BRI2 Protein Regulates β-Amyloid Degradation by Increasing Levels of Secreted Insulin-degrading Enzyme (IDE)*§*

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Background: The British precursor protein (BRI2) influences amyloid precursor protein metabolism.

Results: BRI2 lowers β-amyloid peptide levels by increasing levels of secreted insulin-degrading enzyme (IDE) in both cells and mice.

Conclusion: BRI2 as a receptor protein regulates IDE levels and in turn promotes β-amyloid degradation.

Significance: Targeting the regulation of IDE may lead to new approaches to therapeutically address sporadic Alzheimer disease.

The amyloid precursor protein (APP) is one of the major proteins involved in Alzheimer disease (AD). Proteolytic cleavage of APP gives rise to amyloid-β (Aβ) peptides that aggregate and deposit extensively in the brain of AD patients. Although the increase in levels of aberrantly folded Aβ peptide is considered to be important to disease pathogenesis, the regulation of APP processing and Aβ metabolism is not fully understood. Recently, the British precursor protein (BRI2, ITM2B) has been implicated in influencing APP processing in cells and Aβ deposition in vivo. Here, we show that the wild type BRI2 protein reduces plaque load in an AD mouse model, similar to its disease-associated mutant form, ADan precursor protein (ADanPP), and analyze in more detail the mechanism of how BRI2 and ADanPP influence APP processing and Aβ metabolism. We find that overexpression of either BRI2 or ADanPP reduces extracellular Aβ by increasing levels of secreted insulin-degrading enzyme (IDE), a major Aβ-degrading protease. This effect is also observed with BRI2 lacking its C-terminal 23-amino acid peptide sequence. Our results suggest that BRI2 might act as a receptor protein that regulates IDE levels that in turn influences APP metabolism in a previously unrecognized way. Targeting the regulation of IDE may be a promising therapeutic approach to sporadic AD.

Alzheimer disease (AD) is a disease of progressive dementia and neuron loss characterized by the deposition of β-amyloid plaques and the formation of neurofibrillary tangles in the brain (1). The main components of amyloid plaques, the amyloid-β (Aβ) peptides, are derived by sequential proteolytic cleavage of the amyloid precursor protein (APP) (2). Although mutations in the APP or the PS1 or PS2 genes, leading to alterations in APP processing, are genetically linked to familial cases of AD (3), increased levels of amyloidogenic Aβ peptides are believed to play a major role in disease pathogenesis (4). However, besides Aβ generation, Aβ clearance is of equal importance in maintaining Aβ steady-state levels. The metalloproteases neprilysin, endothelin-converting enzyme (ECE-1 and ECE-2), and insulin-degrading enzyme (IDE) are the main Aβ-degrading enzymes in the brain (5, 6) and reduced clearance of Aβ peptides may be more related to cases of sporadic AD (7).

Recently, the British precursor protein (BRI2, ITM2B, and E25) (8–10) has been implicated to influence APP processing in cells and Aβ aggregation in vivo (11–15). Although mutations in the BRI2 gene have been linked to familial Danish dementia and familial British dementia (10, 16), the physiological function of wild type BRI2 is not known. The 266-aa type II transmembrane protein is processed by several proteases to shed a 23-aa peptide via furin-like cleavage (17), and a fragment, including the BRICHOS domain (18), into the extracellular space (19). In familial Danish dementia, an 11-aa longer protein, ADan precursor protein (ADanPP), is expressed due to a 10-nucleotide duplication before the stop codon of the BRI2 gene causing a frameshift and elongation of the open reading frame (16). Furin-like cleavage of ADanPP yields a 34-aa peptide prone to amyloid formation (16). BRI2 can interact with APP and has been proposed to inhibit secretase cleavage of APP (11, 13–15). Other studies report an interaction of the 23-aa

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3 The abbreviations used are: AD, Alzheimer disease; APP, amyloid precursor protein; Aβ, amyloid-β; IDE, insulin-degrading enzyme; ADanPP, ADan precursor protein; tg, transgenic; EGFP, enhanced green fluorescent protein; aa, amino acid; AICD, APP intracellular domain.
BRI1 peptide, or the BRICHOS domain, with Aβ in vitro, and a potential inhibition of Aβ aggregation in vivo (12, 20). We previously showed that even the disease-related form of BRI2, ADanPP, reduces plaque deposition in mouse models of AD (21). Given that these two dementia-related proteins can interact and influence each other, this prompted us to further investigate the underlying mechanisms of the observed Aβ plaque reduction. We suspected that not only Aβ generation but also Aβ clearance by degrading proteases may be affected, because Aβ as well as BRI2–derived peptides can be degraded by IDE (22), one of the major Aβ-degrading enzymes in the brain.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The cDNA encoding the human WT form of BRI2 in vector pCR2.1 (provided by R. Vidal, Indianapolis, IN) was mutated by standard mutagenesis using PfuUltra High Fidelity DNA polymerase (Stratagene) to introduce the Danish mutation (10-nucleotide insertion TTTAATTTGT). BRI2 and ADanPP cDNA inserts were liberated by digest with BamHI and introduced blunt-ended into the EcoRV cloning site of pcDNA3.1/Zeol (+) (Invitrogen). To generate the BRI2Δ construct, a stop codon was introduced following the triplet encoding aa 243 of BRI2 by site-directed mutagenesis of the pcDNA3.1/Zeol (+)-BRI2 construct. All cDNA constructs were verified by sequencing. The CMV wild type βAPP695 cDNA construct (23) was used to express APP695 (24). A human PS1 cDNA mutated at L166P (25) was inserted into pcDNA3.1/Zeol (+) (Invitrogen). pEGFP-C1 (Clontech) was used to express EGFP.

