PAR-4 Is Involved in Regulation of β-Secretase Cleavage of the Alzheimer Amyloid Precursor Protein*

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Mounting evidence indicates that aberrant production and aggregation of amyloid β-peptide (Aβ)-(1–42) play a central role in the pathogenesis of Alzheimer disease (AD). Aβ is produced when amyloid precursor protein (APP) is cleaved by β- and γ-secretases at the N and C termini of the Aβ domain, respectively. The β-secretase is membrane-bound aspartyl protease, most commonly known as BACE1. Because BACE1 cleaves APP at the N terminus of the Aβ domain, it catalyzes the first step in Aβ generation. PAR-4 (prostate apoptosis response-4) is a leucine zipper protein that was initially identified to be associated with neuronal degeneration and aberrant Aβ production in models of AD. We now report that the C-terminal domain of PAR-4 is necessary for forming a complex with the cytosolic tail of BACE1 in co-immunoprecipitation assays and in vitro pull-down experiments. Overexpression of PAR-4 significantly increased, whereas silencing of PAR-4 expression by RNA interference significantly decreased, β-secretase cleavage of APP. These results suggest that PAR-4 may be directly involved in regulating the APP cleavage activity of BACE1. Because the increased BACE1 activity observed in AD patients does not seem to arise from genetic mutations or polymorphisms in BACE1, the identification of PAR-4 as an endogenous regulator of BACE1 activity may have significant implications for developing novel therapeutic strategies for AD.

Amyloid plaques, one of the characteristic lesions found in the brains of patients with Alzheimer disease (AD), are primarily extracellular deposits of amyloid β-peptide (Aβ) (1–12). Aβ is produced when amyloid precursor protein (APP) is cleaved by β- and γ-secretases at the N and C termini of the Aβ domain, respectively (3, 5–8, 13–36). The β-secretase is membrane-bound aspartyl protease, named BACE1 (β-site APP-cleaving enzyme-1; also known as Asp2 or memapsin-2) (1, 5, 6, 16, 18, 34, 37–75). The γ-secretase is a protein complex consisting of presenilin, nicastrin, Aph-1, Pen-2, and possibly other protein subunits (19, 22, 35, 76–88). Aberrant processing of APP leading to increased production and aggregation of Aβ is a neurotoxic event and may play a central role in the pathogenesis of AD (1–88).

The complete coding sequence of human BACE1 mRNA is ~1.5 kb long and encodes a pro-BACE1 protein, which is further cleaved by furin and other members of the furin family of convertases to remove the N-terminal propeptide domain within the trans-Golgi network (5, 48, 49). The cleavage occurs at the sequence RLPRΔE, a furin recognition motif. Mature BACE1 is formed when cleaved immature BACE1 is further modified by N-linked glycosylation (46, 55, 57, 66). BACE1 mRNA is found in neurons of all brain regions (47, 64, 72). Cleavage of APP by β-secretase (BACE1) generates a soluble N-terminal fragment (soluble APPβ) and a membrane-bound C-terminal fragment (known as CTF99). Cleavage of CTF99 by γ-secretase produces Aβ (1–88). In vitro, BACE1 was shown to be able to generate two APP C-terminal fragments (CTFs) starting at either Asp1 (CTF99) or Glu11 (CTF89) of the Aβ sequence (47, 49). Alternatively, cleavage of APP at the α-secretase site generates a soluble N-terminal fragment (soluble APPα) and leaves the C-terminal fragment (known as CTF83) containing the APP transmembrane domain and cytoplasmic tail (89–93). BACE1 is a type I membrane protein with a single transmembrane segment linking a luminal catalytic unit to a C-terminal cytosolic tail. The newly synthesized pro-BACE1 is processed to mature protease by furin during transit through the secretory pathway to the cell surface (1, 5, 6, 16, 18, 34, 37–75). The observation that mice with a targeted deletion of BACE1 are viable and fertile but fail to produce any Aβ indicates that BACE1 is required for Aβ generation (6, 40, 51, 62). Because neither genetic mutations nor polymorphisms in BACE1 seem to be responsible for the increased BACE1 activity observed in AD patients (1, 42, 47, 51), the identification of factors that effectively regulate BACE1 activity is apparently a highly promising approach to treating AD.

