoh, A., Wada, M., Takahashi, K., Nakashima, H., and Takai, Y. (1998) Biochem. Biophys. Res. Commun. 244, 258–262
32. McLoughlin, D. M., Standen, C. L., Lau, K. F., Ackerley, S., Bartnikas, T. P., Gitlin, J. D., and Miller, C. C. (2001) J. Biol. Chem. 276, 9305–9307
33. Setou, M., Nakagawa, T., Seog, D. H., and Hirokawa, N. (2000) Science 288, 1796–1802