**Generation of Transgenic Mice Expressing Wild Type Human BRI2 (wtBriPP)—**BRI2 cDNA (see above) was introduced into the blunt-ended SalI cloning site of the cosmid-based Syrian Hamster prion protein expression vector (provided by S. Prusiner, San Francisco). After removal of vector sequences by NotI digestion, microinjections of the purified construct into C57Bl/6N pronuclei yielded several putative founders. NotI digestion, microinjections of the purified construct into the blunt-ended SalI cloning site of the cosmid-based Syrian Hamster prion protein expression vector (provided by S. Prusiner, San Francisco). After removal of vector sequences by NotI digestion, microinjections of the purified construct into C57Bl/6N pronuclei yielded several putative founders. C57Bl/6N-NotI digestion, microinjections of the purified construct into the blunt-ended SalI cloning site of the cosmid-based Syrian Hamster prion protein expression vector (Stratagene) to introduce the Danish mutation (10-nucleotide insertion TTTAATTTGT). BRI2 and ADanPP cDNA inserts were liberated by digest with BamHI and introduced blunt-ended into the EcoRV cloning site of pcDNA3.1/Zeol (+) (Invitrogen). To generate the BRI2Δ construct, a stop codon was introduced following the triplet encoding aa 243 of BRI2 by site-directed mutagenesis of the pcDNA3.1/Zeol (+)-BRI2 construct. All cDNA constructs were verified by sequencing. The CMV wild type βAPP695 cDNA construct (23) was used to express APP695 (24). A human PS1 cDNA mutated at L166P (25) was inserted into pcDNA3.1/Zeol (+) (Invitrogen). pEGFP-C1 (Clontech) was used to express EGFP.

**Transgenic Mice Used for Cross-breeding**—The APPPS1–21 mice used for cross-breeding with wtBriPP transgenic mice have been described previously (25). APPPS1–21 mice have been generated and maintained on a pure C57Bl/6J background. All mice analyzed were hemizygous for the transgene(s) of interest. All animal experiments were performed in accordance with the current German animal welfare law and licensed by the local veterinary authority (Regierungspräsidium Tübingen).

**Histology and Immunohistochemistry**—Brains were removed upon sacrifice and immersion-fixed in 4% paraformaldehyde. Immunohistochemistry was done on 25–μm thick coronally cut cryoprotected free-floating frozen sections, using standard immunoperoxidase procedures with Elite ABC kits (Vector Laboratories) with Vector SG (Vector Laboratories, Burlingame, CA). The CN3 polyclonal antibody to Aβ was used as described previously (26).

**Stereology and Quantification of Pathology**—Stereological analysis was performed using a microscope equipped with a motorized x-y-z stage coupled to a video microscopy system (Systems Planning and Analysis, Inc., Alexandria, VA). Neocortical brain regions were defined using a standard mouse brain atlas (51). Quantification was done on the left hemisphere. Analysis of Aβ amyloid load was done on a series of coronally cut 25-μm free-floating sections (every 24th section for the neocortex). Thus, all analyses included 8–10 sections per animal. The amyloid load (percentage) was determined by calculating the areal fraction occupied by immunoreactive Aβ, in two-dimensional sectors at a single focal plane at 20×/0.45 numerical aperture.

**Cell Culture, Transfection, and Treatment**—HEK293 cells (ATCC), HEK293 cells stably expressing wild type βAPP695 cDNA (HEK293-APPwt cells) (23), and HeLa cells (ATCC) were cultured in DMEM 4.5 g/liter glucose with l-glutamine (Lonza) supplemented with 10% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Transient transfection of cells was carried out using FuGENE HD (Roche Diagnostics) or Lipofectamine LTX with Plus Reagent (Invitrogen) according to the manufacturer’s instructions. Cells transfected with pcDNA3.1/Zeo (+) vector served as a control. One day after transfection, cells were supplemented with fresh medium and incubated for 24 h to obtain conditioned cell media. 10 mg/ml insulin solution (human), cell culture tested (Sigma), was added to the cell culture medium at 10 μM concentration during the incubation period as indicated. After gathering conditioned cell media, the relative number of cells was measured using AlamarBlue® cell viability reagent and fluorescent detection according to the manufacturer’s protocol (Invitrogen), to adjust for differences in cell numbers.