PAR-4 (prostate apoptosis response-4) is a leucine zipper protein that was initially identified to be associated with neuronal degeneration in AD (94). The levels of Par-4 mRNA and protein were found to be increased in tissue from vulnerable brain regions of AD patients compared with age-matched control patients. Double labeling analysis using antibodies against phosphorylated tau (antibody PHF-1, a marker of neurofibrillary tangle-bearing neurons) revealed that ~30–50% of tangle-bearing neurons are also PAR-4 positive (94). The levels of PAR-4 in vulnerable neurons are effectively induced by insults relevant to the pathogenesis of AD, such as trophic factor withdrawal or aberrant elevations in intracellular calcium levels. Additional data suggest that PAR-4 exerts its cell death-promoting action in the early stages of cell death prior to caspase activation and mitochondrial alterations (94). PAR-4...
also increases the production of neurotoxic Aβ in transfected neural cells (95, 96). An essential role for PAR-4 in aberrant Aβ production and neuronal apoptosis was demonstrated in studies of cultured primary rat hippocampal neurons and PC12 cells since inhibition of PAR-4 activity significantly diminishes Aβ production and attenuates apoptosis induced by Aβ or Alzheimer presenilin-1 (PS-1) mutations (95, 96). Of importance, the adverse effects of PAR-4 seem to require its interaction with other proteins since a deletion mutant of PAR-4 lacking a protein/protein interaction domain in its C-terminal region does not enhance Aβ production, and overexpression of this domain of PAR-4 blocks PAR-4 activity in a dominant-negative fashion (95, 96). Apoptosis-antagonizing transcription factor (AATF), another leucine zipper protein, is an interaction partner and potent inhibitor of PAR-4 activity (95, 96). AATF was initially identified as an interaction partner of Dlk (DAP-like kinase), a member of the death-associated protein kinase family of pro-apoptotic serine/threonine kinases (96–98). AATF binds directly with PAR-4 via the leucine zipper domain and blocks the activity of PAR-4 in enhancing Aβ production (96). These data strongly suggest that PAR-4 may, via protein/protein interactions, regulate not only apoptotic pathways, but also amyloidogenic processing of APP. We now report that the C-terminal domain of PAR-4 formed a complex with the cytosolic tail of BACE1 and increased β-secretase cleavage of APP. In hippocampal neurons, RNA interference (RNAi)-mediated silencing of Par-4 resulted in decreased APP cleavage activity of BACE1.

MATERIALS AND METHODS

Transfection of IMR-32 Cells and Trophic Factor Withdrawal—The methods used were described in our previous studies (94, 96, 99–101). In brief, human neuroblastoma IMR-32 cells (American Type Culture Collection) were maintained at 37 °C in an atmosphere of 95% air and 5% CO2 in Eagle’s minimal essential medium supplemented with non-essential amino acids and 10% heat-inactivated fetal bovine serum. A full-length Par-4 cDNA was subcloned into the expression vector pRcCMV, yielding the recombinant construct pCMV-Par-4, which encodes a 1.2-kb RNA species and a full-length 38-kDa PAR-4 protein (94, 102). AΔCTF, which contains the C-terminal region of PAR-4 (156–318), was similarly subcloned into the pRcCMV expression vector, yielding the recombinant construct pCMV-Par-4ΔCTF, which encodes an ~700-hp RNA species or the N-terminal half (i.e., ~18 kDa) of the PAR-4 protein (94, 102). A cDNA fragment containing the complete human pro-Par-4 (from a 700-bp RNA species) was obtained from Dr. Joseph Schachner (University of California, San Diego) and inserted into the pEGFP vector (Invitrogen). The recombinant construct pEFP4-BACE1 was constructed using full-length human BACE1. Human IMR-32 cell lines stably expressing Par-4 and Par-4ΔCTF were established by transfection using Lipofectamine 2000 reagent (Invitrogen) with pCMV-Par-4 or pCMV-Par-4ΔCTF. Transfected cells were selected with G418 (400 µg/ml) for 4 weeks, and surviving clones were selected. IMR-32 cells expressing human BACE1 were similarly established, except that the transfected cells were selected with hygromycin (400 µg/ml). Additional double-transfected IMR-32 cell lines were generated in which two proteins (PAR-4/BACE1 and PAR-4ΔCTF/BACE1) were coexpressed. For control purposes, parallel cultures of IMR-32 cells were stably transfected with pRcCMV and pEFP4 vectors alone. After the cells became confluent in the culture flasks, the culture medium was replaced with fresh medium and incubated for 48 h at 37 °C to condition the medium for Aβ measurement. Trophic factor withdrawal was initiated by washing cultures four times with Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl2, 1.0 mM MgCl2, 3.6 mM NaHCO3, 5 mM glucose, and 5 mM HEPES, pH 7.2) with subsequent incubation in 1 ml of Locke’s buffer.