**Antibodies and Western Blot Analysis**—The following antibodies were used: monoclonal antibody 6E10 specific to human Aβ (crude ascites; Covance); anti-APP C-terminal rabbit polyclonal antibody A8717 (Sigma); monoclonal antibody 8G4 to GAPDH (HyTest Ltd.); chicken polyclonal antibody to ITM2B raised against amino acids 1–60 (Abcam); rabbit polyclonal antibody to insulin-degrading enzyme (IDE) (ab25970, Abcam), used for Western blot detection of IDE; rabbit polyclonal antibody IDE-1 (27), used for detection of IDE in cerebral spinal fluid; monoclonal anti-IDE antibody (ab25733, Abcam), used for immunoprecipitation of IDE; 56C6 anti-neprilysin monoclonal antibody (Novocastra); monoclonal antibody NT1 raised against the N-terminal region of PS1 (28); and HRP-conjugated secondary antibodies (Santa Cruz Biotechnology). Synthetic CTF-50, a 50-amino acid peptide resulting from the γ-secretase cleavage of the C terminus of APP at Leu270–Val271 (Calbiochem) was used as a molecular weight control for AICD. For detection of proteins from mouse brains, brains were frozen, homogenized at 10% (w/v) in homogenization buffer (0.32 mM sucrose in PBS, including complete protease inhibitor tablets (Roche Diagnostics), aliquoted, and stored at −80°C until further use. For detection of proteins from cell lysates, cells were lysed in RIPA buffer supplemented with protease inhibitors (10 μM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1× complete inhibitor mixture (Roche Diagnostics), 5 mM EDTA, 2 mM 1,10-phenanthroline (Sigma)) followed by centrifugation at 16,000 × g for 15 min. For purification of cellular membranes, cells were incubated in
hypotonic buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1× complete inhibitor mixture, 2 mM 1,10-phenanthroline) for 10 min on ice. Cells were homogenized, and nuclei and debris were removed by centrifugation at 1500 × g for 10 min. The supernatant was then centrifuged at 100,000 × g for 1 h. The membrane pellet was resuspended in RIPA buffer. Protein concentrations were determined using BCA protein assay reagent (Thermo Scientific), and equal amounts of protein were analyzed by SDS-PAGE. Conditioned cell media were centrifuged at 900 × g for 5 min at 4 °C to eliminate cells, and supernatants were supplemented with 1× complete protease inhibitor mixture (Roche Diagnostics) and stored at −80 °C. APP, IDE, neprilysin, wild type, and mutated forms of BRI2 (ITM2B) were analyzed by 10 or 12% Tris glycine SDS-PAGE. To detect recombinant IDE, 9 wtBriPP/APPPS1 mice, frozen brain hemispheres were homogenized using a Dounce homogenizer following the manufacturer’s instructions. Samples were mixed with α-cyano-4-hydroxycinnamic acid (Sigma) and spotted onto a metal plate. Spectra were recorded in the linear mode at a laser frequency of 20 Hz within a mass range from 1,000 to 6,000 Da. Each spectrum is the result from an average outcome of at least 300 laser shots collected in 30 shot steps. FlexAnalysis 1.0 software (Bruker Daltonics GmbH, Germany) was used for visualization of the mass spectra performing smoothing and base-line subtraction.

RESULTS

BRI2 Promotes Aβ Degradation by IDE

—A mouse monoclonal antibody against IDE (ab25733, Abcam) was coupled to M-280 sheep anti-mouse IgG Dynabeads (Invitrogen). Conditioned cell media from BRI2-transfected HEK293 cells were incubated with IDE-coupled Dynabeads or uncoupled Dynabeads as a control. Immunoprecipitations were carried out according to the manufacturer’s instructions. Subsequently IDE in cell media and precipitated IDE were analyzed by Western blot. Media were transferred to HEK293-APPwt cells for 24 h, and secreted Aβ was analyzed by Western blot and immunoassay.

Immunodepletion of IDE from Conditioned Cell Media—Conditioned cell media were analyzed for Aβ38, Aβ40, and Aβ42 levels where indicated by an electrochemiluminescent-based sandwich immunoassay using MS6000 human (6E10) Aβ-3-Plex kit and the Sector® Imager 6000 (Meso Scale Discovery) according to the manufacturer’s instructions. Each sample was measured in duplicate, and the mean was taken. Values were adjusted for the relative number of viable cells measured by AlamarBlue® cell viability assay. The concentration obtained for controls was set to 100%, and% Aβ relative to controls was calculated. To measure human Aβ in wtBriPP/APPPS1 mice, frozen brain hemispheres were homogenized in 50 mM Tris, pH 8, 150 mM NaCl, 4 mM EDTA containing protease inhibitors complete (Roche Diagnostics) and 2 mM 1,10-phenanthroline (Sigma). Aβ was extracted with diethanolamine (DEA) as described previously (21), and Aβ levels were measured by immunoassay as described above.

Aβ Degradation in Vitro—Conditioned cell medium from BRI2 or pcDNA3.1/Zeo(+) control transfected HEK-APPwt cells was prepared as described above, mixed with 10 μM Aβ(1–40) (American Peptide Co.), and incubated for 1 h at 37 °C as described earlier (30). Aliquots were taken prior to (0 h) and following the incubation period. For degradation of Aβ with recombinant IDE, 9 μM Aβ(1–40) was incubated with 0.6 ng/μl IDE (R&D Systems) in Tris/NaCl buffer (50 mM Tris, 1 mM NaCl, pH 7.5) for 1 h.

Mass Spectrometry Analysis—Molecular masses of intact Aβ(1–40) peptides and the proteolytic products upon degragation of Aβ by recombinant or cell-derived IDE were determined using the matrix-assisted laser desorption ionization-time-of-flight mass spectrometer (MALDI-TOF MS, Biflex III, Bruker Daltonics GmbH, Germany). Snap-frozen samples from Aβ degradation assays were thawed and desalted using C18 ZipTips (Millipore) following the manufacturer’s instructions. Densitometry was performed with ImageJ software to quantify the intensity of the individual bands.
BRI2 Promotes Aβ Degradation by IDE

FIGURE 1. BRI2 reduces amyloid plaque deposition in the APPPS1 mouse model of AD. A and B, Aβ immunostaining of 4-month-old wtBriPP+/APPPS1+ double tg mice compared with single tg wtBriPP+/APPPS1+ littermates reveals a decrease in Aβ plaque deposition. Higher magnification is shown in the right panels. C, stereological quantification of neocortical Aβ load reveals a remarkable decrease in Aβ deposition in the double tg mice (**, p < 0.001; all females; n = 11 single tg, n = 7 double tg). D, Western blotting of APP, C99, and Aβ in wtBriPP+/APPPS1+ and wtBriPP+/APPPS1− mice reveals no change in levels of APP and C99 but a decrease in Aβ in double transgenic mice. Shown are three mice for each genotype. GAPDH serves as a loading control. E, quantification of C99 levels by densitometric analysis of band intensities normalized to GAPDH (p = 0.683; n = 5 single tg, n = 5 double tg). Scale bars, 500 and 100 μm. F−I, analysis of APP, C99, and Aβ levels in 1.4-month-old pre-depositing wtBriPP+/APPPS1+ double tg mice compared with single tg wtBriPP+/APPPS1+ littermates. F, Western blot analysis with antibody 6E10 reveals no differences in APP or C99 levels. Shown are three mice for each genotype, all females. GAPDH serves as a loading control. G, quantification of C99 levels by densitometric analysis of band intensities normalized to GAPDH and APP (p = 0.220; n = 5 single tg, n = 5 double tg, all females). H, Western blot analysis with antibody A8717 reveals no differences in α- or β-cleaved APP C-terminal fragments (APP-CTFs) (upper panel) or AICD (lower panel, stronger exposure of the same blot). The two bands represent the nonphosphorylated and phosphorylated form of AICD (AICD/p-AICD). I, immunoassay of DEA extracted brain homogenates shows a significant decrease in Aβ40, Aβ42, and Aβ38 comparing double tg with single tg littermates (*, p < 0.05; n = 9 wtBriPP+/APPPS1+; n = 5 wtBriPP−/APPPS1−, all females).