Immunoprecipitation and Western Blot Analysis—The levels of expression of PAR-4 and BACE1 were determined by Western blot analysis as described (94, 96, 101). The antibodies used that specifically recognize PAR-4 were polyclonal rabbit and mouse monoclonal antibodies raised against full-length rat PAR-4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). These antibodies react with PAR-4 of mouse, rat, and human origin and recognize both full-length PAR-4 as well as the deletion mutant of PAR-4. The anti-APP antibody used was a rabbit polyclonal antibody raised against a 22-amino acid synthetic peptide derived from the C terminus of human APP (Zymed Laboratories Inc., South San Francisco, CA). The anti-BACE1 antibodies used were rabbit antiseraum raised against mature human BACE1 (a generous gift from Dr. Jordan Tang) and rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acids 458–501 of human BACE1 (Chemicon International, Inc., Temecula, CA). For immunoprecipitation, aliquots of cell lysates containing 200 µg of protein were incubated for 1 h at 4 °C with appropriate dilutions of anti-PAR-4 or anti-BACE1 antibody in immunoprecipitation buffer (150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 2 µg/ml peptatin A, 0.25 mM phenylmethylsulfonyl fluoride, and 50 mM Tris, pH 7.6). Protein A-Sepharose resin (30 µl/sample; Amersham Biosciences) was then added to the extracts for collection of the immunocomplexes, which were then washed three times with immunoprecipitation buffer and solubilized by heating in Laemmli buffer containing 2-mercaptoethanol at 100 °C for 4 min. The solubilized proteins were separated by electrophoresis on a 4–12% gradient SDS-polyacrylamide gel and transferred to a nitrocellulose sheet. For Western blot analysis, the nitrocellulose sheet was blocked with 5% milk, followed by a 1-h incubation in the presence of anti-APP, anti-BACE1, or anti-PAR-4 primary antibody. The membrane was further processed using horseradish peroxidase-conjugated secondary antibody, and immunoactivity was detected by chemiluminescence using the ECL system (Amersham Biosciences). To examine whether PAR-4 interacts with PS-1, an affinity-purified rabbit anti-PS-1 polyclonal antibody (103) was used in the immunoprecipitation/Western blot analysis. Equal loading was verified by probing the blots with anti-tubulin antibody (Sigma). Western blot images were acquired and quantified using Image Station 2000R and Densitometry 1D Version 3.6 software (Eastman Kodak Co).

Hippocampal Neuronal Cultures and Quantification of Aβ(1–40) and Aβ(1–42) by Sandwich Enzyme-linked Immunosorbent Assays (ELISAs)—Dissociated hippocampal cell cultures were prepared from postnatal day 1 mouse brains using methods similar to those described previously (100). Briefly, hippocampi were removed from mouse brain and incubated for 15 min in Ca2+ - and Mg2+-free Hank’s balanced salt solution (HBSS) containing 0.25% papain and triturated by trituration and plated into polyethyleneimine-coated plastic or glass-bottom culture dishes containing minimal essential medium with Earle’s salts supplemented with 10% heat-inactivated fetal bovine serum, 2 mM α-glutamine, 1 mM pyruvate, 20 mM KCl, 10 mM sodium bicarbonate, and 1 mM HEPES, pH 7.2. Following cell attachment (3–6 h post-plating), the culture medium was replaced with Neurobasal medium (Invitrogen) containing B27 supplements and incubated in a humidified atmosphere of 95% air and 5% CO2 at 37 °C for 2 days. Cultures were stained with a monoclonal antibody directed against the N-terminal region of human Aβ and two other monoclonal antibodies directed against Aβ(1–40) plus Aβ(1–42) (Sigma). The C terminus-specific sandwich ELISAs use a monoclonal antibody directed against the C-terminal domain of human Aβ and two other monoclonal antibodies directed against Aβ(1–40) plus Aβ(1–42) (Sigma) to total Aβ (Aβ(1–40) plus Aβ(1–42)) was used to measure the changes in the relative amount of Aβ(1–42) secreted from transfected IMR-32 cells (95, 96).

Pull-down Assays—The methods used were essentially the same as those described previously (69). In brief, the peptide from the BACE1 C-terminal cytosolic tail (referred to as BACE1 CT, -CLRQHDDFAD-DISLLK) was covalently linked by thioamide to SulfoLink coupling gel (Pierce) according to the manufacturer’s instructions. Full-length PAR-4 was expressed in IMR-32 cells; the cell lysates were prepared; and 200 µg of protein samples were incubated with 100 µl of gel bearing immobilized peptide in 1.5 ml of phosphate-buffered saline (PBS) at room temperature for 2 h. The gel beads were pelleted by centrifugation at 750 × g for 1 min and washed three times with PBS. The proteins on the gel beads were eluted with SDS sample buffer and subjected to SDS-PAGE. PAR-4 immunoreactivity was identified by Western blotting using anti-PAR-4 monoclonal antibody. A cysteine-blocked gel was used as a negative control.