detected by Western blot with an APP C-terminal specific antibody (Fig. 1H).

Taken together, these results suggest an influence of BRI2 on Aβ generation or turnover that occurs prior to amyloid deposition. Because levels of C99, C83, and AICD remained unchanged, and levels of all Aβ species were reduced, we suspected that rather than having an effect on APP processing by secretases, BRI2 reduced Aβ levels post-processing, potentially by enhanced Aβ clearance. To further investigate the molecular mechanism of the influence of BRI2 and ADanPP on APP, and Aβ metabolism in particular, we conducted cell culture experiments.

BRI2 and Its Mutant Form ADanPP Reduce Levels of Secreted Aβ and Increase Levels of C99 in HEK293-APPwt Cells—HEK293 cells stably expressing APP695 (HEK293-APPwt cells) were transfected with constructs to co-express either BRI2 or ADanPP. We found that both BRI2 and ADanPP clearly reduced levels of secreted Aβ in transfected cells, whereas levels of APP remained unchanged (Fig. 2A). Quantification of Aβ peptides from conditioned cell media by immunoassay revealed a decrease in both Aβ40 and Aβ42. BRI2 and ADanPP significantly reduced Aβ40 to levels 46.0 and 52.2% that of controls and Aβ42 to levels 27.1 and 31.0% that of controls, respectively (Fig. 2B). No significant differences were found between the two constructs in their effect on Aβ. To test the influence of both proteins on APP processing, C-terminal fragments of APP were analyzed. We found a strong increase in the β-cleavage-derived C99 fragment with both constructs, in line with previously published results for BRI2 in cell culture (11, 13). No major changes in levels of the α-secretase cleavage product C83 nor in levels of the γ-secretase cleavage product AICD were observed (Fig. 2C and supplemental Fig. 1). Therefore, the decrease in secreted Aβ does not seem to result from an inhibition of γ-secretase cleavage of the C99 fragment. Still BRI2 might modulate γ-secretase activity specifically, such that Aβ and AICD generation are affected differently. As an example, certain nonsteroidal anti-inflammatory drugs selectively lower Aβ42 and increase Aβ38 levels without inhibition of AICD generation (31, 32). To investigate this possibility, we specifically analyzed levels of all three of the major secreted Aβ forms by immunoassay in conditioned cell media of BRI2 and control transfected cells. BRI2 leads to a decrease of all three Aβ forms. Levels of Aβ38 in BRI2-transfected cells dropped below the detection limit, and no selective increase in Aβ38 was observed (Fig. 2D). Taken together, these results do not point to an inhibition or modulation of γ-secretase cleavage of APP by BRI2.

Effects of BRI2 on Aβ and C99 Are Independent of the BRI Peptide—In investigating plaque load reduction by BRI2, it has been proposed that the shed 23-aa BRI peptide may inhibit extracellular Aβ aggregation and deposition (12). We therefore asked whether the reduction in Aβ levels observed in cells was due to the involvement of the BRI peptide. BRI2 was cloned and
modified, such that a shorter BRI2 protein lacking the peptide sequence (aa 244–266) was expressed. In transient transfection experiments, this BRI2 variant, BRI2/H9004, led to a similar decrease in secreted Aβ/H9252 and increase in C99 as seen for the expression of full-length BRI2 and ADanPP (Fig. 3). These results suggest an influence of BRI2 on Aβ/H9252 levels, which is independent of its secreted peptide.

**BRI2 Increases Levels of Secreted IDE**—The observed Aβ reduction did not seem to result from inhibition or modulation of γ-secretase cleavage by BRI2 or sequestration of soluble Aβ by the BRI peptide. The balance between anabolism and catabolism of Aβ determines its steady-state levels, such that a reduction in Aβ may also be caused by enhanced Aβ degradation. Two major Aβ-degrading enzymes in the brain are neprilysin (33) and IDE (34). Thus, we next analyzed the expression of neprilysin and IDE in BRI2-transfected cells to test for a possible involvement in increased Aβ degradation. Levels of neprilysin were not significantly changed in the membrane fraction of HEK293-APPwt cells transfected with BRI2 compared with controls (Fig. 4A). IDE can be secreted from cells, where it can degrade Aβ (35) and also stay in the cytoplasm, where it is involved in the degradation of AICD (34). To discriminate between these different pools, we analyzed IDE levels in cell supernatants and cell lysates of cells transfected with the different BRI2 constructs. Expression of BRI2, ADanPP, and BRI2Δ led to a significant increase in secreted IDE in conditioned cell media by 2.6-, 3.2-, and 2.9-fold, respectively, compared with controls. Levels of intracellular IDE were not sig-