Immunocytochemistry and Fluorescence Microscopy—For immunocytochemical analysis of PAR-4 expression, the cultured cells were fixed for 4% paraformaldehyde and PBS was permeabilized by incubation in 0.2% Triton X-100 in PBS. Cells were incubated for 1 h in blocking serum (5% normal goat serum in PBS). Cells were then exposed overnight at 4 °C to a 1:100 dilution of mouse anti-PAR-4 monoclonal antibody, followed by incubation for 1 h with fluorescein-labeled anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA). Images of PAR-4 immunofluorescence were acquired using a Nikon T5 100 fluorescence microscope. The average pixel
PAR-4 Regulates BACE1-mediated Cleavage of APP

FIG. 1. Increased BACE1 cleavage of APP induced by PAR-4. a, PAR-4 increases \( \beta \)-secretase cleavage of APP. Representative Western blot analysis was carried out with rabbit polyclonal antibody raised against a 22-amino acid synthetic peptide derived from the C terminus of human APP, showing increased levels of the \( \beta \)-secretase cleavage products CTF99 and CTF89 in IMR-32 cells transfected with PAR-4. The lower bands represent the C-terminal \( \alpha \)-secretase cleavage product CTF83, which was not significantly altered by PAR-4. C1 and C2, clones C1 and C2, respectively. b, PAR-4 significantly increases secretion of A\( \beta \)-(1–42) from transfected IMR-32 cells following trophic factor withdrawal. Cultures of the indicated clones of transfected IMR-32 cells were deprived of trophic support for the indicated time periods, and the A\( \beta \)-(1–42)/total A\( \beta \) ratios in the conditioned culture media of transfected IMR-32 cells were measured by sandwich ELISAs. Note that overexpression of PAR-4 drastically increased the A\( \beta \)-(1–42)/total A\( \beta \) ratios in the conditioned media following trophic factor withdrawal (TFW). ***, \( p < 0.001 \) compared with the corresponding A\( \beta \)-(1–42)/total A\( \beta \) ratios in untransfected and vector-transfected control cell groups. Similar data were obtained from at least three separate transfected cell lines. c, cotransfection of BACE1 and PAR-4 increases the relative amount of A\( \beta \)-(1–42) secreted by IMR-32 cells under basal culture conditions. Whereas transfection of PAR-4 alone significantly increased the levels of A\( \beta \) production following apoptotic insults, coexpression of BACE1 with PAR-4 altered A\( \beta \) production under basal non-apoptotic culture conditions. The percentage of A\( \beta \)-(1–42) as a fraction of total A\( \beta \) (A\( \beta \)-(1–40) plus A\( \beta \)-(1–42)) secreted under basal culture conditions by the indicated lines of transfected IMR-32 cells was quantified by sandwich ELISA. The cell lines used were as follows: untransfected parental cell line (Untransfected), a cell line transfected with empty vectors (Vector alone), a cell line overexpressing full-length PAR-4 (Par-4), a cell line overexpressing BACE1 (BACE1), and a cell line co-overexpressing full-length human BACE1 and PAR-4 (BACE1+Par-4). Similar data were obtained from at least three separate transfected cell lines. **, \( p < 0.05 \) compared with the A\( \beta \)-(1–42)/total A\( \beta \) ratios in the untransfected, vector-transfected, and PAR-4-transfected cell groups; ***, \( p < 0.001 \) compared with the A\( \beta \)-(1–42)/total A\( \beta \) ratio in the PAR-4-transfected cell group.

Intensity of PAR-4 immunoreactivity per cell was determined using LSM 510 software (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Throughout the experiments, the specificity of the immunoreactivity was confirmed by subjecting additional samples to the immunostaining procedures without primary antibody.

**PAR-4 Knockdown by RNAi**—The methods used have been described in our previous study (104). In brief, small interfering RNAs (siRNAs) were generated by in vitro transcription using a Silencer™ siRNA mixture kit (Ambion Inc., Austin, TX) following the manufacturer's instructions. T7 promoter sequences were added to a DNA template (a cDNA fragment containing nucleotides 511–990 of the mouse Par-4 coding sequence) by PCR using the following primers: forward primer, 5′-taataagctactaattgagaltagatgagcagca-3′; and reverse primer, 5′-taataagctactaattgagaltagatgagcagca-3′. The transcription reaction was assembled, and the resulting cRNA was annealed according to the manufacturer's instructions for maximal duplex yield. The RNA samples were treated with DNase/RNase A to remove DNA, and single- and double-stranded RNAs were then purified using a transcription reaction filter cartridge (Ambion Inc.). An siRNA mixture was obtained by RNase III digestion and further purified using an siRNA purification unit (Ambion Inc.). Primary neurons were transfected with siRNA mixtures at a concentration of 100 nM using TransMessenger transfection reagent (Qiagen Inc., Valencia, CA). To determine the transfection efficiency, siRNAs were fluorescently labeled with Cy3 using a Silencer™ siRNA labeling kit (Ambion Inc.) according to the manufacturer's instructions. Labeled siRNAs were then transfected into cells and analyzed by fluorescence microscopy. The effectiveness of the RNAi experiments under our experimental conditions was confirmed using a glyceraldehyde-3-phosphate dehydrogenase-positive control DNA template (Ambion Inc.) according to the manufacturer's instructions. A
**Results**

**cotransfection of PAR-4 Increases BACE1 Cleavage of APP and Production of Aβ**—We previously found that overexpression of PAR-4 increases the production of Aβ(1–42) following apoptotic insults (95, 96). This effect of PAR-4 is effectively inhibited by AATF, an interaction partner of PAR-4 (95, 96). These results suggest that PAR-4 is involved in regulation of APP processing, and this effect of PAR-4 is likely mediated through protein/protein interactions. Because BACE1 cleaves APP at the N terminus of the Aβ domain, it catalyzes the first step in Aβ generation. To test whether PAR-4 alters BACE1 cleavage of APP, we examined the relative levels of CT99/CTF99, two major APP C-terminal products of BACE1 cleavage, in IMR-32 cells transfected with PAR-4. As shown in Fig. 1a, although the levels of both CT99 and CTF99 were significantly increased by overexpression of PAR-4, the increase in the levels of CTF99 was much more pronounced compared with CT99. The levels of CT99, the C-terminal α-secretase product of APP, were not significantly altered. These results indicate that overexpression of PAR-4 increases β-secretase cleavage of APP. To further examine whether a PAR-4-induced increase in β-secretase cleavage of APP leads to increased production of Aβ(1–40) and or Aβ(1–42) in these cells, we measured the Aβ(1–42)/total Aβ ratio in the conditioned culture medium of transfected IMR-32 cells using sensitive sandwich ELISAs as described previously (95, 96). We found that although expression of PAR-4 alone did not significantly alter the basal levels of Aβ(1–42), the secretion of Aβ(1–42) was significantly increased following trophic factor withdrawal (Fig. 1b). Transfection of BACE1 alone in IMR-32 cells increased the production of both Aβ(1–40) and Aβ(1–42) under basal culture conditions (Table I). The levels of Aβ(1–42) seemed to be altered more dramatically than those of Aβ(1–40) by overexpression of BACE1, which led to a slight but significant increase in the Aβ(1–42)/total Aβ ratio (Fig. 1c and Table I). Of importance, cotransfection of PAR-4 with BACE1 further enhanced the production of Aβ, resulting in a significantly exacerbated secretion of Aβ(1–42) under basal and non-apoptotic culture conditions (Fig. 1c and Table I). Taken together, these results demonstrate that PAR-4 alters BACE1 cleavage of APP and increases Aβ production in transfected neural cells.