**FIGURE 2.** BRI2 and ADanPP reduce levels of secreted Aβ and increase levels of the C99 fragment in cells. HEK293-APPwt cells were transfected with BRI2 or ADanPP expression plasmids or pcDNA3.1 vector as a control (co). A, secreted Aβ was prominently decreased by BRI2 and ADanPP, whereas APP levels remained unchanged. Expression of BRI2 constructs was confirmed by Western blot with the ITM2B antibody, and detection of GAPDH serves as a loading control. B, quantification of secreted Aβ40 and Aβ42 by immunoassay from four independent experiments reveals a significant reduction in BRI2- and ADanPP-transfected cells relative to controls (***, p < 0.0001; BRI2 versus ADanPP p > 0.05). C, C-terminal fragments of APP were analyzed in cell lysates. An increase in levels of C99 fragments (middle panel, short exposure) but no change in levels of AICD (lower panel, longer exposure of the same blot) are observed. APP levels remain unchanged. D, HEK293-APPwt cells were transfected with BRI2 or pcDNA3.1 vector as a control (co). Aβ40, Aβ42, and Aβ38 from conditioned cell media were analyzed by immunoassay from three independent experiments. All three Aβ forms are decreased in cell media of BRI2 transfected cells. Secreted Aβ38 from BRI2 transfected cells is below the detection limit.

**FIGURE 3.** BRI2 effects on Aβ and C99 are independent of the BRI peptide. HEK293-APPwt cells were transfected with BRI2 or BRI2Δ, a construct that expresses BRI2 lacking the C-terminal peptide (aa 244–266). Both BRI2 and BRI2Δ lead to a decrease in secreted Aβ compared with controls (co). Western blot detection with the antibody A8717 shows an increase in C99 but no change in AICD or APP levels. Expression of the BRI2 proteins was confirmed by Western blot using the ITM2B antibody. Detection of GAPDH serves as a loading control.
**BRI2 Promotes Aβ Degradation by IDE**

As an additional control, for overexpression of an unrelated transmembrane protein, we transfected cells with the presenilin 1 mutant PS1L166P. This mutant associated with familial AD leads to increased Aβ42 generation (36). In contrast to BRI2, the overexpression of PS1L166P did not lead to increased IDE secretion (supplemental Fig. 2, A and B) or a decrease in Aβ (supplemental Fig. 2C) showing that this effect is specific for BRI2 proteins. Expression and functionality of PS1L166P were confirmed by the expected effect of increased Aβ42 generation (supplemental Fig. 2C). Cell viability was not affected by overexpression of BRI2, ruling out a possible increase in levels of IDE due to cell death (supplemental Fig. 2D). As a second control, overexpression of an unrelated protein, EGFP, did not influence levels of secreted IDE or Aβ (supplemental Fig. 2, E and F). Taken together these results suggest that the BRI2-mediated reduction in Aβ may be due to increased degradation by IDE.

**Insulin Treatment Inhibits the BRI2-mediated Aβ Decrease**—To test if the BRI2-mediated Aβ decrease could indeed be caused by increased levels of IDE, we analyzed if the effect could be blocked by the addition of insulin. Because insulin is a substrate with higher affinity for IDE than Aβ, its addition in excess inhibits Aβ degradation by IDE (35). To this end we transfected HEK293-APPwt cells with BRI2 or vector control and compared Aβ levels in conditioned cell media with or without the addition of insulin during the incubation period. We found that the presence of insulin in the culture medium of BRI2-transfected cells significantly blocked the BRI2-mediated reduction in Aβ, as shown by Western blot and immunoassay measurements of Aβ40 and Aβ42 (Fig. 5, A and B). BRI2 transfection significantly reduced secreted Aβ40 and Aβ42 to levels 11.3 and 11.2% that of the control (p < 0.0001, n = 5). Insulin significantly blocked this reduction (p < 0.0001, n = 5), such that only a minor reduction to 80.6 and 66.7%, Aβ40 and Aβ42, was observed (p = 0.0019/p = 0.0015). Thus, inhibition of Aβ degradation by IDE repressed in large part the Aβ-lowering effect of BRI2 expression. The addition of insulin did not change levels of APP or APP C-terminal fragments (Fig. 5, A and C). Interestingly, the BRI2-mediated increase in C99 was not blocked by insulin (Fig. 5C), indicating that the Aβ decrease and C99 increase are not linked to each other.

**Transfer of Conditioned Cell Medium from BRI2-transfected HEK293 Cells to Untransfected HEK293-APPwt Cells Causes a Decrease in Secreted Aβ**—The above results strongly suggested a major role for secreted IDE in the Aβ reduction by BRI2. The secreted form of IDE is contained in the cell medium. Thus, we asked if the transfer of conditioned cell medium from BRI2-transfected cells would have a similar effect on HEK293-APPwt cells as overexpression of BRI2 in these cells (for an overview of the experiment see Fig. 6A). To do so, we first transfected HEK293 cells with BRI2 or control vector and harvested conditioned cell media 48 h post-transfection. BRI2 expression in these cells and an increase in secreted IDE in the conditioned cell media were confirmed by Western blot (Fig. 6B). Equal amounts of conditioned cell medium from control or BRI2-transfected HEK293 cells were then transferred to HEK293-APPwt cells and incubated for 24 h to allow Aβ secretion. In addition, insulin was added to one set of cells to inhibit Aβ-degradation by IDE. Secreted Aβ was clearly reduced in the BRI2 media-treated cells compared with the controls (Fig. 6, C and D). Quantification by immunoassay revealed a significant reduction in secreted Aβ40 and Aβ42 to 55.9 and 63.0% that of control levels (p < 0.0001, n = 4). Thus, the transfer of conditioned cell medium of BRI2-transfected cells (BRI2-CM) leads to a similar effect as BRI2 overexpression. In addition, when insulin was added to the conditioned cell medium during incubation with the HEK293-APPwt cells, its Aβ reducing effect was completely blocked (p < 0.0001, n = 4) (Fig. 6, C and D). APP expression in HEK293-APPwt cells remained unchanged by the addition of BRI2-CM or insulin (Fig. 6H). In a second set of experiments, we immunodepleted IDE from the conditioned cell medium prior to its addition to HEK293-APPwt cells. Immunoprecipitation with an IDE-specific antibody effectively