**PAR-4 Is Associated with BACE1 in Transfected Neural Cells and Primary Neurons**—To examine whether PAR-4 alters β-secretase cleavage of APP by interacting with BACE1, we performed co-immunoprecipitation/Western blot experiments using specific anti-PAR-4 and anti-BACE1 antibodies. Because the basal levels of BACE1 and PAR-4 in untransfected IMR-32 cells are relative low, co-immunoprecipitation studies were performed first on homogenates of transfected IMR-32 cell clones overexpressing PAR-4 and BACE1. When immunoprecipitation was performed using anti-BACE1 antibody, a 38-kDa PAR-4 band was clearly detected on the immunoblot (Fig. 2a). The reverse order of immunoprecipitation/Western blot analysis of the same transfected cell lines showed similar PAR-4/BACE1 complex formation (Fig. 2b). To exclude the possibility that the PAR-4/BACE1 complex formation was due to an artifact of overexpression of both proteins, we performed further immunoprecipitation/Western blot analysis in primary hippocampal neurons expressing physiological concentrations of these proteins. As shown in Fig. 2 (c and d), similar PAR-4/BACE1 complex formation was clearly observed. Note that anti-BACE1 antibody recognized predominantly the mature form of BACE1 in both transfected IMR-32 cells as well as primary neurons. These results demonstrate the interaction between endogenous PAR-4 and BACE1 and indicate that PAR-4/BACE1 complex formation is physiologically relevant. A control antibody against AATF failed to precipitate BACE1 (Fig. 1f, lane 3), indicating the specificity of the PAR-4/BACE1 interaction. To further confirm the specificity of the PAR-4/BACE1 interaction, we examined whether PAR-4 would interact with presenilin-1 using a specific anti-PS-1 antibody. As shown in Fig. 2e, immunoprecipitation/Western blot experiments in transfected IMR-32 cells expressing PAR-4 showed that both full-length PS-1 and its cleavage products were detected in proteins from total lysate, but not in those immunoprecipitated with anti-PAR-4 antibody, indicating that PAR-4 does not interact with PS-1. Similar data were obtained in the reverse order of immunoprecipitation/Western blot analyses of the same transfected cell lines (data not shown). These negative control experiments confirmed the selectivity of PAR-4/BACE1 complex formation.

**Mapping of PAR-4/BACE1-interacting Domains**—Next, we performed additional pull-down and co-immunoprecipitation/Western blot experiments to determine the interacting domains in PAR-4 and BACE1. Because BACE1 is a type I membrane protein and because PAR-4 is primarily cytosolic, we expected PAR-4 to be associated with the C-terminal cytosolic tail of BACE1. To examine this possibility, we employed the BACE1 CT peptide (-CLRQRHHDFFADDSSLK) and examined whether PAR-4 could be pulled down by the BACE1 CT peptide immobilized on gel beads (see “Materials and Methods”). The BACE1 CT peptide was covalently linked to SulfoLink coupling gel. The cell lysates from PAR-4-transfected IMR-32 cells were
incubated with the gel-linked BACE1 CT peptide. The gel samples were washed and then subjected to SDS-PAGE and Western blotting for PAR-4. As shown in Fig. 3a, PAR-4 was clearly and specifically pulled down by the BACE1 CT peptide. To further determine which domain(s) of PAR-4 are involved in the interaction with the cytosolic tail of BACE1, we performed co-immunoprecipitation/Western blot analysis using full-length PAR-4 as well as a C-terminal half-deletion mutant of PAR-4 lacking nucleotides 541–1267 of PAR-4 cDNA (Par-4ΔCTF). IMR-32 cells were cotransfected with BACE1 and Par-4 or Par-4ΔCTF. Cell lysates from cotransfected cells were precipitated with anti-BACE1 antibody, followed by Western blotting with anti-PAR-4 antibody (Fig. 3b). Note that BACE1 interacted only with full-length PAR-4 (Fig. 3b, lane 2), but not with the C-terminal half-deletion mutant of PAR-4, indicating that the C-terminal half of PAR-4 is necessary in the interaction with BACE1. The reverse order of immunoprecipitation/Western blot analysis of the same transfected cells confirmed that deletion of the C-terminal half of PAR-4 abolished the interaction between BACE1 and PAR-4 (data not shown).

Taken together, our results indicate that the C-terminal half of PAR-4 is necessary for association with the cytosolic tail of BACE1 and that the PAR-4/BACE1 interaction exacerbates BACE1-mediated cleavage of APP.