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**FIGURE 4. BRI2 increases levels of secreted IDE.** HEK293-APPwt cells were transfected with BRI2, ADanPP, or BRI2Δ expression plasmids or empty vector as a control (co). A, levels of nephrilysin in the membrane fraction of cells remained unchanged by BRI2. APP in the membrane fraction is shown as a loading control. B, secreted IDE was increased in the conditioned cell media from all cells transfected with one of the BRI2 constructs. In contrast, intracellular IDE detected in cell lysates remained unchanged by BRI2 expression. C, quantification of secreted IDE by densitometric analysis of band intensities. The 3-fold increase in secreted IDE relative to control (co) from three independent experiments is shown (***, p < 0.0001 relative to control; BRI2 versus ADanPP, BRI2 versus BRI2Δ, ADanPP versus BRI2Δ, p > 0.05).
**BRI2 Promotes Aβ Degradation by IDE**

![Image](67x546 to 231x575)

**FIGURE 5. Insulin treatment inhibits BRI2-mediated Aβ decrease.** BRI2- or vector control (co)-transfected HEK293-APPwt cells were supplemented with fresh medium 1 day after transfection and incubated for 24 h to generate conditioned cell medium. Where indicated, 10 μM insulin was added. A and B, without addition of insulin BRI2 expression prominently decreases levels of secreted Aβ. Inhibition of IDE activity with insulin prevents this Aβ decrease almost completely. A, levels of secreted Aβ are shown in two exposures of the same blot. Secreted IDE is increased with BRI2 expression and remains unchanged by the addition of insulin. In addition, APP expression remains unaffected by insulin. GAPDH serves as a loading control. B, Aβ40 and Aβ42 from conditioned cell media were analyzed by immunoblot assay from five independent experiments. Graphs show % remaining Aβ relative to controls in BRI2-transfected cells and BRI2-transfected cells treated with insulin. Insulin significantly blocked the BRI2-mediated reduction in Aβ40 and Aβ42 (*p < 0.0001). C, addition of insulin does not alter levels of APP C-terminal fragments.

Taken together, these results suggest that a secreted factor in the medium of BRI2-transfected cells confers the BRI2-mediated Aβ decrease and that this soluble factor is IDE, because the effect can be blocked by insulin and, more specifically, by immunodepletion of IDE. Next, we were interested if the transfer of conditioned cell medium would also lead to an increase in levels of the C99 fragment in the recipient cells. As shown in Fig. 6H, C99 levels were not changed in these cells, although Aβ was reduced. Thus, as seen in the experiment above, the observed Aβ decrease and C99 increase are independent effects.

**BRI2-mediated Aβ Decrease by IDE in HeLa Cells—** Next, we used HeLa cells to confirm our results in a different cell line. HeLa cells had been used previously to study the effects of BRI2 on APP processing (13). We co-transfected APP695 and BRI2 into HeLa cells and analyzed levels of APP cleavage products and IDE. Again, BRI2 led to a reduction in secreted Aβ (supplemental Fig. 3A) paralleled by an increase in secreted IDE, with no changes in cell viability (supplemental Fig. 3, C and D). Similar to that in HEK293-APPwt cells, an increase in C99 levels was observed, while AICD levels remained the same (supplemental Fig. 3, B and H). To test if the increased levels of secreted IDE also caused the Aβ decrease in this cell line, we included the addition of insulin as variable in the experiment. As in the HEK293-APPwt cells, in HeLa cells almost all of the Aβ-lowering effect was blocked when Aβ degradation by IDE was inhibited by the addition of insulin (supplemental Fig. 3, E and F). BRI2 transfection significantly reduced secreted Aβ40 and Aβ42 to levels 38.1 and 12.7% that of the control, as measured by immunoblot assay (*p = 0.0021/p = 0.0019, n = 3). Insulin significantly blocked this reduction (*p = 0.0002/p = 0.0003, n = 3), as only a minor reduction to 79.4 and 82.5% Aβ40 and Aβ42 compared with control levels was observed (*p = 0.0029/p = 0.0301) (supplemental Fig. 3, E and F). As a control, levels of APP and APP C-terminal fragments were examined and shown to remain the same following the addition of insulin (supplemental Fig. 3, G and H). These results confirm the major influence of IDE in the BRI2-mediated Aβ decrease.

**Enhanced IDE-mediated Degradation of Aβ(1–40) in Cell Media of BRI2-transfected HEK293-APPwt Cells—** To specifically test for IDE-mediated degradation of Aβ, we compared the Aβ proteolysis pattern obtained after its degradation by recombinant IDE and by conditioned cell media from control and BRI2-transfected HEK293-APPwt cells. Following incubation with synthetic Aβ(1–40), very similar MALDI-TOF spectra were obtained for conditioned cell media from BRI2-transfected cells (Fig. 7, E and F) and recombinant IDE (Fig. 7, A and B). Both contained peaks corresponding to individual Aβ fragments generated after its degradation by IDE (Table 1) (30). Incubation with cell media from control-transfected cells did not show an increase in IDE-specific Aβ-degradation products (Fig. 7, C and D). This evidence strongly supports that BRI2 mediates increased Aβ degradation through secreted IDE.

**Increased IDE Levels in Cerebral Spinal Fluid of wtBriPP Mice—** Next, we wanted to test if the BRI2-mediated increase in secreted IDE may also be involved in the observed Aβ decrease and plaque load reduction in the transgenic mice. As expected, no significant differences in IDE levels were detected in the...
total brain homogenates of wtBriPP-tg mice compared with controls (Fig. 8, A and B). Here, both the secreted and the intracellular pools of IDE are mixed together such that changes in only the secreted form may be masked by the relatively overwhelming level of intracellular IDE. To specifically analyze secreted IDE, we collected cerebral spinal fluid from wtBriPP-tg and non-tg littermates. Western blot detection of IDE revealed significantly higher levels of IDE in the cerebral spinal fluid of tg mice compared with the non-tg littermate controls (Fig. 8, C and D).

**DISCUSSION**

The British precursor protein BRI2 is argued to influence APP processing and Aβ deposition in cells and in vivo by...

**FIGURE 6.** Transfer of conditioned cell medium from BRI2-transfected HEK293 cells to untransfected HEK293-APPwt cells causes a decrease in secreted Aβ. A, a schematic overview of the experiment. HEK293 cells were transfected with BRI2 or empty control vector (co). Conditioned cell medium from these cells was transferred to untransfected HEK293-APPwt cells with or without addition of 10 μM insulin as indicated and incubated for 24 h. B, parallel to BRI2 expression, increased levels of IDE were detected in conditioned cell media from BRI2-transfected HEK293 cells compared with controls. C and D, HEK293-APPwt cells treated with conditioned cell medium from BRI2-transfected cells (BRI2-CM) show decreased levels of secreted Aβ compared with cells treated with control medium (co-CM). This effect is inhibited by the addition of insulin (+Ins). C, detection of secreted Aβ by Western blot. D, analysis of secreted Aβ40 and Aβ42 by immunoassay from four independent experiments. Graphs show % remaining Aβ relative to controls incubated with BRI2-CM compared with BRI2-CM-incubated cells additionally treated with insulin. BRI2-CM significantly reduced secreted Aβ40 and Aβ42 relative to controls (p < 0.0001), while insulin blocked this reduction completely (**, p < 0.0001). E, IDE in conditioned cell medium from BRI2 transfected HEK293 cells was immunodepleted using an IDE-specific antibody (IDE-IP) or only beads as a control (co-IP). IDE levels in immunodepleted or control cell media as well as the precipitated IDE from control and IDE-specific immunoprecipitation is shown (precip.). F and G, conditioned cell media were then transferred to HEK-APPwt cells, incubated for 24 h, and secreted Aβ analyzed by Western blot. Immunodepletion of IDE reverts the BRI2-mediated decrease in Aβ. G, quantification of total Aβ by immunoblot from three independent experiments shows that immunodepletion of IDE from the cell media of BRI2 transfected cells blocks its effect on Aβ decrease (IDE-IP-CM versus co-IP-CM, **, p = 0.0098; BRI2-CM versus co-CM, ***, p < 0.0001). If treatment with BRI2-CM did not lead to an increase in levels of the C99 fragment in HEK293-APPwt cells. The addition of insulin does not alter levels of APP, APP C-terminal fragments, or AICD. GAPDH is shown as a loading control.
BRI2 Promotes Aβ Degradation by IDE

FIGURE 7. Enhanced IDE-mediated degradation of Aβ(1–40) in cell media of BRI2-transfected cells. A and B, degradation of Aβ(1–40) by recombinant IDE. Synthetic Aβ(1–40) (9 μM) was incubated with purified recombinant IDE (0.6 ng/μl) for 1 h at 37 °C. Samples obtained before (A) and after (B) the incubation period were analyzed by MALDI-TOF MS. Representative spectra indicate peaks at m/z value of 4332.8 and half-m/z value of 2165.5 corresponding to single and doubly ionized Aβ(1–40) in samples prior to incubation (A). Following incubation with IDE, several degradation products of Aβ(1–40) are detected (B). The annotation of peaks to the corresponding peptides is given in Table 1. C–F, HEK-APPwt cells were transfected with control vector (pcDNA3.1) or BRI2 expression plasmid. Conditioned cell media were collected and incubated with Aβ(1–40) (10 μM) for 1 h. The Aβ levels in control and BRI2-transfected cell medium before (C and E) and after (D and F) incubation were analyzed by MALDI-TOF MS. Enhanced IDE-mediated degradation of Aβ(1–40) is evident in cell medium of BRI2 transfected cells (F) as compared with control cells (D). The Aβ degradation products in the conditioned cell medium are similar to that from recombinant IDE (B), further confirming the IDE-mediated degradation in the conditioned medium. a.u., arbitrary units.

BRI2 Promotes Aβ Degradation by IDE

varying mechanisms (11–15). Although the previous studies have suggested that mutations in the BRI2 protein may relate to a loss of BRI2 function and its effect on APP (37, 38), we have recently described that the mutant form of BRI2, ADanPP, decreases Aβ plaque load in transgenic mouse models of AD (21). In this study, we show that wild type BRI2 causes a similar plaque load reduction in the APPPS1 mouse model. We also confirmed previous results that overexpression of BRI2 decreases levels of cell-secreted Aβ (11, 13) and showed in addition that the mutated form ADanPP reduces Aβ levels to a similar extent in HEK293-APPwt cells.
Aβ degradation by competing for IDE (35), completely abolished the Aβ-lowering effect of both BRI2 transfection and the transfer of conditioned cell media from BRI2-transfected HEK293 cells. Furthermore, the more specific removal of IDE, by immunoprecipitation from the conditioned media, abolished the Aβ-lowering effect on the recipient cells, ruling out a major contribution of other soluble factors from the conditioned cell media. In addition, incubation of synthetic Aβ(1–40) with conditioned cell medium from BRI2 transfected cells showed enhanced Aβ-derived degradation fragments very similar in their MALDI-TOF spectrum to those derived from incubation with recombinant IDE, strongly supporting that BRI2 mediates Aβ degradation by secreted IDE.