RNAi-mediated Par-4 Gene Silencing Diminishes BACE1 Cleavage of APP—RNAi has been proved to be a very effective oligonucleotide-based gene silencing technology that allows sequence-specific gene suppression in a variety of organisms and cultured cells. The application of RNAi techniques to primary neurons, a setting in which genetic manipulations have traditionally proven difficult, has become a versatile tool to study gene function and protein expression in neuronal cells. To further determine the crucial role of PAR-4 in regulating β-secretase cleavage of APP, we examined whether knockdown of PAR-4 expression by RNAi would result in a significant decrease in β-cleavage of APP and in Aβ production. One drawback of the RNAi technology is the need to design, synthesize, and test several siRNAs before an effective sequence can be identified. However, this problem can be avoided altogether with the Silencer™ siRNA mixture kit employed in this study.
In this system, a population of several siRNAs (instead of a single siRNA sequence) was generated by digesting long double-stranded RNA with RNase III, which cleaves double-stranded RNA into 12–30-bp double-stranded RNA fragments. Single siRNA sequence) was generated by digesting long double-stranded RNA with RNase III, which cleaves double-stranded RNA with RNase III, which cleaves double-stranded RNA into 12–30-bp double-stranded RNA fragments. The termini and overhangs of RNase III cleavage products are thus the same as those produced by Dicer in the in vivo RNAi pathway. This effectively eliminates the need to design and screen individual siRNAs. The successful application of this RNAi protocol to knockdown PAR-4 expression in primary neurons has been described (104). The transfection efficiency of siRNAs in neuronal cultures was determined by fluorescence microscopy of neurons transfected with siRNAs fluorescently labeled with Cy3 (104). Under our experimental conditions, an average of 78.6 ± 6.2% siRNA transfection efficiency has been achieved (104). To confirm that the siRNA mixture targeted against PAR-4 would effectively inhibit aberrant induction of PAR-4 expression, primary hippocampal neurons from wild-type mice were either mock-transfected (control) or transfected with siRNAs against PAR-4 at a concentration of 100 nM. 48 h after siRNA transfection, cells were treated with either vehicle (control) or 50 μM glutamate for 8 h. The cells were then processed for PAR-4 immunoreactivity using fluorescence microscopy. As shown in Fig. 4a, the induction of PAR-4 expression induced by glutamate, as assessed by immunofluorescence microscopy, was completely knocked down by siRNAs targeted against PAR-4, but not by the non-silencing control siRNA. Further Western blot analysis confirmed that PAR-4 expression was largely knocked down by siRNAs targeted against PAR-4, but not by a validated negative control siRNA against GFP (Fig. 4, b and c). To determine the specificity of the siRNA mixture targeted against PAR-4, the same protein samples were probed with antibody against another leucine zipper protein, AATF. As shown in Fig. 4 (b and c), neither the siRNA against Par-4 nor the siRNA against GFP altered the expression of AATF. These results indicate that the Par-4 siRNA mixture specifically and effectively targets Par-4 mRNAs for degradation by RNAi.

To examine whether PAR-4 is involved in regulating β-secretase cleavage of APP, we investigated whether RNAi-mediated silencing of Par-4 would lead to a significant decrease in the levels of the β-secretase cleavage product CTF99 in hippocampal neurons expressing APPwt or APPmut by siRNAs targeted against Par-4 (Fig. 4, d and e), but not by a negative control siRNA against GFP (data not shown). Interestingly, whereas a small amount of CTF99 was detected in APPwt neurons, the levels of CTF99 seemed to be significantly diminished in APPmut neurons (Fig. 4d). The significance of this observation is not immediately clear. The levels of CTF99 in APPwt- and APPmut-expressing neurons were not significantly altered by siRNAs targeted against Par-4 (Fig. 4d).

We next examined whether RNAi-mediated depletion of PAR-4 would reduce Aβ production in hippocampal neurons expressing APPmut. As shown in Table II, the levels of Aβ, particularly of Aβ-(1–42)) in the conditioned culture medium of primary hippocampal neurons expressing APPmut were significantly reduced by Par-4 siRNAs.

**DISCUSSION**

Mounting evidence indicates that aberrant production and aggregation of Aβ-(1–42) play a central role in the pathogenesis of AD (1–88). Aβ is produced when APP is cleaved by β- and γ-secretases at the N and C termini of the Aβ domain, respectively. The observation that mice with a targeted deletion of BACE1 are viable and fertile but fail to produce any Aβ indicates that BACE1 is strictly required for Aβ generation and that inhibition of BACE1 activity might be a safe and effective treatment option for AD (6, 40, 51, 62). Because the increased BACE1 activity observed in AD patients does not seem to arise from genetic mutations or polymorphisms in BACE1 (1, 42, 47, 51), the identification of factors that effectively regulate BACE1 activity is apparently a highly promising approach to treating AD. PAR-4 is a leucine zipper protein that was initially identified to be associated with neuronal degeneration in AD (94). Subsequent studies found that PAR-4, via protein/protein interactions, also increases production of neurotoxic Aβ in transfected neuronal cells, indicating that PAR-4 may regulate both apoptosis and amyloidogenic processing of APP (95, 96). Because BACE1 cleaves APP at the N terminus of the Aβ domain, it catalyzes the first step in Aβ generation. These results suggest that PAR-4 might be involved in regulating BACE1 cleavage of APP.

The data presented in this study indicate that PAR-4 is indeed associated with BACE1 and specifically increases β-secretase cleavage of APP. This notion is supported by several lines of experimental evidence obtained in this study. 1) Transfection of PAR-4 in IMR-32 cells increased the levels of the β-secretase cleavage products CTF99 and CTF89 without
significantly altering the C-terminal α-secretase cleavage product CTF83. 2) Cotransfection of BACE1 and PAR-4 increased the amount of Aβ-(1–40) and Aβ-(1–42) secreted by IMR-32 cells under basal culture conditions. 3) PAR-4 was specifically associated with BACE1 in co-immunoprecipitation/Western blot assays in both transfected cells and primary hippocampal neurons. 4) Additional pull-down experiments and immunoprecipitation assays showed that the C-terminal half of PAR-4 was

FIG. 4. Inhibition of BACE1 cleavage of APP by RNAi-mediated Par-4 gene silencing. a, the siRNA mixture targeted against Par-4, but not a non-silencing siRNA, inhibits PAR-4 expression induced by glutamate in primary hippocampal neurons. Primary mouse hippocampal neurons were either mock-transfected (Control) or transfected with siRNAs against Par-4 at a concentration of 100 nM. A non-silencing siRNA (see “Materials and Methods”) was used as a negative control. 48 h after siRNA transfection, cells were treated with either vehicle (Control) or 50 μM glutamate for 8 h. The cells were then processed for PAR-4 immunoreactivity using fluorescence microscopy. Note that the induction of PAR-4 expression was completely knocked down by siRNAs targeted against Par-4, but not by the non-silencing siRNA. b, representative Western blot analysis showing specific knockdown of PAR-4 expression by the siRNA mixture targeted against Par-4 in hippocampal neurons. Upper panel, primary hippocampal mouse neurons were either mock-transfected (Control) or transfected with siRNAs against Par-4 or with a validated negative control siRNA against GFP (see “Materials and Methods”). 48 h after siRNA transfection, cells were treated with either vehicle (Control) or 50 μM glutamate for 8 h. Proteins were then prepared from the neurons and subjected to Western blot analysis of PAR-4 immunoreactivity. Note that the induction of PAR-4 expression was largely knocked down by siRNAs targeted against Par-4, but not by the siRNA against GFP. Middle panel, the same protein samples were probed with antibody against another leucine zipper protein, AATF. Neither siRNAs against Par-4 nor the siRNA against GFP altered the expression of AATF. Lower panel, the same blot was stripped and reprobed with anti-β-tubulin antibody to confirm equal protein loading. c, quantitative analysis of PAR-4 knockdown by siRNAs in hippocampal neurons as assessed by Western blotting. Values are the means ± S.E. (error bars) of determinations made in at least six separate experiments. ###, p < 0.001 compared with the value in the control group; ***, p < 0.001 compared with values in the glutamate, GFP siRNA + glutamate, and control groups. d and e, RNAi-mediated silencing of PAR-4 leads to a decrease in β-secretase cleavage of APP in primary neurons expressing APPwt or APPmut. d, representative Western blots showing that depletion of PAR-4 by RNAi reduces the production of CTF99 in hippocampal neurons expressing APPwt or APPmut. e, densitometric analysis of Western blots of CTF99 in primary hippocampal neurons expressing APPwt and APPmut. ***, p < 0.001 compared with corresponding values in control (non-siRNA-treated) neurons (analysis of variance with Scheffe’s post hoc tests). Values are the means ± S.E. of determinations made in six separate experiments.
that notion is further supported by several recent reports showing cascades are initiated, indicating that apoptosis and aberrant \( \text{N}_{\text{atal}} \)-linked glycosylation at Asn153, Asn172, Asn223, -secretase activity seems to be dependent on the extent of \( \text{N}_{\text{-link}} \)-linked glycosylation (1, 5, 6, 16, 18, 34, 37–75). Therefore, it is also possible that binding of PAR-4 to BACE1 may regulate \( \beta \)-secretase cleavage of APP by altering intracellular trafficking and/or maturation of BACE1.

Because BACE1 cleaves APP at the N terminus of the \( \alpha \)-domain, it catalyzes the first step in \( \alpha \)-generation (1, 5, 6, 16, 18, 34, 37–75). The observation that mice with a targeted deletion of BACE1 are viable and fertile but fail to produce any \( \alpha \)-indicates that inhibition of BACE1 activity might be a safe and effective treatment option for AD (6, 40, 51, 62). Because the increased BACE1 activity observed in AD patients does not seem to arise from genetic mutations or polymorphisms in BACE1 (1, 42, 47, 51), the identification of PAR-4 as an endogenous regulator of BACE1 activity may therefore have significant implications for developing novel therapeutic strategies for AD.

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