As an alternative mechanism, it has been proposed that BRI2 could lower Aβ levels through direct inhibition of γ-secretase cleavage of the APP C99 fragment (11, 13, 14). In line with this hypothesis, we also found increased amounts of the C99 fragment in cells. However, in our experiments levels of the γ-secretase cleavage product AICD remained unchanged. These results are contradictory to previously published results (13) and may be due to the direct detection of the AICD fragment in our experiments, as opposed to use of an indirect method of measurement (13). In our cell experiments, the main factor responsible for the decrease in Aβ was contained in conditioned media and could be inhibited by insulin. Our results indicate that the increase in C99 is independent of the Aβ reduction, because it was not observed in cells that received the conditioned cell medium from BRI2-transfected cells. In addition, we did not observe changes in C99 levels in vivo, in APPPS1 mice crossed with wtBriPP mice. This is in line with the described increase in Aβ in BRI1 knock-out mice without changes in APP C-terminal fragments C83 and C99 (14).

In mouse models, the 23 amino acid long BRI peptide, which is shed by furin-like cleavage, is proposed to inhibit Aβ aggregation and thus reduce plaque load in transgenic mice (12). With a shorter BRI2 protein lacking the peptide sequence (BRI2Δ244–266) we still observed increased levels of secreted IDE and decreased Aβ. Thus, this function of BRI2 seems to be independent of the BRI peptide sequence. This observation is in line with results from others reporting that aa 46–106 of BRI2 were sufficient for the reduction of Aβ40 levels in vitro (11).

IDE is a metalloprotease that cleaves insulin as well as other peptide substrates (39). The enzyme is primarily located in the cytosol, but a fraction of the enzyme is found in peroxisomes and on the plasma membrane. In addition, the enzyme is secreted by an unconventional pathway (40), can be found in the cell media of several cell lines, and is shown to degrade extracellular Aβ (27, 35). Here, we show that BRI2 influences only the cell-secreted form of IDE, whereas intracellular levels of IDE remain relatively unchanged. To date, it is not known exactly how these different pools of IDE are regulated. A potential association with exosomes is discussed (30, 41), and further work will be needed to investigate the involvement of BRI2 in these processes in more detail.

Aβ levels are elevated in the brains of IDE-deficient mice (34, 42), and in mouse models of amyloid deposition, increased levels of IDE, through overexpression, reduce amyloid plaque load (43). Because 95% of IDE is contained in the cytosol and only a

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**TABLE 1**

MALDI-TOF MS analysis of the cleavage products of Aβ(1–40) peptide by recombinant insulin degrading enzyme

| Peptide fragment | Calculated mass (Da) | Observed mass (Da) |
|------------------|----------------------|-------------------|
| 1–13             | 1560.7               | 1562.6            |
| 1–14             | 1697.7               | 1698.9            |
| 1–15             | 1825.9               | 1827.1            |
| 1–18             | 2166.0               | 2164.9            |
| 1–19             | 2313.1               | 2313.7            |
| 1–20             | 2460.2               | 2460.8            |
| 1–27             | 3134.3               | 3133.6            |
| 1–28             | 3260.5               | 3264.2            |
| 1–33             | 3674.0               | 3675.6            |
| 15–28            | 1580.8               | 1581.6            |
| 14–40            | 2784.5               | 2785.2            |
| 15–40            | 2647.4               | 2648.6            |
| 20–40            | 2032.1               | 2032.9            |
| 1–40             | 4327.2               | 4332.8            |
| 1–40*            | 2163.6               | 2165.5            |

*1–40* represents doubly ionized full-length peptide (m/z2 instead of normal m/z).
small fraction is secreted (39), small changes within this pool prove to be difficult to detect in the mouse brain. We did not find significant differences in IDE levels in total brain homogenates of wtBriPP tg mice compared with control mice. To specifically analyze secreted IDE in the brain, we collected cerebral spinal fluid and found that IDE was significantly increased in the cerebral spinal fluid of wtBriPP tg mice compared with non-tg littermates. These results suggest that BRI2 can mediate an increase specifically in secreted IDE in both cells and mice.

A second line of evidence that indicates that enhanced IDE-mediated degradation may be responsible for the plaque load reduction in mice comes from the fact that levels of the major secreted Aβ forms, Aβ40, Aβ42, and Aβ38, are equally reduced in wtBriPP/APPPS1 mice compared with single transgenic APPPS1 mice early, prior to amyloid deposition. The reduced plaque load in older mice may thus be caused by lower Aβ levels, rather than by an inhibition of Aβ aggregation. Taken together, our results suggest that BRI2-mediated changes in the secreted form of IDE in the brain are responsible for the observed decrease in amyloid deposition. It remains possible that in vivo other proposed mechanisms (11–14) may work in concert with this previously undescribed function of BRI2.

At present, the physiological role of the BRI2 protein is not known. It is cleaved by several proteases, whereby extracellular parts are shed and an intracellular domain is generated (19), similar to proteins involved in signal transduction by regulated intramembrane proteolysis (44). Having these properties, BRI2 resembles receptor proteins involved in signal transduction. Like many receptors, BRI2 can form homodimers (45). Thus BRI2 signaling may regulate levels of extracellular IDE. Several pathways can regulate IDE levels in different experimental systems. The PI3K pathway (46), insulin receptor signaling (47), pathways can regulate IDE levels in different experimental systems. BRI2 signaling may regulate levels of extracellular IDE. Several pathways can regulate IDE levels in different experimental systems.

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